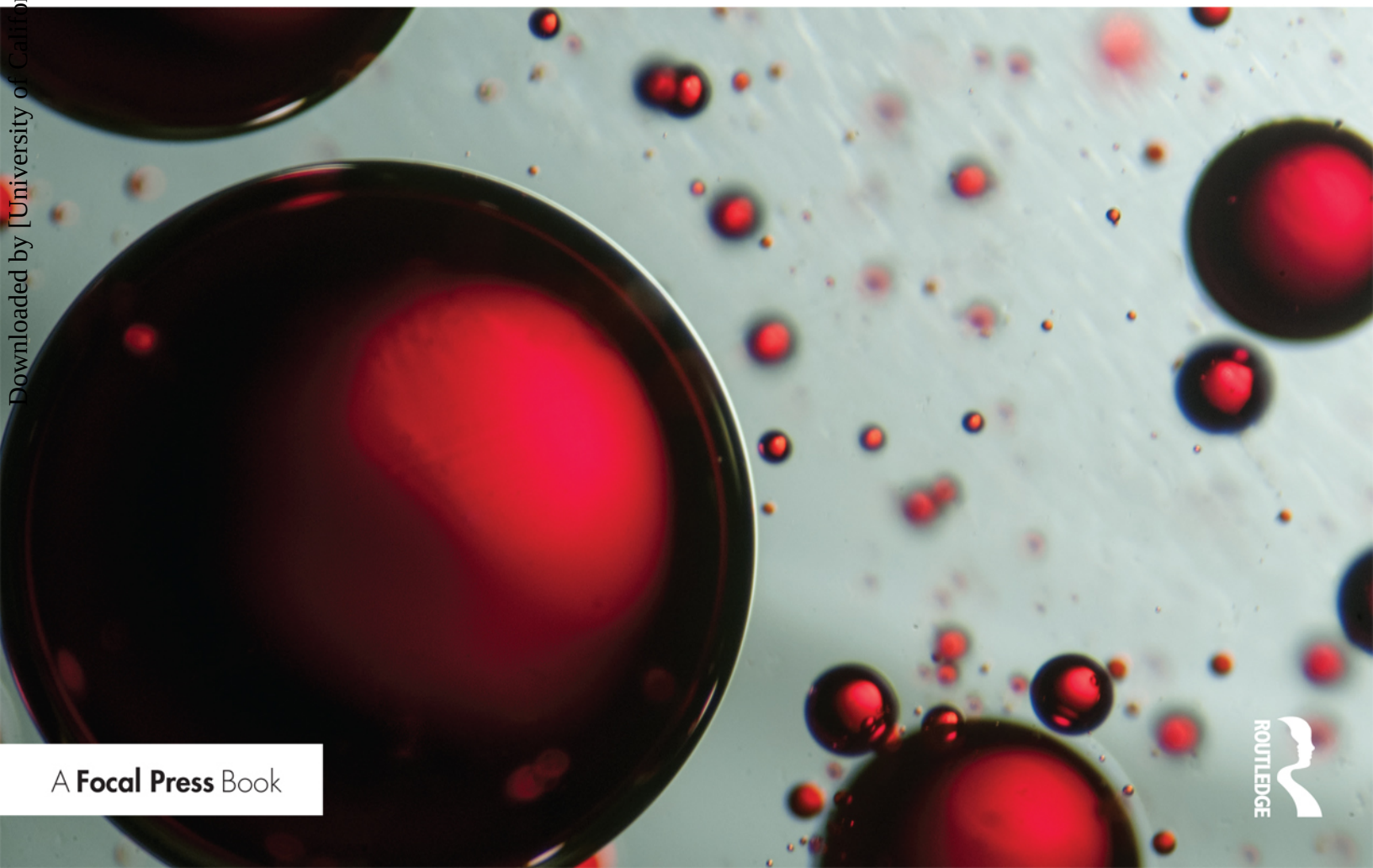
Applications in Scientific Photography
Series Editor: Michael Peres

Laboratory Imaging and Photography

Best Practices for Photomicrography and More

Michael Peres
with James Hayden, Staffan Larsson and Ted Kinsman



A **Focal Press** Book

LABORATORY IMAGING AND PHOTOGRAPHY

Laboratory Imaging and Photography: Best Practices for Photomicrography and More is the definitive guide to the production of scientific images. Inside, the reader will find an overview of the theory and practice of laboratory photography, along with useful approaches to choosing equipment, handling samples, and working with microscopic subjects. Drawing from over 150 years of combined experience in the field, the authors outline methods of properly capturing, processing, and archiving the images that are essential to scientific research. Also included are chapters on applied close-up photography, artificial light photography and the optics used in today's laboratory environment, with detailed entries on light, confocal, and scanning electron microscopy. A lab manual for the digital era, this peerless reference book explains how to record visual data accurately in an industry where a photograph can serve to establish a scientific fact.

Key features include:

- Over 200 full-color photographs and illustrations
- A condensed history of scientific photography
- Tips on using the Adobe Creative Suite for scientific applications
- A cheat sheet of best practices
- Methods used in computational photography.

Michael R. Peres is the editor-in-chief of *The Focal Encyclopedia of Photography*, fourth edition, and former chair of the biomedical photographic communications department at the Rochester Institute of Technology. Since 1986, he has taught photomicrography, biomedical photography, and other applications of photography used in science. Prior to joining the RIT faculty, Peres worked at Henry Ford Hospital and the Charleston Division of West Virginia University as a medical photographer. He is the recipient of the RIT Eisenhart Outstanding Teaching Award and the Schmidt Medal for Lifetime Achievement in the Field of Biocommunications.

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Contents

ABOUT THE AUTHOR

MICHAEL R. PERES joined the teaching faculty of Rochester Institute of Technology's School of Photographic Arts and Sciences in 1986. He is a professor of biomedical photographic communications and teaches photomicrography, biomedical photography, and other related applications of photography used in science. He served as the chair of the biomedical photographic communications department for more than twenty years. Prior to joining the RIT faculty, Peres worked at Henry Ford Hospital and the Charleston Division of West Virginia University as a medical photographer.

Peres has enjoyed a varied photographic career that began in 1973. He is an educator, author, artist, and innovator. He has led workshops worldwide and has been publishing most of his career. In 2007, he served as the editor-in-chief of *The Focal Encyclopedia of Photography*, fourth edition. Some of his professional activities include co-coordinating the R•I•T Images from Science project and the RIT Big Shot project, and chairing the Lennart Nilsson Award nominations committee (1998–2011).

Peres has received numerous awards including RIT's Eisenhart outstanding teaching award and the Schmidt medal from the BioCommunications Association for lifetime achievement in the field of biocommunications. He holds a master's degree in instructional technology and bachelor's degrees in biology and biomedical photographic communications. He is also a registered biological photographer.

ABOUT THE CONTRIBUTORS

JAMES HAYDEN is managing director of The Imaging Core Facility at The Wistar Institute in Philadelphia, Pennsylvania. Hayden earned a BA in biology and biophotography and was employed by the University of Pennsylvania School of Veterinary Medicine in 1983. He also ran a freelance photography business called Bio-Graphics. He was hired by The Wistar Institute in 2002. Although specializing in photomicrography, he also photographs everything from cells to people. Hayden's responsibilities include customized imaging, consultation on experimental design, advanced imaging training, and quantitative image analysis. Hayden has lectured extensively and led workshops in both domestic and international venues. He has won numerous awards, as well as appearing in books and on the covers of such journals as *Nature*, *Science*, *PNAS*, and *Cell*. Hayden's images have been featured in the Nikon International Small World photomicrography competition where he has also served as judge. His written publications range from technical papers to contributions in scientific research journals. He is a Registered Biological Photographer, Fellow, and Louis Schmidt Laureate of the BioCommunications Association. Hayden also serves as an Adjunct Professor at the University of the Sciences in Philadelphia.

TED KINSMAN earned an AS degree in Optics, a BS degree in Physics, and an MS in Science Education. He has worked as an optical engineer, a physicist, and a physics instructor before joining the faculty at Rochester Institute of Technology in the fall of 2013. Kinsman is one of the few active scientific photographers specializing in scanning electron microscopy and high-speed photography. Recently, Kinsman's work has expanded to include radiography where he continues to produce images for books and magazines. His work has appeared on The Discovery Channel, ABC, NBC, PBS, CBS, and the British Broadcasting Corporation. He has also contributed imagery to the *Frozen Planet* series, and James Cameron's *Avatar* movie. Kinsman is currently an assistant professor for Rochester Institute of Technology's School of Photographic Arts and Sciences where he specializes in Photographic Sciences.

STAFFAN LARSSON lives in Stockholm, Sweden, and earned an AS degree in photography and holds a national certificate in digital image workflow delivery systems. Larsson has forty-two years of experience in medical and scientific photography. He has worked at Karolinska Institutet Medical University and Stockholm County Council Healthcare. In 2009, he joined the Royal Institute of Technology's School of Technology and Health faculty and Clinical Science Intervention and Technology (CLINTEC) at Karolinska Institutet. Larsson has also worked in the arts, photojournalism, and in nature. As a photographer his work has illustrated countless science and medicine articles featuring images created using new and interesting ways. He has led workshops and courses for students all across Sweden and the US. Larsson was pivotal in the formation of the Lennart Nilsson Award in 1997 and developed initiatives supporting its mission for more than fifteen years. He was an author in *The Focal Encyclopedia of Photography*, fourth edition, and is currently a research engineer in medical and scientific photography at the School of Technology and Health, Royal Institute of Technology, where he teaches and assists scientist working groups as a consultant for communications and public relations.

ABOUT THE SERIES

APPLICATIONS IN SCIENTIFIC PHOTOGRAPHY

Like all fields, there are many subspecialties within the broad field of scientific photography. While each seemingly specialized, there are areas within the individualized fields of science where photography is practiced in generalized ways as well. The **Applications in Scientific Photography** series was produced to become an industry resource, in homage to the seventeen books created for the Time Life Photography series in the 1970s, which outlined various aspects of photography for the working professional and student, including technology, methods, applications, tools, and important photographers.

The first book in the **Applications in Scientific Photography** series is, *Laboratory Imaging and Photography: Best Practices for Photomicrography & More*. This book covers industry-accepted practices and standardized approaches to laboratory imaging and photography, including imaging philosophies, equipment selection and operation, and digital imaging workflow choices required in this era of unprecedented change and new product choices. Readers will learn from recognized experts how to select and operate equipment, optimize sensors, and how to profile files for research, publishing, data delivery and the critical task of archiving. Special attention will be given to light microscopy and related techniques.

Each subsequent title in the series has been designed to share the best practices from each discipline using applied and practical strategies. Each book in the series will deconstruct and emphasize how images are considered scientific data and the end facts of an experiment or photographic documentation/survey.

Titles in the Series

Laboratory Imaging and Photography: Best Practices for Photomicrography & More
Michael Peres, Rochester Institute of Technology

ACKNOWLEDGEMENTS

I am deeply indebted to many people who played a role in the creation of this book. The book was written during a twenty-month period that began in the spring of 2014; however, many people contributed to this book during the forty-plus years that I have been a photographer, educator, artist, and innovator.

While writing this acknowledgement, I happened to be watching the movie *A Good Lie*, and in the credits I read: "In Africa we having a saying: If you want to go fast, go alone. If you want to go far, go together." Those words have epitomized the journey of this book, and truthfully of my career. I am sorry if this acknowledgement seems longer than most, but this is an important chance to recognize those who have paved the way for me, enabling me to accomplish this project.

I want to first thank Taylor & Francis for allowing me the opportunity to publish this book. This journey began at a chance meeting in New York City in the fall of 2013 with Kimberly Duncan Mooney, my former acquisitions editor, who worked for Focal Press at the time. I am very appreciative to the guidance and work of Kimberly and Anna Valutkevich, both of whom have left the company after finding new opportunities. Galen Glaze, this book's editorial assistant, has been a valuable contributor of suggestions and managing the content, helping me to get ready for submission. (During the course of the book's creation, Focal Press was purchased by Taylor & Francis.) I also wish to acknowledge the expertise of the copyeditors and designers at Taylor & Francis. They have helped me achieve my vision for this book. A special thank you also to Rochester Institute of Technology for supporting a period of professional leave. Having a dedicated block of time in the fall of 2015 to work on the book provided an enormous opportunity.

I would also like to recognize the work of Gordon Brown who meticulously read my final—but not formatted—manuscript, ensuring everything was correct, understandable, and ready to go. Dr. Richard Zakia, a long-time author of many Focal Press books and a good friend to Gordon and me, would be proud of our partnership.

Below is a list sharing those people and organizations who played a role in the creation of this book. Some have shared encouragement, some have shared knowledge, and many have shared their writing, expertise, and photographs. There is not enough space, or words, to adequately thank you all. Your contributions will define this book.

Contributing Authors

James Hayden • Ted Kinsman • Staffan Larsson

Photographers and Organizations Who Have Photographs in This Book

Berenice Abbott • Albertina Museum • Sarah Alharbi • Jace Artichoker • Anna Atkins • Norman Barker • Boston Medical Library in the Francis A. Countway Library of Medicine, Harvard University • Jonas Brane • Jordan Briscoe • Gordon Brown • Dr. Stanley Burns and the Burns Archive • Cajal Legacy—Instituto Cajal • Canon, USA • Carl Zeiss Microscopy • College of Liberal Arts Office of Information Technology, University of Minnesota •

Frank Cost • Joseph Cowan • Torgeir Dahlen • Hans Danzerbrink • David Winton Bell Gallery at Brown University • D-Kuru • Todd J. Dreyer • Adrian Dyer • Eastman Kodak Company • Eye of Science, Oliver Meckes and Nicole Ottawa • J. Adam Fenster • Tim Flach • Abe Frajndlich • The Hagströmer Medico-Historical Library, Karolinska Institutet • Adam Hartley • James Hayden • Michael Horvath/SharkD/ • Ted Kinsman • Grayce Scott Koppey • Kevin Langton • Staffan Larsson • Jacques-Henri Lartigue • Fabrizio Lazio and the École polytechnique fédérale de Lausanne • Library of Congress Prints and Photographs Division • Dr. Jeff W. Lichtman and Joshua R. Sanes, Harvard Medical School • Clay Patrick McBride • Lainie Maier • David Malin • Torey Miller • *National Geographic* • National Library of Medicine • National Library of Medicine of the National Institutes of Health • Martin Oeggerli • Nate Pallace • Phred Peterson • Lindsay Quandt • Howard Radzyner • Amanda Rebbechi • John Retallack • Rochester Institute of Technology • Patti Russotti • Nanette Salvaggio • School of Photographic Arts and Sciences • Josh Shagam • Laura Solomon • Geoffrey Stein • Nancy M. Stuart • Joseph Territo • Time Life/Lennart Nilsson • Kristen Toohey • Lynne Tseng • Bill Watterson/GoComics.com • Sue Weisler • Susanne K. Williams • Joel Peter Witkin/ Catherine Edelmann Gallery.

People and Organizations Who Made a Difference on My Journey

Barbara and Richard Peres (my parents), thank you for encouraging me to follow my passions and study photography • A heartfelt thank you to all of my students—past and present—from RIT and Henry Ford Hospital. I have learned so many important lessons from each and every one of you • Thank you to the BioCommunications Assn for providing me a forum to share my work that began in 1979 • Thank you to colleagues and institutions from near and far for the important opportunities to collaborate • Thank you Professors Emeritus Bill DuBois and Andrew Davidhazy, Larry Koffer, Rick Kozak, Dr. B.J. Mathis, Dr. Lennart Moeller, Dr. Therese Mulligan, Milt Pearson, Sally Robson(d), Rudi Rottenfuser, Coach Joe Stowell, Frank Cost, Tom Zigon, Christye Sisson, and Willie Osterman for your friendship, sharing your knowledge, encouragement, opportunities, and guidance.

PREFACE
IN THE BEGINNING

March 2014, when I submitted the proposal to write this book, it occurred to me that perhaps the seeds were planted decades earlier. By chance, I was introduced to scientific photography when working on a BA degree in biology. At the time, I was attempting to photograph through a microscope because I wanted to try. Alone and without guidance, the results I achieved were awful but the exposure to the challenge of photographing nearly invisible things was thrilling. As I think back to that time, I believe I discovered the power of curiosity and became further addicted to photography. It was a transformative time.

Figure P.1 One of my first recollections of science photography was the use of Lennart Nilsson's picture featuring human development on the cover of Life magazine in 1965 (left). A photograph from the initial publishing was published again in 1996 (right). Within days of the initial publishing, eight million copies were sold. Image courtesy of Time Life/Lennart Nilsson.

The Importance of Curiosity

While at Bradley University, one of the many jobs I held was student photographer for the university's audio-visual department. The best part of the job might have been that I had access to the university's darkroom twenty-four hours a day. When the department was closed, I developed an interest in figuring out how special films made by Kodak, Ilford, and Agfa worked. I can remember long hours spent evaluating how color was reproduced using Kodalith® film, Line Positive Direct® film, or Vericolor Print® film. The more I experimented, the more I wanted to try. I bought my first book, *The Manual of Close-Up Photography* written by Lester Lefkowitz, and I joined the Biological

Photographic Association, now the BioCommunications Association. As I progressed with experimenting, photographing technical subjects remained challenging but it became easier using the new knowledge that I had gained from the investigations.

More than forty years have passed since those early beginnings. Following the completion of my biology degree I went back to school and earned two more degrees: one in biomedical photographic communication and a master's degree in instructional technology. I worked in a custom film laboratory, a commercial studio, and in three large teaching hospitals located in three different states. I became a registered biological photographer and I worked as a freelance photographer. Now, after teaching biomedical photographic communications in Rochester Institute of Technology's School of Photographic Arts and Sciences for more than three decades, this seems like the right time to write this book.

Figure P.2 While everything has changed in more than three decades, it also seems like nothing has changed. Scientific photography requires passion, curiosity, and persistence. Left to right: My first photomicrograph featuring algae (1976), various packages of specialized Kodak® emulsions designed specifically for scientific photography applications at the time, and a 1975 self-portrait taken while a student at Bradley University.

An Important History

Today the use of film is virtually non-existent in science and images can be made in ways that I could never have imagined even just a few years ago. For decades, important books and articles were written about this subject; however, many of these publications were written at the end of the film era. Today there is no shortage of websites—some great and some not so good—dedicated to the subject of photography, but not many are dedicated to the subjects included in this book. While the Internet is nothing less than extraordinary, I believe there is still a place for a concise and modern resource book that contains—in one volume—practical suggestions useful for creating the best imaging practices in science laboratories. This book has been written to co-exist with the Internet and I hope will be considered as a useful lab manual or handbook.

It has been said so many times that some might consider it trite, but no one achieves success without the help and encouragement of many along the journey. Sometimes there are cheerleaders and other times collaborators that contribute valuable assistance

and opportunities. The cumulative decades of experiences gained in photographing challenging subjects and working beside some of the best scientific photographers in the world has served as the foundation of my career and for this book. It is interesting to note that much of the important work created during the Internet's infancy is relatively invisible on the web. Not much of the work or publications from important authors and scientist photographers such as Dr. Leon LeBeau (d), John Gustav Delly, Dr. Roger Loveland (d), H. Lou Gibson (d), Professor Emeritus Nile Root (d), Alfred Blaker (d), Martin Scott, Dr. Richard Zakia (d), Dr. Leslie Stroebel (d), or Jack Vetter (d) has found its way online. Maybe that will be one of my "next" projects but for now I hope that in some small way the entries in this book will give a voice to some of their life's work, which served as the foundation of knowledge for countless scientist photographers.

I also invited a few expert collaborators to share their unique knowledge, including James Hayden, Ted Kinsman, and Staffan Larsson. Between the four of us, there are nearly 150 years of experience spanning across many diverse imaging experiences and backgrounds.

Traditions and Practices

I am sure when historians chronicle the history of science from this era, it will be described as the "Age of the Image" because every aspect of science now includes imaging. Every publication and area of research is heavily invested in images and imaging. There is, however, nothing new about using images in science. Science adopted photography as soon as the technology and materials needed to make images permanent were discovered. What is absolutely new is how people now create images. The advancement of technology will not slow down in the foreseeable future and developing new attitudes about the fast-paced changes and skills is an attitude needed for success.

Anna Atkins, a British botanist and photographer, is considered by some to be the first to publish a book dedicated exclusively using science pictures to convey science data rather than the traditional drawings—the norm of the time. Images made in the mid-1800s were singular pieces, because there was no simple way to make multiple copies. Each image was an original that could not be duplicated and so the integration or distribution of images to audiences was challenging. Today, I am continually amazed that images can be shared electronically to worldwide audiences nearly synchronously with their creation. It is simply fantastic!

Figure P.3 Scientific illustrations can serve to inspire and preserve scientific data. This photograph is a cyanotype of *Pteris aquilina* produced by Anna Atkins for her book, *Photographs of British Algae: Cyanotype Impressions*, originally published in October 1843. Image courtesy: http://commons.wikimedia.org/wiki/File:Anna_Atkins_-_Pteris_aquilina_-_Google_Art_Project.jpg.

A New World of Opportunity

Photography and imaging are playing increasingly new roles in science and in society in general. At a recent conference, it was shared that Facebook is currently the world's largest picture archive, and it goes without saying that smartphones have become the most frequent type of camera used today. I have seen smartphones used to document surgery and on microscopes. New apps are being released weekly—or so it seems—and the things people can do with imaging continues to advance in immeasurable ways.

For this reason, it becomes important to recognize that today's working "imaging" vocabulary is also evolving. New terms are being formed and adopted by users. Consider for a minute that Adobe Photoshop® software has become a verb. It is now common to suggest someone to Photoshop something in or out of a picture. Google® and Xerox® also benefit from such associations. A web search is now frequently called "Googling it" and making an electrostatic copy is frequently described as making a Xerox copy. Terms like "computational photography" are also the direct result of new technologies. Recently, "selfie" became an accepted word in the English language, and in 2013 the *Oxford English Dictionary* designated "selfie" as the word of the year. While not specifically scientific terms or concepts, these examples speak to the evolution of imaging and its related vocabulary, which is the new normal. Terminology, devices, applications, and users create new words and expressions that find their way into the working vocabulary of the imaging industry.

When the topical outline for the book was being created, there were many email exchanges and debates. I wish there was unlimited space and that I could have included video content, but it was not possible. Given the rate of change and constant release of new products, it was decided to write this book sharing global topics rather than specifics about a particular camera or software. You will also find numerous illustrations and photographs in the book that were created as references. Shared freely in this book is also work produced by some of the world's foremost science photographers. Each image has been attributed to its maker; however, if there is no attribution, the image has been provided by the author.

Working to a Solution

I have found that having a positive attitude is critical to successful imaging outcomes. While often not suggesting that they are photographers, scientist photographers must have the desire to make good images or it will not happen. A real commitment to the process of making good images is at the foundation of any imaging initiative. Without an interest to work hard at photographing and improving, the creation of quality images will be just lucky accidents. Unfortunately, there are no short cuts to success.

The mission of this book is to share useful and practical methods to science photographers. Given the space constraints, every facet of every subject including image processing has been covered as completely as space would allow. Making things visible is a recurring challenge for photographing scientific subjects. I hope you will enjoy and benefit from this book.

Michael R. Peres

Professor Biomedical Photographic Communications
Rochester Institute of Technology

DEDICATION

This book is dedicated to my wife Laurie and my children, Jonathan and Leah.

Thank you Laurie Peres for your constant encouragement to try new things.
Jonathan and Leah, thank you for your love and frequent suggestions for my projects.
You all inspire me to be a better person every day.

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Introduction

The Imaging Chain

The Product is the Sum of its Parts

Photographing subjects that are sometimes created in a scientific laboratory comes with different challenges and responsibilities than making photographs for art or advertising purposes. In the advertising world, the photographer's role is to make things look better than they do. Embellishment and amplification of an object and its characteristics is—on some level—assumed to be part of the plan. One might argue the role of an advertising photograph is to entice a buyer to make a purchase based on psychological or emotional strategies rather than creating a photograph that reflects absolutely truth.

In science, the embellishment of characteristics or the making of photographs that share false truths is not ethical. Science photographs must be absolute reproductions of an object to the extent that the photographic process will allow. Photographs made of scientific subjects require an absolute verisimilitude. A neutral point is fundamental and needed to create images that have no bias and are mandated for careful study, and for the preservation of scientific data. In Chapter 12, author James Hayden deconstructs this very important aspect of scientific photographic practices.

The process of making photographs for science requires the use of the appropriate tools and best methods. Because of the continual release of new products—with ever-increasing capabilities—there will always be constant challenges to stay current and evolve with the tools and software. There will be constant pressure to join the bandwagon and to use the latest and greatest methods to make images. However, the objective in scientific photography has remained the same since photography's invention, and that is to make things visible using tools and accepted practices current with the times. Frequently, this is heavily dependent on tools and technology.

Making things visible can be accomplished using specifics lights; using lighting in effective ways; or selecting optical methods including interference or special methods such as fluorescence or polarization techniques. These methods—useful in photographing difficult subjects—are considered to be "image forming" events. Once an image has been formed, it can be recorded. Samples too will play a role in imaging outcomes, and the ultimate goal remains the same for the science photographer: to reveal and record characteristics of a subject in highly precise and accurate ways. The first half of this book will explore features and characteristics of applied physics, camera equipment, optics, and theoretical foundations required for the production of highly accurate scientific illustrations. These chapters have been written to provide a thorough overview of how and why things work. The later chapters in the book will explore applications and methods used across many subjects that will provide the "how to" do things in the book.

The Imaging Chain

It might be useful to consider that there are many steps and pieces of equipment required to form and see images. Without overly deconstructing the process, the sequence of events that leads to the formation of an image might be referred to as an imaging chain. An imaging outcome is the result of the various decisions made by an operator, the use of the right tools, and the operator's ability to form "optimized" images during the various

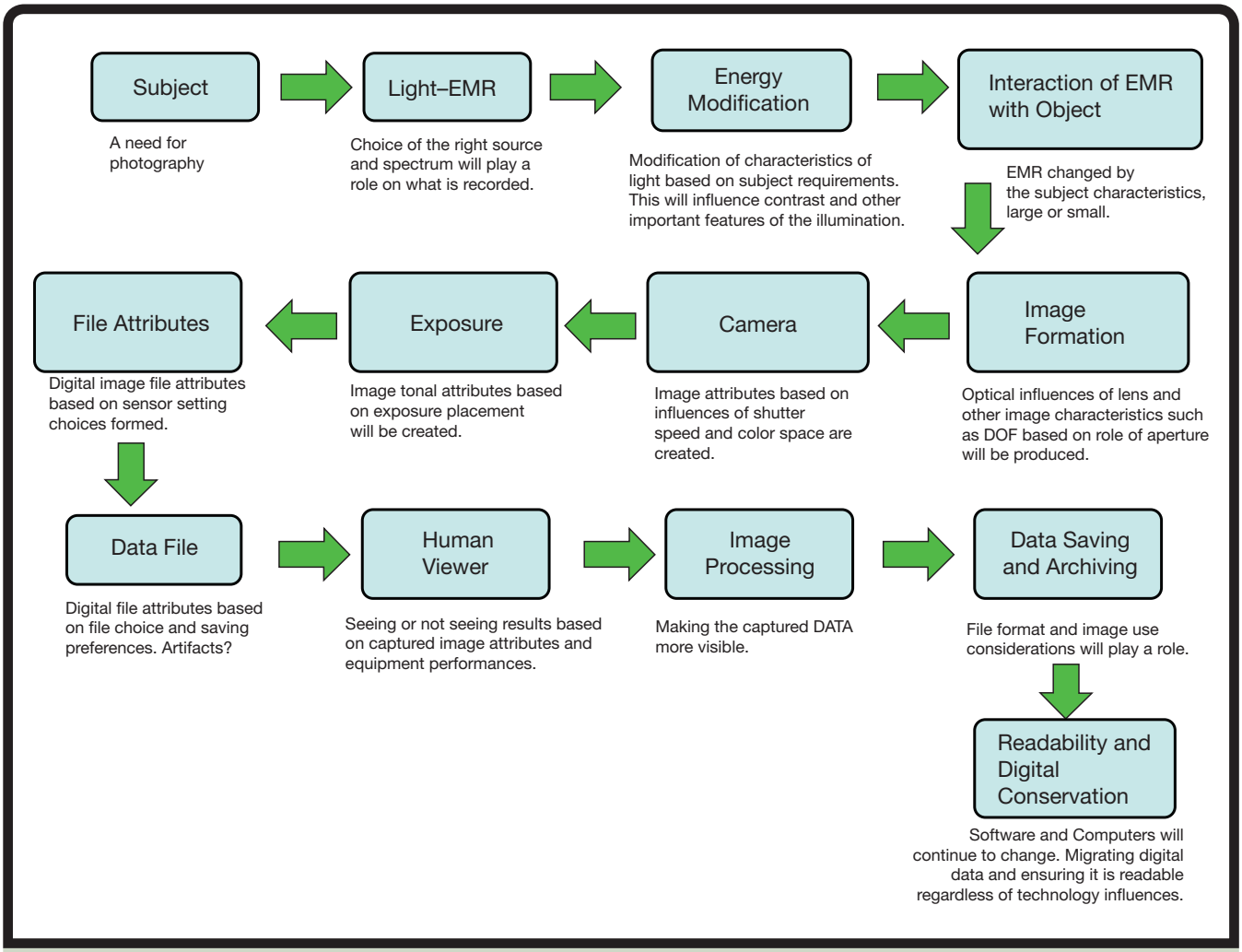

Figure I.1 There are many steps and factors that play a role in the process of forming, capturing, and producing a permanent image. Although composed of independent steps or locations in a continuum of the process, each step contributes to the characteristics of images including enhancement or degradation. The execution of decisions and methods at each stage of formation/processing will influence the quality of the final image. At each juncture, constructive or destructive influences can be introduced that affect the features of the image, such as its sharpness or optical resolution, along the way. While each location may only affect minor changes to quality, when compounded over the entire sequence of events, degradation or other changes to image quality can occur. Attention should be paid to optimize everything in each step to preserve image structure and integrity.

steps in the process. Scientist photographers must select methods that maximize what is possible and that are needed to create visibility before then recording the image. They must also work within accepted practices of the discipline. These considerations and strategies used for imaging might be called best practices. Best practices are grounded in equipment, knowledge, imaging objectives, and using accepted norms. The creation and use of standardized approaches is a fundamental objective for a scientific photographer. In Chapter 1, considerations about scientific methods, standardized practices, and repeatability are discussed.

The best practices used in imaging laboratories would be characterized by a series of independent but linked events. These events are grounded in reality and are based on everything that contributes to an image being formed and recorded. Human physiology, viewing conditions, and photographer/scientist expectations are among other functions that play a role in this continuum of events. Throughout this book, the fundamentals and applications of equipment and processes will be described. When taking a holistic view of photography applied to science, the graphic in Figure I.1 can summarize why—at each location—an image of an object is affected by influences of the process, the sample, or an operator, or all of them to a varying percentage.

Photography Defined

It is a frequent debate what the word "photography" means. "Photography" has come to mean many things in a constantly changing world. The term "imaging" is also a word that has come to mean photography, among other definitions. These two words have become interchangeable when used by authors in today's publications. "Pictures," "snapshots," "images," "photographs," "digital assets" are also terms are used to describe the outcomes of the photographic process. For this book, the authors will try use the term "photography" to describe single-shot applications as much as possible. The term "imaging" will also be used to describe outcomes that include the use of many images, software, or computing devices. It is important to accept that there is truly no absolute definition of "photography" accepted by all. In most cases the words are nearly interchangeable.

The outcomes of taking pictures can also be variable. For many factors, the often-dynamic situations surrounding science imaging are influenced by a myriad of factors. I suspect that there are many photographs made today without great concern for accuracy or quality. Because image-making technology is everywhere and so easy to operate, I think we sometimes simply take it for granted and get lazy or sloppy. H. Lou Gibson (1906–1992), a noted authority on scientific photography who worked from the late 1940s to the mid-1980s as an editor of Kodak technical publications, often shared, "the production of high quality photographs requires care and attention to detail." An example of Gibson's work is shared on the cover page of Chapter 12.

Part 1

Foundations, Fundamentals, Principles, and Theory

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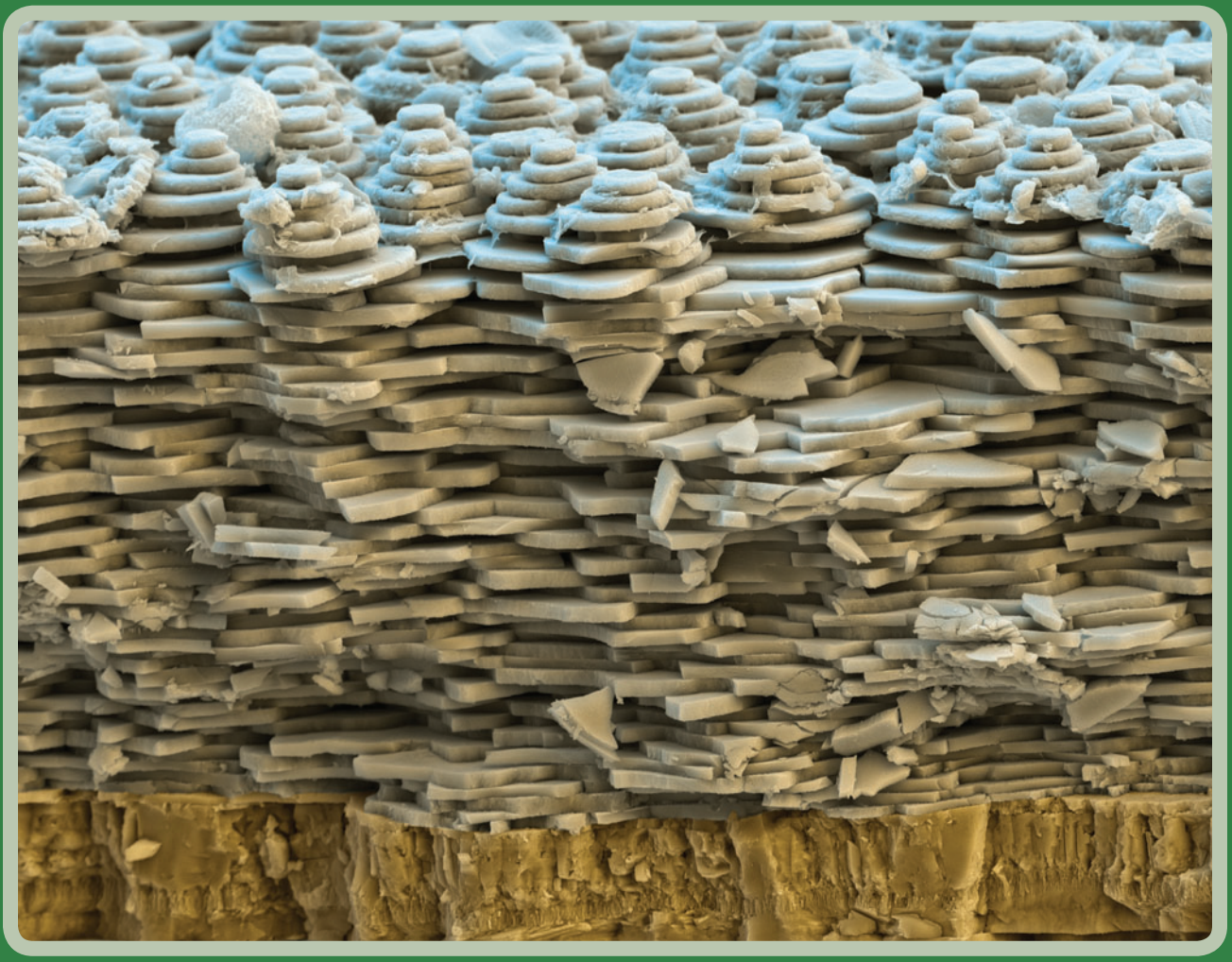
Chapter 1 Defining a Science Image



Science or art? This scanning electron photomicrograph features the shell of the white-lipped snail, *Cepaea hortensis*. The shell is composed of lime. The photograph reveals the layers of the shell, including where the inner wall forms new crystal platelets, and is graphically very interesting. This image is a composite from three detectors, one secondary detector and two backscattered electron-detectors. Compositing and coloring was accomplished using Adobe Photoshop software. The scanning electron microscope used 10 kV with a 13 mm working distance and 2500:1 at 15 x 13 cm. The shell was photographed September 2012 by Nicole Ottawa. Nicole and Oliver Meckes are the owners and photographers of Eye of Science. Image courtesy of Eye of Science, http://www.eyeofscience.com.

A Frame of Reference for the Image in Science

This book has many chapters written to explore various facets of scientific photography. Before delving into the technical chapters, it might be useful to consider the science image as a part of a "bigger conversation". Beginning with the earliest images produced from a time long gone, people have exhibited an interest in chronicling observations using whatever tools were available. Museums and other cultural heritage institutions have countless examples of these wonderful treasures. Making pictures has always needed and will always require tools, materials, and skills/knowledge. Beginning with the discovery that was needed to make permanent silver-halide photographic images in the early nineteenth century, photography's technology has experienced a huge transformation to the current digital world we live in. What has not changed during this time of evolution is the motivation as to why people make pictures in the first place. That motivation remains the same. This chapter was written to share some highlights of photography's evolution specific to science, the challenges and solutions when photographing in science, some key inventors, some important equipment, and some fundamental practices that have spanned more than 150 years.

Today, photographic documentation in science has become a science unto itself. It is difficult to identify an environment or industry not using imaging or images. The idea to use photography in science is by no means new. Making a photograph of an object or event implies a certain importance of that object or the event itself. Many of today's practices have their origins at the time of photography's invention and even before. Pictures record history. Pictures can be called photographs or images. Images in science are considered data or facts and they have become an integral part of exploration, discovery, and certainly publishing. Images can record what is not visible to the human visual system and they can make permanent records of transient events to serve as visual notes if needed at a later date. Pictures chronicle things and situations where words might be inadequate and images remember what time will forget. All these outcomes are pretty impressive.

Digital tools have migrated into every discipline and so have the challenges of staying current and relevant for both users and manufacturers alike. With all of the technological advancements, the creation of images that were never possible can easily be produced with the right digital equipment and knowledge. The rapid adoption of the newest technologies is a direct consequence of the simplification of operation of digital tools, the reduction in the costs of all of the necessary equipment, as well as the continual release of easier-to-use software. Simply consider the explosion of smartphones and tablet computing as a microcosm of the digital space that science operates in.

The Science Image: A Point of Departure

Identifying scientific images from other types of photography requires the use of industry-accepted criteria. Before proceeding with that analysis, one characteristic fundamental to any picture is whether a photo itself is a good or a bad picture based on its technical merits. While seemingly an easy task to accomplish, there can be contributing factors that come into play when photographing challenging subjects or in difficult situations. Without doubt, many factors influence outcomes and it has become increasingly easy to operate a modern camera in the automatic mode and get an acceptable result. Sometimes it is actually a rather good result. Making a photographic recording of a subject has never been easier.

Criteria Used to Identify Good Photography:

- Proper or correct exposure: there is detail in both the dark and light areas of the subject.
- Effective isolation of the subject: clear and simple framing and magnification.
- Proper selection and use of shutter speed and aperture based on the sample requirements leading to an adequate range of focus and object free of blur.
- Effective use of light and lighting: subject characteristics are made visible.
- Appropriate sample treatment: the image has a neutral perspective.
- Effective use of focus and/or depth of field (DOF).
- Emphasizing details that are in agreement with the scientific intent.

Additional criteria specific to science photographs:

- Use of a standardized treatment
- Proper use of a scale.

There are a number of other factors that separate good photographs from bad ones that go beyond simply the effective operation of the equipment. These will also play a role in scientific imaging outcomes and include:

- the duration of the event and the photographer's ability to synchronize the photography with it;
- the frequency of the event and the photographer's ability to record what is needed to make a useable recording of it;
- access to (the location of) the event.

Making high quality photographs in science can be difficult for these and other reasons. That being said, "making an image is better than making no image." To be successful, science photographers must be able to innovate and control a multitude of variables that affect the outcome.

Figure 1.1 These photographs clearly illustrate why the parts of the imaging system matter and skills can make a difference. Both photographs feature a live *Daphnia magna* (sp). Exposure, sharpness, composition, treatment, and the recording of data are the criteria for a successful photomicrograph. The bottom photograph exhibits proper focus, exposure, isolation, and magnification. The upper photograph is awful.

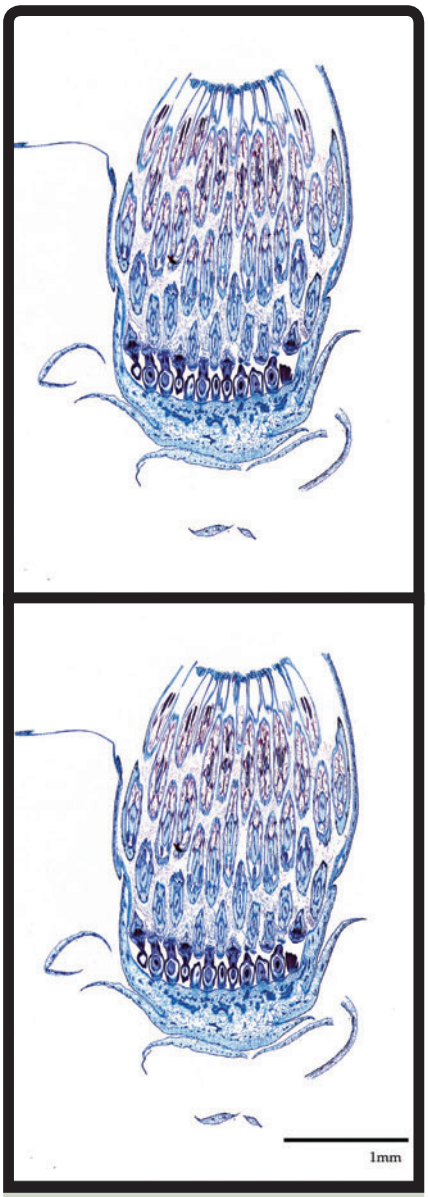


Figure 1.2 These brightfield photomicrographs feature the flower bud of *Taraxacum officinale* (dandelion) shown in a longitudinal section. It is reproduced without a scale (top) and with a scale (bottom). The inclusion of a scale clearly implies a science image. Without the scale, a viewer might see the image's characteristics such as its shape, color, and design before assigning the image a science image.

Science Photographs Require a Scale

Categorizing one thing from another is what people do. People identify things based on experience, criteria, and prior knowledge. On the surface it might be considered easy to identify one thing from another but that is not always the case. Many things do not fit into just one category. Nuances and other factors come into play for identification. Images in particular can operate on many levels and in many environments. Human perception will also play an important role in what is seen, observed, and finally what is believed to have been seen. When a photomicrograph is made, it can operate as a science image or sometimes as art based on a viewer's point of view. Astronomical photographs are similar in that way. Scientific photography might be described differently depending on who is asked for the definition. It might include references to the equipment that was used, such as in photomicrography, or it might be described by the application that was used, such as in radiography. One absolute requirement for the delineation of a science photograph from other types of photography is the need for the inclusion and proper use of a ruler or other type of scale. Whether a ruler is used for a size comparison or a date or timestamp is included, the inclusion of a scale immediately delineates a science image from other types of photography.

Photographer's Intent and Subject Matter

For many, the intent of the photographer might also be used to discriminate scientific photography from other types. Sometimes subtle and not prominent elements play a role in this process. Sometimes the need to identify an image from another type is important while at other times it is not. It is actually quite easy to photograph science subjects in non-scientific ways and it is also possible to evaluate science images—after creation—using a different point of view than when the image was created. The images in Figure 1.3 were produced for very different reasons and at very different times by two different photographers. In both images, the subject might be considered the same. A quick assessment of the images might lead a viewer to conclude they are both medical photographs because of the subject. The image of the *Dissected Head on a Soup Plate* was made by Dr. Howard Brundage in 1905 and was produced as a medical gross specimen photograph during an autopsy at the State Hospital, Columbus, Ohio. The other image, *Head of a Dead Man*, was made in 1990 by Joel Peter Witkin as a fine art photograph. While the content in both images, made decades apart, is similar, the intent of the photographers could not be more different. Consequently a subject by itself cannot be used as the sole criterion for the categorization of an image.

Intent can be at the foundation of legal cases as well. In 1993, I participated as an expert witness in a trial where Dr. William Zink, a Florida pediatric orthopedic surgeon, was charged with four counts of child molestation between 1987 and late 1993. Part of the charges included the taking of

sexually explicit photographs of the children and other male patients. The physician was ultimately acquitted after a long trial. Fundamental to the case was the need to create a legal definition of a clinical medical photograph. The trial focused a lot of attention on "what discriminated medical photographs featuring children that contained nudity from child pornography." It took many long weeks of testimony to create a working legal definition for the jury. In the end, the case ultimately came down to trying to determine the physician's intent. Not specific to this case, it can be difficult if not impossible to measure the intent of an image-maker.

A Picture is Worth a Thousand Words

The need for and the importance of scientific illustrations has a well-documented history. It is easy to find historically important drawings from the seventeenth and eighteenth centuries and sometimes earlier. Leonardo da Vinci, Michelangelo, and early astronomers produced countless drawings of science and in some cases paintings of important discoveries that chronicled—to the best of their abilities and that of the materials of the time—the current scientific frontiers. Initially created as scientific illustrations, these drawings have now become valuable pieces of art over time. Mug shots, early medical photography as well as numerous other types of scientific photographs from the late eighteenth century have become collectibles and treasured items in today's society. While they were originally created to chronicle science explorations and for the documentation of things, in modern times they have taken on different roles.

Figure 1.3 Which is the medical photograph and which is the fine art photograph? Often the boundaries of distinction are not clearly defined. The subject in both photographs is a human head. The top photograph was made by Dr. Howard Brundage in 1905. The lower photograph was made by Joel Peter Witkin in 1990. Upper image, courtesy of Stanley B. Burns and the Burns Archive. Lower image, courtesy of Joel Peter Witkin/ Catherine Edelman Gallery.

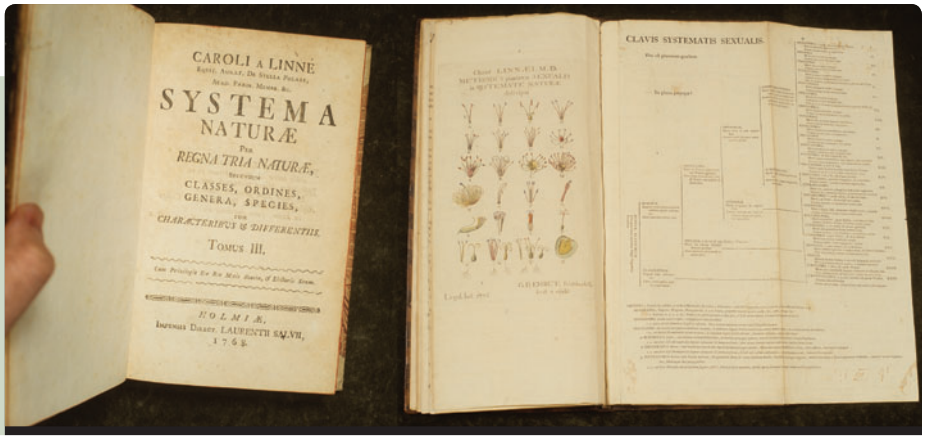


Figure 1.4 This illustration features the title page and page spread from *System Naturae* by Caroli Von Linne', 1768. It is a notable example that shares the importance of scientific methods used for illustration in the pre-photographic period. Image courtesy: Hagströmerbiblioteket, The Hagströmer Medico-Historical Library, Karolinska Institutet.

Figure 1.5 *A View from the Window at Le Gras*, produced by Joseph Nicéphore Niépce, was the first permanent photograph. Image courtesy of Visual Resources and Digital Content Library, College of Liberal Arts Office of Information Technology, University of Minnesota, United States/USA.

Figure 1.6 One of the earliest known solar photomicrographs is this early daguerreotype from 1840. It features a cross-section of clematis stem taken by Andreas Ritter von Ettingshausen. Image courtesy of the Albertina Museum, Vienna, Austria.

The Beginnings of Permanent Photographs and Scientific Photography

The earliest known examples of photographic images using light-sensitive materials were made by Thomas Wedgwood at the turn of the nineteenth century. Wedgwood and Sir Humphry Davy published the results of their experiments in the *Journal of the Royal Institution* in 1802. Wedgwood and Davy made pictures using an emulsion of silver nitrate coated onto leather, and white paper. In the experiments, leaves were placed onto the light-sensitive material, which was then exposed to sunlight and darkened over time. Unfortunately their photographs could not be made permanent. They did learn, though, that by adding sodium chloride to the mixture, they could increase the emulsion's sensitivity to light. This shortened the very long exposure times, but even with faster materials the process was too slow to make images of moving objects. In the end, they were unable to develop a process that would make the images permanent. Their discovery did however serve as the foundation of silver halide photography for the next 150-plus years.

Permanent silver halide images were first achieved in 1826 or 1827 and attributed to Joseph Nicéphore Niépce. His picture entitled *A View from the Window at Le Gras* was the outcome of years of experimentation and used a process that coated bitumen onto a highly polished pewter plate. His associate Louise-Jacques-Mandé Daguerre was also working on a process later named the daguerreotype. Numerous daguerreotypes of scientific work have survived. William Fox Talbot was also a pioneer of a photographic positive/negative processes and he developed the calotype process. Talbot published much of his early work in his book, *Pencil of Nature*.

Photography depicted things with a precision that unskilled artists and drawings could not achieve, and soon after photography's invention scientists, researchers, and physicians began incorporating photography into their work documenting the subjects of their research. From the earliest beginnings, science used more and more photography. The earliest scientist photographers believed that a photomechanical process would be viewed as more credible than drawings as they communicated their ideas to the professional societies of the times.

Anna Atkins' book *Photographs of British Algae: Cyanotype Impressions*, produced in October 1843, is considered the first book to use photographs. Working with Sir John Herschel, she invented the cyanotype process. Blueprints, as they were later called, are still produced for artistic reasons at this time but the slightly different blueprint process used for architectural drawings has been mostly retired. One of her illustrations can be seen in Figure P.3.

As photographic knowledge grew and materials improved, adoption expanded. Photographs allowed for a careful visual inspection of things difficult to observe in real time. Permanent accurate images provided an opportunity for critical review after an event was long over and could be accomplished in a controlled environment. While many events are transitory, photographs provided a new tool for the collection of scientific facts that was semi-permanent. Early adopters of photography could be found in all disciplines of science, research, and medicine.

Carl Curman (1833–1913), a Swedish professor of medicine and also a science photographer, worked at the Karolinska Institutet in Stockholm, Sweden, in the mid-1800s. He was a very early adopter of photography and used it to collect and preserve scientific facts about many of his medical subjects and interests in science. He, like many, realized photographs could preserve scientific facts in an objective manner and record the tiniest of details. It was certainly much faster than trying to draw the same object when trying to achieve this level of detail. In Figure 1.7, it is interesting to see how Curman placed objects against dark or light backgrounds, which influenced the perceptual tones of the object.

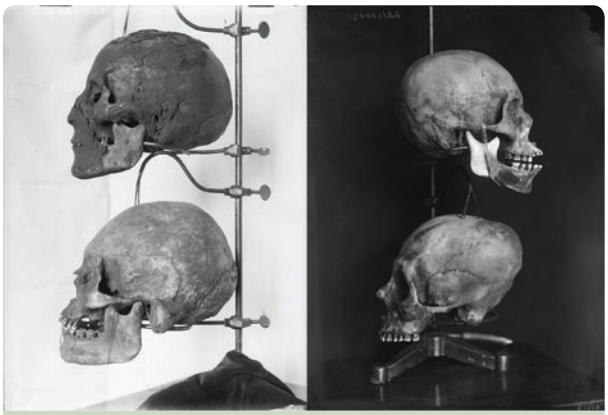


Figure 1.7 Early examples of laboratory photography made by Swedish physician and medical photographer Carl Curman, Karolinska Institutet, circa 1865. Image courtesy of the Archives of Karolinska Institutet.

Making the Invisible Visible

Scales, subject matter, and intent all play a role in discriminating scientific photography from other types, but there are other factors. Creating images that contain empirical facts (data) will always define an image as science (at the time of its making). An important early practitioner of informational photography was Eadweard James Muybridge. Muybridge was hired in 1872 by Leland Stanford to make photographs of a galloping horse in the hopes that the photograph might show all four feet of the animal off the ground at the same time. Muybridge successfully captured this outcome in 1877 by assembling a string of cameras that created consecutive exposures at regular and programmed intervals. In 1879, he synthesized motion of this event and other photographs with his zoopraxiscope, which used drawings from serial photographs. This discovery resulted in the first animated movie. He published eleven volumes of his motion analysis work in 1887, which contained 780 plates of the first serial photographs of humans and animals in motion.

Figure 1.8 *The Horse in Motion* by Eadweard Muybridge, 1877. Frames two and three in the top row show all four of the horse's feet clearly off the ground. Image courtesy of Library of Congress Prints and Photographs Division

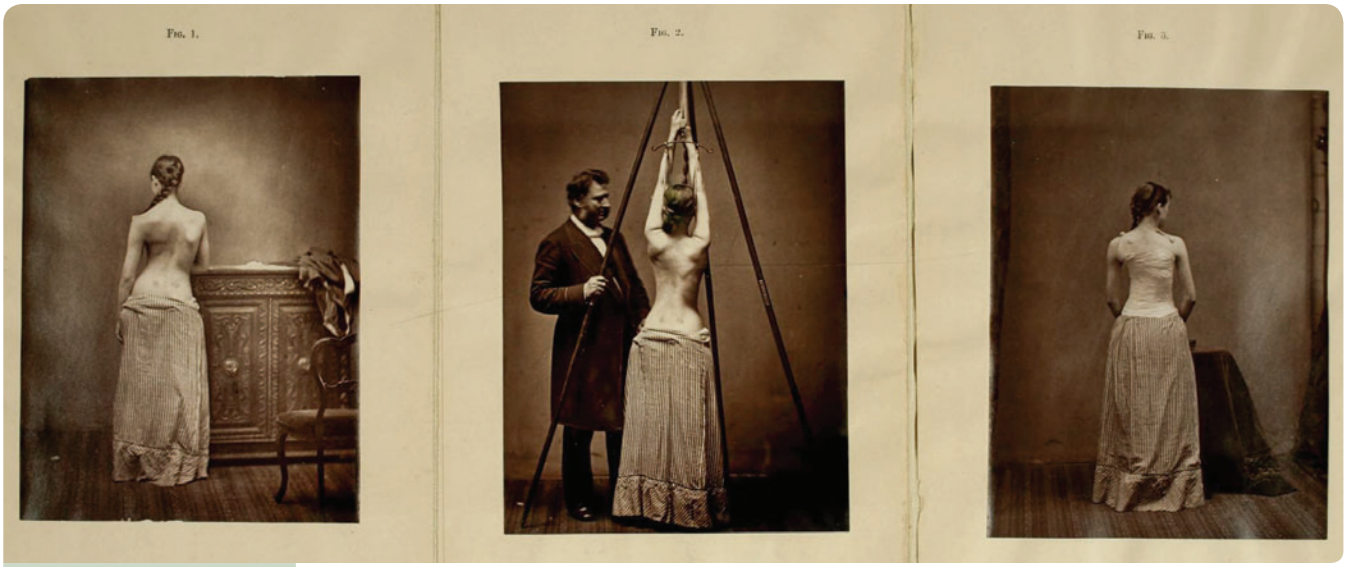


Figure 1.9 This series of photographs features a patient with scoliosis and the related spinal curvature. The middle image demonstrates treatment by suspension and the use of the plaster of Paris bandage developed by Lewis A. Sayre, MD. This work was originally published in London by Smith, Elder & Co., 1877. Reproduction courtesy of the Boston Medical Library in the Francis A. Countway Library of Medicine, Harvard University.

Historical Images and a Contemporary Point of View

Possibly it is now apparent that images can be evaluated using different criteria and that images are not always easy to categorize. It is important when evaluating images from the nineteenth century to use the proper frame of reference for the era. The tools, materials, and skills of scientist photographers were very different and evaluating historical images using a twenty-first-century point of view can lead to inaccurate conclusions. In the series of images shared above, one might conclude that the photographs are representations of early American erotica, but the actual subject of the photographs is a patient with scoliosis. Seeing the furniture and other elements included in the photograph(s) could easily suggest the images to be something else. They have been reproduced in art books as such. The furniture and other elements included in the photographs were necessary to stabilize a person during the photography because of the long exposure times that were dependent on the brightness of the daylight when made.

Standardized Approaches and Repeatability

When making scientific images, the use of scientific methods is a requirement. Using standardized practices leads to the making of high quality and consistent results over time. Defining scientific methods might be easier to suggest for some scientific disciplines but the subjective nature of some photographic practices can make this difficult.

It goes without saying that people will bring individual approaches to problem solving and thinking. Innovation and problem solving as a concept suggests that solutions will be unique and lead to departures from standard protocols. Human thought is full of personal bias and distortions and based on frequently incorrect assumptions. That is what differentiates people from computers. The quality of work produced by a person who works

in science will be heavily influenced by their experiences and biases. Having good analytical skills is crucial for success and is required for systematic methods. This is imperative in the formation of successful and repeatable imaging solutions.

There are fundamental approaches required for all scientific experiments and these play a role in experimental outcomes. Dr. Richard Paul and Dr. Linda Elder suggested in their *Miniature Guide to Scientific Thinking* (Foundation for Scientific Thinking; www.criticalthinking.org, ISBN 0-944583-18-0) that these approaches include:

- Scientific method requires a predetermined purpose for the investigation.
- Scientific method requires appropriate reasoning in the pursuit of resolving a problem or the answering of a question.
- Scientific reasoning is based on predetermined knowledge and assumptions.
- All scientific approaches, no matter how neutral, depend upon the conception that there will be a point of view.
- All analysis of experimentation must be grounded in data, evidence, or information.
- The analysis of experimentation must contain some inferences and interpretation leading to conclusions.

Photographing for science requires the elimination of variables and the creation of repeatable methods. For photography this would include:

- use of the same camera and related sensor as well as settings for photographing the same object;
- use of the same lens;
- use of the same lights and lighting;
- use of the same aperture required for the production of the same image depth of field;
- implementation of a standardized treatment of the object creating a neutral perspective, including where focus is placed, etc.

The only thing that should change should be the time between photographs. Additionally, creating a neutral point of view and treatment of the subject is core to scientific images. This approach leads to the introduction of minimal distortion or embellishment in the result. In science photography, the image needs to be as precise a facsimile of the object that the equipment and operator can create. In science, photographs are facts, not fiction.

Figure 1.10 Subjects can be photographed in many different ways that use both objective and subjective approaches. In these photographs, the author was photographed using non-standardized and standardized processes. The line on the right views shares what is called a Frankfurt plane. Images courtesy of, left to right, (1) Joshua Shagam, (2) John W. Retallack (3, 4, and 5), Clay Patrick McBride.

Father of Standardized Imaging

Many would argue that Alphonse Bertillon (1853–1914), a French police officer who suggested using the anthropological techniques for law enforcement photography, was the father of standardized photographic practices. His work led to the creation of an identification system based on physical measurements of suspects and was called anthropometry. It became the first scientific system used by police to identify criminals, but over time was replaced by fingerprint photography. As part of this initiative, Bertillon became the inventor of the mug shot system still used worldwide to this day. Photographing of criminals began as early as the 1840s, but it was not until 1888 that Bertillon standardized the process, and it remains as valuable today as the day it was proposed.

Figure 1.11 The top photograph was from Alphonse Bertillon's photo album featuring his display, which included his equipment, at the 1893 World's Columbian Exposition in Chicago. The middle photograph is from Bertillon card 20472, produced November 21, 1908, and from the New York City Municipal Archives. The bottom photograph is a Bertillon card featuring Charles Clark who was arrested for burglary, December 2, 1908. All images courtesy of National Library of Medicine.

Figure 1.12 This pair of time-based photographs, sometimes called chronophotography, demonstrate why standardized imaging approaches are valuable. These two photographs were made thirty days apart and feature *Quercus* sp. acorns (Oak). The acorns were cut open to reveal the seed coat, endosperm, and tree embryo. Dehydration, shrinkage, and color changes are evident between the two views.

Innovators and Technological Progress

As photographic materials improved, so did photographic outcomes. Initially emulsions were sensitive only to blue light. These emulsions would be described as orthochromatic or red blind. In the late nineteenth century, technologists and chemists were working to develop silver halide materials that could record more than simply blue light. Herman Vogel is credited with creating the initial breakthroughs to increase color sensitivity across the green and orange colors, and Josef Maria Eder, an Austrian photochemist, teacher, and photographic historian, was also actively working to increase the photographic responses at that time. He made outstanding

Figure 1.13 Josef Maria Eder's portraits were made by Alphonse Bertillon in the late 1800s and presented on a Bertillon card. Bertillon cards were designed to collect data about a subject and were used in his early anthropomorphic studies. Image courtesy of the Albertina Museum, Vienna, Austria.

contributions to photography, photographic chemistry, and the photomechanical reproductive process. In 1879, his thesis on "The Chemical Action of Colored Light" was published. Eder's work was influential in the creation of sensitizing dyes, which enabled emulsions to have sensitivity to green and later red light.

Photographs describe what words cannot. The expression "a picture is worth a thousand words" refers to the notion that complex ideas and subjects can be shared using a single picture. This expression also characterizes the main goal of visualization, a process of making it possible to view significant amounts of data easily using photographs. In an assignment that I give to my students, I ask them to describe the color blue using only words. I actually believe this to be impossible because there are too many variables, but the ensuing conversation serves to share the complexity and variables of this problem in the classroom and reveals the real challenges of communication.

While photography was rapidly improving at the end of the nineteenth century, Santiago Ramón y Cajal (1852–1934), a Spanish pathologist, histologist, neuroscientist, Nobel laureate and medical artist, was hard at work drawing his observations rather than

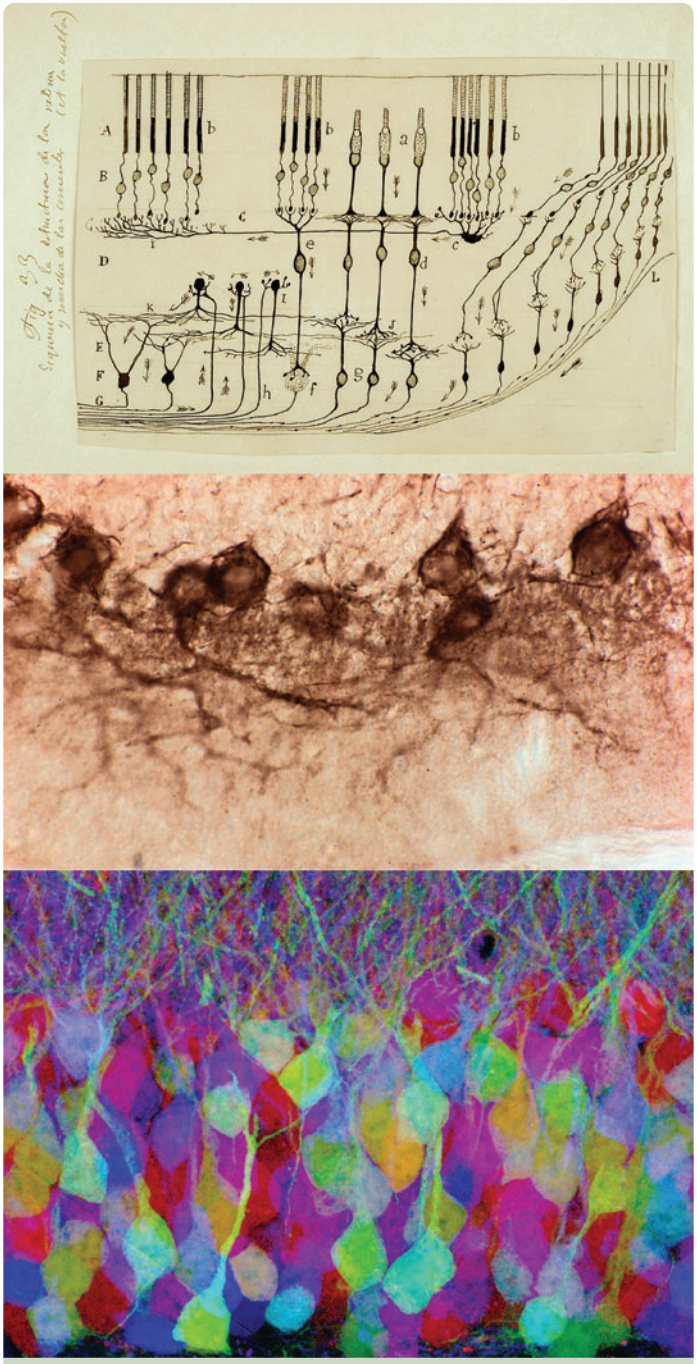


Figure 1.14 The upper photograph is a retinal drawing by Santiago Ramón y Cajal, c. 1900. Image courtesy of the Cajal Legacy, Instituto Cajal, CSIC, Madrid, Spain. The middle photograph is a traditional photomicrograph and reveals neural process bodies in the cerebrum, made by the author. The bottom photograph shares a brainbow, a term used to describe an image where individual neurons of a brain have been stained with fluorescent proteins or markers. These compounds allow the neurons to fluoresce individually based on their specific colors and the excitation energy used. By controlling and varying the amount of red, green, and blue derivatives of green fluorescent protein, it is possible to map each neuron with a distinctive color. The technique was developed in the spring of 2007 by a team led by Jeff W. Lichtman MD, PhD, and Joshua R. Sanes, both professors of Molecular and Cellular Biology at the Harvard Medical School. Image courtesy: Livet, Weissman, Sanes, and Lichtman, Harvard University.

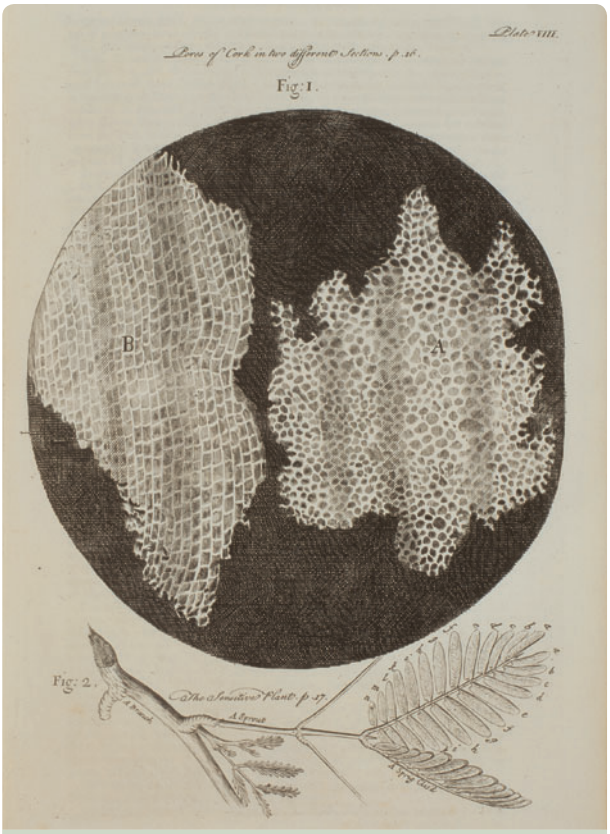


Figure 1.15 This is a drawing of cork from Robert Hooke's *Micrographia*, 1636. Image courtesy: Hagströmerbibliioteket, The Hagströmer Medico-Historical Library. Karolinska Institutet.

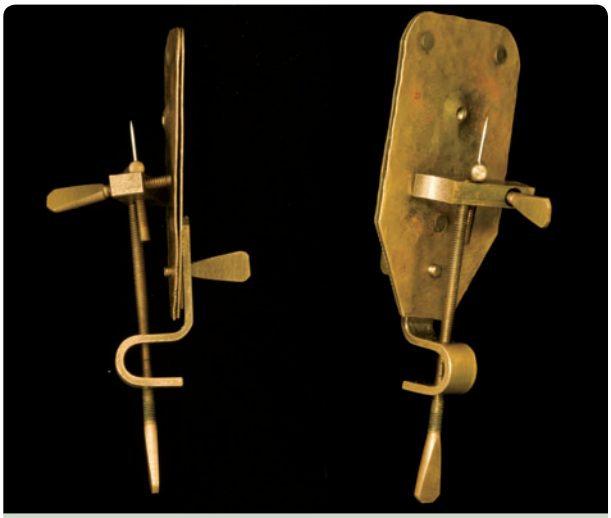


Figure 1.16 This photograph is a replica of Anthony Van Leewenhook's microscope, c. 1673. Leewenhook was an expert lens maker, and by the end of the seventeenth century he was one of the only persons performing microscopic study and discovery. Photograph by Stephanie DeSantis. Image courtesy of Michael R. Peres.

describing or attempting to photograph them. His investigations of the structures of the brain allowed many to consider him the father of modern neuroscience, but his skills as an artist were also recognized. He created hundreds of drawings that illustrated the delicate cells and structures in the brain. The time it took to create these masterpieces was significant. They truly are remarkable in the level of accuracy and detail they achieved. Because there was no simple or practical color photographic process at the time, the color photography that did exist was being produced on a very small scale using primitive materials. This might have influenced his efforts to make color illustrations. Because he frequently used color in his drawings, his work might be viewed as seminal when considering the value of color that is so useful for learning and seeing cells within tissues. One critical characteristic of talented photographic scientists and researchers is their ability to critically observe objects.

Instrumentation

It can be argued that art—because of its interpretative nature—has always been about creating a new voice for ideas or emotions. Scientific photography, on the other hand, is about veracity and telling whole truths. Image integrity is an absolute expectation in science. There are various limitations to the human visual system and imaging systems as well; however, the pursuit of seeing more has been fundamental to discovery. Whether increasing or extending the sensitivity of a sensor, or an instrument's ability to form more resolution, there have always been limitations to what can be accomplished. The need for extending vision has been a constant challenge for photographing the invisible since the invention of photography itself.

Two early and vitally important optical instruments that added to scientific exploration and the renaissance of discovery were the telescope and microscope, both invented in the early 1600s. These instruments predated photography by nearly 200 years. While predating photography by centuries, these instruments contributed significantly to the creation of detailed drawings of observations and new knowledge. Robert Hooke was an early pioneer of applied microscopy and is credited with suggesting the term "cells" used to describe the things he observed under the microscope. He referred to the structures as cells because they reminded him of monk's cells.

Microscopy and Carl Zeiss

Contemporary microscopy remains a powerful tool for research, discovery, and imaging. Carl Zeiss Microscopy remains a world leader in this field after nearly 160 years of a high level of achievement and innovation. Optical Instruments was the name of the first company founded by Carl Zeiss (1816–1888), initially opened as a German lens manufacturer in 1846. Zeiss became a notable lens maker because he was able to produce high quality lenses that formed very large and bright images. Zeiss made contributions to lens manufacturing that aided the production of modern lenses (at the time) and specialized in the production of microscopes that were later adapted for photography. In 1872, physicist Ernst Abbe joined Zeiss; he is well known for work that corrected lenses and produced noticeably improved sharpness and contrast. Abbe made numerous significant contributions to the field, and most notable was his groundbreaking work on diffraction theory and image resolution that still remains relevant even today. At the time of this seminal work, the quality of the glass was not adequately corrected to fully test his hypothesis and Abbe recruited Otto Schott to join the Zeiss team. In 1886, they produced a new type of glass that allowed for Abbe's theories to be properly evaluated. This resulted in a new and very high quality optical glass that transformed glass making. This new discovery made possible the production of a new type of microscope objective, the apochromatic lens, often referred to as an APO in today's jargon.

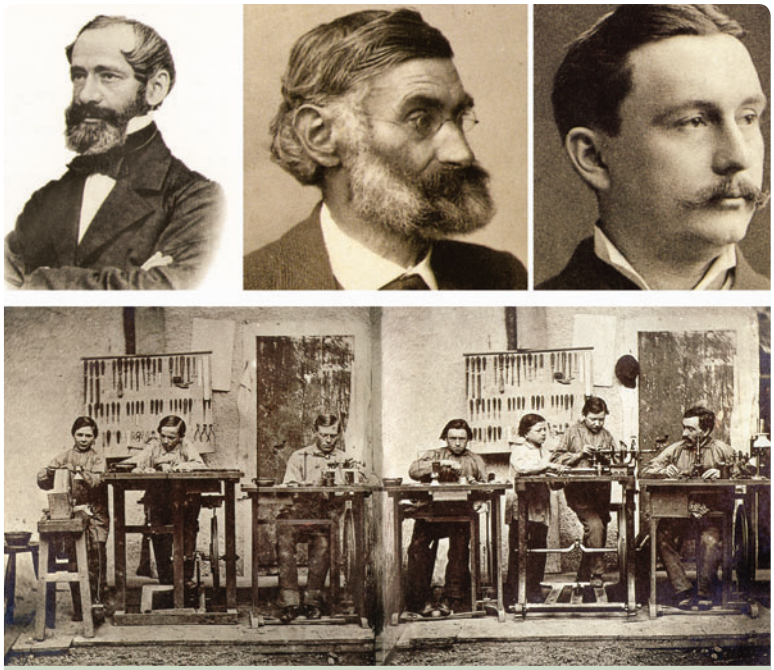


Figure 1.17 Portraits of Carl Zeiss (left), Ernst Abbe (center), and Otto Schott (right). The photographs feature the Zeiss outdoor optical grinding laboratory, c. 1846. Images courtesy of Carl Zeiss Microscopy.

The Invisible Spectrum

Infrared radiation (IR) was discovered in 1800 by astronomer William Herschel. Herschel became aware of this radiation because of the effect the heat had on his thermometer, and the discovery of ultraviolet (UV) radiation was also made through observation one year later. In 1801, German physicist Johann Wilhelm Ritter discovered UV when watching silver salts darken when exposed to sunlight. Ritter's curiosity arose when silver chloride-soaked paper darkened more quickly when in the sunlight than when he used violet light by itself. Ritter called the result "oxidizing rays" and emphasized the chemical reactivity required to distinguish UV from visible radiation. Initially he thought the changes were the result of "heat rays" that had been discovered the year before.

Photographing using the invisible spectrum was nearly impossible at that time because emulsions were incapable of recording near UV or IR radiation. The first infrared photographs were made and published in 1910 and were taken by Robert W. Wood. Wood's photographs were taken using an experimental film using very long exposures. The IR photography was accomplished using plates that contained special emulsions created by Charles Edward Kenneth Mees, who worked at the photographic company of Wratten & Wainwright. The Wratten & Wainwright company was later purchased by Eastman Kodak in 1912, and Mees became the director of the Kodak research labs located in Rochester, New York.

This book will not cover radiography in any meaningful way; however, the discovery of X-ray radiation and its ability to see within an object was significant tool for science and imaging. Wilhelm Conrad Röntgen (1845–1923), a German scientist, produced and detected electromagnetic radiation of short wavelengths and high energy today known as X-rays. At the time of their discovery in 1895, they were called Röntgen rays. Röntgen referred to the radiation as "X" because it was an unknown type of radiation. Röntgen earned the first Nobel Prize in Physics in 1901 for this discovery. Radiography was immediately embraced as a powerful tool for seeing the invisible and the inside of solid materials, and medical images were being produced one year after its discovery. The use of radiography has been an enormous and powerful imaging tool in modern times. Outside of medicine, radiography plays a vital role in learning about objects and their internal structure without performing destructive preparations on objects.

Figure 1.18 The radiograph (left) is the hand of Mrs. Wilhelm Röntgen, captured in the first X-ray image, made in 1895. This image was originally reproduced in *Otto Glasser, Wilhelm Conrad Röntgen and the early history of the Roentgen Rays* (London, 1933). Image courtesy of the History of Medicine Division, National Library of Medicine of the National Institutes of Health. The middle radiograph was made by Josef Maria Eder and Edward Valenta. The X-ray was exposed after improving Röntgen's apparatus. Eder was a pioneer in the field of photochemistry and in the development of specialized photographic films and papers. At the time, these pictures were designed for the general public and were not diagnostic but shared spectacular views into objects normally invisible. Image courtesy of Hagströmerbiblioteket, The Hagströmer Medico-Historical Library, Karolinska Institutet. The photograph on the right was made by author and notable natural science photographer Ted Kinsman, who explores the boundaries of visible and invisible spectrums. By combining X-ray images with visible light photographs. Kinsman's work can facilitate better understanding of structural and external features of subjects. Image courtesy of Ted Kinsman.

Advancements in Film Technology —Kodak, Agfa, Ilford, and Polaroid

Cameras, lenses, radiated energy as well as a user's abilities all played important roles when photographing around 1890. The capability of the recording materials available at the time ultimately determined what photographs could be made and what could be recorded. The Eastman Kodak Company, commonly known as Kodak, might arguably be the most iconic company that made film. There were other giants, including Agfa, Ilford, Fuji, and Polaroid, who also produced important products for science applications.

Kodak opened in 1888 after seven years in business as the Eastman Dry Plate Company. In 1881 the Eastman Dry Plate Company opened for business using methods Eastman pioneered to create "dry" film. Prior to this invention of dry plate technology, photography was accomplished primarily using the collodion process, which used wet emulsions on glass plates. By 1885, Kodak had invented flexible roll film and in 1888 the Eastman Kodak Corporation was founded. Kodak is best known for its photographic film and paper products. During most of its 133-plus year history, Kodak owned most of photographic film and paper sales in the United States and heavily influenced what was possible. Their increasing interests in innovating new and special films and related photographic processes played a profound role in scientific imaging.

As photography became mainstream at the turn of the twentieth century, the invention of new products and services by Kodak was explosive. Owning the US personal photography market as a consequence of the Brownie camera, Kodak aggressively explored the development of products used to push the boundaries of what was possible using silver halide technology. In 1912, Kodak opened the Kodak research labs, organized solely for silver halide research. New discoveries allowed film to be used in more demanding environments, such as in science. Kodak significantly improved the film base materials and gelatin required to suspend the light-sensitive salts. This discovery revealed that gelatin was crucial to silver halide material responses as well as film aging. This became a huge breakthrough and allowed emulsions to be coated onto numerous new materials. Compounded with chemical development, the products could now feature improvement to contrast, resolution, and sensitivity (ASA, light speeds, or now called ISO). Image stability was also greatly improved and would continue for the decades that would follow.

In 1913, the first of many new products came out of the research labs, including a high contrast film and faster X-ray film emulsions. A lot of work was put into the creation of experimental astronomy plates. In 1914, Kodacolor film was released. It was—at the time—a color negative film. Two emulsions were used in this color negative film. One emulsion was sensitive to green light and other to red. These emulsions were later bleached and then dyed in complementary colors.

Eastman Kodak purchased the Fredrick Wratten Company because of its high quality filter line, and the first infrared sensitive emulsions were commercially produced in the 1920s. Spectroscopic plates soon followed. Camouflage detection emulsions were developed in World War I and soon Kodak Aerochrome and Aerographic films were created for World War II. In the 1920s, 35 mm films were created for use in the new Leica miniature cameras and the 1930s brought breakthroughs in optical sensitizing that increased material responses

significantly. Based on many of these innovations, spectroscopic plates for astronomers also improved dramatically and greatly aided discovery in the northern skies. Panchromatic black and white roll films continued to improve, and in 1935 the Kodachrome slide film was invented. The late 1940s saw the invention of Kodacolor negative film. Kodak Tri-X roll film was released November 1954. It was a derivative of Super XX. In 1963, the Lunar BIMAT emulsion was created for NASA and John Glenn's orbit around the earth. Ektachrome was invented in the late 1970s, and E-6 processing was invented to support that emulsion.

Electron microscopy emulsions were released in the 1960s and UV emulsions found their way to the market in the late 1960s. In 1971, Photomicrography Color Film was invented and was followed shortly thereafter by Technical Pan Film, an extremely high resolution emulsion. In 1982, Kodak created tabular grain silver halide films that resulted in the production of a whole new generation of silver halide products at the height of film photography's usage.

In 1986, Kodak scientists created the Kodak M-1 1.4MP CCD sensor, based on the work of Steve Sasson (Figure 1.22). Following that time, many other film products followed, with only minor improvements as the market moved towards a digital world. After going through bankruptcy reorganization in January 2012, the Eastman Kodak silver halide business became part of the newly formed Kodak Alaris Company in the fall of 2013.

While Kodak was predominantly in the US market, Agfa and Ilford were large players in the European markets. Agfa originated from a German company that produced chemicals and dyestuffs in the early 1900s. In the early 1920s, the manufacturing of photographic products became dominant. Agfa developed a grain screen process in 1916 that enabled color transparencies to be produced. Agfa patented a triple-layer reversal color film in 1935 that went into production as Agfacolor. The new reversal film came in 35 mm and 8 mm sizes. A few years later, Agfa introduced a subtractive color negative film. Agfa manufactured and distributed a sizable amount of its photographic films, processing equipment, paper, and chemicals to the minilab/retail, professional, wholesale, and consumer markets. Some of the notable products from Agfa included: Agfa Contour, Agfa DIA COPEX R, radiography films, Agfa Otho 25, Diazo films, PMT processes, and Scientia 10 E.

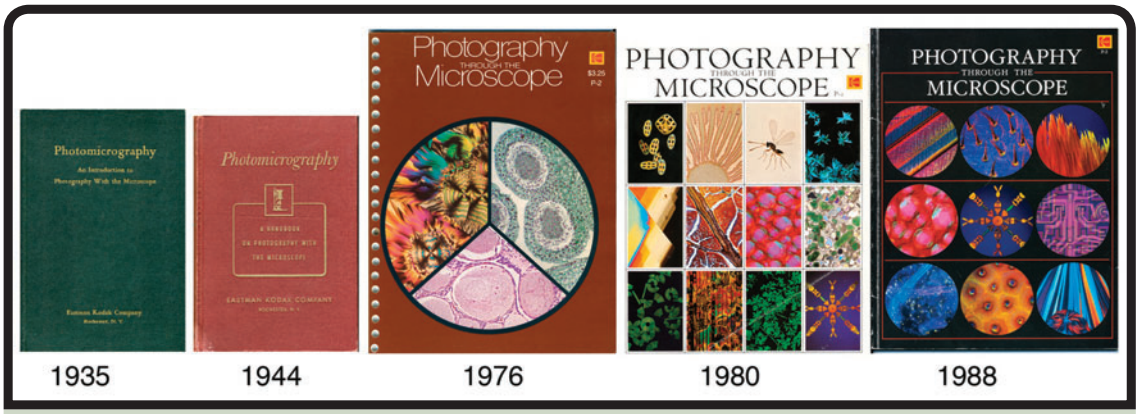


Figure 1.19 The Kodak publication *Photography through the Microscope* was first printed in 1919. Shown here are covers from five editions of this important resource produced by Kodak. Images courtesy of the Eastman Kodak Company.

Short Duration Light, Electric Flash, and Stroboscopes

One of the challenges resident in science photography is the need to create short duration light. In the late 1800s, sparks were used for the sole purpose of stopping the action of an event. In 1917 Etienne Oehmichen invented an electrical stroboscope that was used for examining engines while they were running. By 1935, the Oehmichen system was capturing pictures at a rate of 1100 frames per second. Following the invention of this technology, Augustin and Laurent Seguin discovered that by accumulating electricity using a capacitor they could create a spark with more energy than could be provided from a battery by itself. Until this point, all stroboscopes used a single electrical circuit that ionized the gas, which gave off light when electricity passed through it. Various switches were needed to delivery the electrical energy to the gas-filled tube and the equipment was bulky and hazardous.

The first underwater color photograph, which featured a hogfish, was photographed off the Florida Keys by Dr. William Longley and *National Geographic* staff photographer Charles Martin in 1926 (Figure 1.20). The camera was encased in waterproof housing and pounds of highly explosive magnesium flash powder required for underwater illumination were set off when ready. The flash powder was located on a frame that was placed above the area where the camera was to be located. The explosion of light from the magnesium flash powder allowed Longley and Martin to photograph underwater life for the first time. When ready to photograph, the photographers activated the camera's shutter and triggered the magnesium powder explosion, which illuminated down to 15 feet (4.6 meters). Unfortunately, during this expedition Dr. Longley was burned by the explosion and spent six days in the hospital recuperating following the making of this photograph.

Figure 1.20 This photograph features a hogfish. It was the first underwater color photo ever taken. Image courtesy of *National Geographic*.

Harold Edgerton is considered as one of the most important figures in the history of modern electronic flash lighting. At MIT, although much of the initial research had been started before he was hired, he quickly elevated the research and capabilities of this technology. Short duration flash equipment had proven useful to scientists and the industry by allowing photographing of events that could not be seen any other way. During the 1930s many books were including photographs of short duration events. Kodak improved its emulsion sensitivities to short duration energy and development of color films allowed this work to find new applications. Edgerton's work led to the creation of a flash system that had the same intensity as 40,000 standard 50 W bulbs. This was incredibly powerful at that time.

Figure 1.21 Harold Edgerton was photographed in Cambridge, Massachusetts, in 1986 with his iconic picture of an apple and bullet as the background. Photograph courtesy of @Abe Frajndlich 2011 from the book *Penelope's Hungry Eyes* (New York: Schirmer, 2011).

Figure 1.22 Kodak imaging scientist and engineer Steve Sasson was photographed with his camera, c. 1995. Image courtesy of Eastman Kodak Company.

Most of Edgerton's work was accomplished in a darkened room, which was used to record the effects of the stroboscope using an open shutter. Edgerton is also well known for the development of effective methods for synchronizing short duration events for the timing with flash discharges.

Modern Technologies—Digital and Electronic Photography

In 1975, the first digital camera was invented by Kodak imaging scientist Steve Sasson (Figure 1.22). Sasson worked as an engineer in the Kodak research labs. His invention used a charged couple device (CCD) as the sensor. The non-commercial camera was very heavy and cumbersome. It wrote data files to cassette tapes. The sensor had 10,000 pixels and created a grayscale image. The image took twenty-three seconds to be recorded following the exposure. Much like the first image made in 1826 by Joseph Nicéphore Niépce, this invention would soon transform the photographic world. The first digital camera that created images directly as a digital file was the prototype Fuji DS-1P camera. In 1988, this camera recorded directly to a 16 MB Toshiba internal memory card that required a battery to preserve the data in its memory. The first digital camera to be commercially available was the Dycam Model 1, also available as Logitech Fotoman in 1990. Both models used a CCD sensor, stored the digitals on internal memory and connected directly to a computer for downloading the files.

Electronic pictures were actually pioneered by NASA ten years before Sasson and the Kodak invention. A frame grabber was used to capture one field of video in those early systems. Mariner 4 flew past Mars in July 1965 and used an imaging system that utilized a video camera equipped with a tube. The image was moved from the tube and was sent to a digitizing board. The camera was designed and built by imaging scientists at the NASA Jet Propulsion Lab. The digital image was written to a four-track tape and transmitted to earth using a slow but effective radio communication technology. The spacecraft took eight months to get to Mars. The imaging system produced twenty-two photographs and it required four days to transmit the files back to Houston.

Scanning Electron Microscopy

Manfred von Ardenne invented the scanning electron microscope in 1937. It achieved high magnifications by scanning with a small raster setting coupled with a finely focused electron beam. The first commercial instrument was manufactured in 1965 and was sold by the Cambridge Scientific Instrument Company under the name Stereoscan. Today, SEM technology is used exhaustively in the material sciences and biological sciences environments.

The Confocal Microscope

The development of confocal methods was motivated by the need to see and photograph biological samples located in living tissue with complete focus top to bottom. Marvin Minsky is credited with producing the first working confocal microscope in 1955; he patented the idea in 1957. His methods are now used on all modern confocal microscopes. Minsky's configuration used a pinhole in front of the imaging system that subtracted all of the out-of-focus image. To create an image using a confocal microscope, the focused spot of light must be moved across a sample. In the original Minsky instrument, the specimen was moved on a vibrating stage and not the illumination. In modern systems, the illumination beam is moved using mirrors and the object stage is fixed.

Duality of Images

Images of science often create conflicts for viewers because scientists are taught that science should be factual and should not have an emotive component. Truthfully I find that many science images do inspire, amaze, and still share data. Some science students are taught to look at science in a certain way and to remain objective. This can lead to confusion about how to simply enjoy a science image.

Corey Keller in his essay "Sight Unseen Picturing the Invisible" (published in Corey Keller (ed.) *Brought to Light, Photography and the Invisible 1840–1900*, Cambridge, MA: Yale University Press, 2008) shared that nineteenth-century popularizers of science did not see the lay response of wonderment and pleasure as inappropriate but rather counted on this reaction to encourage scientific interest from the general public. His research suggests that in the early nineteenth century in Britain, the use of pictures in science education was a hot topic of debate.

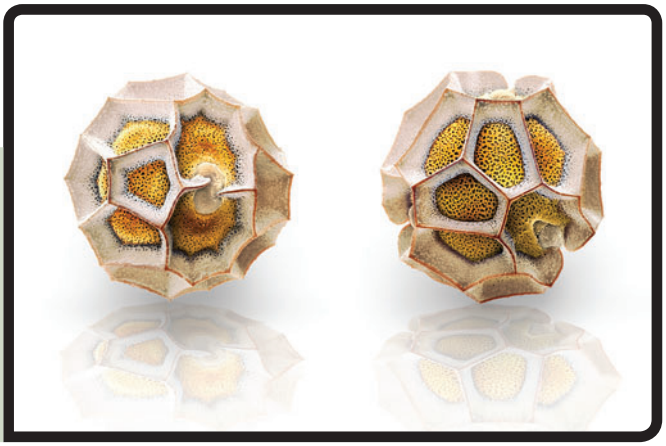


Figure 1.23 This scanning electron micrograph features pollen from the Treasure Flower, *Gazania sp*. It had a diameter of 31 µm. Pollen grains are remarkably diverse, and many are stunningly beautiful and have sizes that vary from 10 to 250 µm. This image was created by Martin Oeggerli, a noted scientist photographer working in Switzerland, with assistance from H. Halbrittner and R. Buchner, University of Vienna. Image courtesy of Martin Oeggerli/ Micronaut; http://www.micronaut.ch.

Anne Secord in her article "Botany on a Plate: Pleasure and the Power of Pictures in Promoting Early Nineteenth-Century Scientific Knowledge" (http://artplantaetoday.com/2010/04/01/botanical-illustrations-promote-scientific-knowledge/) evaluated this phenomenon when she looked at how botanists used illustrations in the late 1880s. These botanists recognized the importance of allowing viewers to experience pleasure while imparting scientific knowledge. Secord's research brought attention to "the role of pleasure in intellectual pursuits." Nothing has changed in more than 130 years and images still create a sense of wonderment and inspiration for many.

Science Images as Art

This essay was originally published in *The Focal Encyclopedia of Photography*, fourth edition, in 2007, and was written by Michael R. Peres and David Malin. It has been adapted for inclusion in this book.

Since its invention, photography has been recognized as both an art and a science, linked by the technology through which its images are captured and then preserved. It is natural that aspects of these three components, in varying degrees, would be evident in all photographs. The extent to which art, science, or technology dominates the photographic expression is in the hands and the imaginative eye and mind of the practitioner. The importance of the motives in photography is as relevant in the digital age as it was for Daguerre and Fox Talbot, both of whom made some of the first photographs of scientific subjects.

Once its potential was realized, the intent of scientific photography has always been to make images without the photographer's personal biases being unduly evident. More on this topic can be read in Chapter 12. However, true objectivity is not possible, since someone has to press the shutter, light the subject, and frame the scene. In addition, the myriad of considerations necessary to convert a 3D world into a two-dimensional image are influenced by the photographer or imposed by the technology. So while the intent may be complete objectivity, subjective influences inevitably find their way into an image.

Most scientific photography is done with visible light and traditional cameras, but may also record invisible objects with dimensions of atomic or cosmic proportions, exploiting almost any region of the electromagnetic spectrum in ways that are unconventional or highly specialized, such as holography or electron microscopy. Scientific imaging also embraces the representation of scientific data that has no visual counterpart, such as a radiograph, or that is purely numerical, such as a fractal. Many of the subjects are recorded specifically because they have not been observed before, cannot be observed directly, or simply because an image is the most convenient way to capture a rich stream of data, as evidenced in an outward-looking astronomical telescope or downward-gazing earth-orbiting satellite. Consequently, a frame of reference is often absent from many science pictures. When images are presented without scale, title, or context, they may appear as abstract and beautiful images to the uninformed viewer.

It is clear that scientific photography offers a vast opportunity for anyone with a creative eye, although many of its practitioners would not consider themselves artists. Indeed, many would not admit to being photographers in any conventional sense either. Nonetheless,

it is hardly surprising that images made for science can be aesthetically pleasing or even inspirational, since they often reflect aspects of the world of nature, of science, and of technology that are not easily observed by the masses. Sometimes this world is inaccessible, unseen, or non-visible, yet can produce images that are mysterious, revealing, provocative, or inspirational to the science community and beyond.

Much of this was foreseen by the French astronomer Arago, who introduced Daguerre's revolutionary invention to the French government in July 1839, with the intention of making the details public in return for a generous life pension for Daguerre. The full text is in Eder's *History of Photography*. It was clear that Arago saw the new process as useful in archeology, astronomy and lunar photography, photometry, microscopy, meteorology, physiology, and medicine, while noting "its usefulness in the arts." Thus from the beginning of photography its value in the sciences was recognized.

The objective of preserving scientific data through permanent images was a key motivation before photography itself was invented. The idea of recording the outlines of leaves and insect wings using light alone was suggested 1802 by the photographic pioneer Thomas Wedgwood. This became a practical reality with Fox Talbot's calotype salt-paper prints and through John Herschel's cyanotype (blueprint) process, invented in 1842. A year later this led to the first book to be illustrated with photographs, Anna Atkins' *British Algae: Cyanotype Impressions*, shared in the Preface.

Atkins' book contained over 400 cyanotypes and appeared a year before Fox Talbot's much better known *Pencil of Nature*. In the preface to her book Atkins wrote,

> The difficulty of making accurate drawings of objects as minute as many of the Algae and Confervae has induced me to avail myself of Sir John Herschel's beautiful process of Cyanotype, to obtain impressions of the plants themselves, which I have much pleasure in offering to my botanical friends.

Despite its prosaic title and unusual subject matter, it contains images of science that are delicate and often quite beautiful, revealing the variety, transparency, and detail of natural forms in a way that no drawing can. One of these beautiful photographs can be seen in Figure P.3.

Atkins' skillful work showed that photographs had the potential to replace the pencil drawings often used for botanical specimens and to provide a new and visually compelling means of expression. It had also convinced some people, uninterested in algae, science, or even photography itself, that the forms and textures captured by this new process could be intriguing or even beautiful. It is in these ways, through inspiration, insight, and expression that images of science may also occasionally, by chance or design, be works of art. It is a rather small departure from this to deliberately make scientific images that are intended to be aesthetically pleasing but that almost incidentally include scientific subjects and use scientific equipment, ideas, or techniques. There were other early practitioners of science photography whose work was groundbreaking in both its photographic results as well as its aesthetic qualities. In her chapter on "The Search for Pattern" in *Beauty of Another Order*, Ann Thomas writes,

> Mid-nineteenth century art critic Francis Wey (1812–1982) while puzzling over whether photography was an art or science, decided ... it was a kind of hyphen

between the two. In fact art-science was the term nineteenth-century astronomer Thomas W. Burr used to describe the recording of magnetic and meteorological data in 1865.

There were many pioneers dedicated to using photography as a means of scientific enlightenment, and initially most were British or European, though the work of New Yorker John William Draper caught the eye of another pioneer, the distinguished astronomer Sir John Herschel. Commenting on *Draper's Experimental Spectrum*, a daguerreotype made in 1842, he refers to "the beauty of the specimen itself as a joint work of art and nature." Later, and exploiting an entirely different property of photography, Thomas Eakins, Eadweard Muybridge, Etienne-Jules Marey, and Harold Edgerton at various times showed how it can be used to stop motion with arresting images. As photographic technology improved, we find photography firmly allied to the microscope to explore the hidden beauty of the very small or to the astronomical telescope to reveal unseen cosmic landscapes.

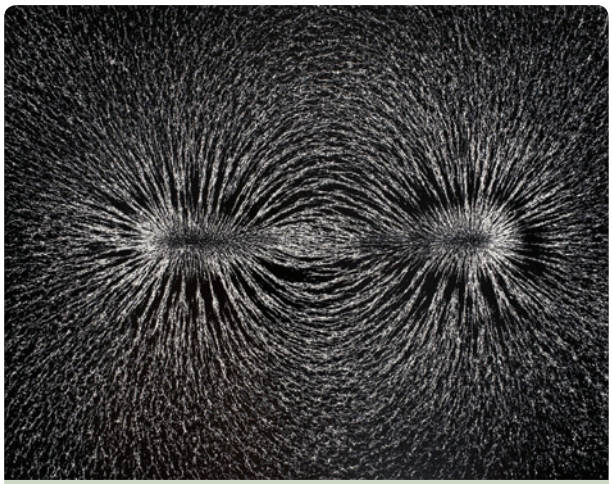


Figure 1.24 This photograph, entitled *Magnetic Field*, was made by Berenice Abbott during a two-year period while she was employed at MIT in the late 1950s. Abbott created photographs that memorably documented the principles of physical science—mechanics, electromagnetism, and waves where she developed innovative techniques for capturing scientific phenomena. Image courtesy of David Winton Bell Gallery, Brown University.

Many of these early practitioners were scientists who turned to photography to add to their understanding. More unusual was the photographer who turned to science for inspiration. A great example of this would be the pioneering photographer Berenice Abbott, who made her reputation with her monumental Federal Art Project documentation *Changing New York* (1935–1939). Berenice Abbott proposed a new role for herself as science photographer, but she found little encouragement for her interest. In later life she turned her considerable talents to capturing scientific ideas in images, and wrote that photography was "the medium pre-eminently qualified to unite art with science. Photography was born in the years which ushered in the scientific age, an offspring of both science and art."

The Abbott photograph of tiny metal shards oriented and energized by magnetic fields demonstrates her interests in photography and science. While many of her scientific images are based on an extended exposure with strobe lights flashing on an object as it moves through space and time, this photograph catches a single moment. Even though the image depicts a static point in time, the curving trails of the metal arranged by the magnetic pull suggest a powerful sense of movement. By pinpointing naturally occurring geometric patterns and rhythms created by scientific processes, Abbott demonstrated her understanding of the aesthetic value of composition and created photographs rich with information.

When we look at scientific images taken 150 or more years ago, many now seem to be minor works of art, partly because of their rarity, but also because many of those who embraced photography in its early days had some artistic training or temperament. Many of the early processes also had a delicacy of tone or color that lends a grace and style rarely seen today; however, few of the early science pictures that we now see as artistic either sought or received the attention that we accord them today. They were exchanged between friends

or colleagues, shown at the meetings of the learned societies of the day, and sometimes exhibited as examples of the art of photography. The world's first photographic exhibition was held in Birmingham, England, in 1839. It consisted of fifty-six photographs by Fox Talbot, of which half were pictures of grasses, seeds, ferns, and other botanical specimens. Many of these "photogenic drawings" have not survived, but by description they are clearly images of science.

Not all practitioners had Fox Talbot's eye for composition, and as photography became more specialized and complex, pictures for scientific purposes were often made without aesthetic considerations. However, as photography became more widely available in the 1880s, largely through the efforts of George Eastman, and more widely published through magazines such as the *National Geographic*, specialists in geography and geology, botany and anthropology, to name but a few, soon found their images were more desirable for publication if they were also good to look at. Thus scientific photography overlapped with photojournalism in that regard. Photojournalism might have reached its zenith in the 1960s, and interest in scientific images as visually interesting artifacts similarly increased.

As Ann Thomas writes in *Beauty of Another Order*, "scientific photography is a subject long overdue scholarly attention and several publications and exhibitions have paved the way for more comprehensive treatment." Among those she lists are *Once Visible* at the Museum of Modern Art, New York, in 1967; *Beyond Vision* at the Science Museum, London, in 1984; and *Images d'un Autre Monde* at the Centre National de Photographie, Paris, in 1991, as well as several other important exhibitions worldwide.

Contemporary scientific photography as art has become very popular. A notable activity designed to promote an aspect of scientific photography as art is the Nikon Small World photomicrography competition, which started in 1974. The exhibition celebrated its fortieth anniversary in October 2014. The winning entries are exhibited widely throughout North America and also included in a printed calendar. The Japanese Society of Scientific Photography has also had an annual exhibition since 1979, and a similar but more recent series of Visions of Science competitions and associated exhibitions are held annually in the UK.

Figure 1.25 Astronomy photographs are nothing short of spectacular. The Hubble Telescope produces remarkable photographs, but so do many ground-based telescopes. Noted astronomer photographer and co-author of this entry David Malin made this photograph of the Horsehead Nebula in Orion, revealing wisps of dust and gas that make up the iconic Horsehead Nebula that lies 1600 light years away. Malin became well known for his methods, which included the making of three photographs using red, green and blue filters on black and white photographic plate emulsions. Image courtesy of David Malin.

Nowhere has the inspirational nature of the scientific image penetrated further into the public consciousness than in astronomy. Since the early 1980s, true-color photographs of distant stars and galaxies from ground-based telescopes have become commonplace.

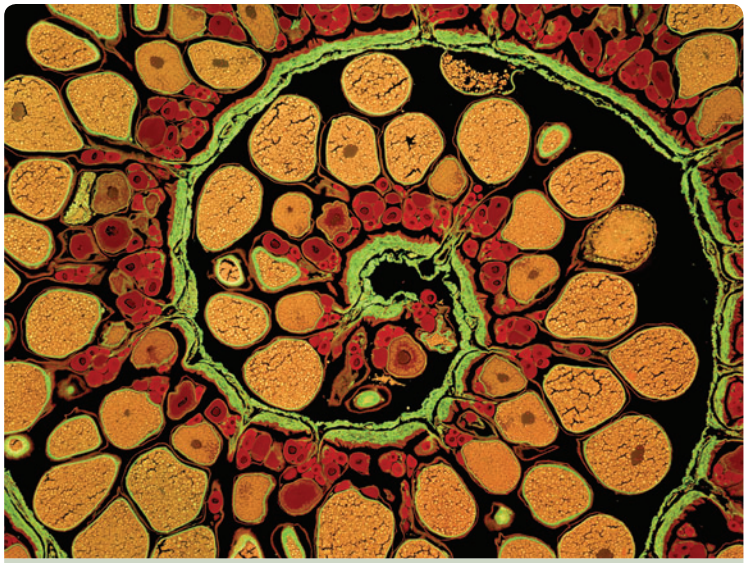


Figure 1.26 This fluorescence photomicrograph by James Hayden reveals the swirling developing oocytes or unfertilized eggs and was awarded the fourth prize in the 2009 Nikon Small World Competition. The magnification at camera was x4. Image courtesy of James Hayden.

Now, several decades later, the stream of stunning images and groundbreaking science from the Hubble Space Telescope have transformed our view of the universe both scientifically and aesthetically. In addition, several generations of probes and satellites have visited the outer solar system. Not surprisingly, the most photogenic of the planets, the ringed world Saturn, provides the most remarkable and haunting images from the joint USA–European Cassini spacecraft. The beauty of these pictures is no accident; space agencies long ago realized fine images and frontline science were both perfectly compatible and complementary.

At the other end of the scale, optical and electron microscopes are also capable of producing striking images, sometimes in the cause of science and sometimes for art's sake. The scanning electron microscope is especially adept at this since its magnification can be so high yet create images of great depth.

A contemporary example of photographers working on the frontiers of art and science is provided by German photographers Oliver Meckes and Nicole Ottawa, whose work is compelling, powerful, and fascinating. One of their photographs can be seen on the title page of this chapter. Their photographs portray their subjects with scientific accuracy and visual elegance. Martin Oeggerli is also a noted scientist photographer working in Switzerland whose work transcends science and who is considered by many an artist. His photographs have been featured in *National Geographic* and reproduced in other internationally important publications. And there are others in this era including Norman Barker, Kenneth Libbrecht PhD, Lennart Nilsson, Viktor Sykora, Charles Krebs, Dr. David Teplica, and Felice Frankel, to name a very few.

More than 187 years after the first science pictures were created, there is still an ambiguity associated with this type of photography. Just as the intent of the photographer decides what category of image will be made, so it is the mindset of the beholder that decides if it is art or science.

SUGGESTED READING

Barker, Norman, and Christine Iacobuzio-Donahue, *Hidden Beauty: Exploring the Aesthetics of Medical Science*. Atglen, PA: Schiffer Publishing, 2013; ISBN-10: 0764344129.

Collins, Douglas, *The Story of Kodak*. New York: Harry N. Abrams, 1990; ISBN-10: 0810912228.

Davidhazy, Andrew, and Michael R. Peres (eds), *Images from Science 2: An Exhibition of Scientific Photography*. Vancouver: RIT Cary Graphic Arts Press, 2008; ISBN-13: 978-1-933360-34-8.

Foster, Giraud, and Norman Barker, *Ancient Microworlds*. No location: Custom and Limited Editions, 2000; ISBN-10: 1881529657.

Gaede, Peter-Matthias, Oliver Meckes, and Nicole Ottawa, *Die fantastische Welt des Unsichtbaren: Entdeckungen im Mikrokosmos*. Hamburg: Gruner und Jahr, 2002; ISBN-10: 3570193721.

Keller, Corey, *Brought to Light: Photography and the Invisible, 1840–1900*. New Haven: Yale University Press in association with the San Francisco Museum of Modern Art, 2008; ISBN-13: 9780300142105.

Malin, David, *Heaven and Earth: Unseen by the Naked Eye*. London: Phaidon Press, 2004; ISBN-13: 978-0714844398.

Moller, Lennart (ed.), *Images in Science*. Stockholm: Karolinska University Press, 2003; ASIN: B004C6J6WQ.

Nilsson, Lennart, *A Child is Born*. Crystal Lake, IL: Delta, 2004; ISBN-10: 0385337558.

Oeggerli, Martin, *Micronavigating between Science and Art*. London: Blurb, 2008.

Peres, Michael R. (ed.), *The Focal Encyclopedia of Photography, Digital Imaging, Theory and Applications, History, and Science*, fourth edition. New York: Focal Press, 2007; ISBN-13: 978-0240807409.

Thomas, Ann, *Beauty of Another Order: Photography in Science*. New Haven: Yale University Press, 1997; ISBN-10: 0300073402.

http://www.nikonsmallworld.com

http://undsci.berkeley.edu/index.php

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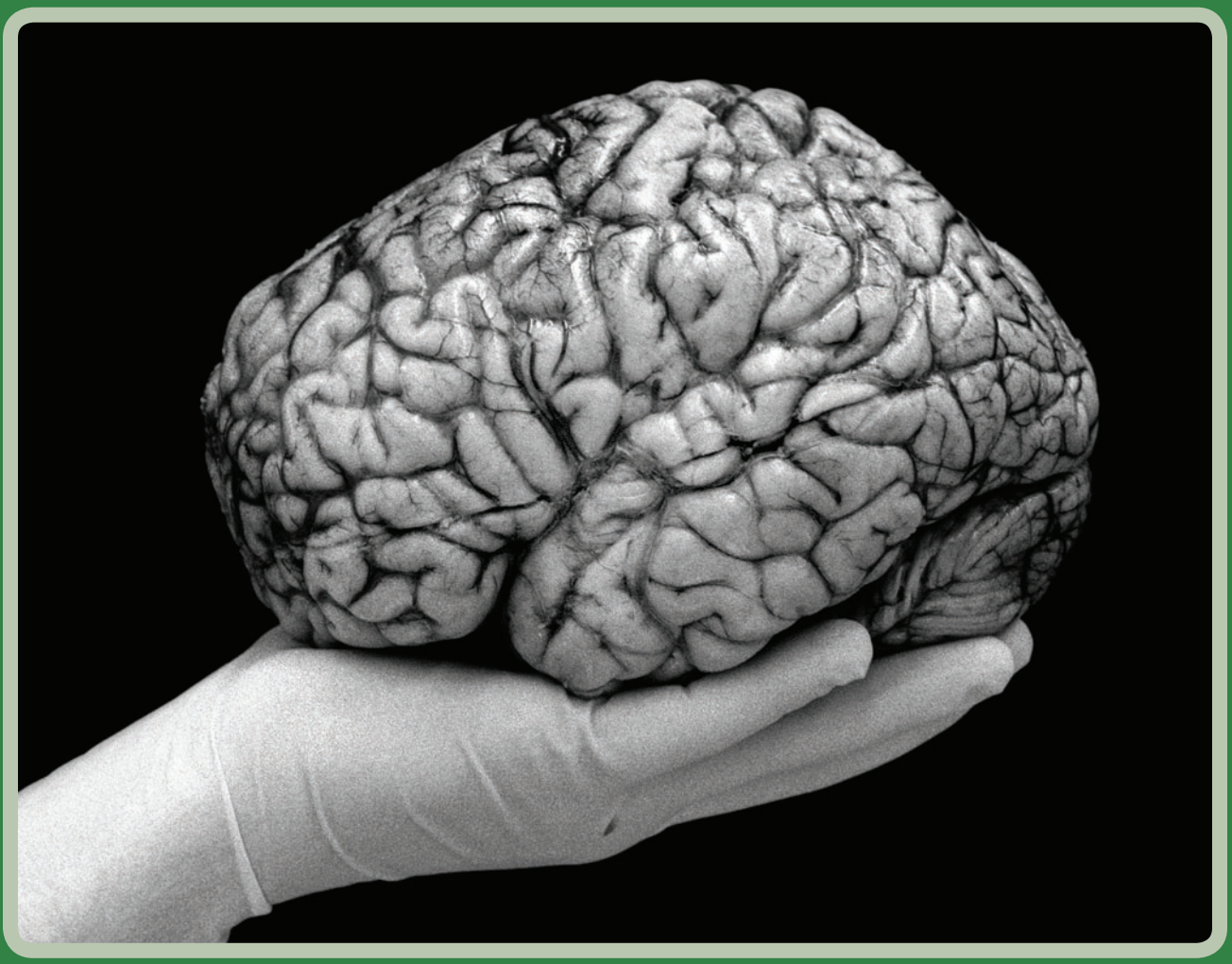
Chapter 2 Human Vision and Perception



This photograph features an adult human brain removed during autopsy. The photograph was made in 2000 for illustrative purposes in the hospital mortuary. Image courtesy of Amanda Rebbechi, Prince of Wales Hospital, Sydney, Australia.

Sometimes overlooked in conversations about imaging is the influence that a human operator plays in the process and ultimately in an imaging outcome. While not a part of the tools, hardware, or software components of an imaging system, the human observer must achieve distinct vision and develop a high skill level in observation that will influence outcomes from start to finish. If an operator does not have good visual acuity, it may not be possible to make good images, ever. At certain times when an operator is fatigued, it is a chore to create effective focus, and in specific situations operators might see mirages or illusions. While not in the desert, mirages/illusions are the result of the human visual system forming "visual" information based on physiology or perception. These outcomes are consequences of the components of the human system including the eye, the sample, how radiated energy behaves in a specific environment, or interpretation of what is seen.

Photographing in a science lab can be a situation that is simply challenging to work in. This may be caused because a camera's viewfinder is very bright or very dim in an imaging laboratory that has a different brightness or when evaluating an image displayed on a poorly profiled monitor that is used in a very bright room. Being aware of an operator's biases—as well as features of the environment where the imaging is being performed—will influence what is possible. Physiologically, the human vision system works in very predictable ways; however, the perceptual process influencing "what-was-believed-to-have-been-seen" is less predictable. Perception is a fundamental component to human vision.

The Imaging Room

The room where imaging is performed is the first place where an operator can affect outcomes based on seeing. A room without windows—or at the least a room where the ambient light can be controlled and/or extinguished—is ideal. Room-darkening shades will provide effective control of ambient light and leading to better visibility. Unwanted ambient light will negatively influence vision by lowering image contrast or brightness or both. Contrast is necessary to see and differentiates structures from one another. In a bright room, a pupil will have a different diameter than in a dimly lit room. When the pupil is enlarged or small, some structures within an object may be less visible than when the ambient brightness is less than ideal. Typically a sample's dark or light tones will become less visible when the pupil adjusts to the brightness or contrast differences as influenced by the ambient light. The inability to see the entire range of brightness at one moment is defined as the dynamic range of the eye. Sensors also have a dynamic range. When a computer screen is near to an ambient light source, the pupil will be conflicted about which light source is dominant. A slight change to the viewing angle to the monitor may cause the pupil to change size and influence what is observed. Monitors also will have numerous surfaces for contrast and reflection control. Controlling "secondary" light sources will lead to better visual acuity. Being aware of light and dark adaptation is enormously important for the fine-tuning of an imaging system.

An "imaging" room should have walls that are painted in neutral colors. Having strong colors in an imaging room will contribute to color adjacency effects, human eye color fatigue, and poor accuracy, and these influences can be evidenced in Figure 2.5. Light and dark adaptation as well as persistence of vision will also play a role in how the room

should be designed. More will be shared about human perception later in this chapter. Human vision is very responsive but has limitations, which can easily be amplified by environmental factors. Subject size, subject contrast, and experiment location will play a role in the operator's ability to see an object or image in proper or improper viewing conditions. Developing—and optimizing for—distinct vision is an acquired skill.

Seeing

> You can't depend on your eyes
> when your imagination is out of focus.
> (Mark Twain)

Humans are very visual and take in enormous amounts of information using their eyes every moment of every day. Seeing has the obvious physiological component but there is also an interpretative component. Vision scientists share that because a person references what is known and familiar, human vision/seeing has developed to its current capabilities over millions of years of evolution. All organisms have different levels of vision depending on the environments where they live and what they eat.

Scientific images in particular may have no frame of reference necessary to understand what is being looked at. The scientist photographer in particular brings unique memories/experiences that are based on personal experiences to many interpretative events in the laboratory. These experiences are full of personal biases and play a role in critical vision and understanding of what is being presented by the process and/or outcome. When an "image maker" becomes more experienced, expectations for quality and what is possible will increase. A frame of reference becomes necessary for understanding and is required for a science photographer to create and trust methods. In science these approaches are imperative for the belief that the results are true and demonstrate integrity. More can be read about the integrity of the process in Chapters 12 and 15.

Basic Structures in the Human Visual System

Photographing in a laboratory environment or when using a photomicroscope, for example, presents many unique seeing and imaging challenges that are found in few other applications. When trying to photograph in challenging environments, there are a myriad conditions that are often present. The environment may be too bright for the observation of dimly lit objects, for example. How the iris responds to a monitor or other inherent ambient light influences will make seeing some things difficult. Objects may be transparent or nearly invisible or the images they produce may be intermittent. Some images may be aerial or poorly defined. Having a working and practical knowledge of how the eye operates can be an important tool when working in less than ideal conditions with complicated imaging problems or when using difficult samples. Learning how to be a critical observer can assist in creating better imaging outcomes.

The human visual system begins with the eye. The eye is a sensory organ and part of the central nervous system. The eye contains numerous components—the lens and a cornea,

to start. Both play a role in the refraction of light that leads to the sharp focusing of a scene onto the retina. The retina is the structure responsible for the detection of light and has pathways for delivering the decoded light information as electric signals for interpretation to the brain. This transmitted electrical information becomes visualized as mental images in the brain, contributing to the formation of the perception of what has been seen or observed. This outcome leads to understanding and assessment.

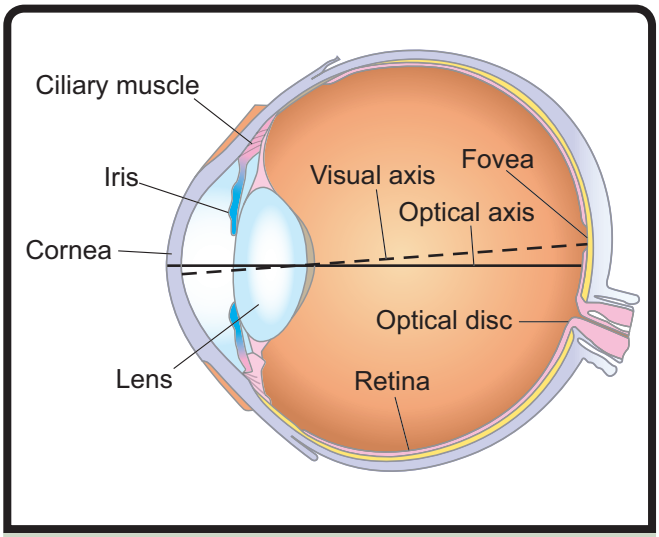


Figure 2.1 This basic drawing of the eye shares all internal parts and nerve pathways.

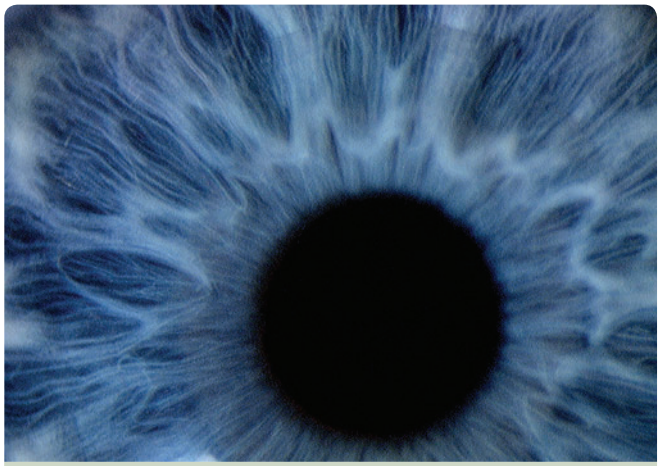


Figure 2.2 A human iris photograph. Image courtesy of Kevin Langton.

The human eye is a circular structure located within the bony orbit of the skull. The eye is held in place by muscles, glands, and fat. The eye muscles move the two eyes synchronously, which allows the eye to capture the most visual information possible during both still and moving events. Eye movements are controlled by the rectus muscles. Eye movements will be either involuntary reflexes or voluntary actions controlled by the brain. Light will enter the eye through the cornea. The cornea is clear and refracts the light into the anterior chamber of the eye where the lens is located. The cornea provides the first and largest amount of refraction necessary for image focusing. Because the eye is soft, its shape can be changed when pressure is applied. Rubbing tired eyes will have an effect on critical focusing and visual acuity. Contained within the globe is a jelly-like substance called the vitreous.

The brightness of the light that enters the eye is controlled by the diameter of the iris or pupil. The human iris is made of various types of cells including colored epithelial or modified skin cells. The iris is mostly opaque to light but can become thin with age and may allow stray light to enter the eye. When the iris thins and allows stray light to enter the eye, this lowers image contrast. The iris diameter is established by the brightness of the environment. It changes size when the light intensity changes or the distance to an object of interest changes, when a person is in pain, or when a person's emotional status changes.

Light will next pass through the lens, located behind the iris, and into the vitreous. The vitreous is a refractive jelly-like substance. The vitreous can have irregular densities within it. These abnormalities are called floaters and are a condition called myodesopsia. It is believed that as a person ages they may develop more floaters, although there can be optical density differences in the vitreous in a person beginning at a young age. Floaters may change over time as a person ages and other physiological changes affect the eye. When using a microscope in particular, floaters can become highly visible when diffraction is created from the microscope's aperture diaphragm.

The human lens is small, approximately 1 cm in diameter. It is composed of various layers of modified epithelial cells that vary in refractive index; refractive index will be discussed in Chapter 3. Refraction defines the lens's ability to bend light rays as a result of the changing of the light's speed and wavelength. Bending light allows for precise focusing of a scene onto the retina—the photosensitive layer of the central nervous system. The retina is located on the innermost surface of the back of the eye, the fundus. The retina is very complex and is comprised of seven layers. The photoreceptors are located in the layer of the retina most near to the lens. Photoreceptors are a type of cell called a neuron. There are two types of neuron cells—rods and cones. Rods and cones are sensitive to a range of wavelengths of light. Color vision is primarily the function of cone cells and rods respond primarily to brightness changes. Rods can detect very small amounts of light. Even a single photon can stimulate a change in brightness once dark adaptation has occurred. Night or low light vision is primarily accomplished by rod cells and leads to monochromatic images. The region of the retina where an individual has the best vision is called the fovea and is located in the macular region. When focusing, it can be useful to align the eye to an effective angle to an event, helping to ensure scene brightness and other aspects of the object's image are most directed to the fovea within the macula.

Located in the retina is the optic disc. The optic disc is often called the blind spot because there are no photoreceptors located in this region. It is where the nerves that travel to and from the brain enter and leave the eye. The nerves that travel to the brain are called axons. They are the electrical pathways for visual information to be transmitted to and from the brain. Glaucoma or optic diseases can cause these pathways to degrade, sometimes leading to blindness. The fovea is the region of the retina where the highest resolution and color vision occurs. Rods and cones are not distributed randomly in the eye. The fovea has a very dense composition of cone photoreceptors. The concentration of cones decreases rapidly as distance from the fovea is increased. There are no rods in the fovea. Rods increase rapidly moving toward the outer edges of the retina. There are 7 million cone cells in an eye and 120 million rod cells. While the capture and initial processing of light occurs in the eye, it is agreed that human vision and visual perception occurs in the brain.

Optics of the Eye and Image Formation

The human visual system has often been compared to a camera. There are similarities, but there are also many differences. Both systems create inverted images or images that are upside down and reversed left to right when compared to the scene or the subject's orientation. This is because of the lens. The diameter of a retina is approximately the same as the 24 mm dimension of a full-sized DSLR sensor. The effective focal length of an average eye is about 17 mm. This creates a depth of field that is slightly greater than most camera systems can produce without using increased DOF methods. The short focal length of the eye combined with the curvature of the retina produces an angle of view of that is approximately 180 degrees. The "normal" angle of view of a lens used on a DSLR camera using a full-sized 36 mm x 24 mm sensor would be approximately a 50 mm lens. Because the vertical (height) and horizontal (width) dimensions of the sensor are different, a camera lens will have a different vertical and horizontal angle of view. An eye does not

have this limitation. Sphincter muscles control the diameter of the iris. These muscles make it possible to change the size of the opening from approximately 2 mm to 8 mm. These diameters correspond to the f-numbers found on a lens aperture and on a 50 mm lens would be approximately $f/8$ when closed and $f/2$ when fully open. The size of a pupil can influence image definition, contrast, and depth of field.

One major difference between the eye and a photographic camera is that the camera's sensor will have uniform pixel resolution across the sensor. There is not a uniform density of photoreceptors across the entire surface of the retina. Because of this distribution, visual perception and sight will differ across the central regions of the eye when compared to the periphery of the retina. Camera images and displays will exhibit the same tone, color, and contrast edge to edge.

Different than a camera, human vision can be degraded by other elements that are located within the eye. Light that enters the eye can be deviated from the image-forming pathway by corneal and lens defects, internal reflections from densities within the vitreous, or the internal scattering of light from cataracts or iris thinning. One could correctly argue that dirty camera lenses also produce this type of degradation. Light that is scattered and not focused will arrive at the retina as veiling light described as flare or non-image-forming light. Flare leads to a reduction of image contrast, which then leads to a lowering of system visibility. Physiological differences in the eyes of individuals may cause corresponding differences in optical quality or visual acuity across users of imaging systems. In system evaluation, there is a concept described as the standard viewer, and engineers build devices for this audience. Standard viewers represent a category of people with visual acuity that represents what "most viewers" will see in a prescribed level of brightness and environment using an average test target.

The lens of the eye can be a significant source of degradation. The lens—when relaxed in an eye with normal vision—is relatively flat. A flat lens will focus light from distant objects onto the retina at what is described as infinity. An adjustable photographic lens will often have an infinity symbol (∞) located on it. Accommodation or changing the eye's lens shape will make the lens more convex and allow focusing to be accomplished at shorter distances. The closest distance a lens can focus is called its near point or working distance. This location for a young child maybe as short as 3 inches, but as a person ages the lens will become less flexible. This condition is called presbyopia. When this happens, a person's near point may become 30 inches or even further from the eye. Because the distance at which the relaxed eye focuses also tends to increase with age, it is not uncommon for nearsighted individuals to find visual acuity will improve with age. This makes focusing on near objects more difficult, a condition that eventually requires a corrective lens with a plus diopter. The human lens may also become yellow with age and this changes a person's color vision and color discrimination. This will be especially evident in the blue region of the spectrum. The lens can also become cloudy with age or form cataracts. Cataracts scatter the light going through the cornea and this leads to the lowering of contrast and visual acuity.

The Physiology of Seeing

Light is detected in the eye as a consequence of a chemical reaction that occurs in retinal photoreceptors. The pigment in the neuron that is light sensitive is called rhodopsin or visual purple. Individual units of radiated energy are called photons and interact with the rhodopsin. When a photon enters the photoreceptor cell, it is absorbed by these specialized pigments located in the cell. The absorption causes a change in the pigment and creates an electrical signal in the photoreceptor. This signal is then transmitted to a specialized neuron that leaves the eye and travels to the brain. Once the signal reaches the visual cortex of the brain it is consciously perceived by the viewer.

Under low light levels, rods are the main contributors to vision. In bright light levels, cones become the main contributors to vision. Rods are specialized for the detection of low light because they possess an increased level of rhodopsin that leads to greater amplification of the signals than in cones. Visual purple can become bleached or washed out and must be recharged. This bleaching can affect vision when changing environments where there is a big change in brightness or when looking at experiments with high degrees of brightness that are changeable. The bleaching of visual purple can take up to forty-five minutes to be dissipated and then recharged to useful levels that are required in low light.

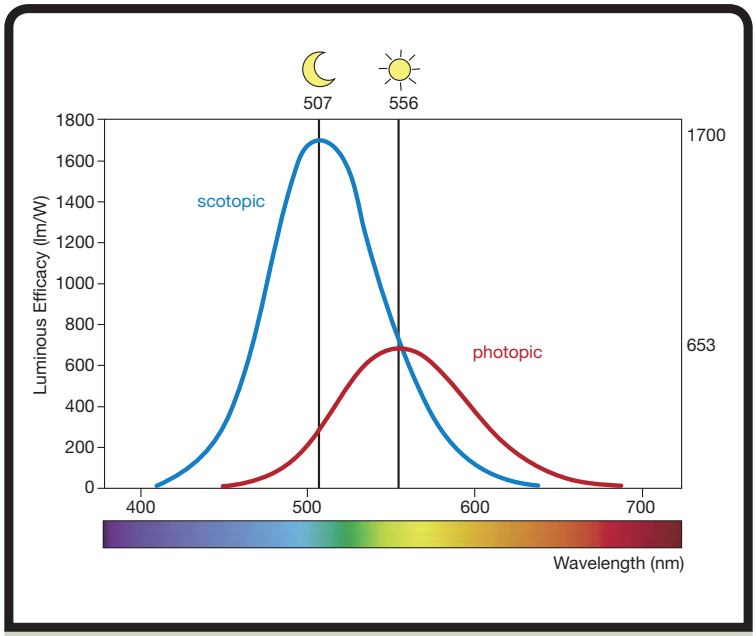


Figure 2.3 This graph reveals how rod (scotopic) vision and cone (photopic) vision differs.

Dominant Eye

One eye will be dominant over the other and will contribute more to vision. Determining which eye is dominant is relatively easy to evaluate. Clasp your hands and place them in your lap. Without conscientiously thinking, quickly raise your clasped hands and point an index finger at some distant target. It is now possible to determine, which eye pointed the finger to the target. Evaluate whether the left or right eye directed the aiming to the target by closing each eye and seeing which eye is locked onto the target. That eye will be the dominant eye. While not identical to right or left handedness, there is a similarity to the concept of dominance. Interestingly, two-thirds of adults are right eyed.

Visual Perception and the Physiological Aspects of Sight

Perception, by definition, is the organization, identification, and interpretation of visual information with the goal of creating an understanding of an environment. It is interesting to note that when people are asked to describe an object's appearance they use shape, color, and size. This should be of no surprise. So when these criteria are applied to one object, individuals will frequently describe slightly different perceptions of what they saw based on factors of their age, experience, and observational skills. The act of seeing will lead a person to believe that something was seen in a certain way. Perception is powerful and influences what someone believes they saw. Another facet of this process is the power of expectation. People will see what they are looking for and perception can lead to a reality for a viewer. The perceptual aspect of human vision plays an enormous role in the seeing and interpretation of things.

Perception of Color

When using color to describe objects, it is assumed that this strategy will lead to greater interpretation and provide more detail useful in gaining better understanding. Most people have good color vision and high levels of color discrimination. The human retina is sensitive to radiant energy emitted in the 400–700 nm range. The 400–500 nm region describes blue spectrum, the 500–600 nm region describes green region, and 600–700 nm describes red region. The human retina is not sensitive to ultraviolet or infrared radiation. Some people have color vision defects, and having defects is surprisingly not uncommon. Color vision defects (color blindness) are categorized as being either protan or deutan. Deutan color vision defects are more common and are found in approximately 6 percent of men. People who are affected by protan color blindness are less sensitive to red light, and deuteranopia affects sensitivity to green light. Within protan and deutan color vision defects, there are also two broad groups, dichromacy and anomalous trichromacy. In these situations, colors are discriminated from one another on the basis of saturation and lightness variations. Anomalous trichromats have slightly better color discrimination, but in most cases the discrimination is significantly different than normal color vision. Monochromats are individuals who are truly "color-blind," and cannot see any color.

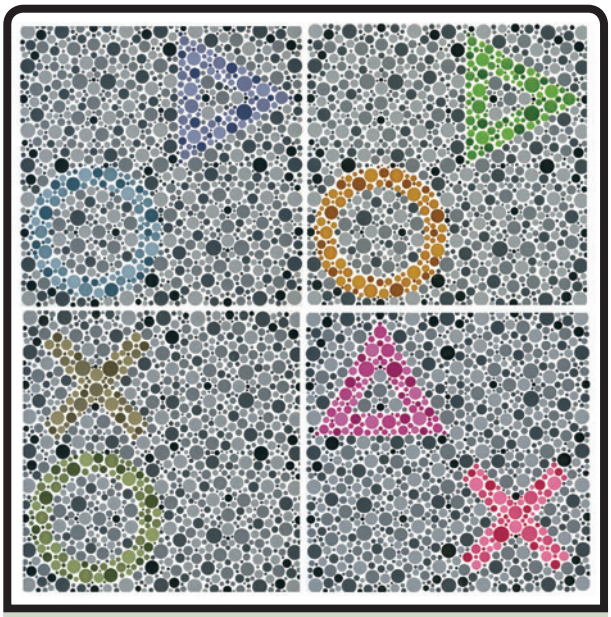


Figure 2.4 This illustration is a composite showing various pseudoisochromatic plates used to evaluate color vision deficiencies. Image courtesy of Richmond Products Inc., Albuquerque, New Mexico.

Color discrimination can be tested using various methods including the use of pseudoisochromatic colored plates. In a pseudoisochromatic plate, simple geometric designs that contain colored circles are placed against a background of gray circles. People with defective color vision are unable to see some of the designs. What colors are seen or not seen will

define the extent of the defect. Figure 2.4 shares four variations of pseudoisochromatic plates where different colors are visible or not.

There are other factors that contribute to seeing colors accurately. For the record, color is a complicated subject. There are a great number of texts on this subject and important new research is being conducted daily. In the fall of 2014, the Nobel Prize for Physics was awarded for the discovery of how to make a pure blue emission in an LED source. Color science and imaging plays an important role in the discriminating of one region of a sample from another region or color. When a neutral gray patch is surrounded by a strong color, it will appear to be comprised of the color opposite to the gray patch. So if a gray patch is surrounded by red, the gray will appear cyan. This outcome is also evident when a colored square is surrounded by black, gray, or white; its color saturation and brightness will appear to change.

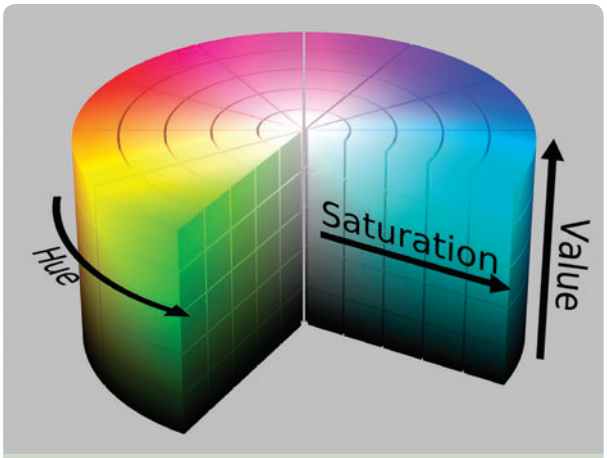


Figure 2.5 Many important systems are used to describe color and these would include the International Commission on Illumination (CIE) chromacity diagram as well as the Munsell color wheel. This illustration shares the Munsell color wheel and uses three attributes, hue, chroma, and value, to describe color. Image courtesy of Michael Horvath/SharkD/, http://commons.wikimedia.org/wiki/User:SharkD.

Persistence of Vision

The human visual system is very responsive to changes and interpretation by the brain. The time delay between responding to photons and the process of perception is very small and from a practical point of view is negligible for humans. The perception of vision and still seeing after light stops falling onto the retina is of great importance for reasons that differ from physiological sight. The persistence of an image on the retina will last only 0.25 seconds after the stimulus has been removed. This phenomenon is important to the formation of the perception of motion and allows a viewer to see pictures that move. If the increment is longer, flicker between frames will be observed. If the time is too short, no movement or change in location will be perceived. Motion perception is important because it provides information necessary to successfully interpret and navigate environments where things are dynamic and not static.

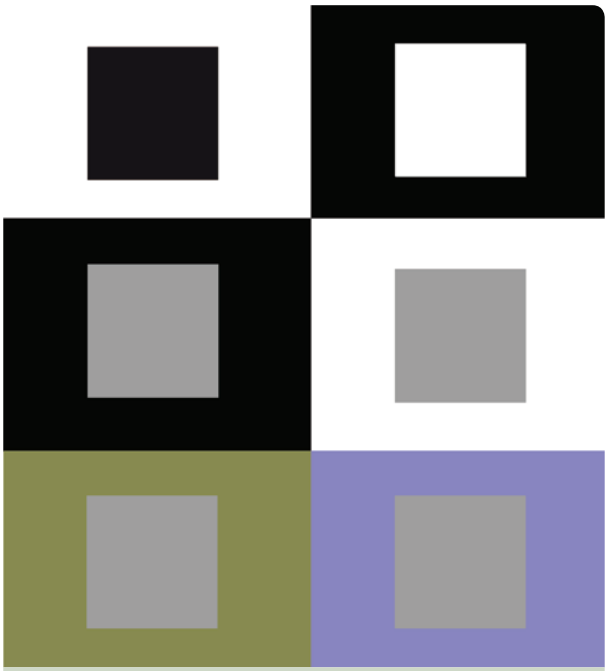


Figure 2.6 A white patch surrounded by black will appear darker because of adjacency effects. Gray patches surrounded by black or white fields will also exhibit this behavior. If gray patches are surrounded by olive and lavender patches, the gray will appear to be two different colors. Because these two colors—olive and lavender—are of similar saturation and value, the gray patch on the olive will look almost like lavender, and the gray patch on the lavender looks almost like the olive. This perceptual outcome is a function of interpretative processes and not a function of the object or physiology.

Afterimage

Another physiological component of sight is the formation of what is described as an afterimage. Afterimages can be white or black. Afterimages occur after looking at a bright stimulus for a prolonged period of time. Typically they are the opposite in brightness to the stimulus. It is common to see these images having both a brightness and color component. When looking

at a bright red light for a prolonged period, a cyan dark afterimage may result. Afterimages can be a nuisance when working with low light imaging or when creating events that have a high brightness in low light situations.

When comparing afterimages to persistent images, the persistent image physiology leads to a positive outcome that is useful in visualizing motion and afterimages lead to lower vision discrimination. If a person attempted to read immediately after looking a very bright light, the afterimage would make seeing letters difficult.

Perception of Depth

The perception of depth is of great importance in human vision. Seeing depth is accomplished by having two points of view and is the result of two eyes from two different locations. It is described as stereoscopic vision. Depth that is observed in photographs is very different than depth observed when seeing 3D world. In reality—and because the human eyes are offset approximately 6 degrees—an object will be evaluated from two different points of view. This allows for the perception of foreground to background difference and allows these differences of distances to be gauged by a viewer.

Three-dimensional scenes do not suddenly become two-dimensional scenes in an image. When a location in space is fixated on using both eyes—and to the extent that both eyes do see exactly the same object—fusion of the image can occur. When fusion of the locations happens, disparity of that location occurs and a person sees differences. This disparity allows for depth and distance clues. This process leads to stereovision and creates depth or the ability for the viewer to establish object distances. This is not the case in a 2D image whether on a display screen or in print.

In two-dimensional images disparity does not occur, and binocular vision does not operate when looking at 2D images of prints or on monitors as it does in the real world. That being stated, different factors help people delineate depth in an image. One component is aerial perspective. Aerial perspective is a condition where elements of a scene further from the lens may exhibit less contrast and are less defined than closer objects in a scene because of the effects of aerial haze. Other clues include that objects that are in the foreground will cover up objects that are in the rear of the scene.

Adaptation

Visual adaptation is the adjustment of the visual system to the environment or to the object. Visual plasticity is something that influences photographers and photography. The visual system has evolved to adapt to changing stimuli to better see for potential changes. These changes might occur in brightness, color, size, motion, orientation, pattern, and sharpness, enabling a person to see and function effectively when these changes occur.

Brightness/lightness adaptation allows sight in environments with large light level differences such as found at midday or midnight. This range is represented by a luminance ratio of about a billion to one. A change in the retina's sensitivity is a gradual process and may require up to forty minutes for complete dark adaptation to occur, for example. The

increase in sensitivity that occurs during dark adaptation is the result of changes in the pigments in the retinal receptors and in the neural processing of the signal. Differences in dark adaptation and light adaptation occur quickly. Because of the changing sensitivity of the visual system during light and dark adaptation, the eye is a poor measuring device for absolute light levels.

More on Perception

Visual perception of the world differs markedly from the rapid flow of two-dimensional information created by the eye. Human vision can lead to cognitive thought and emotional responses, and can lead to understanding based on many factors. The stimuli that are observed and then processed by a photographer might serve as the inspiration to take photographs.

The following paragraph has been shared on the Internet for as long as anyone can remember. It has been attributed to scientists at Cambridge University and clearly demonstrates how a human sees, references, interprets, and then adds information to what is being seen. It a useful example that demonstrates how information and understanding is accomplished when using visual clues.

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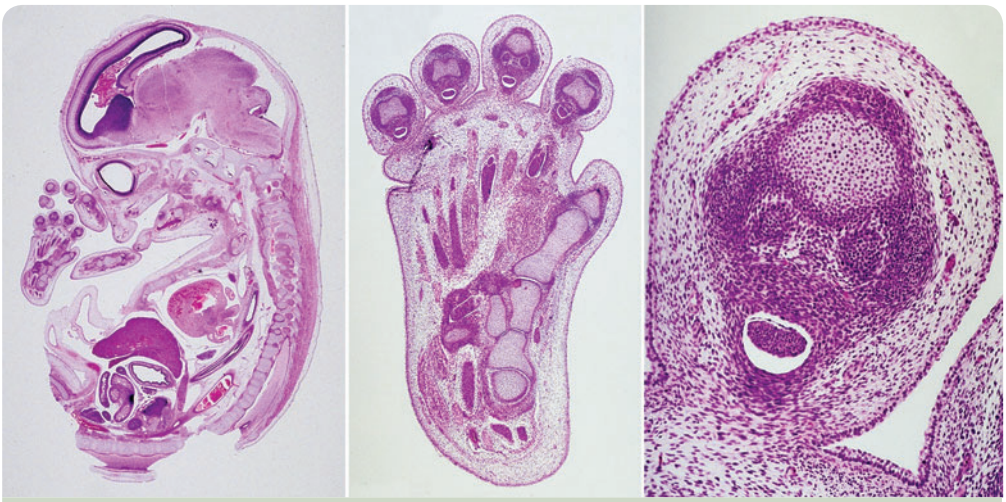

Figure 2.7 Images can be contained within images. This series shows a progression of magnifications that features a common marmoset monkey. The sequence reveals the whole body and goes through to details visible at the cellular level. The original intention was to photograph the whole fetal image; however, when magnified for closer inspection, the intriguing picture in the foot was observed. At a magnification of x12.5, the bizarre likeness of the face in one of the toes was discovered. The three images were combined to record this scientific oddity. Image courtesy of Kristen Toohey, Southborough, Massachusetts, United States.

This example might be useful for gaining a greater appreciation how the cognitive process works but, in reality, rarely are examples this defined. Human vision is powerful and easily discriminates all kinds of information within images. Within an image, content and information is derived in different ways and is often either ignored or moved forward in level of importance. Seeing is easy to accomplish (when vision problems do not exist) but becoming a careful observer of objects and their components is a learned activity.

Figure 2.8 A person will frequently see what they believe they are looking for without looking further. What is perceived to be the subject in this drawing on the left is a face, but it is actually handwriting that has been inverted from its black ink on white paper and oriented in at an angle. Without additional information, the photograph on the right appears to be that of a human palm. Actually, it is the palmar surface of an orang utan's hand. Expectations—and without a frame of reference or caption—will frequently lead viewers to incorrect conclusions. Image of the orang utan provided courtesy of Tim Flach and Tim Flach Studios, London England, http://timflach.com/.

One basic technique fundamental for human vision/discrimination is comparison, grouping and observation. If an object is something new and not familiar to a viewer, with no frame of reference it can be difficult to assess. But when situations are familiar, seeing a sample and its structures will be a more reflexive process that can lead to conclusions. The process of comparison depends on prior knowledge and experience. How strong a memory is and which features or characteristics are embedded will become the foundation of comparison and assessment. Statistics reveal that eyewitness accounts of an accident or crime are often unreliable because, for the most part, people are poor observers of fine details and the interpretive process can lead to the recalling of false data. Because identification is based on prior knowledge, people often see what they were looking for.

Resolution and discrimination that contributes to visibility are two different components of human vision that play a role in perception. A star in the night sky would be considered a point visible because of its brightness and subsequent contrast, appearing bright against the dark sky. Faint stars are not visible to the unaided eye because there often isn't enough brightness to create contrast or visibility. Resolution is not the same as visibility and is defined as the ability to distinguish two objects as separate points that are close together. Sometimes, when looking at a tree from a long distance for example, the leaves appear as simply a large green mass, but as the distance to the tree is shortened, the individual leaves become resolved from the mass.

Seeing is a function of the brain. The mind groups and scans elements in the scene in an almost reflexive fashion trying to identify details. Discriminating minor deviations in patterns can be challenging.

Mirages or Illusions

Unfortunately, the world is not simply lines, letters, or points. Human vision and reality is complex. Frequently things are grouped naturally and but rarely separated into perfect parts. Grouping works for spatial and temporal scales are useful for understanding. In many situations, there are many objects that contain similar or different shapes, edges, and other features, and grouping may or may not allow for the perception of likeness or not. If there are other similar objects, the most important object will be compared with others to form a higher level of discrimination and organization of the object. Sometimes there is confusion for a viewer because of the cues. This can be evidenced with the example of Zöllner lines. Grouping in this case gives rise to viewer confusion. Resolving the confusion is accomplished by seeing only one interpretation and ignoring the others.

Photographers through practices of lighting, composition, and contrast can change the separation between a subject and its background. The term "figure–ground" is used to describe this relationship. This concept of an object's relation to other included elements and their emphasis is an important aspect of Gestalt psychology. In a Gestalt analysis, things are considered as the whole and how the elements operate together rather than as elemental components. When consciously trying to make visibility difficult, the military developed camouflage uniforms to take advantage of blending. In other types of photography, the making of contrast for separation of an objective to its background is the goal.

In the Rubin vase example shown in Figure 2.10, a viewer may perceive the bright object as the foreground; the shape will be

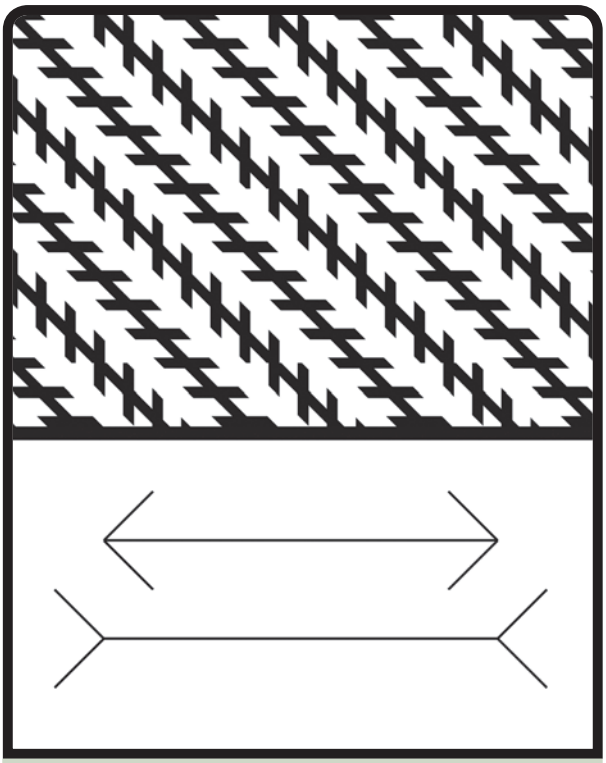

Figure 2.9 The top image features Zöllner lines, named for German astrophysicist Johann Karl Friedrich Zöllner, who discovered them. The cross-hatching of the diagonal lines with short vertical and horizontal ones causes the visual perception of the lines to rotate in the opposite direction. In fact, the diagonal lines are exactly parallel. The bottom image shares another classic example. In this example, the length of the two lines appears to be of different lengths. The Müller-Lyer illusion is an illusion that uses suggestive information to cause a false interpretation by the viewer as to which line is actually longer. Most viewers will chose the example where the fins point inwards. This observation was first made by Franz Carl Müller-Lyer, a German sociologist, in 1889.

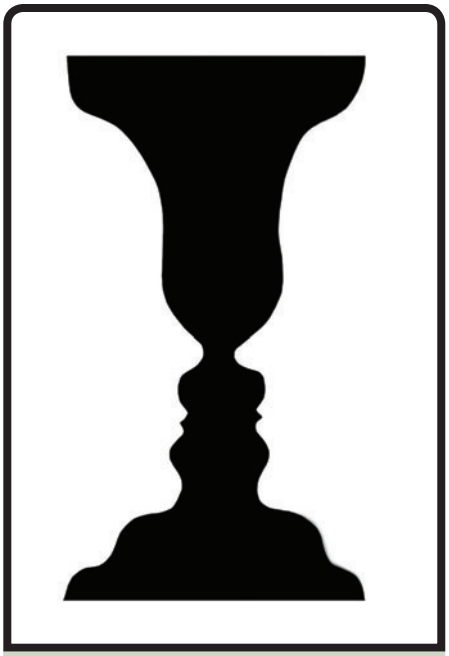

Figure 2.10 A Rubin face or the figure–ground vase is a famous set of ambiguous or bi-stable (i.e. reversing) two-dimensional forms developed around 1915 by the Danish psychologist Edgar Rubin. Some people will see a vase and others will see two faces.

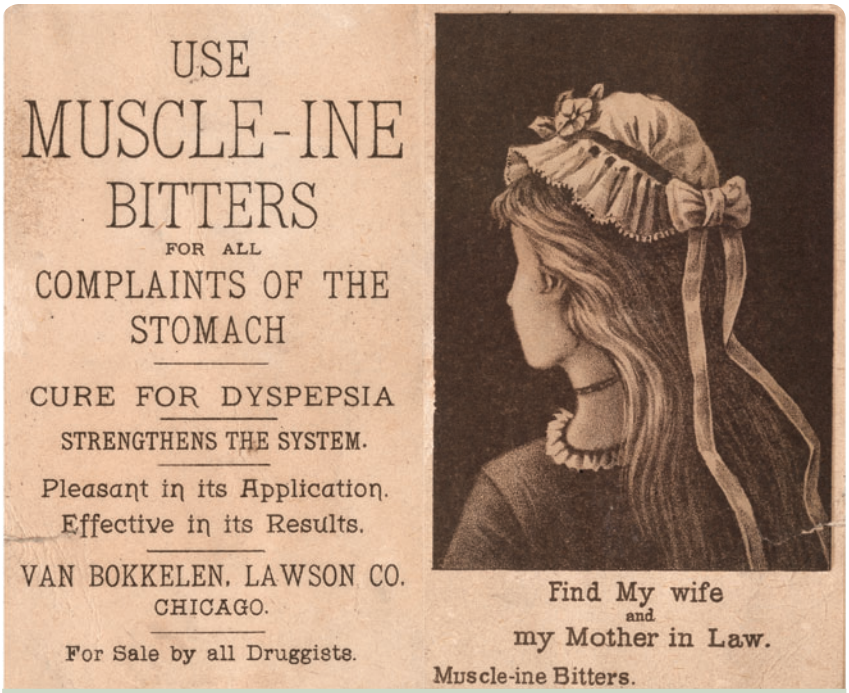

Figure 2.11 This illustration is another classic example of a figure–ground illustration. The illustration was created as a patent medicine card distributed in the early 1900s. Included in the illustration are two women, one old and one young. Which face a viewer sees first will be a function of contrast, brightness and camouflage, and the ability to separate or group other visual clues. Image courtesy of the Michael Torbenson Collection and Norman Barker.

seen as a vase with an irregular shape. If the background around the vase becomes the foreground and subject, the shape emerges as two facing human profiles. Similarly the patent medicine card shared in Figure 2.11 reveals this same phenomenon, based on what shape is stronger to the viewer. It is interesting to watch people try and see the old or young woman in this drawing created in the early 1900s.

SUGGESTED READING

Huxlin, Krystel R., "The Human Visual System," in Michael R. Peres (ed.), *The Focal Encyclopedia of Photography, Digital Imaging, Theory and Applications, History, and Science*, fourth edition. London: Focal Press, 2007; ISBN-13: 978-0240807409.

Snowden, Robert, Peter Thompson, and Tom Troscianko, *Basic Vision: An Introduction to Visual Perception*, second revised edition. Oxford: Oxford University Press, 2012; ISBN-10: 019957202X.

Zakia, Richard, *Perception and Imaging: Photography—A Way of Seeing*, fourth edition. London: Focal Press, 2013; ISBN-13: 978-0240824536.

https://en.wikipedia.org/wiki/Visual_perception

http://micro.magnet.fsu.edu/primer/lightandcolor/humanvisionintro.html

http://visionscience.com

Chapter 3 Applied Physics and Image Formation for the Scientific Photographer

In this photograph, doctoral student Joseph Cho's hand is pictured along with a multidirectional perfect paraxial cloak comprised of four lenses. From a continuous range of viewing angles, the hand remains cloaked and the grid is seen through the device, matching the background located on the wall approximately 2 meters away in color, spacing, shifts, and magnification. The edges of the optics can be seen because this lens group was a small-angle paraxial cloak. The artifact of seeing the edges of the cloak can be reduced by using larger optics and at a longer viewing distance. Optical cloaking configurations were designed by University of Rochester Professor of Physics John C. Howell, along with PhD student Joseph Choi. This photograph was made in Bausch & Lomb Hall September 11, 2014, by J. Adam Fenster/University of Rochester.

Light

One might argue that photography really is all about light. Light may be the singular most important ingredient in a photograph. In most situations there is plenty of light for use when photographing but often that light is not optimized for the uniqueness of the sample or situation. The light is simply present and without character. In many instances it only possesses brightness. A poor analogy but an effective one would be to consider trying to use a butter knife for surgery. The word "photography" means to write with light. Choosing and using light is one of the most fundamental tools in the photographic process and leads to what can be observed in or on an object and subsequently recorded during the photographic exposure. Achieving success when photographing in science requires a complete and practical working knowledge of applied light with its well-understood and predictable behaviors.

Visibility Requires Contrast, Magnification, and Resolution

The ability to see an object—whether large or small—requires the need for light that creates contrast, the use of optics to create magnification for visibility and to form resolution. Other chapters describe the role of human vision and the role of the sample (Chapter 5) in the imaging chain. This chapter will evaluate how radiated energy works and more specifically how light contributes to image formation. A part of this chapter will be dedicated to photographic optics and their influences on the overall quality of an image.

Light emanating from a source will have a physical size, brightness, directionality, and a spectral or color output. These characteristics and others play a role in the appearance of an object as seen by a viewer, which in turn will affect how an object will be reproduced as a photograph. While taken for granted and rarely considered, it is fascinating to deconstruct how the physics of this works down to the particle level. To start, radiant energy that might include visible light leaves a source. This energy might be all light or contain other spectral emissions such as infrared or ultraviolet. Light will have a brightness that might be adjustable and the light's source will have physical size. The light that illuminates an object will come from a specific direction relative to the object's surface(s), which may or may not be in sync with the camera's point of view. The light will interact with the object and become modified in some way. The modification can be a reflection, transmission, or absorption or a bit of each. This in turn creates "reflected" light that is carrying information about the sample and its unique characteristics. Before being "collected" by the eye, some of the light may interact with other light that is leaving that sample or light from other sources or from the light itself. This interaction may result in collisions or interference that can play a positive or negative role in the formation of the structure and contrast within the image. The light is finally collected by the eye or imaging system and contains structural information about the subject, including its size, surface or internal structures, color, and often more. When miniaturized to the particle or photon level, these interactions can be difficult to describe, deconstruct, and subsequently understand. Visualizing "the invisible" might be the most challenging element of scientific imaging. At the end of this linked

sequence of events, data is revealed and recorded on the sensor. It is interesting to note that, often, a sensor will record more than the human observer can see.

Describing electromagnetic radiation (EMR) and how radiated energy behaves has been a challenge to physicists for centuries. Even in 2015, no singular model for describing radiated energy travel has been universally accepted because none explain all aspects of this science. Many historically important theories describing energy travel have been proposed, including the particle and wave theory. Albert Einstein suggested the quantum theory, which utilizes components from both theories. Recently a new theory described as the "string theory" has gained some acceptance. In this model, energy is traveling in what might be considered as a flexible rubber band that is constantly wiggling and changing shape. In Figure 3.2, scientists at the École Polytechnique Fédérale de Lausanne created the first photograph ever made that records both the wave and particle features of light. What is universally accepted in all theories is that light does travel away from its source in some fashion. Sir Isaac Newton first described light travel in the 1600s using his corpuscular theory. More than 300 years later, Max Plank and Einstein each added ideas to the conversation, trying to help explain the phenomenon of light travel. In the quantum theory, light is considered to travel in separate packets or photons. The use of singular photons can effectively be used to describe some aspects of energy travel. Using particles explains straight-line travel fairly easily. Photons are also useful in explaining photographic exposure. A pixel will collect photons in its well, the physical location in the pixel that absorbs photons. A minimum number of photons is required to create an exposure that registers in the pixel moving the sensor from an absolute black level to a shade of the darkest grey. The use of photons does not adequately describe speed changes when the light goes from one medium to another or when trying to describe the light's spectral composition or its colors. The use of the wave theory does a better job of describing these behaviors.

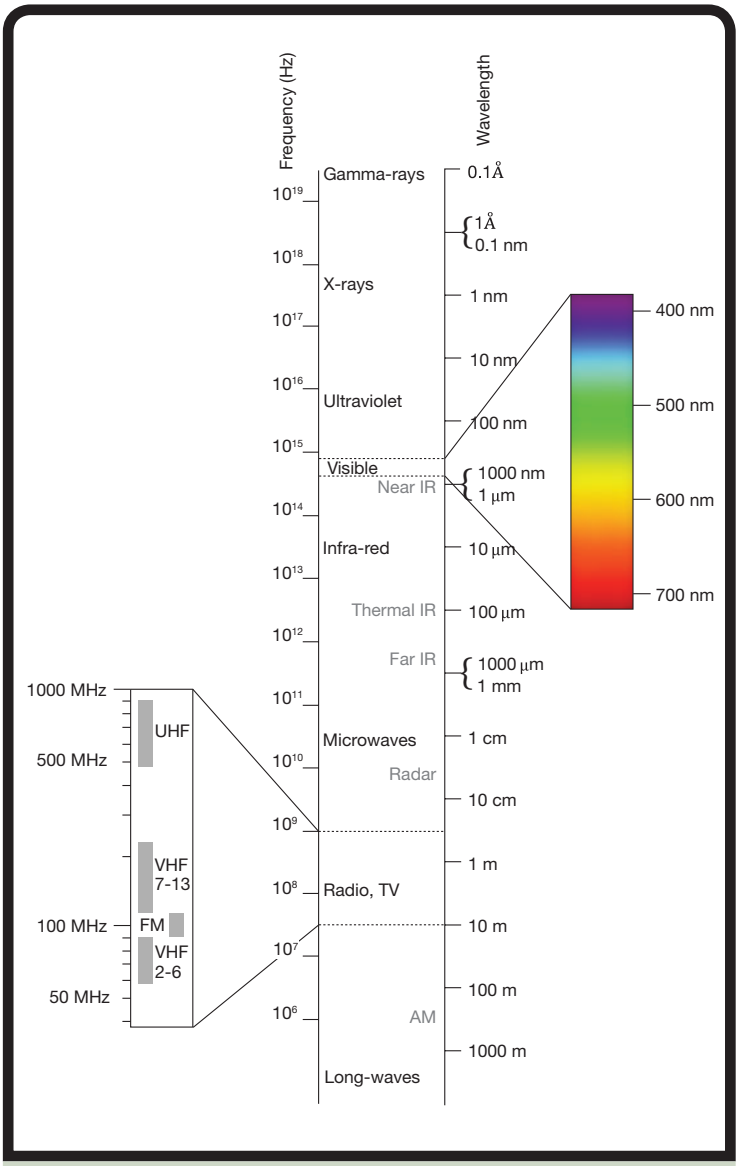

Figure 3.1 Electromagnetic radiation can be visualized on this chart. Short wave energy such as gamma rays are more energized than longer wave energy such as radio or television waves. Each region of emission can play a role in revealing data about an object when used for imaging.

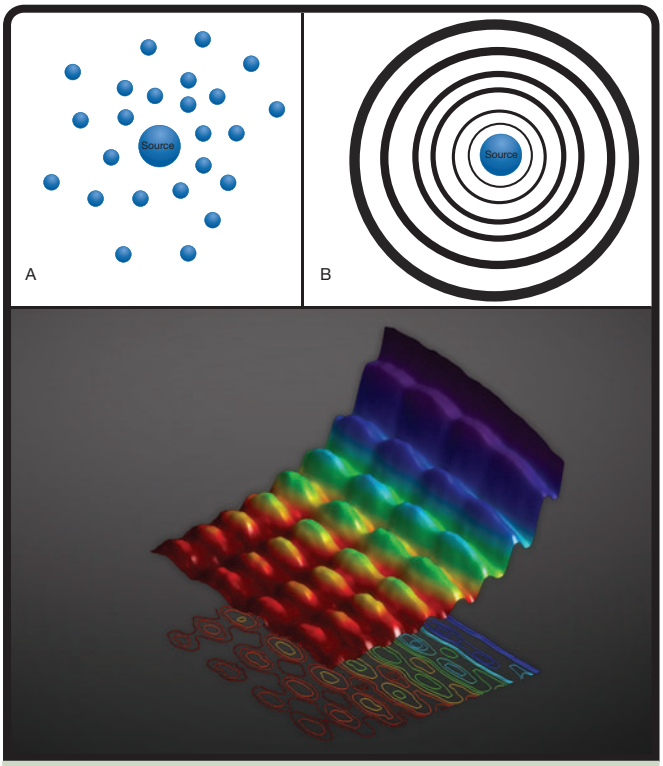

Figure 3.2 Image A represents radiated energy particles similar to raindrops leaving a source and moving in straight lines until the particles interact with objects or other materials. Image B represents waves leaving a source and traveling in sync away from the source by oscillating up and down. They can collide and can lead to interference that creates or diminishes contrast. Bottom image: Since the days of Einstein, scientists have been trying to directly observe both aspects of this light behavior at the same time. Taking a radically different experimental approach, scientists at the École Polytechnique Fédérale de Lausanne were able to take the first ever photograph of light behaving both as a wave and as a particle. Image courtesy of Professor Dr. Fabrizio Carbone, Laboratory for Ultrafast Microscopy and Electron Scattering, Institute for Condensed Matter Physics, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland.

When discussing light, it is practical to consider whether the light has originated from a point or diffuse source. The source of emission plays a role in the characteristics and quality of light. A point light source that is very small would be considered direct light. It creates a dark and well-defined shadow of an object and can be used with higher precision than diffused light. Light that is diffused as a consequence of traveling through a semi-transparent material or reflected off a non-directional surface such as a wall would be described as diffuse or diffused light. Clouds create diffused light and the sun at noon would be considered a direct light. Diffusion changes many aspects of light and its travel and characteristics. When considering particles of light traveling from a point source, it might be practical to consider how a mirror or other flat shiny surface operates to better understand travel behavior. A traveling photon that interacts with a smooth shiny surface will be reflected at an angle equal to the incident angle. The reflection will be nearly identical in brightness to the source and result in a specular reflection. The size of the reflection will also be proportional to the size of the light. If the surface of the subject in the photo is rough, the photon's angle of reflections across its surface will be more scattered, less predictable, and create diffuse reflections. Specular light creates high contrast and clearly delineated surface details. Diffused light will create less contrast and will reveal colors more diffusely.

When waves are used as a method for explanation, aspects of the brightness of light using the wave's amplitude can be practical. Waves are observed naturally in the ocean or in other water environments. Waves have height and possess energy. Considering how a wave operates while standing at the shoreline might serve as a useful example for the movement of positive and negative energy. The elevation of a wave describes the volume of energy and the direction of travel. When the wave moves forward, it pushes forward, but following the raised portion of a wave is the region that is below the surface. That part of the wave would have negative energy and move away from the shore. As illustrated in Figure 3.3, a wave can be drawn as shared and will have specific regions. Crests and troughs are used to describe a wave's amplitude or its brightness. The wave will have a travel direction and exhibit a vibrational magnetic component that is 90 degrees to the amplitude. It will also have a frequency, or cycles per second emission. A wavelength will describe the distance from similar points on the light wave such as a crest to crest or a trough to trough. This distance is measured in nanometers or angstroms that is used to describe the color of the light. For example, red light would be

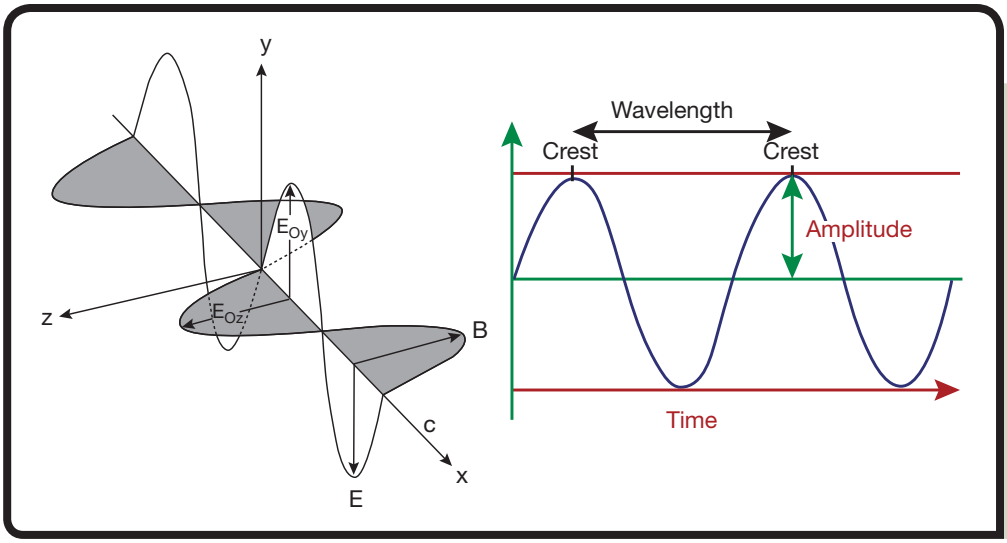

Figure 3.3 Waves are an excellent tool that can be used for explaining brightness and color described using wavelengths. One wavelength is equal to the distance from one crest to another crest or from one trough to another trough. Waves also have a travel direction and a magnetic component that is 90 degrees to the brightness component.

composed of wavelengths from 600 to 700 nm. Human vision can respond to energy that is 400–700 nm and this region is known as the visual spectrum or light. Short wave light such as 400 nm conversely will have more energy than long wave light, which has low energy. "White light" is the term used to describe full spectrum light. White light will have a blue component, a green component, and a red component that will be present in varying percentages. It would be possible but highly unusual to have a source that emits 33 percent of each primary spectrum, red, green, and blue, unless designed to do so.

Sources

Electromagnetic radiation comes from a variety of sources including the sun, incandescent sources such as tungsten and tungsten halogen bulbs, fluorescent lighting, metal halide sources, LED, OLED, laser, HMI, and electronic flash systems. All of these sources are used in photography. Classically described, the energy emitted from all these various sources would be provided using energy (watts) and wavelengths (or color temperature). Gamma rays are much more energetic than radio waves but radio waves will have a longer wavelength than X-rays. Shorter wavelengths of light will produce greater optical resolution.

The main source of light on this earth would of course be the sun. It emits all the known electromagnetic radiation. The sun is used frequently for general purpose photography but not so much for laboratory photography or photomicrography. A few important properties of light would include its intensity or brightness, direction, spectral composition, speed, and polarization. Although dim, there are other sources of light that serve a small role when photographing. Light sources not often thought of would include meteors, stars and other astronomical subjects, the aurorae, bioluminescent organisms, lava and volcanoes, nuclear reactions, chemoluminescence, fluorescence, CRT and LED displays, electric arcs, gas discharge lamps, tungsten lamps, and lasers. All of these will have emissions containing portions of the visible light region of the spectrum. Other sources might also include fire and black body radiation sources.

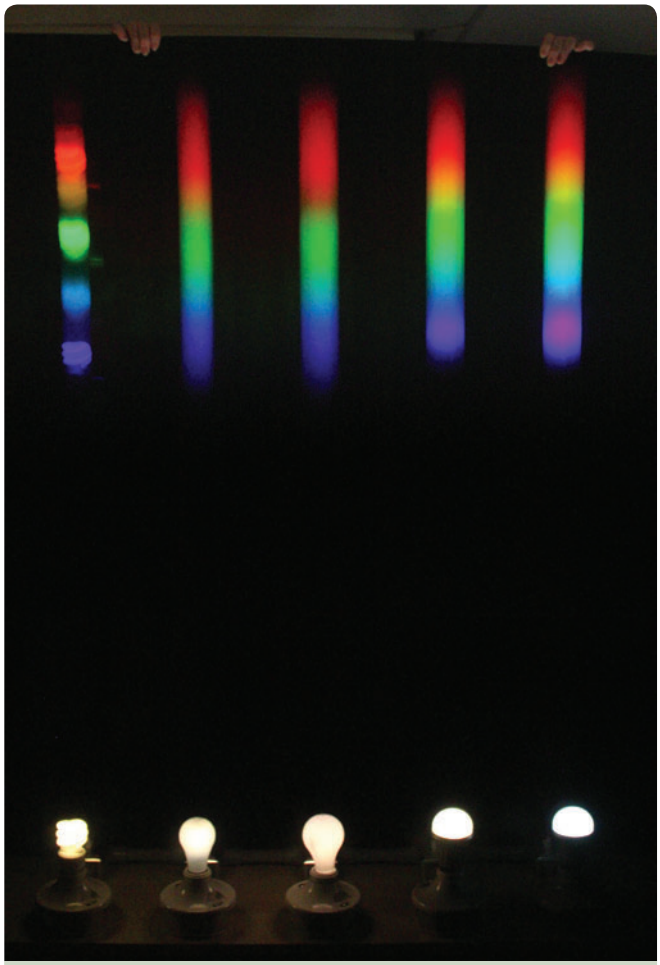

Figure 3.4 Tungsten, fluorescent, and LED lamps all will have different spectral emissions. A tungsten source is considered a continuous source while fluorescent sources would be considered discontinuous sources. This characteristic is evidenced in the bulb on the left, which has an emission of lines or chunks of color and is not smooth. The compact fluorescent bulb had a color temperature of 2850 K. Bulbs 2 and 3 are halogen bulbs and were operated at 2850 K. The fourth bulb is a 3000 K LED bulb and the bulb on the right is a 5000 K LED bulb. The bulbs were photographed through a diffraction grating useful in revealing the spectral power distributions of each bulb. Image courtesy of Nanette Salvaggio and Josh Shagam.

Continuous and Discontinuous Spectrums

When a source emits its various compositional components, the emission can be continuous or discontinuous. A continuous spectrum includes the emission of wavelengths smoothly over a broad range. Incandescent sources such as tungsten halogen bulbs or the sun would be demonstrative of a source that emits its energy in a continuous fashion. There are other continuous sources, including an electronic flash. A discontinuous spectrum source conversely would be characterized by emissions that are not smooth but contain discrete wavelengths that, when added together, form a desired color temperature or appearance. An example of this would be a fluorescent bulb, which is characterized by its line spectra composition from the mercury and continuous spectrum from the phosphorous. Fluorescent bulbs can be warm or cool white as well as many other colors. An example of a single wavelength source of non-incandescent visible light would be the sodium-vapor lamps commonly employed in street lighting. These lamps emit a very intense yellow light, with over 95 percent of the emission being composed of 589 nanometer light with virtually no other wavelengths present. This makes for very bright monochromatic images.

Color Temperature

"Color temperature" is the term used to describe the color characteristics of light with applications specifically to photography, astronomy, video, publishing, astronomy, and manufacturing. Light sources that are defined by the concept of color temperature are characterized by a color value shared in degrees Kelvin. This is referenced to a color that corresponds to a black body raised to a specific temperature using the Kelvin scale. A tungsten source using the correct voltage to the bulb would approximate a black body radiator with a color temperature of 3200 K. Daylight composed of sunlighted skylight has a color temperature of 5000 K. These colors and temperatures have been assigned after comparison to the color emission from a correlated black body radiator. Color temperatures above 5000 K would be characterized as possessing more blue light and referred to as cool. Color temperatures that are below 3200 K would possess more reds and yellows and would be characterized as warmer colors. Cameras use this information when being white

balanced. A camera will have pre-set values including daylight, tungsten, fluorescent, or custom sources among others.

Temperature	Source
1700 K	Matches
1850 K	Candles
2700–3200 K	Incandescent sources
3000 K	Soft warm white fluorescent bulbs
4100 K	Moonlight
5000 K	Daylight, cool white fluorescent bulbs
5500 K	Noon daylight and electronic flash sources
6200 K	Xenon sources
6500 K	Cloud-covered daylight
6500–10,000 K	CRT or LCD monitors

Continuous or Pulsed Emission

When light leaves a source, it can be emitted as a continuous emission, as with the sun or an incandescent bulb. It can be discharged over time or in a short duration discharge such as from an electronic flash. A continuous source allows for a wide range of shutter speeds to be used to make an exposure. However, sometimes a subject will move during the timed exposure and create a blur in the image. Continuous sources are quite common. Fiber optic light sources, metal halide bulbs and other similar sources would be considered to have a continuous emission. A short duration or pulsed source is quite different. Pulsed light sources are used to produce single or periodically repeated bursts of light lasting from a fraction of a second to milliseconds. Pulsed light sources can be divided into two categories. The first category includes sources that use the light radiation from a low temperature plasma source such as a condensed spark discharge in gases or exploding wires. The second source is characterized by the short excitation of a phosphor as a result of the passage of an alternating current electric across it or irradiation by an electron beam. Optical frequency oscillators or pulsed lasers are also considered pulsed light sources. Electronic flash lamps with an efficiency of conversion of electric energy into light energy of up to 50–70 percent are pulsed light sources and have found wide application for short duration imaging applications. The power of a pulsed source is often shared with a user in watt/seconds or guide numbers. A typical 160 GN electronic flash unit at full power would create a duration on average of 1/300 second. Subsequent power reductions will shorten the light's duration and allow more frequent discharges to be made with shorter recycling times. This information can be learned from reading the equipment's spec. sheet. Below are the durations from a hypothetical electronic flash unit to be used an example.

- Full power—1/300 second
- ½ power—1/600 second
- ¼ power—1/1200 second
- 1/8 power—1/2400 second
- 1/16 power—1/4800 second
- 1/32 power—1/9600 second
- 1/64 power—1/19,800 second

Light Behaviors

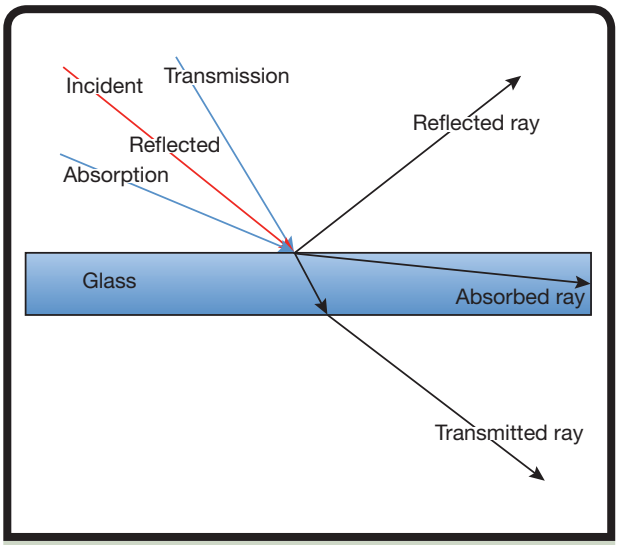

Figure 3.5 When light strikes a piece of glass, three things can happen depending on the angle of incidence. If the angle is great, transmission occurs. If the angle is low, there is a reflection, but if the angle is just right, the light will become trapped in the glass causing flare. This angle is called the critical angle.

Three things can happen to radiated energy (light) when it interacts with a new material: it can be reflected, refracted or absorbed. These three outcomes are influenced by the angle at which the light strikes the surface of the subject. Depending on that angle, a reflection can be produced, or the light can be transmitted (refraction), or the light can be absorbed. These three outcomes will occur to varying degrees by a minor adjustment of the light or the angle of the sample's surface to the light. These outcomes occur at air–glass boundaries or in situations where two materials of differing refractive indices are adjacent to one another, such as water or glycerin. All of these behaviors can lead to image degradation or improved edge definition and should be carefully controlled when possible.

Making technical pictures requires a general awareness of the behavior of light in air, in glass, and glass–air interfaces. A good science photographer anticipates how to control the light when it changes mediums and what occurs when these changes take place. There are exhaustive resources available online as well as in traditional resources that share more completely these principles of light travel and behaviors.

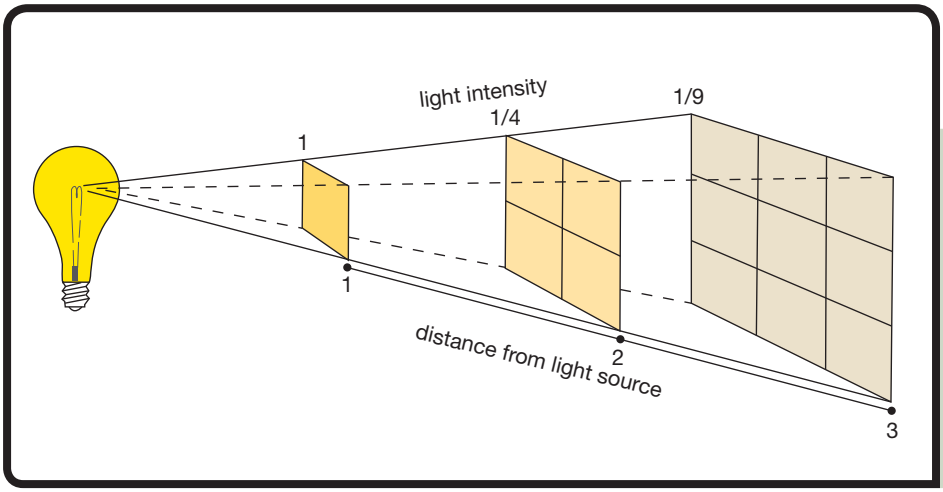

Figure 3.6 A light source will have a brightness associated with it. The brightness can be increased or decreased as needed. A light's brightness will fall off in predictable ways. The light fall off is described using the inverse square law. For every doubling of the distance, the light will be one quarter of the amount.

Reflection

The concept of a reflection might be one of the more simple concepts of energy travel to understand. A reflection can be described using Snell's Law, named after the Dutch mathematician Willebrord Snell, who first described this behavior. Snell's Law describes the reflective behavior of light in this way: "A beam of light that strikes the surface of a

material will be reflected at an angle equal to the angle of incidence." This assumes the surface of the material is highly polished. This angle is measured from a hypothetical perpendicular axis to the reflecting surface, called the normal. A reflection might occur anywhere or off any surface. Reflections can occur within a microscope or other optical instruments that contain multiple lenses or optical elements. Reflections from external sources such as windows or from overhead lighting, although rare, might find their way into photographs of shiny or wet samples made in laboratories. To minimize reflections in general, room lights or other accessible extraneous lights should be turned off when photographic exposures are being made.

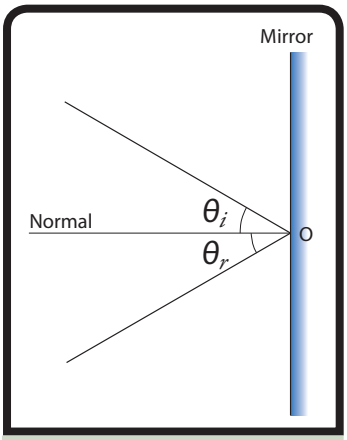

Figure 3.7 When light strikes a reflective or shiny surface, it is reflected at an angle equal to the angle of incidence: *qi* = *qr*

Absorption

When light becomes trapped in a material, it is often because of the light's angle of incidence to the object's surface or characteristics of the surface itself. This angle has been named the critical angle. Absorption occurs when the light cannot escape the material on the side opposite to its entry into the system. See Figure 3.5. Simply stated, absorption results in the creation of non-imaging forming light in the system, which is called flare. Flare light will lower contrast and result in diminished image visibility. Flare frequently is created by poorly managed reflections from smooth surfaces. An example of flare might be found by considering what happens when facing and driving toward the sun with a dirty windshield. The windshield will become hazy because the dirt will provide a screen for the sunlight to focus onto. The elements from the scene will appear washed out and without detail or contrast against this flare light in

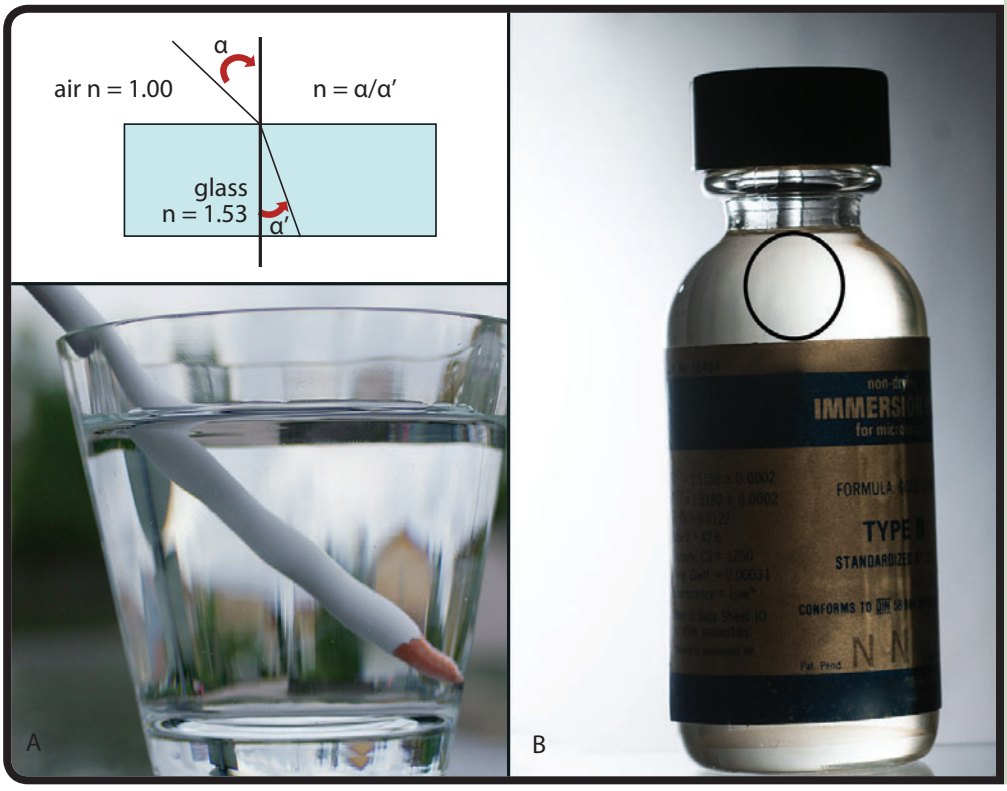

Figure 3.8 When light encounters a material of differing refractive index, it experiences a speed change. This is evidenced as displacement. The graphic drawing at the top shows how, as the incident ray enters the glass, it is slowed and bent towards the normal. When the light leaves the glass, it speeds up and is bent away from the normal. It will be traveling in the same direction as the incident ray, but is displaced. In image (A), this displacement is evidenced by the appearance of the pencil, which looks bent in the photograph because the refractive index of the water is different than that of air and glass. In image (B), featuring a bottle of immersion oil, the applicator wand disappears in the oil (evidenced by the circle) and is visible in the air, the region above the oil.

the windshield. Absorption by optical elements might occur in dirty lenses or prisms or other elements in the system as well. Light simply needs a surface to interact with and it can effectively change the contrast of an entire optical system. This change can be subtle or enormously evident. Keeping lenses clean and free of dirt and oils will add greatly in contrast control.

Transmission

Transmission or, in this case, refraction plays an important role in image formation by focusing light, and it is particularly important in microscopy. Some would argue that refraction is at the foundation of an optical system's performance. Refraction is the bending of light as a consequence of a speed change that occurs when the light enters a new medium of a different optical density. This situation occurs when light goes from air into glass or when light enters water or other optical materials such as immersion oils from air. A type of refraction is dispersion, where each of the spectral components of light is slowed by differing amounts resulting in the separation of light into its primary ROYGBIV spectral components (red, orange, yellow, green, blue, indigo, violet), commonly described as a rainbow.

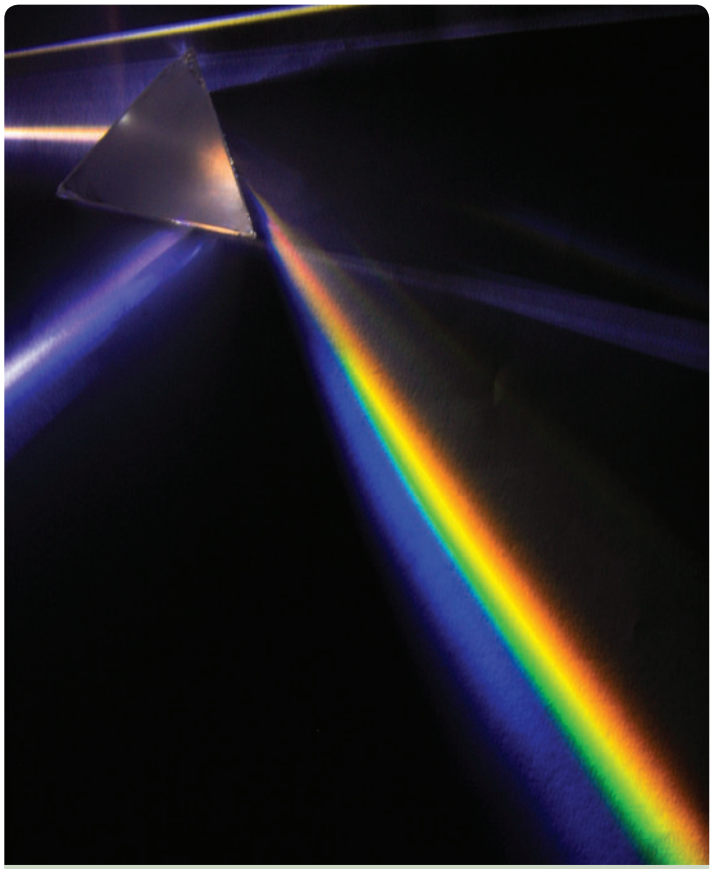

Figure 3.9 In this picture, light is refracted differently within a prism made of flint glass. The red, green and blue components are spread and cause dispersion, evident as a rainbow. The light source in this example was a mercury-vapor lamp. Image courtesy of D-Kuru.

Materials will have different refractive properties and these are shared or categorized as a material's refractive index. A refractive index is a number that describes the light-bending ability or optical density (to air) of the material and how it will affect the speed of the light in that new material. Air has a refractive index (RI) of 1.00 and is considered to act as a vacuum to light and exerts no changes on speed. Matter in air may, however, affect light travel. Water is denser than air and has an RI of 1.33, and glass has an RI of 1.53. Refraction is the behavior which influences a lens's focal length or an object's magnifying power. Shorter focal lenses will have greater refractive powers and longer focal length lenses less refractive properties based on lens design.

Dispersion

Dispersion occurs when light is broken up into its primary colors by the refractive properties of the optical material. A prism provides an excellent example of dispersion, and so are diffraction gradients. When light enters the prism from air, the speed change for each region of the spectrum will be refracted differently. Consequently, some wavelengths will be more greatly affected. This variance of speed results in the bending or refraction differences of light containing RGB. Dispersion is a negative quality for a precise optical system. Lenses that are not highly

corrected will disperse light in higher degrees than more highly corrected lenses. Lenses that are highly corrected are characterized as being apochromatic and have low dispersion problems. Macro lenses are frequently apo lenses. Dispersion will lead to flare and will result in a general lowering of the image contrast. In microscopy, lenses will often have an Abbe number associated with them. An Abbe number is an indication of the degree of correction for an objective lens and is based on the glass's dispersion potential. A higher Abbe number indicates the lens has less chromatic aberration. Having high dispersion is not a positive characteristic of a lens except for soft focus lenses used for high-end advertising portraiture.

Interference

Since energy travels as waves, frequent collisions of waves occur. When waves travel in phase or in sync, they are described as coherent. Waves can also be out of phase or are described as incoherent. When multiple waves collide, interference will occur. Interference occurs when two or more radiated energy waves are present simultaneously at the same location in space. This occurrence produces a new wave that will be the sum of the parts of the contributing waves. Interference can either be constructive—sometimes called additive (positive) or destructive (negative). In constructive interference, the wave energy will be

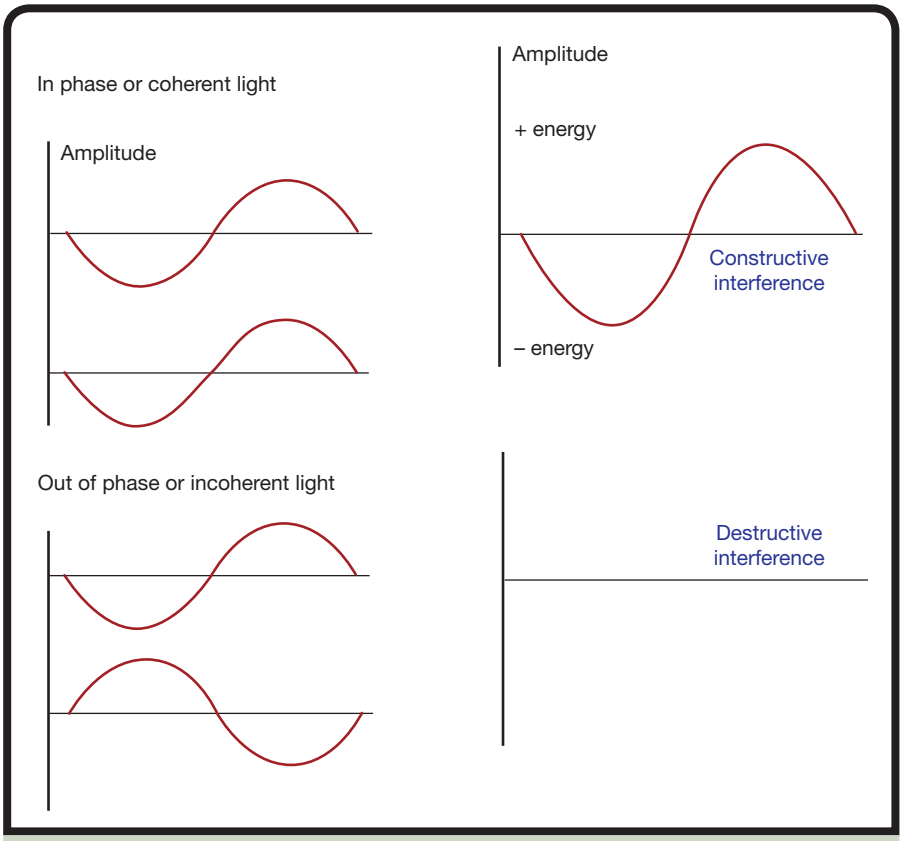

Figure 3.10 In this illustration, waves can be traveling in or out of phase. When the waves collide, interference occurs. When waves are coherent and interference occurs, it creates more brightness (amplitude); when they are out of phase, interferences cause energy loss and diminish brightness. Interference plays an integral role in image formation and contrast.

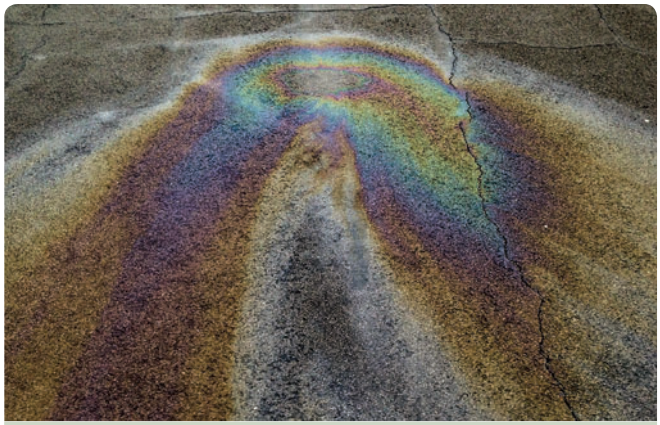

Figure 3.11 In this photograph, interference is evidenced by the various colors revealed in the oil on the wet roadway. The oil, when mixed with water, creates various layers of materials. As light enters the material, it bounces back and forth between the many parallel surfaces, resulting in interference and producing this rainbow image.

greater as a sum of the two energies and will posses more energy. Effectively this increase in wave energy creates more brightness and sometimes produces a change in color. When destructive interference occurs, the total of the energies from the waves will be reduced. This will diminish image brightness by reducing the energy of the new wave, which may also create a color change. In microscopy, interference is an important element of image formation and is needed to create or preserve image contrast required for visibility. Phase contrast microscopy and differential interference contrast techniques take advantage of this behavior, making the invisible, visible without the use of staining.

Lenses

It goes without saying that a lens is a critical element for use with a camera. It might be called a photographic lens or objective as well. Rarely do lenses operate without being on a camera and there are a variety of ways to couple lenses to camera bodies, including bayonet and C mounts. Lenses contain small openings called apertures. Lenses used for video, still photography, or other applications such as astronomy are for the most part somewhat similar in their basic functions. What is different across the choices is the degree of correction, focal length, light transmission capabilities, and angle of view. Lenses can also be corrected for vibration reduction.

A lens has a focal length with a corresponding angle of view. A lens's focal length also takes into account the image magnification or reduction that is associated with the lens. The focal length, typically shared in millimeters, describes the angle of view of the lens relative to the coverage of the circle of good definition that it produces required to cover the sensor. For example, if a sensor were 24 mm x 35 mm, the diagonal of the image sensor would require the circle to be approximately 43 mm, and the normal lens for this example sensor would be approximately 50 mm. Any focal length lens in this example that is shorter than a 50 mm lens would create a wider angle of view and would be characterized as a wide-angle lens, and any lens with a longer focal length used on that sensor would be characterized as behaving like a telephoto.

Apertures are placed into lenses to control the amount of light transmission and affect exposure. In modern lenses apertures remain in the fully open position until the shutter is depressed. This allows ease in viewing for composing and focusing. If the aperture were to close early, the image would be dark when composing and focusing. When the shutter is activated, the aperture blades close down to the selected aperture setting. This feature is described as an automatic lens. A lens where the aperture closes when the f-stop is engaged is called a pre-set or a manual lens.

A lens's maximum aperture is calculated by dividing the focal length of the lens by the largest clear diameter when the lens aperture is fully open. All apertures will encroach

Figure 3.12 In this composite photograph, a skull was photographed using an 18 mm, a 50 mm, and a 200 mm lens. To the extent possible, the skull remained the same size and the camera used the same point of view to make all of the photographs. As can be seen, there is a shape change. The working distance of each picture was different. Located adjacent to where the star was placed, changes are evident in the wide-angle photograph and the telephoto views. The malar and temporal bones have increased separation and visibility in the 50 and 200 mm views when compared to the wide-angle photograph.

into the optical pathway by a small amount. A very fast lens with a high light-gathering ability would be characterized by an f-number of 1.2, for example. On microscope objective, numerical aperture (NA) is used to describe the light-gathering ability of the lens and would be above 1.00. A lens with less light-gathering ability might be characterized as having an f-number of $f/4.0$, for example, or an NA of 0.1. More on NA is covered in Chapter 9.

In addition to affecting light transmission, apertures play an important role in affecting the range of focus or DOF that a lens produces. Depth of field is an important element of an image. Less DOF can be used to optically isolate an area of interest and more DOF can be used to reveal more of the sample by increasing its range of focus. Choosing the correction aperture is influenced by the objective of the photograph and magnification.

Lenses for Scientific Applications

Pinholes served as the original lens because they were able to create focus. Once lenses were discovered in the 1600s, they became valued and useful for obvious reasons. Brightness and image definition is significantly increased in images formed by lenses when compared with pinholes. Seeing detail, structure, and contrast of challenging objects can be greatly improved using a properly corrected lens and other optical elements of a system.

Fundamentals

Choosing the right type of lens to use always begins with the evaluation of where the lens will be used and the image requirements needed for a specific outcome. This outcome might

be for the creation of a flatfield image or a long working distance. Lenses are manufactured using various types of glasses including crown and flint glasses. There are other elements in a lens, including coatings that produce changes in image contrast, color fidelity, and resolution. Lens elements will possess a thickness and will have curved surfaces. The internal elements will be carefully arranged and the position of an aperture also considered, allowing the creation of image depth of field (DOF), depth of focus, and image brightness. Depth of field is located at the subject and depth of focus is located at the sensor. Modern lens designers use many methods to improve lens performance including adding aspheric surfaces, minerals such as fluorite, and gradient elements that create improved performance requiring the use of fewer pieces of glass. Nano-crystals are also being integrated into the newest lenses. Lenses designed solely for DSLR cameras are nothing short of remarkable. Image sharpness is experiencing a period of unparalleled excellence, which includes the use of special rear elements that direct light effectively onto the sensor's surface and more directly uniformly into pixels. Nikon has a VC or violet correction, and many lenses are vibration reduced.

A simple lens can be categorized as being either positive or negative. A positive lens is thicker in the central region and may also be called a convex lens. This lens produces real and inverted images that are focusable. Negative lenses are thinner in the center and would be called diverging lenses. This type of lens produces a virtual image that appears to be larger and in front of the viewing lens. Almost all photographic lenses contain both positive and negative lenses. A lens made of many individual lens elements is called a compound lens and must always remain positive to be used to form focusable images in the camera.

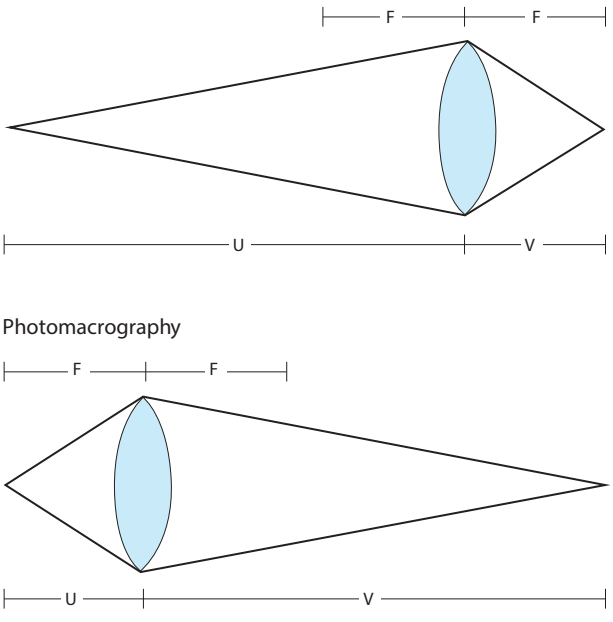

Figure 3.13 In this illustration, the typical lens conjugates found in more traditional applications that make images that are smaller than the object are shared in the top view. The lower view shows the lens conjugates resident in macro applications where the image size is larger than the object. U is the object distance, V the image distance, and F the lens focal length.

Lenses will all have common shapes. A simple positive lens can have two convex surfaces or one surface that is convex and the other surface flat. A biconcave lens is a single element lens with both of its surfaces curved inward towards the center. This lens is thicker at the edges than in the central region. A meniscus lens is a single element lens that has both spherical surfaces curved facing in the same direction.

Lens conjugates describe locations in front of and behind a lens. Conjugates predict where a lens will focus an image or where an object should be located. Lens conjugates play a role in design and refractive considerations but, more importantly, how the lens is used in application. A lens will have a principal focal length, and this is the location where an image is focused when the lens is focused at infinity. There will also be a front focal point for a lens. When a lens is used for reduction applications the object will be larger than the image, and the distance from the scene to the lens

will be large and the distance from the lens to the sensor will be small. When the image size equals the object size or life size, the front and rear image distances will be equal and will be two times the focal length. When the image size is greater than the object size, the object distance will be small and image distance will be large. This situation is evidenced in Figure 3.13.

Image size will always be proportional to focal length. An increase in focal length will produce a larger image. Depending on the focal length and maximum aperture required, various lens configurations can be used to increase image size. A 100 mm lens will make a larger image than a 35 mm lens if the photographer does not change working distance. A telephoto lens uses a compact configuration to achieve a longer focal length using different lenses in a specific order. In a telephoto lens, a negative lens is placed behind the front positive component. This increases image size and makes for a small and lighter weight design.

Teleconverters

A teleconverter is a precision optical accessory that can be mounted in between compatible lenses and the camera body and increases the focal length of the primary lens. This configuration creates a longer working (object) distance for a photographer and leads to the same magnification. If a teleconverter is used in combination with a 200 mm close-focusing lens, for example, it will yield a life-size (1:1) image and does so while maintaining a 71 cm or 28 inch minimum object distance. This amount of working distance is advantageous for many biomedical, industrial, and natural history applications.

Working Distance

Working distance is defined as the distance from the front of the lens to the subject. It plays a role in image size or magnification. Using a larger working distance allows for freedom in arranging lighting and photographing subjects that are challenging. In photographs where the object will be larger than the image, changing lenses and working distances will influence image perspective. Perspective describes how the elements in a photograph appear in the two-dimensional rendering of a scene. Foreground and background magnification differences are included in this process, as well as how straight lines are rendered. Images can have a strong or weak perspective based on the location of the camera in a scene. Subjects located within the scene will have different magnifications and locations within the frame if the location where the camera has been placed is changed. As the magnification increases, the working distance will decrease.

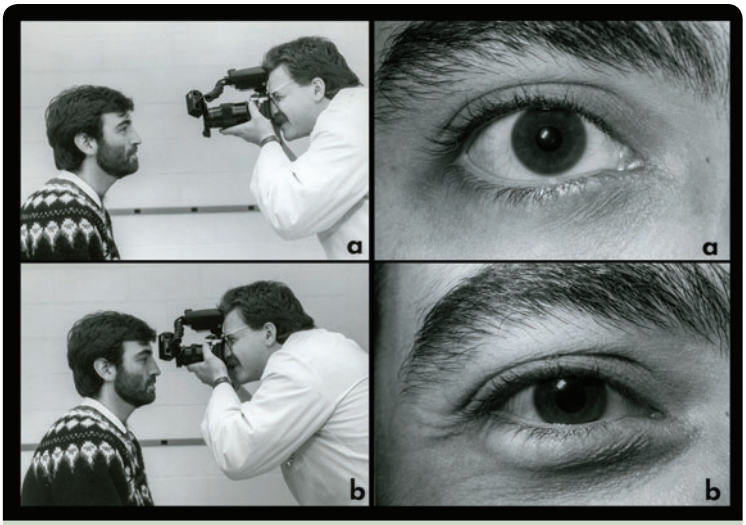

Figure 3.14 In this illustration, a 100 mm (top left) and 50 mm (bottom left) lens were used to create the corresponding images on the right side of the figure. The working distance when photographing at 1:1 is twice the focal length of the lens. While the images are relatively the same magnification, there are differences as a result of the camera's distance to the subject.

Close-up Lenses

Many relatively inexpensive digital cameras are fitted with zoom lenses that are adequately corrected for use in moderate close-up distances; however, DSLR cameras will have a wide variety of close-up or macro lenses available, both in fixed focal lengths and in zoom lens types. A zoom lens with close-up abilities is often described as macro zoom. True macro lenses are designed to produce magnifications of 1:1 or greater.

Expensive macro lenses have elaborate internal mechanisms that allow groups of lens elements to move independently as the lens focusing is extended to close distances, and create excellent life-size (1:1) or slightly larger images. These lenses will always have reproduction ratios inscribed on the barrel, possess modest maximum apertures ($f/2.8$ to $f/4$) and come in various focal lengths used to achieve various working distances. An image magnification of 1:1 is 1:1 regardless of focal length. Important optical characteristics are designed into macro lenses, including excellent image definition. They have been highly corrected for color and spherical aberration as well. Their most important feature is their ability to produce edge-to-edge sharpness when used for small working distances, especially when used with flat subjects.

Figure 3.15 This figure includes three different lenses that can be used for close-up or macro work. A Canon 24–60 mm macro zoom is featured on the left, Canon 50 mm prime macro lens in the middle and a Canon 65 mm macro on the right. They have all been designed for different applications and should not be considered equal, but do allow for close-up applications.

Supplementary Lenses

The design of most fixed focal length camera lenses has been optimized for long camera-to-subject distances and often they will have a minimum focus distance of approximately 50 cm or 127 inches. This distance can be reduced and the image size increased by using a supplementary or close-up lens. A close-up lens is a high quality simple magnifying lens. These lenses will either be a single lens or an achromatic doublet that attaches to a lens like a filter. These lenses are sometimes called plus lenses or plus diopters. Using a diopter is the easiest way to achieve close-up images. It is always best to attempt image creation without the addition of lenses when possible, since any additional lenses to a primary lens can degrade optical performance. The optical power of supplementary lenses increases as

the diopter number increases, for example +1, +2, up to +20. The diopter number of a lens is the reciprocal of its focal length in meters which is expressed:

$$D = 1000 \text{ mm}/f \text{ or } f = 1000/D \text{ or } D = \frac{1}{f} \text{ or } f = \frac{1}{D}$$

So, if using a +2 close-up lens on a focal length of 0.5 meter (500 mm) the lens will have a focal length of 0.20 meter (200 mm). Alternatively, it is possible to convert all values to diopters and then convert their sum to focal length. For example, a 50 mm lens has a power of 20 diopters. Add a 2+ supplementary lens and the sum is 22 diopters. The combined focal length is 1000 mm divided by 22, or, about 45.4 mm.

It is necessary to know the combined focal length of the camera lens and supplementary if it is required to calculate the lens-to-subject distance using the following common lens equation:

$$u = \frac{v - f}{f}$$

where u is object distance and v is image distance.

Supplementary lenses can be combined to obtain even greater magnification. However, when combining close-up lenses, the strongest should be closest to the camera lens. Image degradation will increase dramatically when more than two supplementary lenses are combined. Supplementary lenses can also be used in combination with extension rings and bellows in order to minimize the total amount of lens extension. Shorter lens extension produces shorter exposures. Shorter exposures may, for many subjects, offset the theoretical advantage of using a camera lens with extension tubes alone. Using a small lens aperture may minimize the aberrations introduced when using simple, positive close-up lenses.

Supplementary lenses can be combined to obtain even greater magnification. However, when combining close-up lenses, the strongest should be located closest to the camera lens. Image degradation will increase dramatically when more than two supplementary lenses are combined.

Mirror Lens

A mirror lens is a special lens and is comprised of a catadioptric optical system where refraction (lens) and reflection is combined into an optical system or lens. Mirror lenses are sometimes called CAT reflex lenses, or mirror lenses, and have long focal lengths. Because of the way the image is created there is a big reduction in the physical length of the optical assembly owing to the folded optical path. Mirror lenses typically have focal lengths of 250 mm and beyond 1000 mm. CAT lenses are much shorter and more compact than telephoto lenses and are lighter as well. Because of the limited lenses in the lens chromatic aberration is well managed by the catadioptric optics.

Because CAT lenses have the central element, a reflection mirror, as part of the folded optic design, they do not have an adjustable diaphragm This means the lens has a fixed f-number. One of their most characteristic image artifacts is the annular shape of defocused areas of the image, giving a doughnut-shaped "iris blur" or bokeh, which is the result of the entrance pupil. "Bokeh" is a modern word that has replaced "circles of confusion."

Telecentric Lenses

A special lens used in technical photography is a telecentric lens. Telecentric lenses produce orthographic images. The true advantage of these lenses is that the image magnification remains the same independent of an object's distance or position in the field of view. These lenses are used for making semiconductor chips, for optical lithography, and for metrology. Because their lenses maintain magnification, they are also very useful in machine vision systems. They can be very expensive.

Photographic Filters

Filters are important optical elements that are used in imaging. Filters modify light through absorption, transmission, or interference mechanisms. Filters can also modify light using refraction or diffraction methods. A filter that is part of an image-forming system must be of high optical quality. Filters that are used for illumination systems can be of a lower quality since image formation will be unaffected by their presence except for heat-absorbing filters. Filters come in many materials and sizes, and can be made of gelatin, cellulose acetate, polyester, plastics, glass, or multilayer dielectric coated materials.

White light is composed of UV, B, G, R, and IR. Absorption filters are common. With this type of filter, absorption occurs and the remaining light is transmitted as modified by the filter. The amount of light absorption that occurs in a filter is typically wavelength dependent. Often absorption filters are composed of primary colors, which include red, green, and blue. A red filter will transmit red light and absorb green and blue light. All filters will remove light and lower image brightness. Exposure compensation will be required to a varying degree when a filter is present in an imaging system. Primary colored filters were very common in film photography and now have found use in Bayer filters located directly on digital image sensors used in DSLR cameras. See Chapter 4 for more on this topic.

There are two types of absorption filters, broad and narrow band filters. A broadband filter transmits a wide range of wavelengths. For example a #25 Red filter would transmit the energy that is ~600 nm or longer, while 400–599 nm wavelengths will be absorbed by this filter. Broadband absorption filters are used to separate red, green, and blue colors required to form full spectrum color digital images. The filters are located on pixels used in CCD and CMOS sensors that are actually C, Y, and M combinations. Red, green, or blue filters were frequently required for use in film photographic applications to create differences in black and white image tonality since reds, greens and blues all reproduce as medium gray. Red filters will darken blue and green subjects and lighten reds. Green filters will darken red and blue subjects and blue filters will darken green and red elements of a scene.

A narrow band filter might transmit 440–460 nm wavelengths, for example. Narrow band filters are frequently manufactured as interference filters and are not absorption filters, but serve that same purpose. Narrow band filters are key elements of exciter filters and cubes that are used in fluorescence and confocal microscopy. Using modern technology, it is now possible to produce a filter whose transmission contains just two wavelengths.

Polarizing Filters

Light is believed to travel in waves that are oscillating in all directions. To help visualize this, consider the light to be oscillating north and south and containing a second component that is oscillating east and west. Using filters, light can be modified to oscillate in one direction only. When this condition is achieved, the light is described as being polarized. Polarization occurs naturally as reflections from flat surfaces. These might include the surfaces of water, leaves, roads, parts on cars, windows, and in the atmosphere 180 degrees opposite the sun. A polarizing filter is a type of filter that has rows of aligned molecules that transmit light waves traveling in only one plane and blocks the light that is oscillating in a direction 90 degrees to the transmitted light's orientation. Linear polarizing filters can be used to darken blue skies by removing the light polarized (aligned) by the atmosphere and to increase image contrast of a scene by removing of surface reflections from glass, metal, wood, and plastics. The light that makes up a reflection has been reflected by a material and is traveling in only one orientation. In Chapter 7, methods used to create a polarizing imaging system are shared. See Figure 7.12.

Linear polarizing filters cannot be used for autofocusing cameras or with in-camera metering systems because they remove the polarized light before it reaches the light meter. A circular polarizer is required for these applications. Circular polarizers contain a linear polarizer as the first element of the filter that orients the light in one direction. The linear polarizer is then followed by a quarter-wave plate, which modifies the now linearly polarized light. This modification allows the light to travel in a circular motion and remain polarized before entering the camera, and this light does not affect the meter.

Neutral Density Filters

ND filters are used to lower image brightness in the red, green, and blue spectrums evenly. ND filters have no color component to them and are used to remove brightness only. ND filters typically come in variance densities. A filter of a 0.3 increment will effectively remove 50 percent of the light that goes through the filter. Filters are sold with numerous densities including 0.1, 0.6 and 0.9, for example. Microscopes routinely come with ND filters built in. In landscape photography, ND filters are often used to allow the use of long exposure times. This is desirable for making running water smooth, for example, or for other more aesthetic reasons. In microscopy, ND filters are used to make the illumination comfortable for viewing or imaging.

Interference filters differ from absorption or polarizing filters and are created using multilayer dielectric (MLD) coatings designed to create constructive and destructive interference leading to the production of specific wavelengths. Their transmission will depend on the number of layers, their thicknesses, and chemical composition. A dichroic filter contains an MLD coating to transmit primary colors and reflect complementary bandwidths or wavelength ranges. Dichroic filters are used as mirrors or beam splitters in fluorescence and confocal microscopy imaging systems. These filters reflect unwanted color and transmit complementary colors.

An infrared filter is a visually opaque filter that transmits only a very small percentage of the far red light, but transmits the near-infrared region up to 1000 nm. Near infrared would

be characterized as 700–1000 nm. Certain other filters transmit ultraviolet radiation and block the visible spectrum. Another spectrally related filter is a haze filter that is a pale yellow filter used to absorb scattered UV and extreme blue light produced by atmospheric scattering. A camera barrier filter is used for fluorescence photography and is used to absorb the excitation energy, allowing only the fluorescent wavelengths to enter the lens and be recorded. (More on fluorescence can be read in Chapter 7.) This filter would be located somewhere before the fluorescent light enters the camera or before it leaves the microscope.

Aberrations

All lenses are prone to flaws that can affect performance and these shortcomings are described as aberrations. Aberrations can be broken into categories that affect definition, shape, brightness, and color. The most common aberrations are curvature of the field, barrel and pincushion, coma, spherical and chromatic aberrations, and astigmatism. There are numerous other aberrations. In the context of this text, space will not allow a complete listing or discussion of all aberrations.

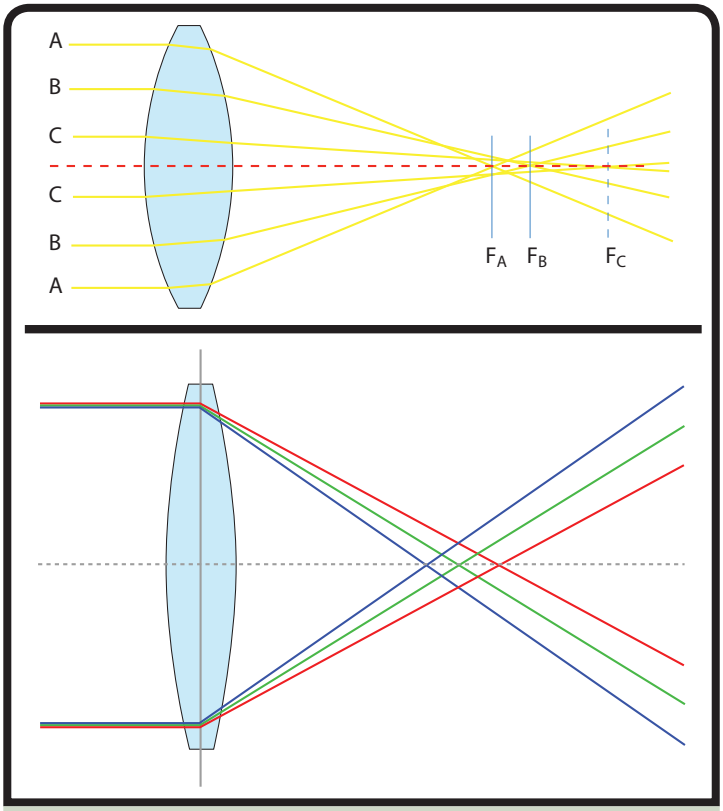

Figure 3.16 Top: Spherical aberration refracts central and peripheral light energy differently leading to images that are lower in contrast and noticeably soft. Portrait lenses by design contain spherical aberration. Bottom: When chromatic aberration is present, red, green and blue light is refracted to different points of focus producing colored halos, or leads to softness around hard, edged objects.

Curvature of the Field

Lenses that have this aberration form images that are curved and not flat. Often when using a microscope, the subject has been prepared as a thin section. General photographic lenses are designed to photograph a world that is equidistant from the front surface of the lens. Curvature of the field is the natural result of using lenses that have curved surfaces. The microscope's objective could not focus in the center and the periphery simultaneously because of the differences in distances to the sample field of view without correction. Consequently, the image produced by this lens unless corrected will exhibit focus in the center or the outside, but not in both regions at the same time. When a microscope is being used for inspection purposes, this is not a problem because the focus can continually be changed. When photography is required, this aberration is a problem. The lens must be corrected for use with flatfield applications. See Figure 9.6.

Spherical Aberration

Spherical aberration is a condition where a lens refracts points of light differently in the middle of a lens than in the periphery. This is a refractive problem and forms an image that seems low in contrast and definition. To create optical resolution,

a lens must collect at least two data points. A lens with spherical aberration will focus the central and peripheral data points in different image planes and will make the image appear more blurry than crisp. The resultant image will lack inherent contrast and will display haziness. Spherical aberration can be controlled a bit by using a lens aperture or a microscope's aperture diaphragm. Portrait lenses are designed with spherical aberration, which is useful to create a mood and softness.

Chromatic Aberration

Chromatic aberration is a condition where a lens will focus red, green, and blue at distinctly different image planes. In this condition, a lens will refract shorter wave energy (blue) to a shorter focal distance than the longer waves, e.g. red. The blue will come to focus first and be the largest image, while the red will focus furthest from the lens and will exhibit the lowest magnification. Chromatic aberration can often be observed in a microscope. It would be evidenced at edge of the focused blades of the field diaphragm that has been centered and remains partially visible in the eyepieces. Unfortunately, stopping a lens diaphragm down does little to resolve chromatic aberration. Regrettably, when photographing, there is only one solution when using a lens with chromatic aberration and that is to use only one spectrum such as green.

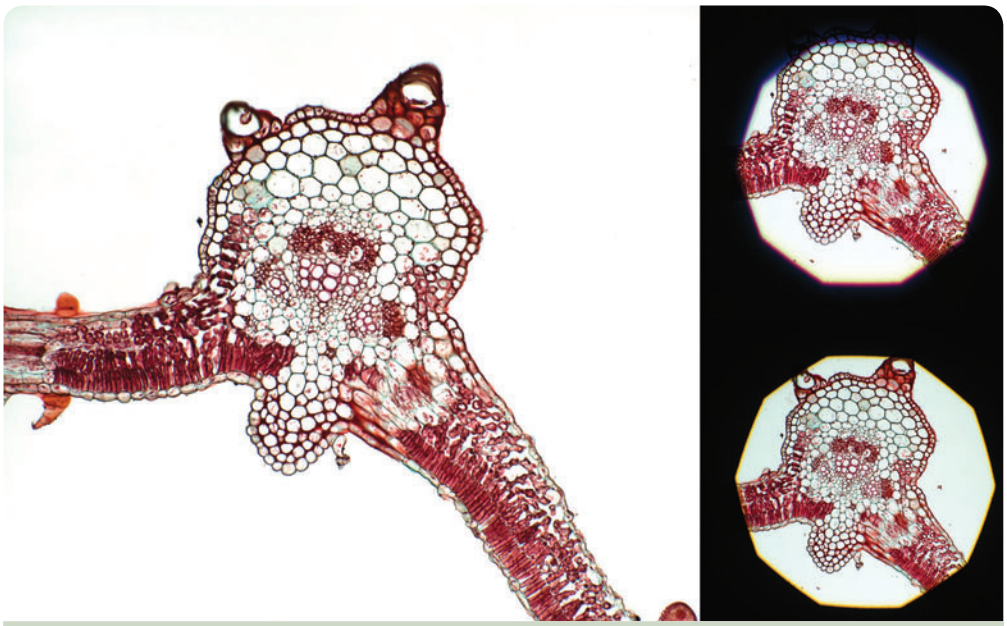

Figure 3.17 In this illustration, the appearance of chromatic aberration can be seen in the edges of blades of the field stop in images produced from two different substage condensers. These two images reveal the quality of two different condensers. On the top right, the Abbe condenser created an interesting outcome with red on one half and blue on the other side. An achro-aplanic created a much more "clean" image with the appearance of less chromatic aberration.

Image Depth of Field

The range of sharp focus in a picture is referred to as its depth of field. It is a function of image magnification and the influence of the lens aperture. Assessment of how large an acceptable range of focus an image exhibits is subjective and viewer dependent. As the image magnification changes so does the amount of available depth of field. Higher magnifications will produce a smaller range of focus. Using less magnification will lead to more DOF. When the magnification of a scene is reduced, there tends to be more depth of field behind the subject than in front. In close-up photography, though, the DOF distribution is more equal in the front of—and behind—the subject. The depth of field is the distance in front of and behind the object plane that is in acceptable focus. DOF can be controlled by use of the lens's aperture. A smaller aperture will create an image with a large DOF. DOF is located in the image. Depth of focus is located at the rear focal plane. In an imaging system that has a small DOF, such as a microscope, the system will have a large depth of focus. If there is a large DOF, there will be a shallow depth of focus.

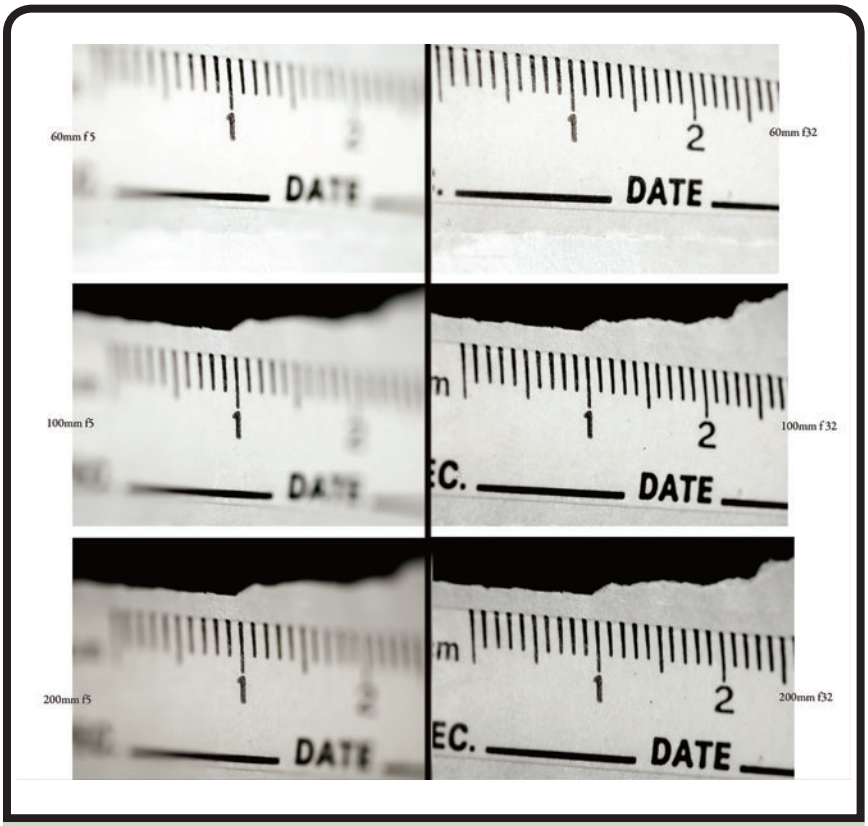

Figure 3.18 In this illustration three lenses of different focal lengths were used to create an image of 1:1 magnification. On the left is a series of photographs made at the maximum opening and on the right the corresponding photographs were made at the smallest apertures for each lens. In each pair, the resultant images are identical in the DOF. Sometimes there is a misperception that longer working distances or wider angles will make more DOF but this illustration shows this is not true.

Diffraction

An image's depth of field will increase as a lens is stopped down, but unfortunately there is a fundamental limit to how far a lens can be stopped down without degrading the image. The behavior that decreases resolution or sharpness is called diffraction. Diffraction can be evidenced when there is the bending of light as it squeezes through the aperture. In everyday photography, an image's DOF can be increased by using a small aperture setting, but as the magnification of 1:1 is achieved or surpassed, the use of very small apertures will produce diffraction. This results in the lessening of image sharpness much like a pinhole aperture would do. Diffraction effects are dependent on a sample's characteristics. Some objects have a potential to create more diffraction effects and others not so much.

Resolution of the image is *f*–stop dependent and assumed to be diffraction limited. This means that points on either side of the specimen focal plane will be blurred by the combined effects of optical and diffraction blur.

Figure 3.19 In this illustration, a butterfly wing exhibits varying degrees of sharpness based on aperture setting. The image on the top was photographed at 5x and using an aperture of *f*/2.0 and it is the most sharp. The middle images exhibit more DOF and are still sharp but lose critical edge definition because of diffraction. The image on the bottom has the most range of focus but is the least "crisp."

SUGGESTED READING

Compton, John, Ira Current, Richard Zakia, and Leslie Stroebel, *Photographic Materials and Processes*. London: Focal Press, 1986; ISBN-10: 0240517520.

Ray, Sidney, *Applied Photographic Optics*, third edition. London: Focal Press, 2002; ISBN-13: 978-0240515403.

Salvaggio, Nanette L., *Basic Photographic Materials and Processes*, third edition. London: Focal Press, 2008; ISBN-13: 978-0240809847.

Stroebel, Leslie, *View Camera Technique*, seventh edition. London: Focal Press, 1999; ISBN-13: 978-0240803456.

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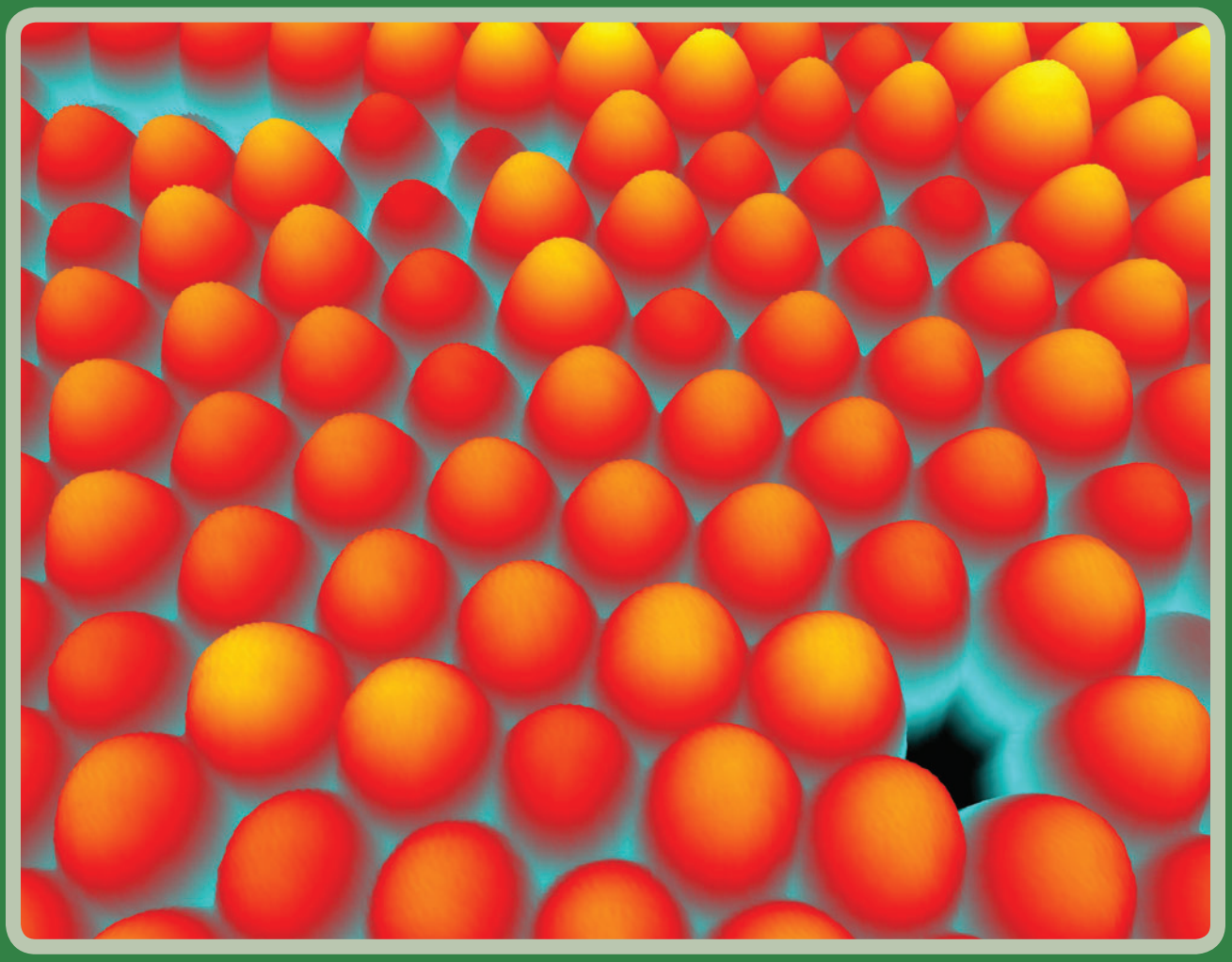
Chapter 4 Digital Cameras, Images, and Strategies



This image features an atomic force photomicrograph that reveals dislocations in a photonic crystal arrangement of polystyrene nanospheres. It was magnified approximately x350,000 at capture and features a sample that was approximately 1.5 µm × 2.5 µm. In the sample, photonic crystals were grown as self-assembled structures from a colloidal solution. The nanospheres each measured about 200 nm in diameter and are approximately 300 times smaller than a human hair, but approximately 500 times larger than atoms. Thorsten Dziomba of Physikalisch-Technische Bundesanstalt provided the image data and Dr. Frank Marlow of Max-Planck-Institut für Kohlenforschung supplied the sample. WSxM software was used to carry out the post-capture colorization. Image courtesy of Hans U. Danzebrink, Physikalisch-Technische Bundesanstalt, Braunschweig, Germany.

The Role of the Camera

The transformation of cameras from simple black boxes used for film into today's powerful imaging tools continues to be remarkable. The camera phone or smartphone is but one example of the numerous iterations the camera has taken in its evolution into the future. Even with all the advances cameras have experienced, the ultimate goal of a camera remains the same, and that is to take a picture. In science, this is might be more aptly called the recording of data.

Cameras come in many brands and varieties. Each type has various advantages, disadvantages, and features. Smartphones (cameras), DSLR cameras, compact digital cameras, and instrument cameras are but a few of the varieties a scientific photographer might select from many different reasons. The only common feature among all of them is that they all use a sensor to record an image (not considering the previous generations of film cameras). All sensors are not equal in performance. Costs, supporting software, application design, the transferring of images and other features will vary greatly across brands and types. This chapter will deconstruct cameras and what should be considered for their use in the scientific arena. The characteristics and features of digital image attributes will also be evaluated relative to the goals of making highly accurate and precise photographs. It will not be possible to include content specific to the hundreds of products on the market.

Figure 4.1 A pinhole camera was built and loaded with Kodak color negative film with an ISO of 400. The pinhole aperture was ƒ/512 and had a 80 mm focal length distance. A mechanical moveable shutter was designed, built, and added to the cardboard box pinhole camera that was loaded onto a drone. Once the drone was in position, the mechanical shutter was triggered remotely producing a one-second exposure. Image courtesy of Rochester Institute of Technology's Aerial and Drone Photography Class, spring 2015.

Camera Components

All cameras—even the most basic ones—will have the same types of components and controls and each is used for the same reason. That is to record light values using a predetermined increment of time. The role of the camera's housing is to isolate the sensor (film) from ambient light. The camera might simply be thought of as a sort of modern-day black box where the sensor is protected from the influences of secondary light sources. Cameras will need a shutter or opening where light can enter the camera and where a lens will be located. A lens is required to focus light onto the sensor that is situated a specific distance from the lens. It remains possible to use a pinhole to make a photograph. This remains common and appealing in the photographic arts. Images made with pinholes will be soft and poorly defined when compared to images made using lenses. See Figure 4.1. Ironically, a pinhole is used in a confocal microscope and contributes greatly to the creation of well-defined and completely focused images.

To make a properly exposed photograph, modern cameras need to measure the amount of light available for photographing, and then suggest and/or determine the proper shutter speed required for the measured brightness.

The brightness of the light will influence the selection of the correct shutter speed needed to achieve a proper exposure of the scene. Each of these components—including the aperture setting and a light meter—play a role in how a camera operates and the quality of the photographic outcomes that can be expected from the device.

All cameras will have controls that will include buttons, knobs, off/on switches, and a method for supplying power. Some cameras may be operated from a computer or phone that is located some distance away from the device. The camera's power may be supplied either using DC with an AC adapter, or through the use of a battery, or both. A camera will also need a port for storage media, or for a USB connection, or have other ports as needed. Modern cameras have ports that include video and audio input and output capabilities. Some cameras will be very basic and offer just a few choices for operation, while other cameras will be very complex offering many precise settings. The possibilities are seemingly endless. Camera technology is experiencing explosive growth and there are frequent upgrades to camera capabilities. Consider the Lytro® camera and its ability to create infinite focus using what is described as light field imaging as one example of what the future will look like.

Camera Modes of Operation

There are several modes in which a camera can be operated, allowing it to make an exposure. These modes include both manual and automatic methods. There are also several secondary modes that control features of the camera's operation. There are reasons why each mode should be selected for use with technical applications. Knowing the equipment and trusting what can be expected is the most fundamental consideration for selecting whether to use a camera in the manual or automatic modes.

Manual Mode

Using a camera in the manual mode of operation provides a scientist photographer total control over the elements of a photographic exposure. There are three components of exposure: the choice of the aperture setting located on a lens or in the optical pathway, the duration of time and corresponding shutter speed choice, and the sensor's sensitivity or ISO to light. When using the manual mode, careful attention must be paid to measuring the light correctly that is present for photographing. Once the light has been measured, the photographer must establish the proper time and aperture settings to produce a proper exposure. A proper exposure will create a file that exhibits detail in the highlights or bright tones and in the dark regions or shadows of a scene. This outcome assumes a scene brightness ratio that is recordable and can fit onto the sensor using a single time. In Chapter 14, high dynamic range photography is discussed as a way to manage large dynamic ranges that are impossible to record using a single time. Once the light has been measured, photographing in the manual mode provides the maximum control of variability from shutter or aperture changes caused by variances in the samples, or other environmental changes. Using manual mode will produce significant advantages in the consistency of exposures created across many images. Files produced using the manual mode—when the conditions do not change—will exhibit a high degree of similarity, useful for batch image processing. When working in challenging environments such as fluorescence applications,

using the manual mode will facilitate tighter exposure control ensuring the creation of files with detail in the important highlights.

Automatic Mode

When a camera is operating in the automatic mode, it will determine some or all of the settings required for the creation of a proper exposure. The camera identifies the correct exposure features by choosing the proper time needed for an exposure, or the possible aperture choices used for an exposure, where to place the lens's focus, how and where to measure light, the correct white balance, and how to adjust the camera's sensitivity. Some cameras will have more modes of operation than others. Within automatic operation, the camera can be operated using an aperture or program priority mode. In the program mode, the camera calculates the shutter speed and aperture setting basing the best choices on a balance of brightness and image stability. Scientific photographers should shy away from the program mode. There are too many locations where lack of control in this capture mode can lead to a wide range of image variances. Aperture priority can be more effectively used in science since the aperture controls the ever-important range of focus. DOF is an important image attribute and a higher priority for controlling than the shutter speed/aperture choice of program mode. A general awareness of the image attributes that come from using long times or with variances in DOF will always play a role in choosing one over the other.

Secondary Modes

Beyond the primary modes of operation for controlling exposure, cameras may have other secondary settings that are useful for other aspects of operation, too. This might include a burst or rapid-fire mode making a number of photographs in quick succession. There is maybe a single-shot mode that takes a single picture at a time when the shutter is tripped. When using autofocus mode, it can operate by either activating a single placement of the lens focus or being continuously active. Single-mode application is especially useful for stationary subjects and when focus is once locked will stay locked in, while a continuous focusing mode is useful for moving subjects. Never use autofocus when photographing science unless there are compelling reasons to do so. Autofocus produces images that are in focus but often the focus might not be located in the region of most interest.

Many cameras may also have a flash mode. Using a camera in flash mode will allow the operator to select between the common flash settings such as fill flash or auto-flash, which allows the flash to be used for weakly lit areas. Red-eye reduction, another setting, discharges a pre-flash once before the actual flash photo is taken, which allows the subject's pupils to become small and thus reduce red-eye. A pre-flash may trigger secondary flash units located in a lab. These units may not be synchronized. Having the flash in the off mode will turn off the built-in flash. Use of an on-camera flash, while convenient, is not an ideal strategy for making highly useable images.

Photographic Exposure

Depressing a shutter and recording light values is photography. The duration of time that light will strike a sensor is one of the components required for a photographic exposure to occur. The intensity of the light is controlled by the lens and/or aperture. The third component of exposure is the sensitivity of the sensor. All of these influence the exposure of an image. An exposure is defined as a function of time and light brightness using the following equation:

$$H = E \times t$$

where H is the total exposure, E is the brightness or volume of the light (controlled by an aperture) and t is the time increment that is selected. Any combination of time and intensity can be used to achieve the correct exposure when matched to the sensor's sensitivity correctly. A correct exposure can contain various combinations of time and intensity to achieve a result. Bright light will require the use of shorter times and low light levels will require the selection of longer times. Consider for a moment that a drinking glass might represent a pixel (sensor) and water (from a faucet) might serve as an example of the energy required to make an exposure. If the faucet is dripping into the glass, the glass will fill, but it could take many hours. If the faucet is opened to its largest discharge, the volume of water will be much greater and the glass will fill in seconds. At the end of each of these situations, the glass or pixel will become filled with water (or photons). All the following combinations of t and E that lead to the same H are considered to be equal and create a condition described as reciprocal exposures.

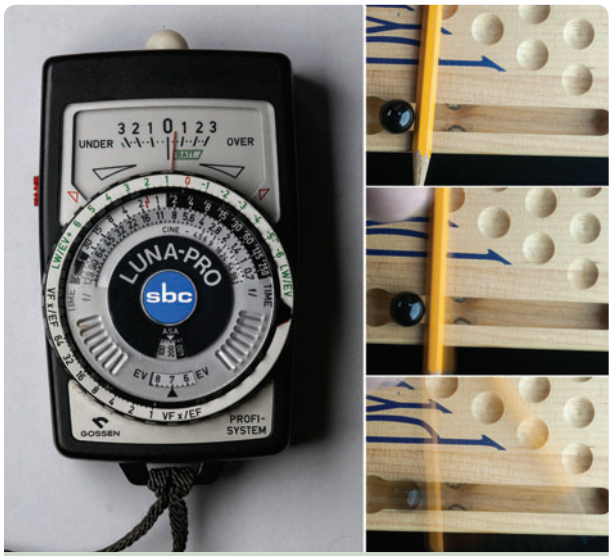


Figure 4.2 Reciprocal exposures deliver the same "amount of light" referred to as an exposure value or EV. Many combinations of time and intensity can create the same volume of light. Visible on the face of this Gossen light meter are the various combinations of time and intensity that create an EV of 7. One of the possible combinations is ½ sec. at ƒ/8. On the right side of this composite photograph are three photographs made using EV 7. The top photograph used 1/30 sec. at ƒ/16, the middle used 1/15 sec. at ƒ/8 and the bottom ½ sec. at ƒ/4. In each photograph, there are variances in image sharpness because the pencil moved and released the marble from its position in the track.

The following times and aperture selections will all create equal exposures; however, the images produced using these various combinations may be different in image attributes. The subject may blur or the image's DOF may vary.

1/1000 sec. @ ƒ/2.8 : 1/500 sec. @ ƒ/4 : 1/250 sec. @ ƒ/5.6 : 1/125 sec. @ ƒ8 : 1/60 sec. @ ƒ/11 : 1/30 sec. @ ƒ/16 : 1/15 sec. @ ƒ/22 : 1/8 sec. @ ƒ/32 : ¼ sec. @ ƒ/45

Light Measurement

Every modern camera has some base ability to measure the amount of light available for photographing. This feature or tool is called a light meter and converts the light to information required for determining a correct exposure. Establishing the proper time and E (aperture setting) is based on the scene or an object's brightness. Meters can be built into a camera or they can be external to a camera. Handheld light meters are now experiencing a reduced role because digital cameras can display a histogram and image,

both on a camera's LCD display, or on a computer monitor, if the camera is tethered in real time. Many cameras also have live view, which enables the image to be visualized during production without the need for external light measurement. Live mode is a powerful tool but will consume battery power to operate.

Handheld meters are capable of measuring light that is falling onto a subject or the light that is reflected back to the camera from the subject. In either situation, a light meter converts brightness to 18 percent gray. This is an important fact to keep in mind. White objects are interpreted by the meter as 18 percent gray and dark objects will also be interpreted by the meter's cell as 18 percent gray. To produce a correct exposure, the information from the meter must be adjusted. When photographing dark objects, it is necessary to reduce the exposure time by a factor of approximately –2 EVs, and when photographing bright objects exposure times must be lengthened +2 EVs. EV stands for exposure value, or exposure stops, and represents the total energy available for exposure, including time and aperture influences; it reduces or lengthens an exposure by a factor of 2.

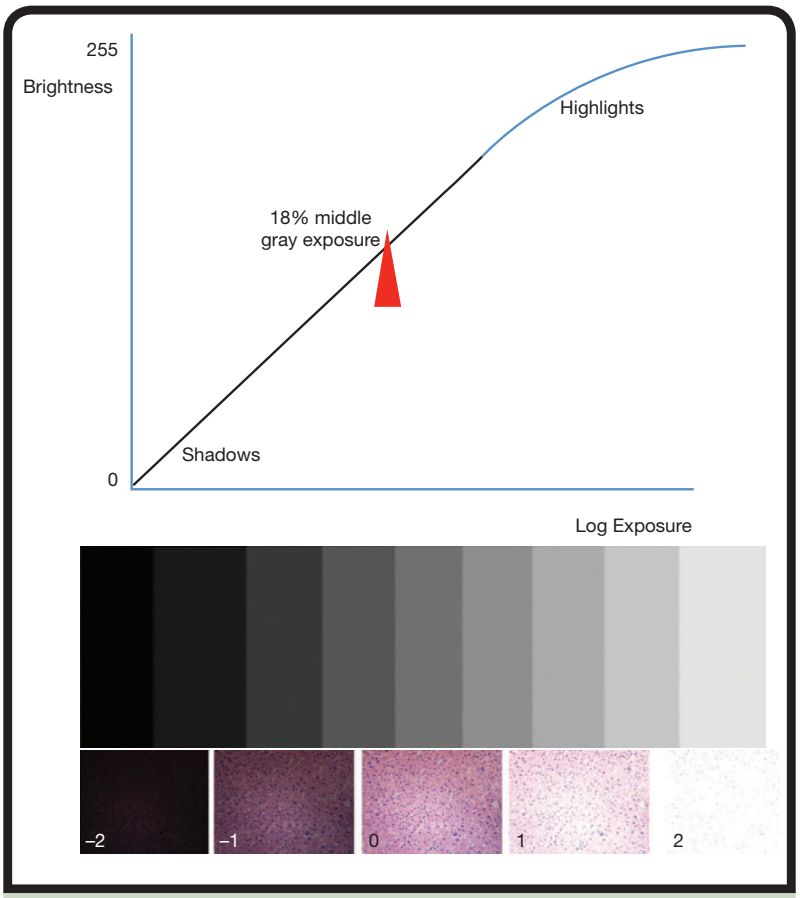


Figure 4.3 In this figure, a characteristic curve shares how tones are reproduced when using a ten-step grayscale image using a digital sensor. A ten discrete tonal scale has been an important photographic tool in photography. A tonal scale is influenced by a camera's bit depth. At the bottom of this figure, five exposures or bracketed exposure ranges are displayed, ranging from a –2 EV setting through the correct or 0 exposure and then on to +2 EV on the right.

Meters can produce readings that are global or average across the entire scene or measure only a small spot in the scene. They can also be center-weighted or measure the amount of light in the periphery of the camera's field of view. In the average mode, the meter will average all of the brightness across the entire field of view. In a center-weighted meter, the primary exposure information will come from the central 60–80 percent region of the field of view. Spot metering is the most precise method and can measure a very small region and a very specific region of a scene, sometimes down to a 1 degree area of the scene. Spot metering is very accurate and is not influenced by other areas in the frame. This method can be useful in challenging imaging situations such as in fluorescence applications. There are spot meter features found in many DSLR and instrument cameras.

Shutters

Regardless of the quality, a camera must have a shutter and a way to activate the shutter. The shutter is one of the most fundamental components of a camera. The camera's shutter will open and close in the light's pathway, allowing or eliminating the ability of light to strike the sensor. When the shutter is opened, the light will strike the sensor for a specific time. The specific time that is used is called the shutter speed. As computers and microprocessors have found their way into cameras, electronic shutters have become the norm. They are designed to deliver any time increment needed and are not limited by constraints typical of mechanical shutters. In the film era, camera shutters were operated at 1/60 sec., 1/125 sec. and 1/250 sec., for example. Each time was a doubling or halving of the adjacent increment. This was how mechanical timing technology worked. Contemporary shutters are capable of producing a continuous range of shutter speeds seamlessly from very long to a very short 1/8000 of a second. When the shutter is closed, the pathway to the sensor is closed, which blocks the light from reaching the sensor.

Types of Shutters

There are three types of shutters used in cameras today: leaf, focal plane, and electronic. There are also capping shutters that can be situated in front of a lens. Those used during the film era and now contained in all DSLR cameras are focal plane shutters and electronic shutters. Large-format cameras and some medium-format cameras use primarily leaf shutters. Smartphones use solely electronic shutters.

Focal Plane Shutters

Modern focal plane shutters have a mechanical component and also electronic controllers. A focal plane shutter might be referred to as a slit shutter. It is called a slit shutter because, as the shutter opens, it behaves like a slit that travels across the sensor. When the shutter is activated, the first curtain or gate opens and at the appropriate time the second curtain or gate begins to close. This allows only a portion of the entire sensor to be exposed to light for a predetermined and specified time but can distort moving objects.

There is only one time when the entire sensor is revealed to light, using the shortest possible duration. This time should be used for the synchronizing of the shutter to the duration of light produced by an electronic flash. This time is called the X sync speed. It might be indicated using an X or with a lighting bolt symbol 7 that is printed on the camera. If the shutter opens for too short a period of time when using electronic flash, a partial exposure to the sensor may occur. This is revealed by the presence of a dark region in the image at one side or the top or bottom (Figure 4.5). This occurs because one of the shutter's curtains has begun to close before the first curtain

Figure 4.4 This photograph—made by Jacques Henri Lartigue in 1913—is entitled *Race Car*. It is a classic example of how a focal plane shutter can affect an image's shape when photographing subjects that are moving. The car's wheel has been stretched because the car was traveling in the same direction as the travel of the focal plane shutter. When the shutter opened while the car drove from the left of the scene to the right, different parts of the wheel were in front of the film for different lengths of time. Image courtesy of http://themotorsportarchive.com/2012/08/23/jacques-henri-lartigue/.

Figure 4.5 When a shutter is opened using a time where the entire sensor is not fully exposed, a shadow from one of the curtains will be cast onto the sensor. In this example a shutter speed of 1/1000 was used for the top left picture; on the top right 1/500 was used; on the bottom left 1/250, and on the bottom right 1/125 sec. The time on the bottom left represents this camera's sync speed.

has reached the other side of the sensor. Once the first curtain has traveled across the sensor, the flash is discharged; however, the second curtain is now in the light path. The curtain will then cast a shadow of itself onto the sensor during exposure. The degree of error will be revealed by how large or small is the shadow that covers the sensor. Using a camera's built-in flash will be controlled by the camera. When using external flash equipment and operating the camera in the manual mode, it is possible to select and use both a correct and incorrect sync speed. Shutters can have travel directions that are up and down or side to side.

Using a longer shutter speed than the sync speed can be accomplished and creates an exposure that contains ambient or secondary lights. This exposure may result in images that have both frozen and blurry components and is referred to as dragging a shutter. Dragging a shutter may produce a ghost image from the ambient light part of the exposure if things within the frame move.

More on Sync Speed and Focal Plane Shutters

An electronic flash is an important tool for science photographers. Flashes can be useful for stopping motion of moving subjects. Using a flash requires that the shutter and the pulsed light be synchronized. The shutter speed that allows the entire sensor to be visible to light at one increment as mentioned is called the sync speed. If the curtain travels the long or horizontal dimension of the sensor, a longer time will be needed because the distance is longer. A vertical traveling shutter will use shorter shutter sync speeds. This can be useful information when working with challenging subjects and very short durations of light are needed.

When the front curtain is used to sync with a electronic flash's discharge, this method is the camera's normal or regular sync setting. In this application, the flash is discharged when the first curtain reaches its maximum opening and reveals the entire sensor prior to the second curtain beginning to close. Electronic flash is useful because it will freeze the motion of the subject or eliminate image shake/vibration from an exposure. It is challenging to use flash for the creation of magnified images, where vibration effects can be most problematic. It is possible when using front curtain sync to use a slower-than-the-suggested sync speed. In this instance, the rear curtain does not close immediately and may remain open for a predetermined time. As mentioned above, this is called dragging a shutter and leads to the creation of additional exposure from the ambient light and the possible blurring of the subject. This blurring is the result of sample movement during the ambient light exposure. With front curtain sync speeds, the blur will appear in front of the subject when the flash fired. Many DSLR cameras may also refer to this as the fill flash setting. This problem can be overcome by opening the shutter separately from the discharge of the flash. Start the exposure using the ambient light and then, when desired, manually discharge the flash

before closing the shutter. In this approach, the blur will be located behind the sharp and well-defined flash exposure.

Rear curtain flash sync describes a condition when the second curtain triggers the flash as it begins to close. When this occurs, the ambient shutter exposure will occur first, causing the blur to be behind the subject when the electronic flash is discharged. The blur in this condition will appear to follow the subject and will appear more natural to viewers. Many DSLR cameras come with rear curtain sync settings that can be selected in the camera menu.

In the film era, shutters were made of cloth, but in the digital era they are thin blades of metal alloys, including titanium. Cloth was primarily used in film cameras but has been abandoned in DSLR cameras because microscopic fibers from the cloth might find their way onto the sensor and weight. The fastest shutter speeds of professional SLR film cameras was 1/1000 sec.; however, modern DSLR shutters are routinely capable of 1/4000 sec.

Digital sensors are fixed within the body, and because of magnetic or static electric fields they are vulnerable to dust and particles that are attracted to them. The more a camera body is open, the more dirt will find its way inside and onto the sensor. Many DSLR camera sensors will have a cleaning function that should be used frequently. This is a very important step in the creation of images that will require the minimum of image retouching. Dust particles will cast shadows onto a sensor and often seem to find themselves in regions of a photograph where removing them is not possible for reasons of scientific veracity. Shadows will appear as black well-defined dots (Fig. 4.6). There are numerous ways to clean a sensor. The easiest method is to use the camera's clean sensor mode. If this is inadequate, there are other ways to clean a sensor but these come with some risk. There are tools and products available for cleaning sensors. The use of pressurized air from a squeezable bulb is desirable over physical contact. Aerosol air should be avoided because the propellant may end up on the sensor and be difficult to clean. There are a number of cleaning brushes that also are possible tools. Use of brushes and moving a cleaning tool across the surface of the sensor is always accompanied by the risk of creating a charge or exposure on the sensor. Cleaning the sensor should be done with the camera pointing downward and turned off to eliminate the static charge. In this fashion, the dust and particulate matter will fall downward.

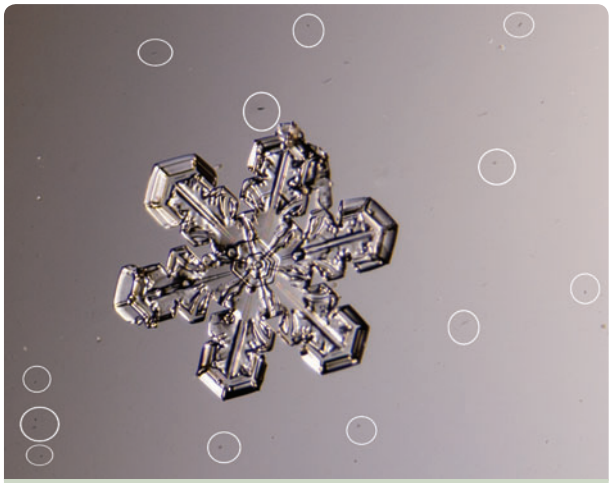


Figure 4.6 In this photograph, dust has found its way onto the sensor and is scattered throughout the image. Dust creates dark spots in the image because it casts a shadow from itself onto the sensor. It is easy to clean dust from the sensor but it can be a hassle or not possible to remove the dust from the image file. The dust has been circled to emphasize its presence.

Leaf Shutters

Different than a focal plane shutter is a leaf shutter. Leaf shutters are located within a lens and not in the camera itself. Focal plane shutters are located at the focal plane of the camera, which is very near to where the image will be focused onto the sensor. For this reason, focal plane shutters are considered more practical than leaf shutters since only one shutter is needed per camera and can be used with various lenses. One leaf shutter is needed per lens

because leaf shutters are located within the lens. Phase® medium format digital cameras and a few other medium format digital cameras still use leaf shutters in their cameras and lenses. Leaf shutters were preferred for studio photographers because leaf shutters could sync at any shutter speed. There is not just one sync speed. Since the shutter is located within the lens, it opens from the center of the lens, then moves to the periphery and the back. In this way, the whole sensor is exposed to light for the entire time of exposure. The duration of time from closed to the maximum opening and then back to closed constitutes the time available for exposure. By opening and closing in this manner, the entire sensor is exposed to the entire field for the whole duration of the event. This allows for greater flexibility for the synchronizing of high-speed events using shorter duration lighting. Leaf shutters were also integral parts of large format camera lenses when 4 x 5, 5 x 7, 8 x 10 and 11 x 14 film formats were common in high-end photographic studios.

Electronic Shutters

Electronic shutters are found in smartphones, mirrorless and DSLR cameras. Electronic shutters operate by turning on and off a pixel's sensitivity to light. It is remarkable how much engineering is in that little chip. Electronic sensors/shutters allow a sensor to create video. Focal plane shutters are used in DSLR cameras primarily to provide an absolute block to light but when making still photographs with a sensor's electronic shutter mode operating in the global mode. The global mode means that the whole sensor creates a simultaneous capture. The focal plane shutter controls the duration of time that the sensor is exposed to light. Focal plane shutters are located in front of the sensor and operate in harmony.

Electronic shutters are controlled by the camera's computer and require power to operate. In some ways, electronic shutters were invented when digital photography was invented. Their origins are directly linked to the evolution of the sensors, including charged couple devices (CCD) and complementary metal oxide semiconductor (CMOS) sensors. The photo site in a sensor is called a pixel. Pixels are unique in the way they work. When a pixel is exposed to light, and unlike film, the resultant exposure signal must be moved from the pixel before the pixel can be used again for a new exposure. This feature is sometimes called the rolling shutter feature. Moving the charge from the pixel allows the pixel to be refreshed so it can be used for the next exposure. Electronic shutters are much different than focal plane or leaf shutters because they are part of the sensor. There are no moving parts in electronic shutters. This can create very immediate responses and triggering. When using mechanical focal plane or leaf shutters, there will be a delay when triggering the shutter. The delay in an electronic shutter is only influenced by the delay in the triggering of the shutter function. Sometimes when a shutter is depressed there is a delay in the actual opening of the shutter. Early digital cameras had shutter lag before the camera would operate and make an exposure. This delay has been greatly eliminated, although it still may be present in the operation of inexpensive cameras; it is a non-factor in high-end cameras. When partially depressing a shutter, many components of the exposure, such as calculation of exposure, white balance, and focus functions, are operationalized, and when pressing the shutter button completely the instantaneous nature of the light recording occurs.

How the exposure is taken off the sensor has also changed over time. Not so long ago, CCD was moved from the sensor across the entire sensor in what would be characterized as a global movement. When some cameras are operating in the still picture mode, the focal

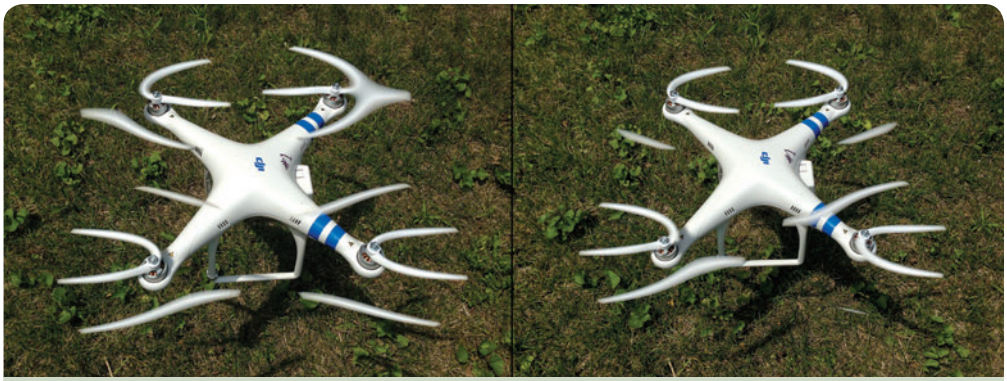


Figure 4.7 The effects of a rolling shutter are evidenced in how the rotating blades of a drone's propellers were recorded in these two pictures. Because of the way the exposure is moved off the sensor when photographing moving objects, sometimes objects can be distorted. In these two photographs, different artifacts have been created. Images courtesy of Ted Kinsman.

plane shutter opens and closes and the sensor records the data. This data is then moved all at once. This is important to allow the sensor to be ready to receive a new exposure. Once a sensor is charged from the exposure, the data must be moved all at once to either a buffer memory or storage media. As sensors have evolved, they have acquired both still and video capabilities. Using a camera for video, the sensor becomes what is called a rolling shutter where data is moved line by line immediately after exposure. The data is moved to a buffer where it is recreated into a contiguous image file that may or may not have existed on the sensor. When exposures are created this way, there is the potential for objects moving in certain directions to experience shape or complete distortion based on speed and travel direction relative to the direction of the data's movement. Pixel data that is offloaded row after row after exposure is characterized as a rolling shutter. Stationary objects do not experience this type of distortion.

Shutter Effects on Images

Since focal plane shutters travel across the sensor, there are times when the whole sensor is not exposed to light at the same time. Various regions of the sensor may actually see slightly different things. For an exposure time that is shorter than a camera's sync speed, a sliver of the sensor will be exposed to the object and a similar but unequal portion of the sensor will be blocked from light. For this reason and in certain situations, focal plane shutters could cause distortion of an object's shape when the subject is traveling at fast speeds. Compression or stretching of a traveling object can occur when a camera uses a focal plane shutter. If an object is traveling parallel to the sensor and is traveling in the same direction as the curtain, it is possible for the subject to travel synchronously with the curtain and become stretched as an image. If the object is traveling in a direction opposite to the travel direction of the curtain, it is possible for the object to become compressed in size. While not a frequent image artifact, it is possible for this to occur in specific situations with subjects traveling at high or slow rates of speed, particularly vertical shutter recording of objects that travel horizontally.

Vibration Effects

Because DSLR cameras use a mirror, the physical movement of the mirror and shutter during the exposure can cause image shake (Figure 4.8). Mirror-induced camera shake will lead to images that are not crisp or clearly delineated. This is most evident in close-up photography and other applications where the image's size is larger than the object's size. When the image size is smaller than the object size, the effects of image shake can sometimes be absorbed by the image and difficult to see. The vibration is present but absorbed by the reduction of the scene. When the image size is larger than the object, the effect of camera or environmental vibrations will create images that lack definition and will be easily noticed. Using shorter shutter speeds will actually increase the apparent loss of image detail from the shutter's mirror. Using longer or slower shutter speeds will actually minimize the affects of mirror shake. When using longer times, the exposure component that creates vibration will be smaller relative to the time of the exposure used to make the base exposure. Using a longer shutter speed can be achieved by using less brightness if image vibration is evident. Using neutral density filters is also an effective way to make a longer exposure time by taking away brightness without affecting the color temperature of the light.

Figure 4.8 This image reveals a lack of sharpness caused by image shake. In this photograph the shake is a result of tripping the shutter using a finger and shows the effects of mirror vibration. The image magnification was x5 at capture.

Image shake can be introduced by the camera's mirror or from other sources. Elevators, subway trains, and computer fans and fans located in lights can all be sources of vibration. Eliminating or dampening vibration should be a high priority for science photographers. Vibration reduction tables are expensive solutions but are excellent tools and very practical for removing vibration. Many, if not all, high-end DSLR cameras will have the ability to lock up the mirror after focusing has been completed. Mirror lock-up is very helpful in minimizing the image shake produced from mirror movements. It might also be practical to trigger the camera using a remote trigger or by tethering the camera to a computer. I sometimes use self-timer for triggering the camera and the delay shutter function. Instrument cameras do not use mirrors or form images and these types of cameras will not exhibit image shake from the camera itself. All cameras and imaging systems are vulnerable to environmental sources of image vibration.

Mirrorless Cameras

The simple fact that a camera's mirror and its mechanisms can be a source of vibration in DSLR cameras has inspired interest in cameras without mirrors for years. A Leica rangefinder was an example of a camera without a mirror. These types of cameras were called rangefinder cameras and operated without mirrors. Because they have no mirrors, they are quieter than DSLR cameras; however, the image and the scene will not be identical in composition. This condition is called a parallax error. Using a mirror and reflex viewing system requires space in the camera body but allows for precise composition. Without the

need for a mirror, cameras can be made smaller and can use various sized sensors as well. Mirrorless cameras can sometimes create images that are sharper because of less vibration since there is no mirror to move. One disadvantage of mirrorless cameras is that they do not have optical viewfinders. The image is viewable using an electronic viewfinder that can be secured as an accessory or is a part of the camera and is visible on the camera LCD panel. Composition is accomplished using the LCD display on the back of the camera. This type of camera might be considered equivalent to a compact digital camera but, unlike compact cameras, mirrorless cameras can use interchangeable lenses. Compactness, quietness, and possible increased image sharpness are all advantages over compact digital cameras when coupled with the ability of the camera to accept interchangeable lenses. Mirrorless cameras can be used effectively in the laboratory using live view mode. Almost all mirrorless cameras have similar sensor attributes to DSLR cameras.

Sensors

There are two main types of sensors used in digital cameras today. One type of sensor is a charged couple device (CCD) and the other a complementary metal oxide semiconductor (CMOS). In the active pixel CMOS sensors, the electrical power design has been re-engineered as the sensors have been improved, enabling power to be used more effectively. A CCD requires ten times more power than a CMOS sensor. This aggregating of electric design lowers power consumption by the sensor and image processing at the sensor. This in turn leads to more effective use of electricity and produces less heat and subsequent noise. A sensor is the most fundamental capture device in the camera and is also referred to as an array. Located on the sensor are photo sites or pixels.

Pixels

The basic element of a sensor is the pixel. It might be described as the photosensitive component of the sensor. Pixels are the basic picture elements of an image and are the smallest component of the picture file that can individually be processed. A digital camera's sensor or array might contain millions of photo sites or pixels that are used to record an image (Figure 4.9). When the camera's shutter button is activated, an exposure can begin. During the exposure, each pixel or photo site is uncovered and is exposed to the influences of photons. The pixel collects and stores the photons that fall on its surface. Once the exposure has been completed, the camera's shutter is closed and each pixel using camera software calculates how many photons have been captured in each pixel's well or structure. The quantity of photons captured in each pixel can then be characterized into various intensity levels or bits, which describe an image's brightness and detail.

Pixels might also be characterized as full frame or interline. CCD sensors primarily are comprised of full frame pixels. A full frame pixel's entire surface area is light sensitive and transports charges, but not at the same time. This data transfer occurs at slower rates than interline pixels. CCD sensors require a shutter but are capable of higher ISO and resolution, producing generally a better signal to noise ratio; they are frequently used in high resolution cameras. Interline pixels have a smaller light-sensitive area because some of the pixel is

used for electronics and transporting charges. An external shutter is not required for this type of pixel. They operate at lower ISO than full frame pixels and create less resolution but have faster frame rates.

The greater the number of tones that a pixel can discriminate, the greater the bit depth of the image, which in turn will reveal more data about an object. A pixel's tonal scale begins at 0 brightness and ends at 255 brightness. The most basic tonal scale would be described as an 8-bit image. A bitmap describes a pixel-by-pixel description of an image that is observable on a monitor or camera screen. When the resolution is high enough, the image is seen as a continuum or image structure; however, when there are insufficient pixels, there will be the appearance of picture elements or pixels. When the image appears to reveal pixels, the condition is sometimes called bitmapping. This occurs when the image display does not have adequate number of pixels required for a smooth display in the predefined size that is used for viewing. Bitmapping is done with sizes of pixels on all number of pixels. This can include both screen and print applications.

Single-shot Area Array Sensors

There are two configurations of sensors or arrays, one that produces simultaneous capture and one that uses scanned capture. Single-shot or instant capture sensors are the most common and are found in DSLR cameras and many other types of camera. In an area array sensor, pixels are organized in horizontal and vertical directions like a piece of graph paper. There are a specific number of pixels found in each direction. In a linear or scanning array camera, a single row of pixels is located in only one orientation. There will be a specific number of pixels in this single row of pixels. The number of pixels is fixed in either type of array. The linear array or pixel wand is moved across the lens's field of view using a stepping motor. A scanner is a common example of a linear array type device. Most pixel areas use rectangular shaped pixels but other shapes have been tried.

Most of today's commonly used DSLR and smartphone cameras are equipped with area array sensors (Figure 4.9). In this type of sensor, each pixel is covered with red, green, or blue colored filters located on top of each pixel. This type of sensor uses what is referred to as a Bayer pattern, which was invented by Eastman Kodak scientist Dr. Bryce Bayer in 1975. Each pixel captures only one channel of the scene as the red, green, or blue component. In this method, the missing RGB data for each pixel will be re-created or de-mosaiced (see below) mathematically by the software referencing the neighboring pixels. This process is called color interpolation. In a Bayer pattern array, the sensor will contain twice as many green pixels as red and blue pixels. This is because human vision is most sensitive to green light. The use of an area array sensor has several advantages. Most importantly, this type of array can be used for both moving and stationary subjects for both continuous and short duration lighting where exposures can be a few milliseconds or longer than a few minutes. There are, in most cases, no mechanical parts associated with this type of sensor. This sensor can also be made into various physical sizes and has been easily integrated into most types of camera, including those being used in security cameras, for example. Pixels too will have different sizes based on a number of engineering factors. An average pixel might be 7 μm in width, and with a few exceptions pixels are primarily square.

A disadvantage of an area array sensor is the simple fact that each pixel records just one channel. Because of this function, a mathematical reconstruction of data not captured from the other two channels is re-created by referencing neighboring pixels in a process called de-mosaicing. This can result in image artifacts. These artifacts are evidenced as color aliasing or color moiré effects. To manage this possible artifact, cameras use optical anti-aliasing filters in front of the sensor. The use of an anti-aliasing filter will create some loss of image sharpness because an anti-aliasing filter is a type of soft focus filter. Another disadvantage of this method is the additional time needed to create and process the data required for the image. Because the image result is influenced by the color information from surrounding pixels, images from different cameras may produce completely different color results.

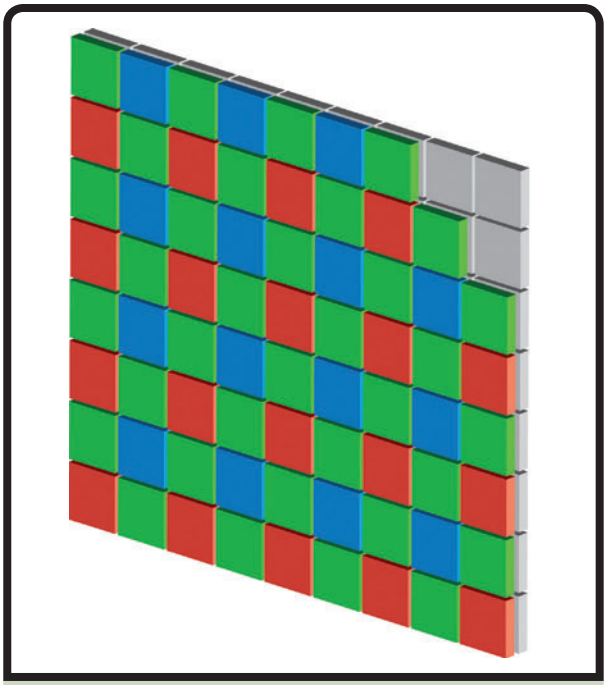


Figure 4.9 This illustration features a one-shot array sensor with Bayer pattern.

Area array cameras can also operate using a multi-shot format and were frequently used on instrument and studio cameras. They are not so common at this time. They were also used where an image might be the result of several photographs of a static object that are recombined into one larger file. A separate red picture, green picture and blue picture could be made and then a new file would be created from those files. There is no moiré pattern produced from this camera type. In this type of file, each channel has the most color data for a given sample and image processing can sometimes produce cleaner and better outcomes.

Another type of multi-shot camera moves the sensor small distances during the exposure, creating a slightly different field of view for each capture. This creates a file with more pixels than a single-shot capture can produce. This method is a common feature in Zeiss, Nikon, and Olympus cameras used on photomicroscopes. In this type of camera, a sensor might have 1300 x 1000 pixels in its single-shot mode, but when using multi-shot capture it creates files with more than 4000 x 3000 pixels. A multi-shot camera is significantly slower to operate and cannot be used with live or moving objects. Area array cameras without a Bayer filter are monochromatic cameras and are used for low light applications found in fluorescence microscopy and astronomy. Since filters remove light, having a Bayer filter on the sensor reduces illumination. A naked chip is most sensitive to light, and color can be added using what is described as pseudo-coloring using various image editing software products.

Linear Array or Scanning Cameras

Scanning cameras were part of the first imaging systems able to create files with enough pixels to be useful in applications where large output files were needed for print. These cameras initially were based on scanner technology. A scanning camera uses a single row of pixels located on the linear array or wand that is moved across an imaging field using a stepping motor. During the exposure, an array travels across the image area recording and

creating an image line by line. The linear array sensor often contained three rows of pixels. Each row of pixels was covered with either a red, green or blue filter or stripe that captured each color in a single pass. The significant advantage of scanning cameras was the ability to make a large imaging sensor at a low cost. Scanning cameras were used most often in studios where subjects did not move and very large files at a low cost were needed. There are not many cameras of this type still sold at this time; the Better Light® scanning back camera is an example of this type of camera and sensor and is most frequently used with large format cameras.

It should be obvious that a scanning camera can be used effectively with a stationary subject. Any movement by the subject or movement of the camera during an exposure will lead to an image with features that are not recorded correctly. Another disadvantage might be the long time required for capturing the entire image as it scans across the field of view of the lens. Bright lights are needed for this type of camera; however, scanners have the light source built into the scanning wand. Studio lights must keep a constant and high level of brightness during the exposure. If the light brightness changes during the making of the scan, the image will exhibit different brightness bands throughout the image file. Tungsten and tungsten halogen lights can change brightness several times per minute. This is called flicker, and if tungsten lights are used with this type of sensor they will create brighter and darker exposure bands in the image. Fluorescent lights are worse and add color variations to the darkness gradient. HMI lights are suggested for use with scanning back cameras.

Multi-shot Area Array Systems

Many early high-end digital color cameras used a monochromatic area array sensor. To create color using these cameras, a filter wheel equipped with red, green, and blue filters was located in front of the lens and sensor. Three exposures, one through each filter, were then created, giving a red, green and blue exposure of a subject that was captured in a series of three separate pictures. It should be obvious that color images could be made of stationary subjects only. Any movements of the camera or object would result in poor registration of the images from the red, green and blue channels. The color filters could be located in front or behind the lens. Both locations have advantages and disadvantages. If filters are located near the lens, flare or other non-image-forming light might affect their performance. If the filters are located behind the lens, the alignment of the filters or dust may be recorded in the image. While monochromatic cameras or cameras without filters remain popular and common in fluorescence microscopy, three-shot cameras are no longer common at this time.

Figure 4.10 This photograph includes the symbol ⊖ printed on a DSLR camera body that indicates where the sensor is located. This is useful for camera alignment in close-up applications. This is useful for camera alignment in close-up applications and important for determining magnification and exposure compensation.

Sensor Sensitivity, ISO, Binning, Gain

Adjusting the sensitivity of a sensor can be a very useful tool. The camera's sensitivity is factory fixed in a smartphone camera and most compact digital cameras. The sensor's sensitivity is adjustable in mirrorless or DSLR cameras. True instrument cameras such as those used on photomicroscopes can also have their sensitivity increased but this adjustment is made in a different manner. All camera sensors are produced with a basic sensor sensitivity setting. In DSLR and mirrorless cameras, sensitivity of the sensor is shared using ISO. ISO stands for International Standards Organization, an organization that oversees standards in various industries including imaging products. This includes sensitivity ratings for camera sensors. When an operator changes an ISO setting, that adjustment changes the camera's sensitivity to light. It can be raised or lowered. Today, it is more common to see ISO settings ranging from 50 to 16,000 or higher. These settings or numbers are direct indicators of the sensor's sensitivity to light. A lower setting makes the sensor less sensitive to light and a higher number, such as 16,000, makes the sensor significantly more sensitive to light. This is a significant range. The only real danger to increasing sensor sensitivity is the potential to create digital noise in the file. ISO is one of the three factors that plays a role in a photograph's exposure. The choice of shutter speed and the brightness, a function of the lens aperture, are the other two components of exposure. ISO can be controlled directly in the camera's menu or is accessible on many cameras as a switch on the camera body.

Instrument cameras also have a base sensitivity but it is not shared as an ISO. In fact, very few instrument cameras share their base sensitivity directly. It is simply a given. Increasing the sensor's sensitivity is accomplished using something called gain. Gain is shared as a factor or number. A factor of 0 would indicate no gain is being used at capture and, dependent on the camera, can be adjusted upwards from there. It is possible to increase the gain significantly. For more on gain see Chapter 10. Electronic amplification of the sensor results in the sensor working harder to record lower amounts of light. When the sensor is operating, it generates heat. Heat is not a positive element in a sensor and can result in what is described as noise. Noise is an outcome where pixels are affected by the heat, resulting in random exposure of pixels. Noise can cause image data to be compromised. If the ratio of signal (good exposure) to noise is too high, the image signal will not be discernible from the noise.

Binning is another feature available in instrument cameras that allows a sensor to collect more light by combining pixels. Light levels can vary across various samples. This can especially be true in fluorescence applications. Gain as described is a method that increases the sensor's ability to respond to lower levels of light using shorter shutter speeds. This can be useful but can create noise. When the noise levels compete with the sample's signal strength, gain is no longer a useful method. When this situation occurs, it might be possible to create an exposure by grouping pixels together. Aggregating pixels is called binning. Pixels can typically be grouped as two, three, four or more. Grouping pixels together increases the surface area of the pixel (group) and collecting area of the now enlarged photo site. Like all things, the disadvantage when binning comes from the down sampling of the total number of pixels available for an image. If a sensor has 1200 x 1000, for example, the setting of a 4 x 4 binning arrangement will cause 16 pixels to behave as one. When the sensor is set up this way, it will create a file that has 300 x 250 pixels. Binning is used when light levels are

so low there is no other way to make an exposure. Remember, any image is better than no image and thus a smaller file becomes better than no file. For more about binning and gain, see Chapter 10.

Noise: Dark, Shot, Sensor, and Evaluating Noise

Electrical devices generate heat, and when sensors are electrified they are vulnerable to heat production. Heat can cause pixels to be affected and behave as if they have been exposed. This artifact is evidenced and called noise. Noise can come from a variety of conditions. When the sensor is operating, the noise that is produced is called shot noise. Shot noise occurs when exposure times are long. Long is a relative term. In the not so distant past, exposure times of 10 seconds would produce noise in some cameras. All cameras are not similar in how they reduce for noise and modern cameras with improved sensors manage the creation of heat and exposure in improved fashions. All digital cameras are vulnerable to noise creation. DSLR cameras have a noise suppression filter or setting. Mirrorless cameras also have this setting. Noise reduction filters at a most basic level soften an image, allowing the noise or pixel information to be absorbed or blended into the image and making it less apparent. Instrument cameras can be equipped with ways to cool the chip and minimize the creation of heat and also have a noise reduction filter. Cooled chip cameras can be very effective for low light imaging and are found in fluorescence microscopy applications and in astronomy.

Because of electrical currents, all sensors make some noise even when not making an exposure. This type of noise is called dark current noise. Engineers have worked hard to improve the performance of sensors in this area but, because they are electrical, heat is an absolute component of a sensor's operation.

It is possible to test a camera for its performance capabilities, including noise production. This can be a very useful test to run. Testing a camera for noise and other image attributes can be undertaken in the following way.

Sensor Evaluation

Evaluating a camera for basic image quality performance, and noise production in particular, can be a tedious but useful task that provides scientific photographers with practical information about a camera's performance in challenging situations. When evaluating a camera for noise and other sensor-created outcomes, it is important to see the data produced from the sensor with minimal image processing enhancement or influence. The evaluation's objective is to reduce the camera's software biases on image attributes, allowing the test to reveal how the image capture attributes are affected in various situations. An ideal way to manage file attributes including noise from the analysis is to use high bit depth RAW files because of their better signal-to-noise ratio.

To start a camera's evaluation, it is important to ensure all capture settings such as image sharpening, noise reduction, and color enhancement are turned off. Noise reduction and other at-capture choices are a function of in-camera image processing algorithms and it is

important to remove them from the assessment. These settings are visible in the camera's menu of operational choices once it has been turned on.

To evaluate a camera's performance, some sort of standard target or sample will be required. For close-up photography, some type of a small color checker can be useful as the target, but when a system produces images that are larger than the object targets are less readily available. A sample needs various tones and fine detail. A useful target should include a black, white and gray region and also contain at the least some primary colors. When possible, a target that has fine details oriented both vertically, horizontally, and diagonally is preferable.

To evaluate a camera, all testing parameters must be kept identical to get valid image data outcomes across the various settings that will be used during the testing. This means everything that is done during the test should utilize identical camera set-ups, except for ISO or other settings choices; identical targets; identical lighting; identical framing; identical optical settings and lens use as well as similar diaphragm settings; identical image processing along with all noise reduction and other optional in-camera processing turned off.

Once the target and location for the evaluation have been determined, set the camera's sensitivity to its lowest ISO, e.g. 100 when using a DSLR. A slight modification to these recommendations will need to be identified based on the type of camera and what type of equipment will form the image. It might be ideal to use manual exposure for this test but an automatic exposure method will also work. In addition to turning off noise reduction and image sharpening, it will be important to select Adobe RGB 1998 or another wide-gamut color space for this assessment.

It is practical to choose a RAW file format for the evaluation but recording TIF files will also be very informative. Do not test sensor performance using JPEG files since data will be lost when the file is compressed. Images should be captured using auto white balance and RAW images can be converted to 16 bit using the image processor's pre-processing feature.

It is also expected that the test will require the use of uniform and even lighting. The lighting should impart no biases to the results.

1. Place a sample or target in front of the camera. Compose and focus the image. Fill the frame or choose a composition that will remain the same for each of the next steps. Focus the image.
2. Set the camera to the lowest ISO and make an exposure that achieves the proper time and aperture setting for the normal outcome or a –0– exposure placement using the auto mode.
3. Change the ISO to a setting that is 2x the initial capture. For example, if the setting was 100, change the ISO to 200, etc. Make an exposure that achieves the proper time and aperture setting for the Normal outcome or a –0– placement using the auto mode.
4. Proceed to make exposures using all the various ISO settings on the camera.

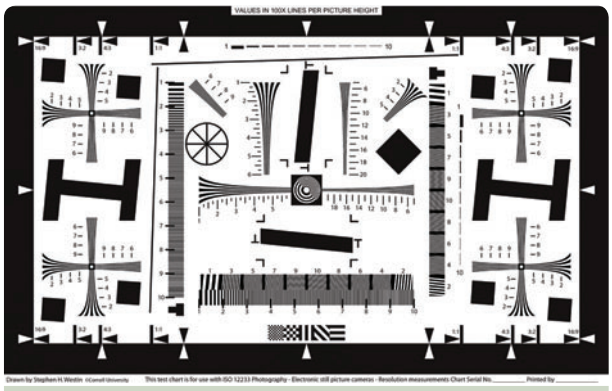


Figure 4.11 A standard test target such as this image resolution test target or a color checker (see Figure 15.2) can be useful when evaluating a camera's performance.

5. Using Adobe Photoshop or other image evaluation software, open all the files and assess the noise or granularity of the images as the ISO is increased. A noticeable degradation of sharpness, color, and other image attributes will be evident.

Bit Depth

In a pixel, a bit is the smallest unit of data that is present. As the basic unit of binary information, a bit can be off and create a value of 0, or be turned on and create a value of 1. The chapter has discussed pixel resolution but a file can also possess color and tonal value or data. This may be described as the bit or color depth of an image. Imaging applications and devices will always create superior image data with greater bit depth. Brightness resolution refers to the number of shades of gray that an image can discriminate and be derived from each pixel. The more bits, the greater the tonal range will be included in the file and this in turn will lead to greater information, useful when processing the file to reveal more.

Since sensors have become so good at collecting photons, the ability to discriminate more tones can be accomplished by increasing the bit depth when possible. The term "bit depth" implies that there are a finite number of tones a pixel can manage. Bits are expressed as an exponent of 2, and an 8-bit image would be described as 2^8. This bit depth will produce a file with 256 discrete tones for each R, G, B color for a total depth of 16,777,216 colors.

Some contemporary sensors record 10-, 12-, 14- or 16-bit images. Increasing the bit depth increases the data available and assists greatly with image processing. Increasing data may increase a sensor's dynamic range but, more importantly, provides more shades of grays, colors, and data from the object. When a file has more data, more can be made visible or revealed when image processing. Processing data changes it: sometimes the data is improved and at other times it can be diminished because of noise or other artifacts. It can be a trade-off. More bit depth leads to bigger files and more data. Less bit depth leads to smaller files and less data. Bit depth can typically be adjusted both at capture and in image processing typically by changing from JPEG to RAW or TIF. When files are down sampled, data is removed, but when files are made bigger, interpolation or false data is created.

Color Space

A color space is a mathematical model or manner in which specific colors are recorded, described, and/or displayed. Color spaces describe a set of physical colors and the corresponding names or numbers that have been created to describe them. A PMS color—a part of the Pantone system of color space descriptors—is an example of an analog model, where the highly structured mathematical descriptions used for color mixing can be found in Adobe RGB 1998. sRGB files are also an example of a type of color space useful for preparing images for web or monitor viewing. There are three values used in an RGB system or four values found in CMYK systems.

By carefully selecting a color space, scientific photographers can establish a detailed mapping function used for capturing color, processing color, and assessing how the color at output will look. This is established within the mathematical color space or an

image's color gamut. The gamut of a color will be part of what defines an image. For example, Adobe RGB 1998 and sRGB are two different color spaces that are both based in the RGB color model. The Adobe RGB 1998 space will display more colors with a wider gamut. sRGB is a smaller color space and will compress or suppress more color information. The most common reference standards for color space, recording, and display are the CIELAB or CIEXYZ color spaces. These systems were designed to mimic the colors that the average human can see. When selecting what color space to select for a camera, use the widest gamut available located in the camera's preferences. This will lead to files with the most information.

Gamma and Contrast

Some instrument cameras allow for the setting of a sensor's capture contrast. No DSLR cameras to my knowledge provide this feature. Adjusting capture contrast would be indicated as gamma and can be found in the camera's menu. Gamma is an important contrast characteristic of all imaging systems. Gamma defines the relationship between a pixel's numerical value and the tonal display from the image's actual luminance when displayed. Without using gamma, all of the shades of gray that have been recorded by a digital camera would not appear as they should and might be described as not having a tone map. Understanding what gamma is and how it works can improve the photographer's ability to create a more accurate exposure, useful in subsequent image editing.

Gamma is important for imaging because human vision does not perceive light in the same way a digital camera does. When twice the number of photons hit the sensor, the sensor will make twice the signal, which is a linear relationship. Human vision is non-linear and a person will perceive a doubling of light as being only marginally brighter. This lack of separation is increasingly less at higher light intensities. When compared to cameras, human vision is more sensitive to changes in dark tones than changes in bright tones. Gamma and tone mapping are what differentiate human eye light sensitivity from that of the electronic systems. Once the recorded file has been saved as a digital image, the gamma information has been encoded and the tonal values will be displayed more closely to what would be expected by a viewer. Human vision is very flexible and digital capture systems are not.

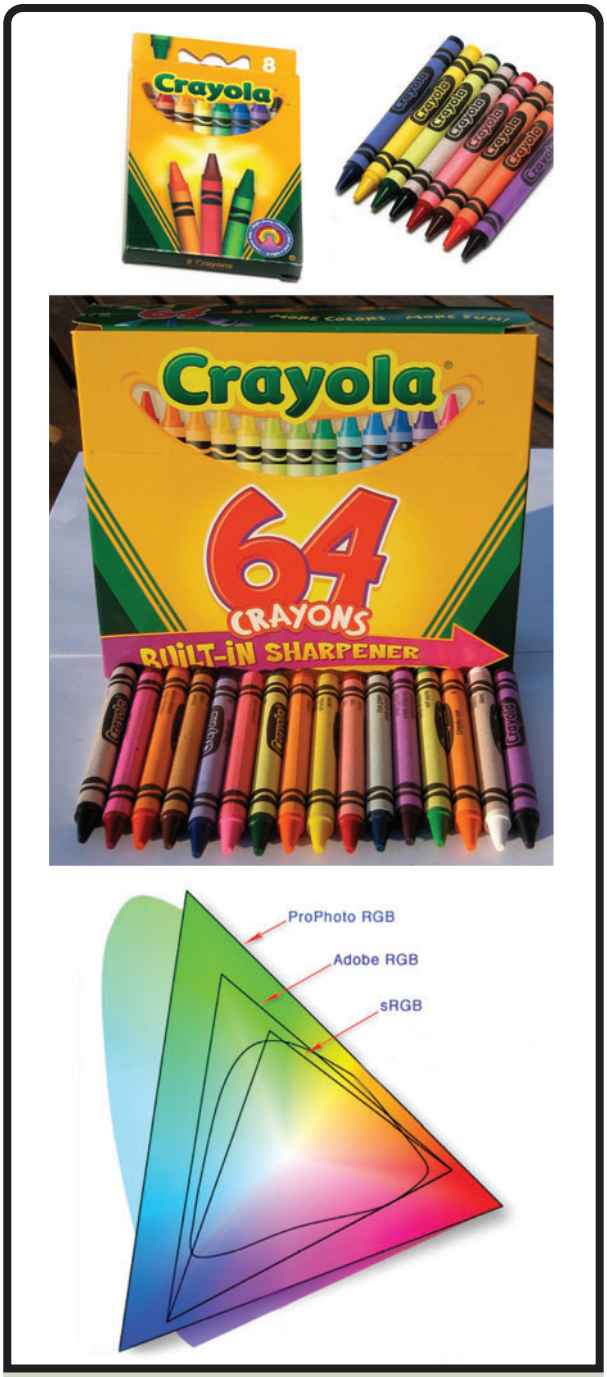


Figure 4.12 A sensor's ability to discriminate colors is influenced by choice of color space. A useful analogy for understanding color space might be found when considering the number of Crayola® crayons found in different sized packages. A package with eight crayons will provide a user with only a few colors when compared to a sixty-four pack. The CIE diagram shared at the bottom shares the approximate color gamut of sRGB, Adobe RGB and ProPhoto RGB, which creates the most colors.

Very few DSLR cameras allow the selection of different gamma recording settings but instrument cameras routinely offer this feature. A gamma setting of 1.0 reflects an average contrast display or recording setting. Gamma is characterized graphically as a reactions rise over run. This predicts what changes in brightness will be caused by a subsequent change in exposure, creating a subject's tonal values. Gamma or $\Delta D = \Delta Exposure / \Delta Brightness$. When an average contrast subject or situation is encountered, a camera gamma setting of 1.0 would be useful. If the sample or situation contains high contrast, such as in fluorescence microscopy, a lower gamma setting is preferable. A high contrast setting would be useful for low contrast samples.

Monitors also have gamma settings. These settings are a bit different than the numbers useful in the camera software. A monitor's gamma range would typically begin at 1.4 and go up through 2.2. An average setting would be 1.8 for a PC and up to 2.2 for a Mac® computer.

Scenes and subjects will contain various brightnesses or reflectances within them. The difference between a high brightness compared to a region of low brightness describes a scene's brightness range, or dynamic range. A sensor can record data more effectively when from a narrow brightness range. A high brightness range creates challenges for a sensor to record the entire range in a single exposure. High dynamic range (HDR) imaging methods have evolved to manage this problem. See Figure 14.5.

White Balance

In photography and image processing, a photograph's color is the result of global adjustments to the intensities of the recorded red, green, and blue color information. The ultimate goal is to render color correctly. The color of an object is affected by the lighting where the object is viewed. Human vision compensates for different types of light allowing a white object to appear white regardless of the light sources with various color temperatures. Digital cameras cannot color-correct objects in the same way and require methods to create neutral colors in different types of lighting and render a white object white. This method of creating neutrality is called setting a gray balance, neutral balance, or white balance. When adjusting color balance, there are changes to the overall mixture of colors in an image that are used for color correction and generalized acceptable versions of the image's color balance.

Image color data produced by a sensor must be transformed from the acquired values to new values that are appropriate for color of the light used for capture and subsequent reproduction and display. An image color balance operates in image editing applications usually by directly modifying the red, green and blue channel pixel values without respect to any color reproduction.

Cameras can have their white balance set in several fashions using auto or manual, or by choosing from several pre-set choices, or custom. Operators can set white balance using the auto mode and the camera will read the scene's various color temperatures and select a setting from its database of pre-programmed choices. This method works well when a scene has lighting that is comprised of one type of light and there are prominent neutral subjects

in the scene. The results when shooting the auto white mode are also affected by changes in the lighting. In most cases the most accurate color rendition requires using the manual white balance setting. Manual settings include incandescent, fluorescent, electronic flash, cloudy, open shade, sunny, and custom Kelvin color temperature choices.

The incandescent setting will be best for tungsten bulbs and used for fiber optic lights and photomicroscopes. The fluorescent setting will prevent common green casts typical in photographs made using fluorescent lights. The cloud setting will add a bit of warmth to the color temperature and the electronic flash setting will add some warmth to the rendered colors. The Kelvin color temperature setting is best for total control where the operator sets the degrees Kelvin required to control how the camera will render the hue and intensity of colors in the scene. Using the Kelvin setting, operators can fine-tune the process creating accurate definition and not skewing the colors. Using a camera in the live view mode will enable operators to see in real time the results of a specific Kelvin color temperature. It can also be useful to locate a white or gray card in the scene to use for white balancing a scene.

Capture File Formats

One of the most important decisions a science photographer must make prior to recording camera exposures has to be what file format should be selected. There are a number of file formats available. All of the choices have advantages and disadvantages. Decisions about selecting an appropriate file format should be primarily decided upon based on which file format will preserve the most data and which one creates the most workflow going forward. One of the first decisions is to choose a format that does (lossy) or does not (lossless) compress captured data. A file format that maintains the full data without compression is called lossless and would be a TIF, BMP or RAW file. It is also correct to write TIFF when describing this file type. There are also formats that offer various degrees of compression or are lossy, such as JPEG, JPEG 2000 or PNG. There is no reason to choose a file format that produces compression at capture because that will compromise the integrity of the file's data even before anything else is done. Data will be changed or lost using compression file formats. There is really only one decision for capture and that is a lossless format. That being said, many wedding photographers do use JPEG because of the advantages for speed and portability of this file format. Rarely should a science photographer use a JPEG file format.

Digital File Structures

As a side bar, there are two broad categories of digital file structures, raster and vector images. A raster image describes the image where the individual pixels can be addressed. It is sometimes called a bitmap file. The other structure for digital files is called vector and is characterized by the file's end points and not the discrete pixels. Vector files can be enlarged or made smaller by dragging or compressing the picture box as a whole unit because of the way their mathematical descriptions allow them to appear smooth at various image sizes. They are very different than raster images. Very few if any capture devices create vector images. Vector files are very useful for desktop publishing, graphics programs and for output but not for capture. Adobe Illustrator® is one such program. Raster image files

are converted to vector files when used in Microsoft PowerPoint®. An EPS (encapsulated postscript) file might be an example of a vector format.

In any type of image file, the size of the file is directly related to the number of pixels, its color space, and its bit depth. Image files can be compressed or they can remain in a file format that does not compress, effectively not changing the captured data. Compression of a file is accomplished using mathematical methods to approximate data or create a facsimile of the original image's data that uses fewer bytes. This process results in the creation of files that are smaller in size than the original captured file. Compressed files can be opened up to their full resolution and in many cases look as they did prior to compression by using decompression algorithms. Considering that, different cameras and software can create different compressions and it is common for two images with the same number of pixels and color depth to have a very different file sizes when using different amounts of compression. Because of the manner in which pixels, color and bit depth are evaluated by the algorithms and are influenced by the variances in the original image subject, compression can result in different file sizes after compression. Within some compression mechanisms, an image's complexity may also result in smaller or larger compressed file size. This sometimes results in a smaller file size for some lossless formats when compared to lossy formats. For example, graphically simple images such as an image with large continuous color or darkness regions, such as a background in a photomicrograph, may be lossless when compressed into GIF or PNG formats. These two file formats may result in smaller file sizes than a lossy JPEG format might make.

There are several file formats that save data files that can be used without compression and are ideal for science. These would include TIF, BMP, RAW. Many instrument cameras also have proprietary file formats. Each of these types of files produce saved files that are identical to captured files. This scenario is ideal for science photography. Any compression of recorded data can cause data loss, no matter how insignificant. There was a time when storage and storage media were a consideration because they were in short supply and expensive. In this era, that is no longer a concern and it is critically important to select a file format without compression whenever possible. Proprietary file formats come with one small advantage, and that is that all of the capture information from the instrument itself is exported with the file as metadata.

The TIF format is considered by many to be the most universal file format. TIF stands for tagged image file format and produces files that contain data that is structured exactly as the data was recorded on the sensor. TIF files can be read across all platforms including LINUX, MAC and Windows operating systems. They are also backwards compatible. That is to say, a TIF file can be opened and read using any device regardless of the age of the operating system and the image processing software. TIF files can also be opened using many image processing software packages without special plug-ins. Plug-ins are special pieces of software that are sometimes required to see and read various file types. TIF files will rarely have challenges when used across various platforms, devices and environments. Most instrument cameras will create TIF files; however, only a few DSLR cameras create TIF files directly.

An ideal file format that makes uncompressed digital files when using DSLR cameras is the RAW file format. The suffix of RAW file formats will vary across camera brands and might be described as NEF, CRW, or others. These file formats save data in an uncompressed

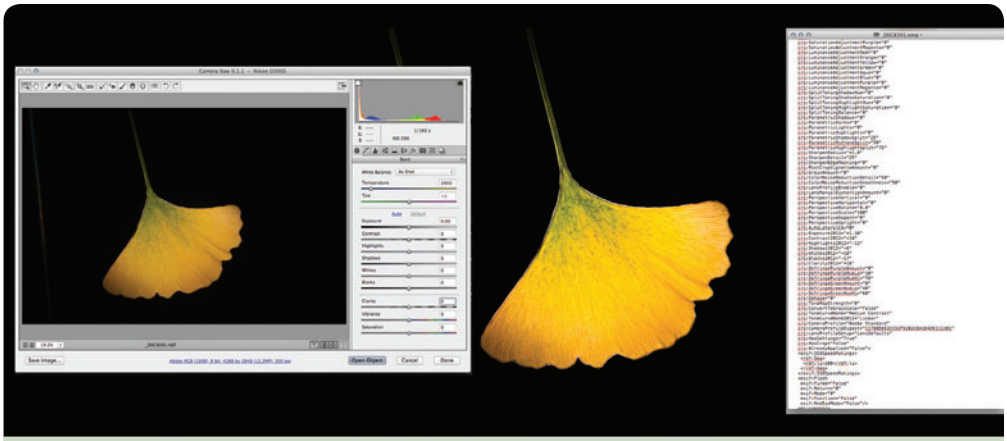


Figure 4.13 In this figure, a RAW file was opened using a RAW file convertor pictured on the left. The center image represents the processed file saved as a TIF file. On the right is the .XMP or sidecar file. Each individual RAW file will have a corresponding .XMP file.

manner and are typically opened using a RAW file convertor after capture or using Adobe Lightroom®. RAW files are very useful and provide great advantages over other file types for scientific photography. Their architecture allows more data to be managed and saved, allowing for challenging subjects and situations. This in turn creates files that are superior for data collection and preservation. RAW files accomplish this by using a file mathematical enhanced structure that allows creation of a greater bit depth and color information to be maintained using the finite exposure range of the sensor. RAW files must be opened and read using a pre-processor program or plug-in called a RAW file convertor. This convertor allows the file to be pre-processed, revealing more than if the image was processed using Adobe Photoshop alone. RAW files will be slightly smaller in file size when compared to TIF files. RAW files are ideal in science because they themselves are never altered and the data display changes are shared with the file in an .XMP or sidecar file. See Figure 4.12. If a scientist photographer were ever asked to testify, the RAW file could be re-opened without image processing information by eliminating the sidecar file.

When a sensor with a Bayer pattern is used, neighboring pixels determine how to create the color for all pixels, but when RAW files are used, the de-mosaicing process is more "cleanly" handled by the RAW file pre-processor. This leads to more precision and control for the photographic process but can be more time intensive. The bit depth of RAW files is also typically greater. A JPEG or TIFF file is frequently 8 bits per channel at capture, leading to 256 tones per pixel color, but it is common for a RAW file to contain 12 bits per channel leading to more than 4000 RGB tones per pixel. RAW file formats are not typically found in instrument cameras.

Figure 4.14 A banana might serve as an effective reference when considering file compression. A banana, when fresh, has a certain appearance and nutritional content rich in vitamins and minerals. When the banana is dehydrated and becomes a chip, it becomes lighter from the loss of water, but still contains all of the nutritional content rich in vitamins and minerals. Attempting to rehydrate the chip does not allow the chip to become what it was. The chip's physical structure will forever remain different. Compression and loss in a digital file structure can cause loss of information.

Other File Formats

Most scientific images can be photographed by using TIF, JPEG, and RAW files but there are other file types. More will be shared about image processing file types such as PSD in Chapter 13. There are, however, a few other file formats that some cameras produce.

BMP describes a bitmapped file format. It is frequently found within the Windows operating system environment. These files are uncompressed and create larger files than compressed file formats. PCT file was an older file format used frequently in the MAC environment. PNG describes a compressed file format called portable network graphics. Its origins were from computer scientists as a free and open source choice rather than the popular GIF, or graphic interchange format. PNG files support 8-bit images so can display 16 million colors. Because PNG uses a lossless compression, it can be useful when image processing is being undertaken. It is particularly useful for use in the web environment where progressive display modalities are used. PNG does an excellent job when text and photos are combined. It is an 8-bit format, however, and uses only 256 colors. Images that are not complex and do not require millions of colors can most benefit from this file format. It is ideal for web use and images with large areas of white or common color. GIF files are universal and can be opened in almost all image processing and display software.

Capturing Using Camera Digital Filters

Scientific photographers when preparing to record images must consider whether to have certain of the image file's attributes enhanced or reduced. This occurs through the use of various in-camera filters or image modifications. The most common of these "at-capture" attributes would include color reproduction, noise, and sharpening. There are various reasons why an operator would choose, or not choose, to use any of these filters. If JPEG files are created, performing some low-level sharpening can be helpful, but if using RAW, the best practice is to de-select sharpening, noise reduction, and color enhancement in the camera.

Sharpening

An RGB camera sensor is manufactured with an IR cut-off and anti-aliasing filter on top of the Bayer filter on the sensor. While these filters are only a few microns in thickness, they still adversely affect critical image sharpness. In fact, many would argue that many contemporary cameras no longer come equipped with anti-aliasing filters. In-camera sharpening is an image processing algorithm that is sometimes chosen because, like all sharpening tools, it increases the localized pixel contrast. This localized increase in image contrast causes an image's fine details to be more delineated. Having files that are electronically enhanced can sometimes produce digital noise over time when future image processing is undertaken. The initially captured image will typically exhibit a smoother gradient of tonal data per pixel, but when sharpness is boosted at capture, images can appear exaggerated. More importantly, if the images are to be used to create a computational result (Chapter 14), sharpening should *never* be selected. Creating files that

have edge enhancements at capture when blended can appear processed and embellished. Perceptually viewers will respond more favorably to images that are sharp and crisp; however, over-sharpened images will appear as such and may not be well received. In fact they may be described as appearing computer generated.

Different cameras provide operators with various levels when selecting to sharpen at capture. A sharpening filter can be applied in an auto mode or by selecting the amount of sharpening that is desired. It can also be turned off and provide no sharpening at all.

Color

Similar to sharpening, an image's volume of color or its saturation can be managed at capture. Since viewers have an expectation of what colors are credible, when choosing how to record the correct color saturation without embellishment and sample dependent, operators can opt to have more or less color data in an image recording. The Kodak research labs performed exhaustive testing in the 1960s to evaluate what consumers desired in color reproduction when using color negative films. After years of conducting viewer surveys, they learned people wanted to see color that had more saturation but that did not offend them. So an individual's preference plays a role in choosing to improve color saturation at capture. All cameras will have a variety of commands to control the amount of color that is captured. An example of these settings could be standard, neutral, vivid, monochrome, portrait, landscape, or flat.

Noise Reduction

There are reasons why noise reduction should be used but as a tool it should be used carefully. Noise filters actually soften edges where pixel differences are located. This softening creates images that will appear less crisp, which is the antithesis of expectations. In general, noise reduction softens an image's appearance and sharpening will increased the apparent contrast, making images appear more sharp. Scientific photographers may all have different biases for the amount of noise reduction that is required. Noise reduction is accomplished by actually blurring the image data a varying amount. The amount of blurring blends image defects that are caused by pixels exposed by heat but not by photons.

Various cameras will have different noise reduction settings. This could be off, low, normal, or—often a camera's default—high. These choices allow operators flexibility in managing the compromise between subject detail and noise levels in JPEG files. Many cameras do not share information on at what ISO or at what long exposure time noise is produced.

Digital Artifacts: Aliasing

Low and medium resolution computer imaging systems normally create and display images with smooth edges. When the frequency of structure of an object matches the pixel resolution correctly, no aliasing occurs. When sample's fine detail is greater than the number of pixels, aliasing can occur and is evidenced in an image appearing as

having jagged edges or bands. Aliasing is often a result of under-sampling or is created when the detail exceeds the rendering ability of the system. I am sure most have seen a reporter's clothing start to have strange moiré patterns when they are wearing clothing with fine patterns and details. Aliasing can be spatial or frequency. In either outcome, it is not desirable and produces images that do not appear correct. It is difficult to predict with certainty when image aliasing will occur. Anti-aliasing filters have been located on a sensor to help manage this image artifact. Unfortunately they make images appear soft.

Connecting Devices

Cameras, scanners, card readers, and any number of peripherals including flash drives need to communicate with a CPU. Almost universal at this time, the Universal Serial Bus (USB) has become an industry standard and was initially developed in 1994. At the time it was defined by the type of cables, connectors, and communication protocols used for connection to power supplies and between computers and other devices. At the time a standardized connection for all things connected to a CPU was vitally needed. This included digital cameras, card readers, printers, and others. In 2000, USB 2 was released. USB was capable of transfer speeds to 1.5 Mbit/sec, and USB 2 was forty times more powerful at 480 Mbits/sec. In 2008, USB 3 was released and could transfer data at up to 5 Gbit/sec.

Memory Cards

Digital image files are stored in buffer camera memory, a temporary location, before being moved to a memory card or to a CPU. Some cameras cannot record images without a memory card and others may operate in either fashion, with or without a card. A memory card is capable of reading and writing, both when located in a camera and when located in a card reader when attached to the CPU. Different names have been given to memory cards, including Smartmedia®, memory stick, or compact flash. Each of these devices is different in storage technology. Differences are evident in size and speed. Different cameras can take both compact flash or SD or XD Media. Media continues to become smaller and faster. It is not uncommon to see 32 GB cards that have a card speed of 32x.

SUGGESTED READING

Allen, Elizabeth, and Sophie Triantaphillidou, *The Manual of Photography*, tenth edition. London: Focal Press, 2010; ISBN-10: 0240520378.

Bilissi, Efthimia, and Michael Langford, *Langford's Advanced Photography* (The Langford Series), eighth edition. London: Focal Press, 2011; ISBN-10: 0240521919.

Kelby, Scott, *The Digital Photography Book: Part 1*, second edition. Berkeley, CA: Peachpit Press, 2013; ISBN-13: 978-0321934949.

Part 2

Applications, Best Practices, and Methods

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Chapter 5 The Sample and its Role in Laboratory Photography

This photograph of *Arabidopsis thaliana* shares the blue light sensitivity of two plants (x2.6 approximate magnification). This image, made in 1993, demonstrates the results of an experiment proving the existence of blue light photoreceptors in thale cress seedlings. There is a normal inhibition response to growth when the photoreceptor detects blue light conditions. The control seedling (right) exhibits stunted growth, indicating the presence of the receptor. The experimental genome (left) had the receptor removed, and the plant does not recognize the presence of the specific wavelength of blue and grows normally. The experimental seedlings were approximately 1 cm in height. Image courtesy of James E. Hayden.

Revealing characteristics of a subject in a highly precise and accurate way is a requirement when photographing for scientific reasons. The upcoming chapters will share practical methods useful in forming and recording scientific photographs that feature laboratory subjects. These applications will include close-up photography, photomicrography, and polarized light technique among many others. There will also be a chapter dedicated to contrast-producing methods of scientific photography including fluorescence, invisible spectrum photography, schlieren, and stereo anaglyphs. There will also be suggestions for the use of scanners as a camera.

The Sample and Photographic Treatments

Effective images are the result of making and executing a good plan featuring sometimes less than cooperative samples. Successful, interesting, and informative images are rarely lucky accidents. It is fundamental to the outcome of the process that the sample will play a critical role in the creation of a successful image. Often scientist photographers are quick to photograph whatever ends up in front of a camera. This might be a consequence of time constraints or not seriously considering the value of the sample's "aesthetics" as it relates to how the sample influences the more permanent image. A bad sample always leads to a less than ideal image. The poor sample or damage within the sample itself may become the focal point of the image.

How to best manage a sample when photographing, or how to best light a sample, or what equipment will be used to photograph the sample will all play a role in the making of effective images. Solutions to these considerations will lead to the creation of what is described as a treatment. Depending on the sample, treatments might be chosen from methods that are proven or something that is untested. Treatments may require a "best guess" approach or some experimentation might be needed to get to the best solution. There are numerous considerations that scientist photographers need to make to get started. Not considering making snapshots, photographing in science requires a careful analysis of the known problems and the development of practical solutions defined by the subject/environment required when making images that are truthful. Chapter 10 includes additional content about subject management.

All samples are unique and present different challenges. Samples can change during imaging or may stop fluorescing during the experiment, for example. Samples can be opaque, transparent or both. Samples can be monochromatic or possess birefringent properties. Birefringence is the display or multiple refractive indices within a sample characterized by colors. A sample could—in the right conditions—exhibit emissions or behaviors (a sort of fingerprint) that are unique to it. Samples can be motile microscopic organisms or without noticeable contrast. Samples may be nearly invisible even when stained. These and other factors must be considered prior to making any photographs because creating sample visibility is integral to making a good image. The treatment must be carefully considered because once it has been started, it might be challenging to retreat. The sample could be damaged, destroyed or changed in negative ways if poor decisions are made. The objective of the scientist photographer is to make images of challenging objects without embellishment or changing the specimen and to include information about a sample that otherwise might

not be immediately evident. It can be very useful to analyze all the various scenarios and to plan for anything to happen. Anticipation can provide a great advantage when preparing how to proceed.

Preparing for Imaging

All of these considerations and subsequent choices play a role in creating successful imaging outcomes. Successful imaging, like many things, requires preparation, much like a camping trip or other activities such as cooking. Collecting and organizing the proper accessories before starting can be of help. Glass slides, hand-held dust blowers, lens cleaning supplies, containers, formatted media cards, charged batteries, warming plates, tape, and other items might make up this inventory of items. Razor blades, needles, scissors, scalpels, pins, toothpicks, dark modeling clay might also be part of the cadre of things located where the imaging activities will be conducted. Other things that might serve a useful role might include Sharpie® markers, aluminum foil, rulers, Petri dishes, silicon caulk, magnets, bibulous paper, pipettes, and syringes. Supplies and accessories will contribute small but important advantages when working on imaging initiatives. It is also important to continually clean any open surfaces prior to working and bringing the samples out. This can be helpful in controlling the addition of dirt or debris to a sample. Dirt in scientific photographs might be referred to as "schmutz," a German word for dirt.

Figure 5.1 Supplies such as lens cleaner, cotton on sticks, and a blower brush are important accessories. Paint brushes, needles and pins, Exacto® knife, tweezers, dissecting needle, scissors, clamps, clay, tape, and other items can be helpful, too. It is imperative to keep these tools and accessories clean. Cross-contamination is a problem when handling small subjects using dirty tools.

Selecting a Sample

A sample is not just a sample. It serves as the foundation from which an image will be formed. The selection of the best possible sample might be one of the most important steps in making an image. Looking for the ideal sample is not a process that comes naturally to everyone. There is variability in nature, in biology, and in general. Selecting the right sample will take additional time but it will be time well spent.

Garbage in will lead to garbage out. Bad samples will lead to ineffective images. Sometimes one sample is all that is available, but when there are choices and flexibility it is invaluable to carefully evaluate all the possible samples. All the imaging skills in the world cannot improve a lousy sample. Frequently science photographers are not tuned into a sample's aesthetics or other properties because it is not part

Figure 5.2 Within this collection of *Acer rubrum* seeds, there is a wide range of attributes to consider for selection. Identifying the best sample from collection when possible will lead to significantly better pictures.

of their training or mindset. Careful evaluation and selection of a sample can lead to noticeably better photographs without improving much else. When samples are prepared for microscopy, often they become swollen or distorted and do not lead to good images. This transformation will create a variety of presentations and care should be taken in evaluating what is available.

Sample Preparation

When samples are poorly selected or prepared, they lead to images that are inadequate. This might be most evident when imaging applications create images that are larger than the objects. In this situation, the elements and characteristics of the sample become the features of the image and not the object itself. Bad preparations of less than ideal samples may lead viewers to see the imperfections as the focal point of the photograph and possibly lead to false conclusions. This is because defects become more pronounced when magnified. Sometimes during sample preparation, damage to a sample occurs. This damage, called an artifact, could be a tear or fold in the material. It might also be a knife or tool mark left in or on the sample when it was being prepared. Whatever the case, any evidence of the dissection or preparation of the sample should be minimized or, better yet, avoided. It is for this reason, and this reason alone, that great care should be taken to evaluate, prepare, and select samples more carefully for imaging than for visual inspection. When simply observing experiments, the process that leads to assessing the presence of a material, cell or other relevant information does not lead to a permanent record. The visual analysis of what is observed is dynamic and imperfections in the sample are not important or relevant. Images are, however, permanent records and will be studied at great length or reproduced in articles. For this reason—and knowing that variability does exist—the selection of the best possible sample from the available choices will lead to better results. This could also be true of locating the best cell in a culture plate or finding the most effective region in a tissue.

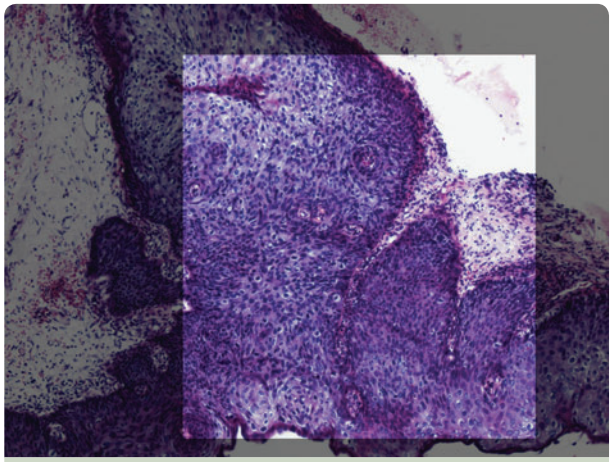

Figure 5.3 When selecting a field of view for photographing, be careful not to include regions that exhibit knife marks or other artifacts. Knife marks are the result of a microtome blade having a nick in the blade. In this figure, moving from the top left corner to the bottom right corner is a white line, an artifact made from the microtome blade.

Isolating the Sample

Once a sample has been selected, how to manage the photography begins. It is not useful to simply plop a sample onto a table and take a picture unless it's the only way to make the photograph. The expression "take a picture or make a photograph" refers to the notion that it takes a bit of effort to achieve good results. In digital photography, there is a common philosophy that artifacts or other unwanted components can be deleted during image processing. This initiative can compromise a sample's integrity, should be avoided, and should not be part of best practice for the making of a scientific image. Sloppy work habits

will contribute to the presence of artifacts in images. It is best to spend a few minutes tidying up on the front side of the work and during the setting-up phase rather than spending a lot of time on the backside of the work to clean up images, something that could have been managed more effectively before pushing the shutter. In some imaging experiments, hundreds of pictures might be made, and if each image requires time to fix problems, it could lead to days of image processing. Spending a few moments in the beginning to prepare and manage samples during the imaging will lead to a scientist photographer's time being better spent.

Composition

One of most effective tools for isolation of a subject is the use of framing or composition in the camera. Frequently, the framing of photographs is not adequately managed. "Less is more" when it comes to isolating a subject within the frame. The frame of a sensor is a rectangle and has an aspect ratio. Many full-size sensors in DSLR cameras have an aspect ratio of 3:2. This number is derived from a 36 mm x 24 mm imaging area. A cropped or smaller sensor camera may have a 4:3 aspect ratio. The first number refers to the width of the sensor and the second number the height of the sensor. A more panoramic sensor will feature an aspect ratio of 16:9. The dimensions of the sensor will play a role in what can be recorded. Filling the frame is an important isolating strategy. Making loose compositions or including extraneous content will diminish the effectiveness of photographs but in some situations maybe unavoidable. Changing magnifications will be expanded on in subsequent chapters, but for the moment using the frame to create an effective image magnification and the removal of competing elements can be a great strategy. Anything left within a composition will be considered by a viewer to be an integral part of a photograph and may be a distraction.

It is interesting to compare how an artist works when compared to how a photographer works. Artists start out with a blank canvas and add information as desired but the science photographer must constantly look for ways to subtract and isolate a subject from its background/environment to create an emphasis and isolation. Properly framing can facilitate isolation. A photographer should always look to locate important features within the frame that will lead to gaining an understanding and not confusion. It is important not to position vital structures of the subject too close to the edge of the frame and to be cautious about cropping out important details. There will always be compromises and limitations.

There are numerous websites dedicated to framing and the composition of effective photographs but key points are:

- Simplify what is included in the frame.
- Consider using the vertical dimension for a composition where useful.
- Do not locate everything of interest in the middle of the frame when possible. Sometimes it is possible to distribute the sample across the whole frame.
- Use diagonals or locate important structures evenly throughout the frame.
- Select and use an effective background.

Magnification

Image magnification can also play a role in isolation. Images with a high magnification may result in the cropping of relevant information. Images that are not magnified enough will not adequately reveal as much data as they might. These aspects of the camera's magnification may play a role in the formation of effective images. Depth of field (DOF) or an image's zone of focus changes with magnification. Higher magnifications have smaller zones of focus and lower magnifications form images with more DOF. Choosing the correct image magnification that contains the most amount of information about the sample should always be the objective. Often this requires compromises required to achieve the best of balance of both.

Controlling Dirt and Extraneous Materials

The most commonly photographed subject in a laboratory when making magnified pictures might just be dirt. There seems to exist the relationship that the more important a subject, the higher the probability that dirt will find its way into the image. Whenever possible, proper housekeeping in the area around the workspace can be helpful in managing dirt and its potential for entering the image. Equipment should be kept covered, the desks and tables routinely cleaned down with lint-free cloths, and the vessels that might be used to hold samples should be cleaned using air blowers or using other methods that are lint free. When using black velvet or other material for backgrounds, it might be useful to use tape as a dust pick-up. This might be considered as a sort of lint brush for surface dirt on the background prior to locating the sample on the background. This process of "sanitizing" might include surface cleaning of the camera lens, removing the dust from the sensors routinely as well as using a soft cloth to polish any laboratory glassware or clean microscope slides that will be used. It is also useful to consider a method to clean dust and dirt from shoes before entering the imaging room. It can also be practical to consider what type of clothing is worn when

Figure 5.4 These two photographs—a snowflake (left) and a bleeding heart, *Lamprocapnos spectabilis* (right)—are classic examples of how dirt can migrate into a composition.

working in the laboratory. Lab coats can be practical to minimize airborne fibers that might come from sweaters or other fabrics that lose fibers routinely. Everything sheds, including you, and often this debris will find its way into the imaging area. This might include head and facial hair as well.

Handling Samples and Preparation

Objects should be handled with great care when they are to be photographed. While many are quite robust and hardy, many are delicate and easily modified by handling. In forensics, the theory of material transfer suggests that whenever two objects meet, content from both will be transferred to the other. This of course is very useful for solving a crime and leads in some cases to the solving of a crime by locating where the foreign material came from. When photographing challenging objects, this is not desirable. Great care should be used when touching and moving objects. Using any method that does not apply pressure to a sample's surface is the ideal but often not practical. I have used pipettes, feathers, small paintbrushes, and special insect tweezers to move samples prior to photographing.

Sometimes a sample will need to be deconstructed or dissected. Care should be taken before engaging in the cleaning or preparing of the sample to consider the risks and advantages of this process. It is very practical to photograph the object as a whole prior to starting any dissection. It can be relatively easy to damage a fragile sample. It can be a waste of time to photograph damaged samples unless the damage is the focus of the photograph. Damage would probably be most evident on the surface of the sample. The simple process of transferring using tools in moving material from one environment to another can leave pressure markings. Toothpicks, needles, pins, brushes, pick-ups, tweezers, and pipettes are useful for transferring but should be used with caution. Try to move or touch things only once to avoid cross-contamination. Once damaged, a sample might be considered useless. Be cautious about touching and moving a sample when required and necessary for the creation of improved images. In the end, getting better results revolves around the controlling of many little things. It rarely is about big improvements but a series of many smaller successes.

Wet Specimens: Distilled and De-ionized Water or Other Fluids

Samples that are wet can be challenging. A fundamental decision resides in whether to keep the sample wet or dry it. Drying can be accomplished in many obvious ways. Sometimes the use of a hair dryer using low heat will expedite drying. Sometimes objects simply need to be left out for a period of time to allow the moisture to leave on its own terms. Accelerating the drying of a sample may not be useful. It is not a great idea to use any type of paper towels since invariably paper fibers will find their way onto the sample. A wet sample that becomes dry will frequently change in its appearance or morphology when not wet. It might get smaller or curl, or it might exhibit a changed color, or it might experience other changes that are not so obvious upon initial evaluation.

If the decision is made to keep the object wet, then there are other things that come into play. Initially, what vessel will be used to hold the sample must be determined. Always use a vessel that is the right size. Never use a vessel that is too large. If the vessel is large relative to the sample's size, the object can float around and present other challenges for photographing. Cleanliness of the vessel is critical. I have found that using a weak acid wash such as white vinegar followed by a light rinse of distilled water can be helpful to manage mineral or other surface contaminants that might find their way into the vessel and subsequently the photograph. Cleanliness of the vessel, and ensuring no dust or particulate matter is present to the extent that is possible, is important. This simple step of housekeeping can make a big difference to the amount of particulate matter that might have to be deleted from the photographs if not removed. Chapter 12 discusses retouching dirt and dust.

One critical activity that requires attention is to minimize the contamination of the sample when adding liquids to keep it wet. In the previous paragraphs, strategies for minimizing the presence of dirt and dust were shared. For wet samples, frequently it is useful to suspend samples in water or other liquids such as mineral oils and glycerin. When using water, the use of de-oxygenated, distilled or de-ionized water is preferred. Tap water will often be full of dissolved air and minerals such as iron and calcium. In a short time, the dissolved oxygen will form bubbles on the sample and vessel walls. Making an effective photograph when this happens will be tough. While sometimes interesting, the bubbles will compete with the subject. Rarely is tap water a good idea for this application but if it is all that is available, allow it to sit for a prolonged period of time before using. I have known photographers to run water through a non-woven coffee filter. On rare occasions, mineral oil or glycerin might be useful for suspensions as well. Another strategy is to locate the sample in clear gelatin such as Knox gelatin, which can also be used to isolate and hold samples in suspension.

Many years ago, in an attempt to photograph a developing root system, seeds were germinated in a chamber filled with clear gelatin, similar to collagen. This material provided an ideal way to isolate the roots and micro-roots. This separation in turn provided an opportunity to effectively light the structure of the roots, which without this treatment would have resulted in the roots collapsing without separation. Allowing the roots to be photographed in air would create morphological changes. The surface of the root will dry as a consequence of heat created when shining lights on the root. This dehydration in turn will result in color changes.

Making Chambers and Welled Slides

Sometimes when photographing wet samples it is required that the sample remain in liquid. In previous pages, strategies were shared for using immersion methods in general. The objective of immersion methods is to allow the sample to remain wet and to control reflections. When a light is shined onto a wet object, many reflections will be produced. These reflections will be random and may possibly obscure important details. For this reason, it remains practical and important to locate samples in water or other liquids. You can see images made using immersion in Chapter 8.

Often it is difficult to find the right vessel for this type of work. Petrie dishes or other laboratory glassware frequently include flaws in the glass formed when the glass was liquid

and then cooled. This creates density differences in the glass. When illuminated, they will appear as varying dark bands in the background. These dark bands can also add false data or artifacts to a sample's appearance. For this reason, it might be desirable to fabricate a chamber using higher quality glass, in the form of microscope slides for example, or small pieces of window glass based on the size requirements of the object.

Fabricating small glass chambers or "aquariums" is a relatively simple procedure but requires care to keep the pieces and parts clean and free from adhesives. The most useful glue for fabricating glass is silicon caulk that is used to seal bathtubs or in other similar waterproofing jobs. To make best use of the adhesive, it is useful to apply it using smaller tubes and lesser amounts. Less will be more. Too much clear caulk may contribute flare in imaging systems. It might be helpful to assemble the sides first allowing them to set up and then adhere the sidewalls to the base. It is of course important to prepare the parts before starting the assembly. Chambers should be sized to the objects they are created for. If they are too small they will compromise the work, and if they are too large the object might possibly drift too much. The area under the lens might be considered the "action field" and creating methods for keeping the sample under the action field can be helpful. Chambers can easily be subdivided using additional pieces of glass cut to size.

Welled microscope slides are useful tools for viewing but have a few drawbacks for imaging. When photographing aquatic invertebrates, a welled microscope slide will keep the organisms in a controlled space but the depth of the slide will allow the organism to swim left and right as well as up and down. Using less water will be helpful in minimizing the depth the organism can move to but the curvature of the well provides other challenges for lighting and imaging. The curvature will introduce some flare and other non-image-forming lighting outcomes. Depending on the direction of the light and whether darkfield or other methods are used, the results may be less than acceptable. Creating a slightly different type of slide using broken coverslips to elevate a traditional coverslip will work much more effectively. The cover slip shards behave like legs of a table.

Staining and Revealing Other Features

It can be useful to add contrast agents to samples for the purpose of enhancing visibility where and when appropriate. Managing treatments where the efficacy of the sample is maintained should always be considered first before adding other materials. All objects are different and may have characteristics that need special attention. When a sample exists in a dry or wet state, it is not unusual for different features to be more or less visible. It might be practical to either dry or add water to a sample. Rehydrating a sample or wetting its surface can be accomplished in many ways. Simply locating the sample in a beaker of water may be practical, soaking it as long as required. This process may also reveal other aspects of the subject. A pine cone when wetted will get darker and the cone's protective seed leaves will retract when wet. Besides soaking, water can be applied using a small paintbrush or spray both selectively and across the whole object.

Live aquatic organisms or other subjects that are alive, such as cheek cells for example, might benefit from the use of a vital stain, a chemical that is inert to biological samples.

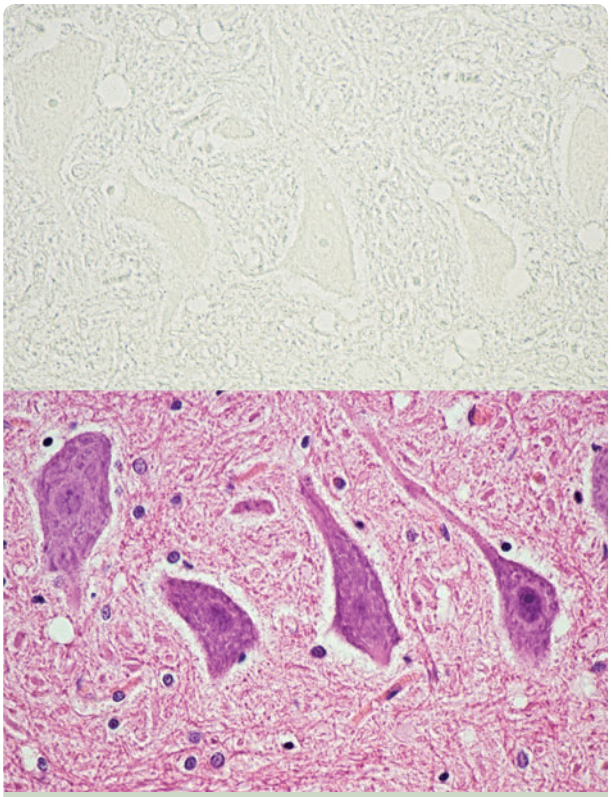

Figure 5.5 This illustration reveals how a section of brain tissue appears unstained and then stained using hematoxylin and eosin. There is a significant change in sample and cell visibility. Staining is a rather complex but important process.

These stains are primarily water-based and contain compounds that are inert and do not affect the morphology of a sample. To use vital stains, the sample to be stained should be prepared as what is described as a wet mount. This is a preparation where water has been added to the preparation and it typically has a cover slip. Once the wet mount has been prepared, it is important to have a supply of bibulous (bib) paper nearby along with the vital stains. Bib paper is a nearly fiber-free paper and very absorbent. There are a variety of sources for bib paper and vital stains. Place one drop of stain next to the cover slip and hold the bib paper on the opposite side of the preparation, drawing the stain through the sample. Once the staining has occurred, it is important to then purge the excess stain out of the preparation. This is accomplished by adding a few drops of water where the stain was located and by placing another piece of bib paper next to the slide. In this fashion, the excess stain will be drawn out of the preparation leaving only the stained material and clear water around the subject. This can be very effective for making structures more visible for examination under magnification. Typically the vital stains come in small dropper bottles and in various colors.

Applying small amounts of liquids to preparations or removing small amounts of liquids can be challenging. Eyedroppers, pipettes and medical syringes can be most helpful for this role and are capable of handling small amounts of liquids precisely. It might be useful to keep a ready supply of various sized syringes for this task.

Specimen Platforms

Much of this content has focused on very small objects, but often some samples are not opaque but transparent or semi-transparent, and they can be large. This type of sample will benefit from having light shined through it. For this reason, it is useful to fabricate a small transparent stage or table to hold the sample. This is most easily accomplished using wooden blocks and piece of double-strength glass with polished edges. In the US, double-pane window glass or ¼ inch is ideal. Polishing the edges is suggested for safety reasons. Depending on the size of the samples that will be photographed, an 11 inch x 14 inch sheet of glass might be useful. The glass can be placed on top of the wooden blocks and light can now be shined from below the sample. Many lighting set-ups will be vulnerable to contamination from extraneous ambient light and reflections. To minimize lighting artifacts, it would be useful to paint the wood blocks black. Maybe an object will have internal facets and surfaces that can refract and reflect light. Surrounding the area where photography will be done with black paper or material will reduce the potential for lighting artifacts to be introduced.

It should be noted that ceiling lights should always be turned off when photographing using glass. Many objects are vulnerable to stray reflections and are comprised of many primarily reflective surfaces. A classic example of this is evidenced in Figure 15.3. Turning off ambient and ceiling lighting will minimize this potential. It is also sometimes practical to photograph through a black piece of cardboard that surrounds the lens. The black cardboard will be reflected in the surface of the sample and will suppress any unwanted reflections in the sample. Holding the black cardboard might be best accomplished by using a clamp, since holding it by hand will compromise what else might need attention during the photographing. As experiences are gained in working the laboratory, additional strategies, tools, and accessories will be collected that will greatly assist in solving problems associated with this work.

Mirrors

Sometimes the orientation of a sample is important. Selecting the top from the bottom can be an important aspect of imaging. Photographing a sample in its proper orientation can be challenging, especially if photographing the bottom or inside is required. When subjects are large, photographing the bottom of an object when there is an orientation concern can be accomplished by raising the subject using a specimen platform (table) to an appropriate height and photographing its bottom. Sometimes, though, the presentation of the characteristics of the sample can be changed when not in their normal state. A useful example of this situation might be found when considering how to photograph feet. It is easy to photograph the bottom of the feet when they are not in a weight-bearing situation. A person could sit on a table and hold their feet out, but this photograph would not be completely accurate since feet bear weight and they will look differently from when they were bearing weight. To photograph feet in a weight-bearing environment would be challenging. A viable solution would be found in the use of a front surface mirror. A person could be situated on a 1 inch piece of plexi-glass located on slightly elevated platform. Under the platform a front surface mirror could be located. Front surface mirrors have their reflective material on the front rather than the rear. Traditional mirrors have their reflective surface on the rear of the mirror. They produce two reflections, one dim reflection off the front of the glass and the primary and bright reflection from the silvering in the rear. Front surface mirrors have only one reflection because the reflective surface is on the front. By using a front surface mirror to reflect the bottom of weight-bearing feet, a highly accurate photograph can be made. While you might not photograph feet, the idea of using a front surface mirror might be useful for various other photographic investigations. Front surface mirrors are used routinely in dental photography, where access to the surfaces of the teeth would be impossible in any other fashion.

Surface Replicas

Photographing the surface of an object can be accomplished in many ways, but when the magnification increases it can sometimes present challenge. A simple strategy can be to create a replica of the surface. Different information about the sample can be revealed in a surface replica. Surface replicating can be accomplished using various materials. Two

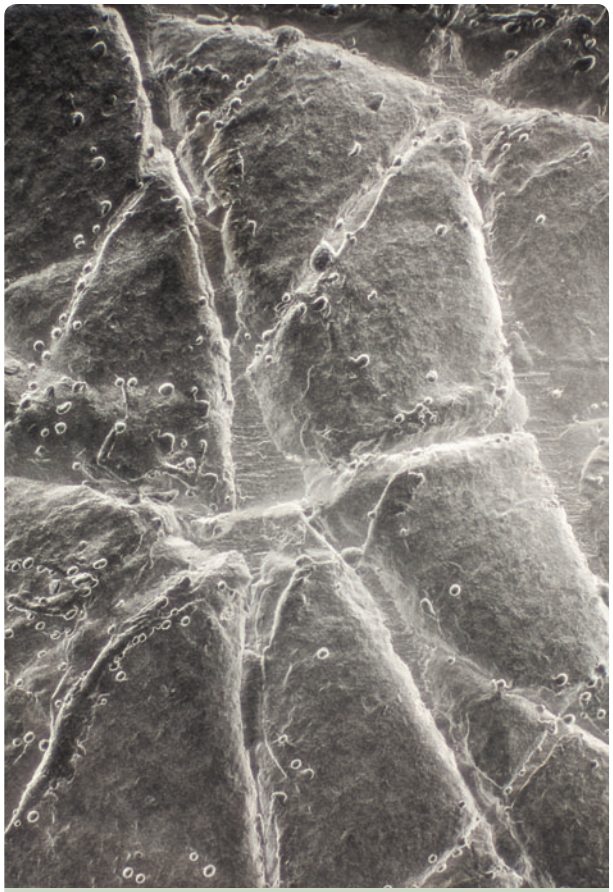

Figure 5.6 This photograph features the surface of human skin. The photo itself is a replica of the surface of the skin. Hot paraffin wax was used to create the surface replica featuring a knuckle, the joint in the finger that bends. The final image is a computational picture comprised of several image slices. The image was photographed using a 4x objective and used darkfield illumination. Image courtesy of Jordan Briscoe.

common materials that are used for surface replicating are NewSkin® and candlewax or paraffin. The latter must be applied when in its liquid state and of course may be quite hot. For that reason, care should be taken when applying it to surfaces.

NewSkin can be applied by painting it across the surface of the sample or by laying an object into a preparation on a clean microscope slide. Allow the compound to dry for a period of time, possibly 10 minutes, for example, before removing. Objects that benefit from surface replicating might include hairs, fibers, leaves or other objects with surface structural information that otherwise would be difficult to see. Skins of various organisms can also be prepared for imaging.

Backgrounds

After a sample has been selected, the choice of the background must also be considered and is no less important than anything else shared in this chapter. In science and certainly other applications, the background serves an important role in isolating a sample in a photograph. Backgrounds need to be simple, neutral, uncluttered, and suited for reproduction or display. Backgrounds for the uninitiated might be an afterthought but should not be. Poor background choices and management of them could lead to lowering image contrast, poor visibility, and/or simply introducing competing elements into the photograph. Choosing the background and how to manage the background's role is an important consideration for making effective scientific illustrations. Wet objects will ooze and interact with papers or towels. There are many things to consider when selecting a background

Figure 5.7 This illustration reveals how one sample can appear when photographed in front of various backgrounds. The seed leaves will look brighter against black and darker when located in front of white. When located in front of a complex background, the various tones and structures in the background compete with the subject for attention.

White, Black, or Gray Backgrounds

White backgrounds can be a logical choice for photographing in science. They are useful when there is a need to isolate an object against a bright tone to communicate the shape and delineate the edges of an object. No matter how white a background looks to the eye, the background in a photograph needs be 1.5 times brighter than the brightness of the light falling onto the sample to produce a white background in the photograph. A difference of 1.5 exposure values (see Chapter 4) will create the correct brightness difference for reproducing the sample's tones against the background without flare creeping into the sample or underexposure of the background's white tone. It is important to properly light both the sample and the background, regardless of the situation, for maximum control and outcomes.

Achieving an even and homogenous lighting for a white background can be accomplished using a light box or other approaches. Even and neutral light is a key element to images with scientific veracity. When using multiple lights, it is important to achieve equal brightness across the entire region that will be located in the background. Using diffusion techniques can be useful to spread the light most evenly across the background. Care should be taken to minimize the spreading of the light used for the background from falling onto the sample. Making a white background can also be accomplished using a light box or by using a laptop's screen to create a uniform white background. Small objects can be located in front of the computer screen. Making an image file with a brightness value of 255 in the R, G, and B channels will be indicative of the background's exposure. More is shared in Chapter 8, which is dedicated to lighting.

It can be easy or difficult to make multiple pictures of samples that all contain the same white tone as their backgrounds and also have them all match in color. More will be shared in Chapters 13 and 15 about color management. The creation of images with the

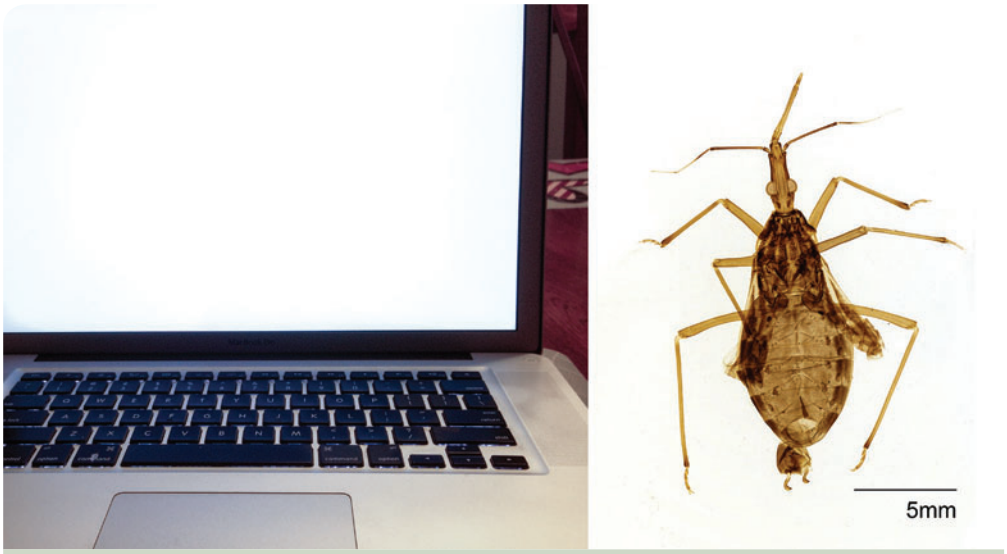

Figure 5.8 A laptop screen can be used as a back light source to make a neutral white background. The image on the right, featuring a whole mount of an assassin bug, was photographed using the computer's screen as the light source and a macro lens set at 1:1.

same tone and color when using a white background has never been easier if the scientist photographer pays careful attention to optimizing the capture settings and subsequent digital image processing strategies required to create files that all contain the exact same tone and color data. When preparing for image capture, all settings should be accomplished using camera's the manual mode. This would include exposure time settings and white balance. Once the images have recorded, a white point in each image's white background can be set to an RGB setting of 240 in each channel. This will lead to a file that will reproduce in print with a touch of gray tone and have just the tonal difference of off-white paper. This can be very important in photographic reproduction in print.

Black can also be an effective choice of background. Unfortunately dark objects have a tendency to blend into the darkness of a black background when there is no edge lighting. Black can be very dramatic and is much easier to manage than white in some applications. For starters there is no variability of color in black backgrounds. White can be vulnerable to secondary influences, whereas black is inert and does not reveal minor changes as easily. When printing photographs in journals or in inkjet prints that use black as a background, it is suggested to use a black point setting of 15 and not 0. Having a printer try and create a 0 brightness value will leave too much ink on the paper and lead to bronzing or other technical issues of the reproduction of black, where the ink pools and dries shiny or has a mirror-like tone when viewed at an angle, especially when printing onto a glossy paper.

Achieving black can be as simple as locating a black material at some distance and location behind the sample. It is ideal not to shine any light on the background or, worse yet, cast a shadow onto the background. A shadow box is an example of not allowing any light to fall on the background. Shadow boxes can be useful in controlling scattered light and its management. For white objects a black background can be very demonstrative of the structures contained within the sample.

Gray backgrounds can be practical but are challenging to use consistently when making numerous photographs. Minor variances in exposure and small lighting challenges are compounded with subtle color changes, which can make gray hard to work with. When

Figure 5.9 All black materials do not behave in the same way when used as a background. In this picture black paper (left), black felt (center), and black velvet were all used as a background. The velvet created the best outcome. This change in black occurs because of reflections and surface structures from the background material itself. Black velvet simply behaves like a sponge for light.

multiple photos are to be reproduced as a group, small variances in color and darkness will become emphasized in the composite. It is far easier to manage black and white for this reason.

The Use of Scales to Indicate Size

The inclusion of a scale in scientific images is an absolute. Without a scale, the image might be considered inadequate by many and not publishable without additional explanations in the caption. The scale is part of the image data. There are some considerations for the use and selection of a scale. The magnification or reduction of an object's size in an image will of course play a role. For close-up photography, it is practical to have several scales available. I like scales that have white markings on black and others that have black markings on white. Metal rulers are not great because of their potential to introduce reflections and optical flare. It is also vitally important to use scales that are calibrated using the metric system at an absolute. An inch ruler would not be practical for selection unless no other options exist. It can also be very useful to have both opaque and semi-transparent rulers. I have found it very useful to also have an inventory of paper scales that have an adhesive back available. There are a number of suppliers of useful products. A quick web search can provide an inventory of vendors for these products. I have also found that forensic supply houses are most able to have a wide range of products.

Using scales requires a few considerations for the method to ensure the magnification of the scale is consistent with the magnification of the object. To accomplish this outcome, it is important to locate the ruler at the correct height or location of the photograph where the focus of the lens is placed. This may require the use of a bracket or third arm to hold the scale at this location in space. The accuracy of a scale located at a different height to the camera will share either more or less magnification. Magnification is in part affected by working distance. Objects placed more closely to a lens while possibly out of focus will be more magnified than objects placed further from the lens.

In addition to locating the ruler scale at the location where the lens focus has been placed, it is also important to have the ruler parallel to the camera's sensor.

Because the DOF of an image might be shallow, having the scale at the proportional height to the sample as well as keeping the scale parallel to the sensor will provide the most meaningful information. Being detail-oriented, I would also suggest the scale is inserted into the field of view in such a manner as to be parallel to the edge of the viewfinder's edges. Keeping all elements of the picture carefully composed and scales properly located will lead to images with more credibility. It takes just as much work to hastily locate a scale than to carefully locate the scale. Good photographers effectively manage all the details.

Scales for photomicrography are a bit more specialized and are called stage micrometers. They can be acquired for use

Figure 5.10 This photograph features an *Acer rubrum*—red maple—seed and a ruler. Scales need to be placed at the correct plane of focus (height) and adjacent to the sample to be most effective.

with both reflected light and transmitted light microscopes. They can be calibrated into various units of microns either in 0.1 mm distances or 0.01 unit distances. They might be a length of 1 or 2 mm distances. They are an integral piece of equipment for calibrating a microscope's internal measurement feature. Many photomicroscopes will come equipped with software that can place bar scales into an image. This feature must be first calibrated before use. Once calibrated (only one time), the software will store the scale's information as long as the hard drive and operating system do not change. The instrument's software frequently is not programmed to determine specific distances but rather the number of pixels in a specific distance. To program the software, a reference distance uses a scale distance of specific distance identifying the specific number of pixels in a specific distance, such as 0.1 mm, for example. Each objective lens needs to be calibrated.

SUGGESTED READING

Blaker, Alfred, *Photography for Scientific Publication: A Handbook*. London: W.H. Freeman, 1965; ISBN-10: 0716706377.

Frankel, Felice, *Envisioning Science: The Design and Craft of the Science Image*. Cambridge, MA: MIT Press, 2002; ISBN: 9780262062251.

Zakia, Richard, and David Page, *Photographic Composition: A Visual Guide*. London: Focal Press, 2010; ISBN-10: 0240815076.

Chapter 6 Basic Laboratory Photography Methods

Close-up Photography, Photomacrography, and Stereomicroscopy

A laboratory-raised *Papilio polyxenes* or black swallowtail (Hodges #4159) caterpillar shows the partial deployment of the osmeterium just prior to girdling itself onto the branch with the production of its first silk loop. The osmeterium is a defensive organ that emits a foul and disagreeable odor serving to repel predators. Photograph courtesy of Todd J. Dreyer.

Because of space limitations, it has not been possible to include everything about photography and how things operate. The next few chapters will share methods useful for the making of photographs that use specialized equipment and techniques. Some assumptions had to be made about the prior knowledge level of the readers of this book. ISO, photographic exposure, white balance, and some other features will be covered as applied to technical applications. If you need additional information about how basic photographic equipment is operated, it would be useful to seek other books or Internet resources.

Overview

Close-up photography and photomacrography—sometimes incorrectly called macrophotography—are techniques that use small camera-to-subject distances. The term "close-up" normally implies that an image will be smaller than lifesize and images will have a magnification that ranges between one-tenth lifesize down to lifesize. These magnifications can also be expressed as image reproduction ratios (image size to object size). Photomacrography typically describes reproduction ratios from 1:1 to about 50:1, or lifesize to fifty times magnification, which is often written as x50. Equipment and techniques beyond 1:1 magnification become increasingly specialized, so close-up photography and photomacrography will be considered separately although there is considerable overlap. Photography above x50 magnification is normally considered to be photomicrography and is covered in Chapter 9.

Close-up Photography

Close-up pictures play important roles in the photo documentation of all kinds of subjects, including technical things, documentary things, artistic things, natural things, or for personal reasons. In medicine, for example, almost all dermatological, dental, and ophthalmic pictures are made in the close-up range. Many applications of close-up photography are also found in botanical, biological, and geological specimens, as well as butterflies, rocks, fingerprints, coins, or postage stamps. There are several ways to make close-up photographs using simple equipment or accessories. In applications where photographs are required for scientific or documentation purposes, it is a good practice to always include a calibration scale near to the object field and at the primary plane of the sample's focus using the highest quality equipment that is available at the time. It is a best practice to use the right tool for a specific job.

Lenses for Close-up Photography

Almost any camera can be used for close-up photography, but using a macro lens on the camera is the critical factor for the best outcome. Many relatively inexpensive compact digital cameras are fitted with zoom lenses that are adequately corrected for moderate close-up distances, despite being designed primarily for use at the infinity focus. This ability is possible because of the digital viewfinder, which bypasses the reflex viewing arrangement

required in DSLR cameras. Smartphones have also become common tools for making close-up pictures, some without focus or structure. However, with a DSLR type camera, there is a wide variety of close-up or macro lenses available, both in fixed focal lengths and zoom lens types. These lenses will lead to excellent and well-defined images. A zoom lens with close-up abilities is often described as macro zoom. It can create very useable results, but a single focal length close-up lens is superior for close-up applications. A prime single focal length macro lens will make better photographs than a macro zoom lens (see Figure 3.15). Important optical characteristics are designed into macro lenses, including excellent edge-to-edge image definition. They have been highly corrected for color and spherical aberrations as well. Their most important feature is their ability to produce edge-to-edge performance when photographing at small working distances. This capability is called flatfield or PLAN. See Figure 6.2. True macro lenses were designed to produce magnifications of 1:1 or greater and operate in situations where the lens-to-sensor distance is greater than the lens-to-subject distance.

Expensive macro lenses have elaborate internal mechanisms that allow groups of lens elements to move independently as the lens focusing extending it to shorter working distances and producing excellent lifesize (1:1) or slightly larger images. These lenses often have reproduction ratios inscribed on the barrel, have fairly modest maximum apertures, and come in various longer—and other than normal—focal lengths producing larger subject-to-lens distances when operated at 1:1. Depending on manufacturer, common focal lengths can be 50 mm, 60 mm, 100 mm or 200 mm, with a maximum aperture frequently of $f/2.8$.

When images are being made at a 1:1 magnification, both the working distance and the image distance will be equal to twice the focal length of the lens. Longer focal lenses provide increased working distances and can be helpful when creating effective lighting or can become important when it is not wise to be near to things such as heat or infectious diseases.

Getting Closer: Supplementary Lenses

Sometimes there is a need to focus at a shorter working distance than a macro/close-up lens will allow. This is because most fixed focal length camera lenses are optimized for long camera-to-subject distances and they are usually are limited in their minimum focus distance. This distance can be reduced and the

Figure 6.1 In this photograph, the reproduction scale on a Nikon 60 mm lens is highlighted. This lens can create a magnification range from 1:1 through 1:10. The corresponding reproduction range is established by positioning the magnification adjacent to the 1: in the focusing window.

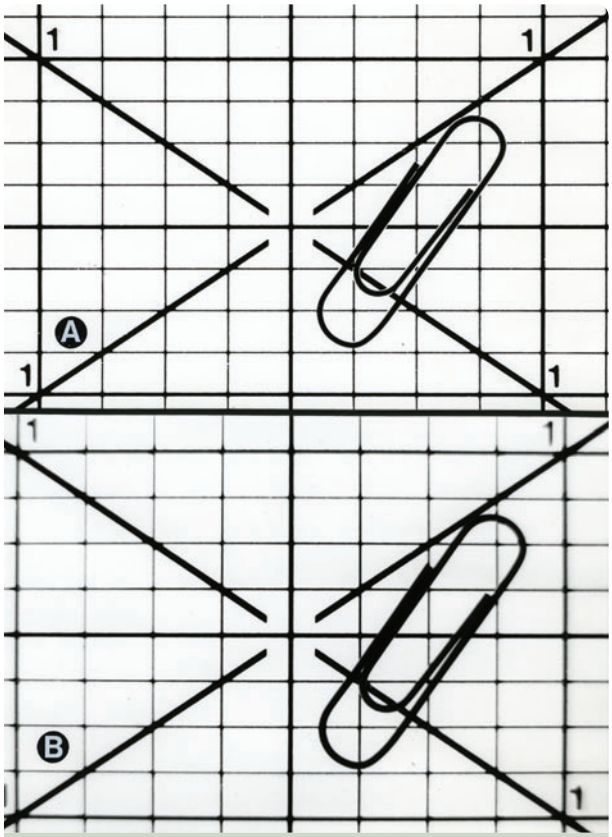

Figure 6.2 This composite photograph reveals the differences between a 50 mm normal lens on the bottom (B) and a 50 mm macro lens (A) on the top. There are considerable quality differences in the edge-to-edge definition of the image structures, such as sharpness and definition.

image size increased by using supplementary or close-up lenses. These lenses will either be a single lens or lens group that attaches to the lens like a filter. These lenses were sometimes called plus lenses or diopters. When used on compact digital cameras, this is the easiest way to achieve close-up pictures using simple cameras. Note, Photojojo® sells small threaded diopter lenses that are useful on smartphones.

The magnifying potential of supplementary lenses increases as the diopter number increases, e.g. +1, +2, up to +20. For more on how this works, see Chapter 3.

A 2+ close-up lens will have a focal length of 0.5 meter (500 mm) and a 5+ close-up lens will have a focal length of 0.20 meter (200 mm).

Alternatively, it is possible to convert all values to diopters and then convert their sum to focal length. For example, a 50 mm lens has a power of 20 diopters. Add a 2+ supplementary lens and the sum is 22 diopters. The combined focal length is 1000 mm divided by 22, or, about 45.4 mm.

Teleconverters are useful precision optical accessories that can be mounted in between compatible lenses and the camera body. Teleconverters increase the focal length of the primary lens and maintain a shorter working (object) distance as well. A teleconverter used in combination with a 200 mm close-focusing lens, for example, will yield a lifesize (1:1) image and does so while maintaining a 71 cm (28 in.) minimum object distance. This amount of working distance is advantageous for many biomedical, industrial, and natural history applications. An increased working distance allows for considerable freedom in arranging lighting or keeping a safe distance from an event. Teleconverters have optical elements and can degrade the resolution of the primary lens if not of a high optical quality.

Getting Closer: Extension Tubes for Close-up Photography

Cameras with interchangeable lenses will also allow for extension tubes or a bellows to be inserted between the camera and the lens, which will extend the lens–image distance. Increasing the image distance rather than changing the focal length will often produce a better optical result than using supplementary lenses when creating lower magnification photographs. Extension tubes are cylindrical tubes of various lengths, used singly or in combination to change the reproduction ratio or image size in fixed steps. Tube lengths may vary from 5 mm to 100 mm, and extensions of 250 mm or more can be used. They are fitted together with threads or bayonet-type mechanisms. Some allow the camera's automatic diaphragm features to be maintained but typically the camera lens must be operated in some of its manual modes. Extension tubes allow one single lens to create magnifications greater than 1:1.

Extension tube sets are relatively inexpensive but are a bit inflexible because they come in specific lengths. This shortfall can be overcome by the use of bellows. A bellows is more costly, but is essential for professional work because a bellows will allow precise control over lens–image distances, which results in more choices and precision in creating image magnification.

When using extension tubes or bellows for close-up work, ordinary lenses should not be used since they are not well corrected for short working distances. A common or normal camera lens has been corrected for long object-to-lens or working distances and uses typically a

short lens-to-detector distance. When this type of lens is used with short working distances, the resultant photos will not be optimal since there are numerous aberrations that will become enhanced when using short working distances. Optical performance can be markedly improved by using the normal camera lens reverse mounted, so that the rear element of the lens is closest to the subject. Manufacturers supply reverse-mounting rings for this purpose but all of the lens's automated features will be disabled. A true close-up lens remains the best optical solution for use in this image size range.

Focusing, Depth of Field, and Diffraction

To achieve a specific image size in close-up photography using a DSLR camera, traditional techniques will need to be abandoned. To achieve critical focus in close-up work, the required reproduction ratio should be determined and (if available) set on the lens barrel. The camera can then be focused by changing the working distance, which involves moving the subject and camera closer or further from one another. This may be done most precisely using a focusing rail. A good rail may have calibration marks and smooth adjustment slides, and will lock for precise control over its movements. The rail should be attached to a tripod to facilitate the most robust structure and create the most precise of outcomes.

A lab jack or other stage can also be a useful tool for achieving precise control over where focus is placed. A quality lab jack is preferable to an inexpensive one. There will be side-to-side movements when the height adjustment is made using inexpensive equipment and this movement will cause minor changes to the photograph's composition. Moving the camera can also be accomplished by adjusting the height of the lab stand or tripod up and down.

If a lab jack is not owned, I imagine everyone has an old lens lying around that can serve as a lab jack used for focusing. Since the helical focusing mechanism in a lens behaves much like a focusing rail, adjusting the lens focus will move the sample up and down. It is

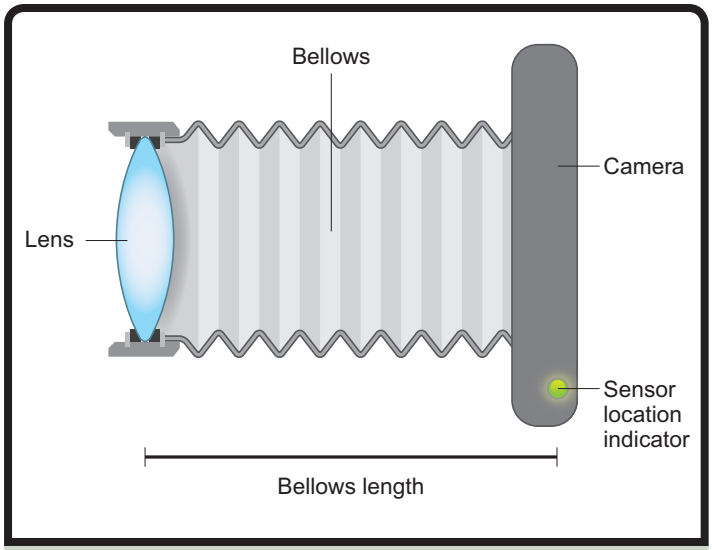

Figure 6.3 The basic parts of a close-up and photomacrography system include a close-up or macro lens, a bellows (or extension tubes), and a camera.

Figure 6.4 Changing the working distance can be accomplished by moving the sample or moving the camera. It is important not to change the reproduction ratio on the lens. A lens can be used as a lab jack. Because of the smooth helical focusing mechanism in a lens, small changes in working distance can be changed very precisely. It can be useful to cover the top of the lens with a piece of black cardboard, as evidenced in this illustration.

important to try and "lock down" the lens's base to avoid it moving around on the table. I have used a large "C"-clamp on the base of the lens to add weight and limit shifting when in use.

The range of sharp focus in a photograph is referred to as its depth of field (DOF). DOF assessment can be rather subjective and viewer-dependent, but in close-up photography depth of field will be noticeably small, and becomes smaller still with increasing magnification. In everyday photography, an image's depth of field can be increased by using a small aperture. As the magnification of 1:1 is approached, a very small aperture—ƒ/16–ƒ/22—will produce diffraction that softens image crispness. Diffraction effects are dependent on a sample's characteristics and magnification. In general photography there tends to be more depth of field behind the subject than in front. In close-up photographs, though, the distribution is more equal in front of—and behind—the subject. DOF can also be increased using Z-stacking or increased DOF imaging. An entry about how to achieve increased DOF using computational techniques is shared in Chapter 14. See Figure 3.18 for additional information about focal length and DOF.

Creating Camera-to-Subject Alignment

Since the DOF in close-up photography is shallow and has a short working distance, it is useful to align the camera to the sample's principal plane of interest to maximize what

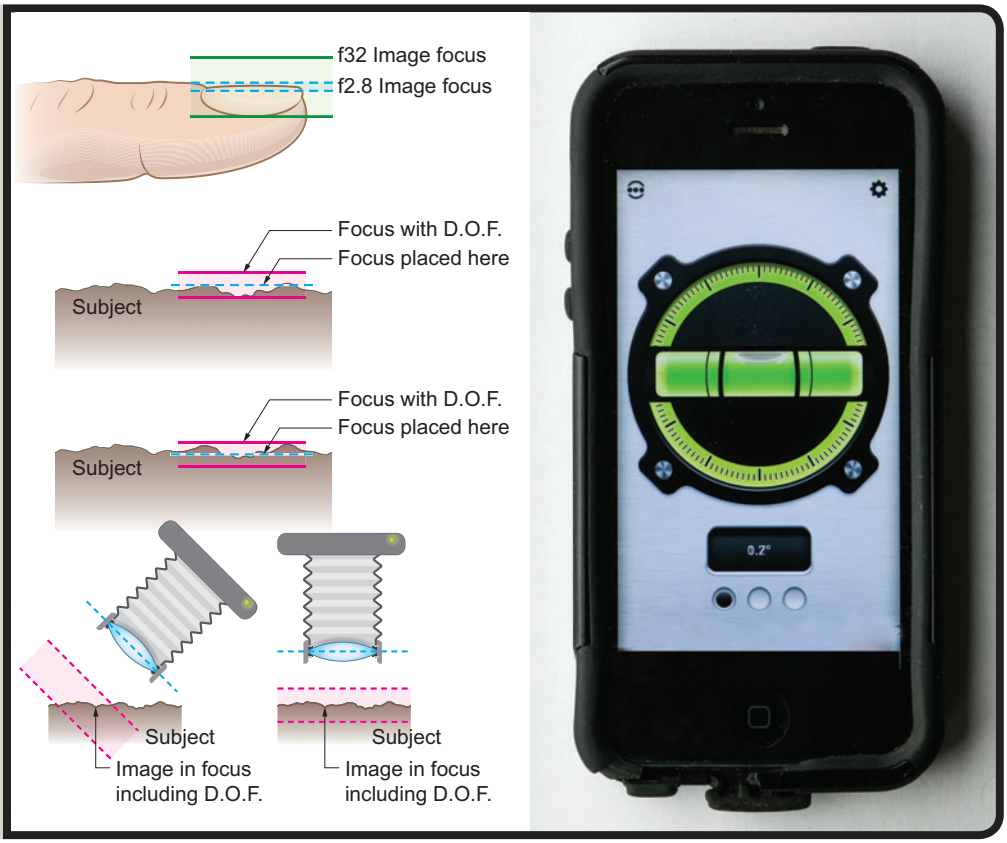

Figure 6.5 Creating and maintaining an adequate DOF in close-up and macro applications can be a challenge. Using an appropriate aperture can be most helpful. In the example illustration, the zone of DOF can be increased proportionately by using ƒ/32 rather than ƒ/2.8. Additionally, rolling the focus into the sample rather than placing it on the top of surface can increase the DOF by a small amount. One of most important tools is camera-to-subject alignment. Keeping the sensor parallel to the sample surface can be one of the most effective of all three strategies shared in this graphic. On the right is an image of an app from a smartphone for a level. A level can be a useful tool aligning the camera to the stage.

DOF is available for recording. Once the reproduction ratio has been set on the lens, the camera should be aligned to the subject. Either the camera or the subject can be adjusted. Figure 6.4 shares a few considerations for this alignment. When focusing, it is important to roll the focus into the sample when working in this way. DOF can easily be wasted in the space above the sample. Using a spirit or electronic level can also be useful to ensure all surfaces are parallel. This will create the maximum DOF possible produced by a lens' aperture choice and effects of magnification.

Selecting the Best Aperture Possible

Camera-to-subject alignment will play a role in the range of focus, but so will the DOF created by an aperture. Using a small aperture will increase the zone of focus but can also introduce diffraction as the image size increases. Using an aperture that is too open will create the problem where not enough focus has been formed to make an effective image. Somewhere between the two extremes is ideal. Figure 3.19 shares the effect of diffraction on details from the scales on a butterfly wing when various settings of the aperture are used.

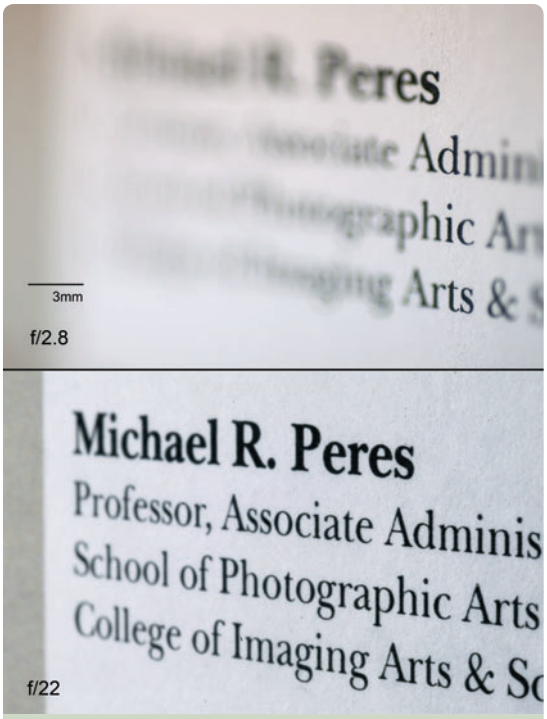

Figure 6.6 This photograph features a business card where the minimum and maximum DOF possible was formed using a magnification of 1:1. The top photo used an aperture of ƒ/2.8 and the bottom one ƒ/22.

Figure 6.7 A close-up lens looks very different than a true macro lens. On the left is a Nikon 60 mm lens, and on the right are three Zeiss® luminar macro lenses with focal lengths of 40 mm, 25 mm, and 16 mm respectively. Notice the macro lenses have no focusing collars, only an aperture adjustment ring. The lenses' relative working distances are indicated in this graphic.

Exposure Compensation and Determination

When increasing the sensor-to-lens distance, light will be lost in the camera and an incorrect exposure will be created. When using today's sophisticated cameras, the information required to calculate the corrected exposure resulting from light loss can be accomplished by the camera without effort. As image magnification increases, the amount of light reaching the detector from a given area of the subject is correspondingly reduced. However, exposure evaluation using through-the-lens metering (TTL) equipment is straightforward. It should be chosen if the system permits and the subject is "average" in its tonal reflection. When using a hand-held and external-to-the-camera exposure meter, allowances must be made for light loss due to the lens-to-detector distance. As an example, at a 1:1 magnification, the rear element of a 50 mm lens may be up to 100 mm from the detector. At this reproduction ratio, there will be two stops of light loss. The amount of light loss for any reproduction ratio can be calculated. There are several equations that can determine the exposure factor.

$$\text{Exposure factor} = (R + 1)^2$$

where R is the image magnification. To calculate a system's reduction or magnification, divide the image size by the object size.

$$\text{Mag} = \text{image size/object size or } i/o$$

In the equation, 1 represents one focal length of any lens used and R is the reproduction ratio expressed as a decimal. Since the reproduction ratio always corresponds to the increase in extension, the R can be thought of as this increase. For example, if the reproduction ratio is 1:5 or 0.2, the exposure factor would be $(0.2 + 1)^2 = (1.2)^2 = \sim 1.4$. Once an exposure factor has been calculated it is applied to the measured exposure, creating the corrected and adjusted time.

Another very quick check on image size relative to object size is to use the viewfinder as a sort of ruler scale. Since the dimensions of the sensor are 1 inch x 1.5 inch for a full-frame camera, the viewfinder will also be 1 inch and it is possible to assess object size by using the 1 inch dimension of the viewfinder for sizing.

Photomacrography

Photomacrography is usually defined as having image magnifications that are in the range of 1:1 (lifesize) up to about 50:1 (fifty times lifesize), but it is more aptly defined by the equipment was used to create the magnification. Photomacrography equipment is generally more elaborate and complicated than the equipment used for close-up work. In photomacrography, the lens is further from the sensor than the lens is from the subject.

There are several ways to produce magnified images or photomacrographs, including using a simple microscope, which uses a single stage of magnification, or a magnifying lens. The best lenses for macro are those designed for this purpose and have special features that will be shared below. These lenses are used with a bellows and lead to images that are larger than the subject. I will share, right up front, that there are very few if any true macro lenses still made by any manufacturer. Many close-up lenses will achieve 1:1 but only the Canon 65 mm macro lens creates magnification all by itself (see Figure 3.15).

Most of the photographic work done in this magnification range is accomplished using a stereomicroscope. Some scientist photographers will also try to use a compound microscope using a 1x or 2x objective to varying degrees of success. The resolution of these objectives used on a microscope will be very low. When making magnified images, the object is placed at a distance equal to the focal length of the taking lens. Additionally, the image must travel a distance to the detector that is greater than two times the focal length of the lens to create magnification.

In this magnification range, it is essential to have a rigid support for the components, ideally equipped with some means of moving each element (object, lens, and detector) precisely along the optical axis and being able to secure the camera parts firmly. This is why photographs in this magnification range are often made with low-power stereomicroscopes instead. Stereomicroscopes will often have a zoom objective lens and possess a relatively low numerical aperture (NA). These instruments are excellent for use with longer working distances and usually come equipped with rack and pinion adjustment of object distance, improving the fine focus. However, photographers should be aware that the tilted optical axis necessarily used in stereomicroscopes results in making photographs that have a similarly tilted depth of field. A binocular or compound microscope does have two eyepieces, but will not produce stereo images directly.

Bellows and Laboratory Set-ups

A bellows is, in effect, a variable length extension tube with a lens board at one end and a location for the camera to be attached (Figure 6.3). The camera and lens boards will be mounted on some sort of a focusing rail system. The bellows extension can easily be adjusted over a very wide range to achieve the correct and precise magnification for the sample under evaluation. Magnification will be limited by the rail's length and the focal length lenses that are available. A bellows is a seemingly simple piece of equipment to operate but it often causes problems for photographers, mainly because the spacing of the key elements (detector, lens and subject) is not fixed, which means that working distance, magnification and focus are independent and interdependent. In a "normal" camera, two of these three variables are usually fixed. Although some bellows can accept a wide variety of cameras and lenses, many are designed for a specific manufacturer's lens mounts.

True Macro Lenses and Optical Considerations

A laboratory photomacrography camera system as described above will usually accept a wide range of apochromatic macro lenses optimized for specific magnification ranges, but will be versatile enough to be used with a true compound microscope and eyepieces. This link between micro and macro has endured for many years and these types of macro lenses are often used with RMS (Royal Microscopical Society) treads used for mounting rather than a bayonet.

True macro camera lenses are special-purpose lenses designed for magnifications greater than infinity. These lenses may have other names reflecting their size, such as short mount;

they are sometimes called thimble lenses because they look like a thimble in size. Macro lenses are unlike close-up lenses in several respects. The first and most obvious difference will be that this lens has no focusing collar because the lenses are designed for use on a bellows. Consequently, most macro lenses will have only a lens diaphragm ring and focus is controlled through changing the working distance as a function of the pre-determined bellows length. The aperture range is usually smaller than for traditional lenses used for other technical purposes. True macro lenses will typically have maximum apertures of $f/2.8$ and minimum apertures of $f/16$ or, at its smallest, $f/22$. At high magnification, small apertures produce undesirable diffraction effects and poor resolution and are rarely selected.

The diaphragm rings of macro optics are seldom marked with conventional f-numbers. The f-numbers on ordinary camera lenses are defined in terms of an object photographed at infinity, which is never the case. These lenses work at short lens-to-subject distances. The effective f-number therefore increases as bellows extension increases. For this reason, some macro lens diaphragm settings are designated by a simple numerical sequence, for example, 1, 2, 3, 4, 5, 6, where each number is a factor of two which creates a one-stop exposure value difference. Other lenses use Stolze numbers, which are similar to f-stops where each number, 1, 2, 4, 8, 15, 30, is proportional to its adjacent stop by a factor of two.

Other Lenses that Can Be Used for Magnifications 2:1 and Higher

Different manufacturers such as Carl Zeiss, Nikon®, Leitz®, Canon®, and Olympus® produced true specialized macro lenses of excellent quality in the past, but that is no longer the case. As previously mentioned, Canon makes a special lens, a 65 mm macro $f/2.8$ that creates an image range of up to 5:1. It is very sharp and very useful for creating image sizes that are larger than object sizes. Regrettably, it is not possible to use this for samples that need to be reduced in image size or less than 1:1.

Camera companies are no longer making true macro lenses but they may still be found as used equipment or on eBay®. Perfectly serviceable lenses made by Bausch & Lomb® or Wollensak® might also be found there. Also, as a consequence of the evolution from motion film to video, wide-angle and normal focal lenses used for 16 mm cine cameras can easily be found and adapted for use in this magnification range. The 16 mm, 25 mm, and 50 mm lenses work well for photomacrography when reverse mounted. Cine lenses are not as well corrected as true macro lenses, but they can make a satisfactory "poor man's" substitute, especially when reverse mounted.

Setting up the System

It can be useful to assess the sample's magnification and compare it to the sensor size to start with. Predicting magnification is quite simple. When using a full sensor DSLR camera, the sensor will be 35 mm or 1 inch in the long dimension. Magnification requirement can be calculated using the equation M = i/o, where the sensor represents the image and the

object will be measured. For example, if a sample were 5 mm, 35 mm/5 mm would require an image magnification of seven times.

The best way to calculate the magnification of a single lens system is by using the equation

$$v = (m + 1)F$$

where v is the image distance, m is the magnification of the system, and F is the focal length used. This equation can be used effectively for magnification calculations in close-up photography or photomacrography, independent of the format of the camera system utilized.

Using the example shared above, approximately 7x is needed. Start by selecting a 25 mm or 1 inch lens. Substituting 7 for the magnification and 1 inch for f, a bellows length of 8 inches or 200 mm would be required.

Next, using the 1 inch lens, create 8 inches of separation between the lens and camera sensor. The sensor's location can be determined by located the circle o on the camera body (see Figure 5.9).

Now locate the subject at a distance of one focal length—1 inch in this example—from the lens and change the working distance to focus the image. Do not change the bellows length.

The camera can be operated in a tethered or live mode and the exposure can be assessed in the preview window. The amount of light loss in the system can actually be calculated using the method shared before.

Finally, focus by changing the working distance.

Exposure Compensation

As the lens is moved further from a sensor required to increase magnification, significant light loss will occur. This principle is defined by the inverse square law of illumination. A camera with an internal metering system will automatically adjust for the light loss; however, with some equipment, it can be helpful to predict the exposure change using an external meter before proceeding. Some events cannot be re-photographed and require establishing settings before beginning to work with a specific sample. Some meters for scientific applications have sufficient sensitivity to make readings using the viewfinder, which would automatically compensate for the loss of light in the system. The light loss in a macro camera system is referred to as the bellows factor and should be applied to the metered reading necessary to obtain a correct exposure when measuring light using an external tool.

Exposure Factor Equations

There are several equations that can be used to determine an exposure factor, but the two most widely used are:

$$\text{Exposure factor} = \left(\frac{\text{image distance}}{\text{focal length}} \right)^2$$

This following equation is equivalent to the previous one because the image distance is the lens focal length plus any extension, making the equation:

$$\text{Exposure factor} = \left(\frac{\text{extension}}{\text{focal length}} + \frac{\text{focal length}}{\text{focal length}} \right)^2$$

but extension/focal length is equal to R, and the second term is 1, brings us back to:

$$\text{Exposure factor} = (R + 1)^2$$

This equation was discussed in close-up photography. The $(R + 1)^2$ version is easier to use if scales or rules are substituted in the specimen plane needed to determine reproduction ratio. The (image distance/focal length)^2 version is easier to use if a graduated bellows is used to determine image distance (remember to add the focal length to the bellows extension reading to get the entire image distance if the bellows scale reads "0" at the infinity focus position). The exposure time indicated for a selected f-stop with hand-held exposure meter is multiplied by the exposure factor.

Depth of Field

The depth of field is the distance in front of and behind the object that is considered to be in acceptable focus. In photomacrographic images, there is relatively speaking no DOF in a photograph. The depth of field will increase as a lens is stopped down, but unfortunately there is a fundamental limit as to how much a lens can be stopped down without degrading the image, even in the plane of sharpest focus. This softening is the effect of diffraction caused by the bending of light as the light passes through a very small slit or opening. This leads to a lessening of image sharpness. More on diffraction can be read in Chapters 3 and 9.

Resolution of an image is assumed to be diffraction limited, and points either side of the specimen focal plane will be blurred by the combined effects of optical and diffraction blur.

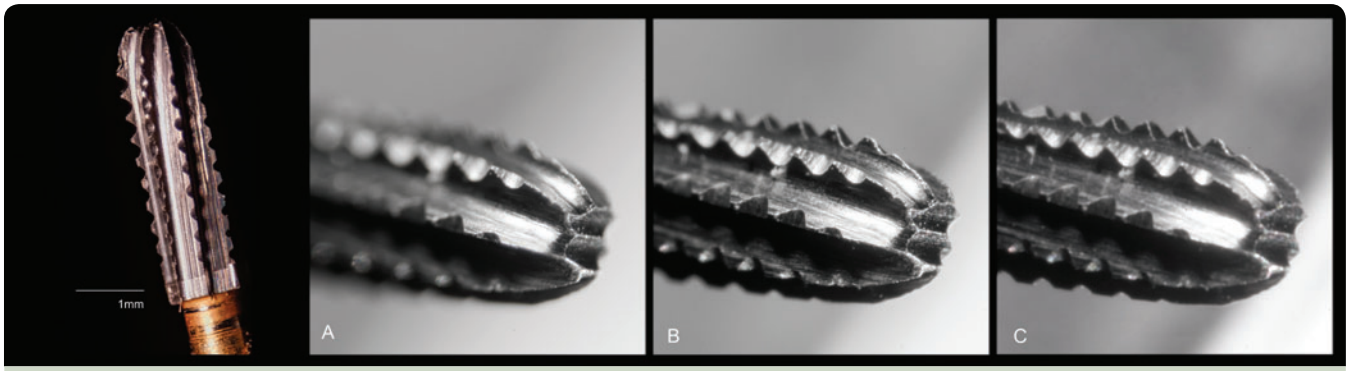

Figure 6.8 This illustration reveals the DOF and sharpness of a dental drill burr photographed with a magnification of approximately 5x. Photograph (A) was made with the lens wide open at ƒ/2.0. It shows the lens's least amount of DOF and most resolution. Photograph (B) was made at ƒ/11 and reveals a modest gain in DOF at no real expense to resolution. Photograph (C) used the lens's smallest aperture, ƒ/22, and reveals what happens to an image's sharpness, negatively impacted by aperture-induced diffraction. On the flip side, there is a considerable increase in DOF.

Subsequent larger magnifications will be more affected. In practice, any aperture can be used when magnifications are less than x5, but as the magnification goes beyond that, image degradation becomes obvious both in the viewfinder and on the subsequent print. In theory all lenses will form their highest resolution when operated wide open or at their maximum opening, $f/1.4$ for example. There are other factors though, such as optical contrast and related optical characteristics of an image.

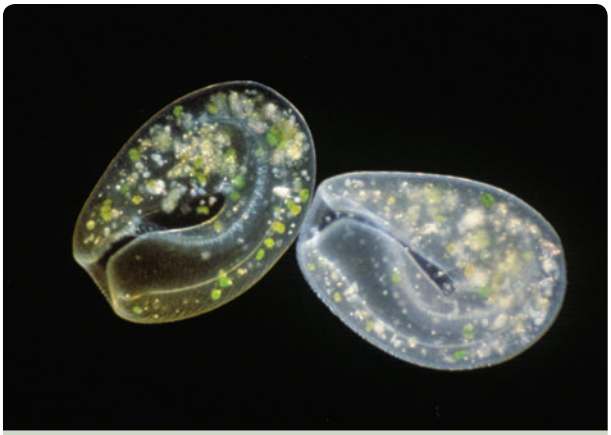

Figure 6.9 This photograph features a live and swimming *Bursaria truncatella*. It was made using a simple microscope equipped with a 25 mm lens. A camera magnification of 4x was used. Darkfield illumination produced from electronic flash lighting was used to maximize image contrast and produce a dramatic and very sharp result.

Stereo Photomicroscopes

Stereomicroscopes seem to have replaced almost all photomacroscopes in science laboratories. It is truly rare to find someone still using a true photomacroscope for low magnification work. Stereomicroscopes are faster to use and are certainly more flexible than macro systems. They provide a range of magnifications and they can quickly be adapted for use with both translucent and opaque objects.

A stereomicroscope is quite different from a compound or upright microscope. It has a primary objective and eyepieces, but that is where the similarities end. Stereomicroscopes do some things very well, but are limited for other reasons. One of the greatest advantages for selection of a stereomicroscope separate from its ease of operation is that the image is oriented correctly to the way it is seen. So the left side of the object is the left side of the image. When moving an object or dissecting the object, this orientation alignment can greatly simplify its use. This makes this instrument ideal for dissection, surgery, and manufacturing, where operators can focus on the work without swapping an orientation. For all other magnified imaging systems, the image and the object are opposite in orientation to one other. If there is a piece of dirt on the left side of an image of a sample when looking into the eyepiece using a compound microscope, the dirt is actually on the right side of the sample. Because of this, an operator must constantly remember this reality, especially when working with delicate materials that can be damaged in handling or when moving them.

Stereomicroscopes form two optical images because there are two optical pathways in a stereomicroscope that create two different points of view for a viewer. This allows the image from a stereomicroscope to produce true stereopsis or stereovision. An image is said to be 3D. The microscope is typically located on a vertical column that is adjustable. This adjustment allows the microscope to be used for evaluation of objects of various thicknesses. The instrument can be quickly raised or lowered on the column and the objective's working distance can be quickly changed.

There are two basic adjustments on a stereomicroscope. One adjustment mechanism is used for focusing or changing the working distance of the microscope and the other is for changing the microscope's magnification. The focusing knob moves the instrument up or down using a very precise worm gear system. Unlike compound microscopes, where the

sample is moved, the stereomicroscope itself is moved. There is only one focus mechanism for a stereomicroscope, not like compound microscopes where there is a fine and coarse adjustment. A stereomicroscope sometimes also uses a Barlow lens. This lens will increase working distances and decrease magnifications.

There are actually two ways that magnification is adjusted in a stereomicroscope. The actual magnification is dependent on the microscope's primary objective. Many stereomicroscopes have various objectives such as 1x, 1.5x, or 2x as the primary objective lens. These lenses are interchangeable. This objective lens forms the initial stage of magnification. Then it is possible to add a magnification adjustment function specific to the instrument. Many stereomicroscopes have a zoom range of approximately 7x to 12x. This adjustment is not fixed and allows an operator to have an entire range of fine adjustments available to optimize just the right amount of magnification. The viewing eyepieces also have magnification. These can range from 2.5x to 10x. More about eyepieces and objectives can be read in Chapter 9. When all the various components of a stereomicroscope are factored as possible elements, these instruments can create images with a magnification of from 2x up to 250x.

Figure 6.10 This illustration reveals how two imaging pathways are formed within the microscope. Located behind the primary objective are two optical pathways that see a sample at small angles. The two images produced by the two lenses are offset by a total of approximately 10–12 degrees.

Stereo is achieved as a consequence of the two optical pathways; however, the imaging system utilizes only one of these pathways. An image produced from a stereomicroscope is not stereo. In fact, since the lenses by design are not at a 90-degree axis to the sample, there can be some minor challenges to manage imaging. Each objective is offset approximately 6 degrees from 90, producing an off-axis view from the left and right side of the object. When imaging, the image is formed from a slightly offset point of view. This can be easily addressed by positioning a sample at a similar angle

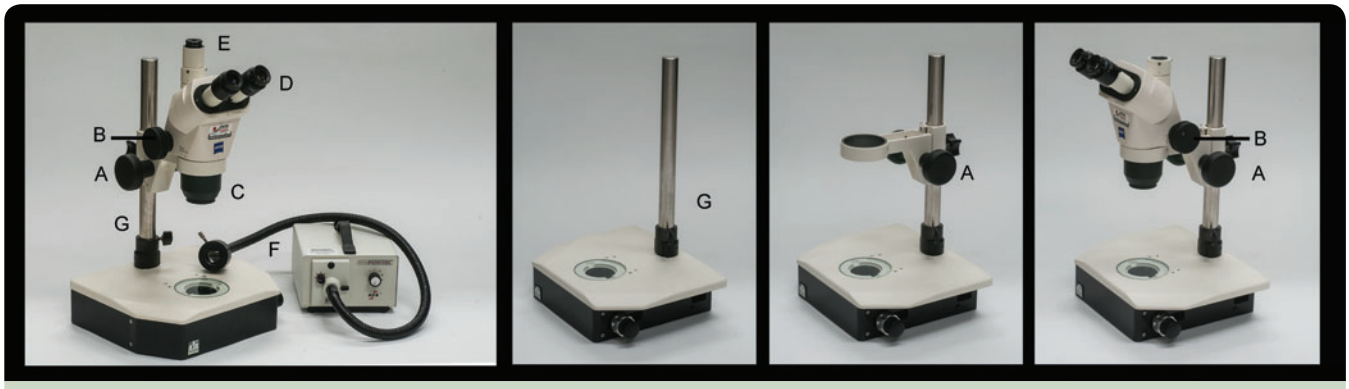

Figure 6.11 A stereomicroscope has different pieces and parts than a simple or compound microscope. In this illustration the basic parts of the stereomicroscope are revealed. (A) features the focus controller which changes the working distance. (B) is the magnification changer. Typically this control will have a 10x zoom range. (C) is the primary objective. (D) features the viewing eyepieces and (E) is the imaging eyepiece. (G) is the instrument column attached to the base that has a built-in illumination port.

of approximately 6 degrees away from the perpendicular, when the sample is underneath the objective lens.

The offsetting of the imaging lens can create minor aberrations since by design the lens is not optically centered over the sample that is being examined. Because of advances in lens design, corrections and higher numerical apertures are more common in stereomicroscopes than in years past. More about numerical aperture is contained in Chapter 9.

Photographing

There are no real unique challenges associated with making photographs or video using a stereophotomicroscope. There will be a beam splitter located in the instrument, which directs a portion, or all, of the image to the camera. The image in the camera will be dimmer than the image observed in the eyepieces since rarely will 100 percent of the image brightness be directed there. There will always be some light loss in an image when relayed to the camera and away from the viewing eyepieces. Often the image will remain visible in the eyepieces but the focus may be different. Many stereomicroscopes have an iris diaphragm located in the optical pathway. Unlike the aperture diaphragm of a compound microscope, the iris diaphragm will limited influence on an image's depth of field. Like all diaphragms, this iris diaphragm will also remove light intensity from the system, which lengthens the exposure times. One of the limiting factors when using a stereomicroscope can be the objective's NA when compared to a compound microscope's objectives of similar magnification, or a true macro lens. Not all images made from lenses of similar magnification will be equal.

SUGGESTED READING

Blaker, Alfred A., *Handbook for Scientific Photography*, second edition. London: Focal Press, 1989; ISBN-13: 978-0240517902.

Gibson, Henry Louis, *Close-up Photography and Photomacrography*. Rochester, NY: Eastman Kodak, 1970; ASIN: B000089208.

Lefkowitz, Lester, *Manual of Close-up Photography*. New York: Watson-Guptill, 1979; ISBN 10: 0817424563, ISBN 13: 9780817424565.

Sheppard, Rob, *Macro Photography*. New York: Pearson Education, 2015; ISBN-10: 0134057457.

Vetter, John Paul, *Biomedical Photography*. London: Focal Press, 1992; ISBN-13: 978-0240800844.

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Chapter 7

Advanced Laboratory Photography Methods

Making Things Visible

These four photographs show how a person with fair and freckled skin might appear using different tones and spectrums. On the top left is the visible light RGB image. That RGB file was then converted to a grayscale version (top right). It is interesting to note how the elimination of color data can change emphasis. On the bottom left is an ultraviolet recording, and on the bottom right the subject was photographed using infrared radiation.

Introduction

Making things more visible is a constant challenge when working in the laboratory. There are a number of methods useful when working with challenging small subjects. Light, lighting, optics, dyes, and behaviors can all be used to reveal things about a subject. Some behaviors are difficult to observe directly because the human visual system is limited in its sensitivity. Light causes visual stimulation in the eye; however, ultraviolet and infrared are radiations that do not cause a visual stimulation to occur. Making photographs of objects—using other than visible light—will reveal characteristic behaviors useful for identifying a material or assessing the presence of a specific behavior. Methods such as fluorescence can also provide information about an object. This chapter will deconstruct theory and practice useful in making invisible things, visible.

Fluorescence

Fluorescence is the emission of light from an object or material, when exposed to short wavelength high energy light or other forms of electromagnetic radiation. The excitation energy causes an object/material to produce a visible emission or light as a consequence of the excitation of specific types of molecules. It is possible for various portions of the entire electromagnetic radiation spectrum to excite a type of molecule causing specific emission of light from the material. Fluorescence is a type of luminescence. Luminescence is defined as the emission of light by a substance that has not been heated. Fluorescent light or other radiation will exhibit longer wavelengths and lower energy compared to the excitation energy that created the fluorescence. If the emission of energy continues after the excitation source is removed, the phenomenon is called phosphorescence. A material's emission can be in the visible, ultraviolet (UV) or infrared (IR) regions of the spectrum. The most striking example of fluorescence might be evidenced when the excitation radiation comes from the ultraviolet region of the spectrum invisible to the human eye. The emitted light from the fluorescence reaction in this example would be visible and behave as a light source by itself.

Fluorescence is used in geology and mineralogy, fluorescence spectroscopy, and fluorescent labeling as a biological detector. It is also used for printing security documents, ink tagging, fluorescent properties of paper money, and credit cards. Fluorescence occurs frequently in nature in some minerals and in various biological states in various organisms including cyanobacteria.

Fluorescence behaviors were first observed by George Gabriel Stokes in 1852. In his paper entitled "Refrangibility (Wavelength Change of Light)," Stokes described how fluorspar and uranium glass had changed when exposed to "invisible light" beyond violet. The glass became blue when it interacted with the invisible light described above. He named this outcome "fluorescence." Today fluorescence applications—and in particular light microscopy—can provide practical methods for studying materials and whether they exhibit fluorescence. Fluorescence can be used to study materials in their natural state (autofluorescence) or to monitor a sample which is infused with a chemical capable of fluorescing when bombarded with short wave energy. When a chemical that fluoresces is added to a material it is described as tagging.

The emitted fluorescent light will possess a longer wavelength and lower energy light than the excitation or absorbed energy possessed. The fluorescence is the result of energy from a source called excitation interacting with a subject. Excitation energy needs to be of a shorter wavelength than the fluorescence emission or the visible light portion of the reaction. When the short wave energy is absorbed and re-emitted as long wave energy, energy is released as light from the energy loss. This phenomenon is known as the Stokes shift. The light emission is the result of the energy loss from the time the photon has been absorbed by the material until the very short increment of time when the light is emitted. The reasons for and the strength of the Stokes shift are very complex. The shift is dependent on the characteristics of a material called a fluorophore (a fluorescent chemical) and the environment/temperature where the material is situated. The release of light is frequently the result of an electron falling back into a lower valence level, or the resting state of the material. This state defines a time when the molecule has its lowest vibrational energy level. A valence is a pathway that electrons use when circling the nucleus. Molecules will have a number of valence levels depending on their composition. A Jablonski diagram (Figure 7.1) is a classic illustration used to share the physics of fluorescence. It can be helpful to gain a better understanding of fluorescence. The illustration was first created by Professor Alexander Jablonski in 1935.

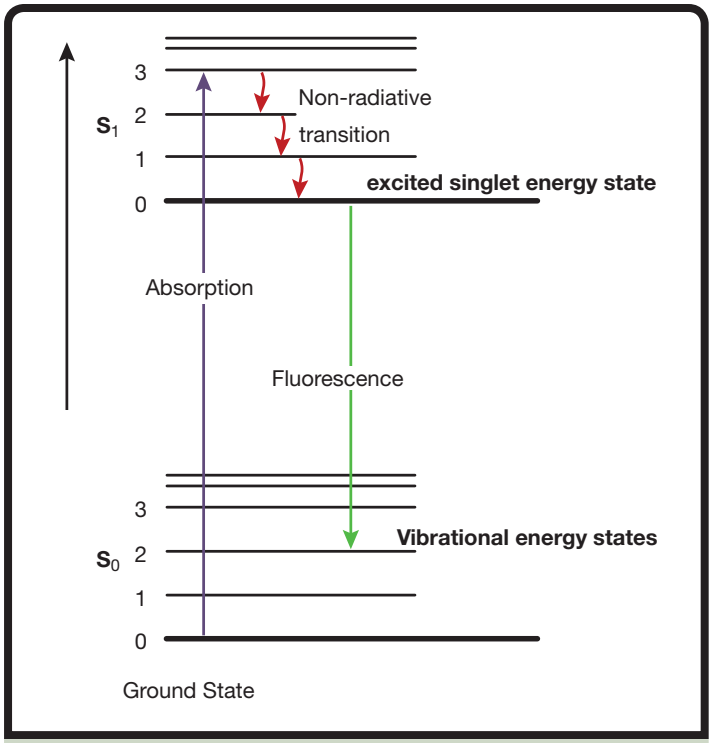


Figure 7.1 The Jablonski diagram displays the basic ground electronic state of a material and is shown using parallel bars in So including where the singlet first level would be situated in the resting state. Electrons in each energy level can exist in a number of energy levels, represented by the various lines identifying each electronic state. When excitation energy is applied to a sample indicated by a purple blue line, an electron jumps from S_0 to S_1. The excitation energy will typically be comprised of short wave energy and is not permanent. When the excitation energy dissipates, the electron falls down and an emission of longer wave energy (light) indicated by the green line is produced.

When molecules are at rest, and before excitement, the electronic configuration of the molecule is described as being in the ground or resting state. When exposed to a photon or photons of higher energy, electrons may be raised to a higher energy level and enter a vibrational excited state. This occurs within a very short time. When a fluorophore is added to a material and absorbs light energy, it is usually excited to a higher vibrational energy level, described by physicists as the first excited state (S_1), before rapidly falling back to the lowest energy level. This occurrence is called "vibrational relaxation."

Ultraviolet and Short Wave Blue Excitation

When interested in evaluating a material's potential to fluoresce, scientific photographers frequently start the evaluation using short wave blue or UV energy. It is possible to use a "black light," an alternate light source, a UV-rich source or an electronic flash unit if the UV removal filter has been removed. It is important to remain cautious when using UV energy: it can be dangerous to your eyes and skin.

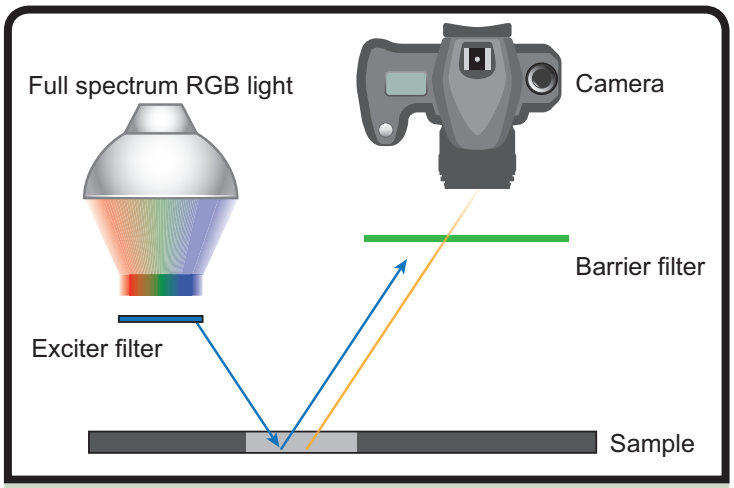


Figure 7.2 There are several elements needed for a fluorescence imaging system. These would include a bright and high energy light source, an exciter filter of appropriate size, and a barrier filter. Fluorescence can be both observed or photographed.

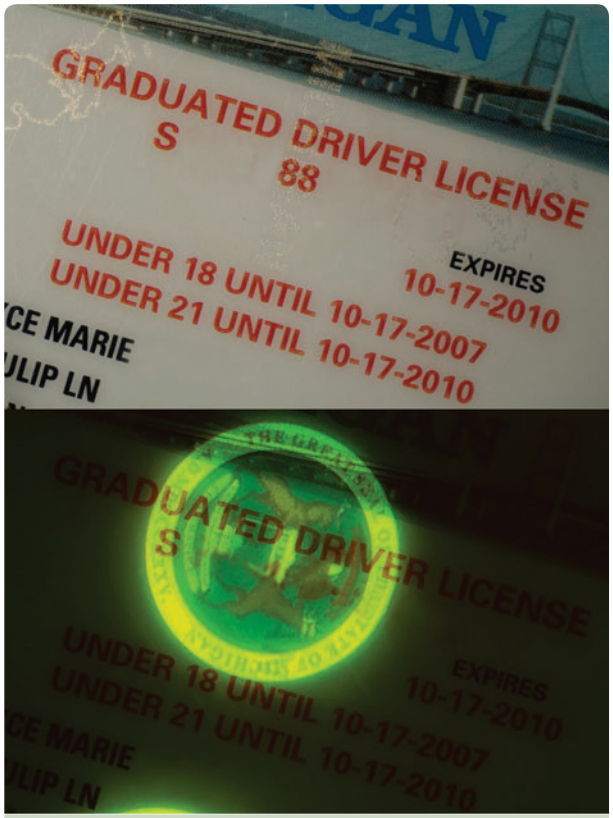


Figure 7.3 An example of fluorescence is evidenced in this driver's license. The top photograph shows the document in its normal state, and the lower photograph shows the fluorescence of a license impregnated with special ink called a marker or authentication element. Image courtesy of Grayce Scott Koppey.

When a sample fluoresces without the addition of fluorescent dyes it is referred to as autofluorescent. If this is the case, the fluorescence is a characteristic behavior of the sample naturally. If the sample does not fluoresce, the sample will need to be infiltrated with a dye (marker), often called a fluorochrome or fluorophore, if desirable. The selective staining of specimens with fluorochromes is important in fluorescence microscopy and biological research used for tissue identification or confirmation that a behavior has occurred. There is significant ongoing research in the development of new fluorescent markers and more can be read in Chapters 9 and 12. Autofluorescence is frequently observed in minerals, plastics, papers with brighteners, or clothing that has been washed in detergents that contain brighteners. Paints, toothpaste and a number of other products including credit cards with security inks will also fluoresce.

The Fluorescence Photography System

Two filters are required in a fluorescence photography system. One filter must be used to create short wave excitation energy that is shined onto a subject and the other is a barrier filter that is used on the camera lens. The barrier or emission filter needs to be mutually exclusive to the excitation energy in its transmission characteristics. For example, if the excitation energy will be 400–460 nm, the barrier filter at the camera should block any light 460 nm and shorter wave energy from entering the camera and it should transmit any light that is 465 nm and longer. This prevents the excitation energy from reaching the detector. The excitation energy is often brighter than a fluorescence emission. It is possible for the excitation energy to overpower the fluorescence. Sometimes coatings and other elements in a lens may also have fluorescent properties. A barrier filter will prevent the lens from fluorescing by subtracting the excitation energy. Since exposures will be made using visible light, traditional digital cameras will work just fine. Long exposure times should be expected. The calculation of exposure times often will need to be estimated. Fortunately DSLR cameras have a preview screen or live view feature. It might be useful to use a high ISO or sensitivity. Select an ISO that has the highest possible sensitivity and does not create measurable digital noise in a file.

Photographing with the Invisible Spectrum

There are unique opportunities and challenges when photographing using energy that is invisible to the human eye. Photographing with either ultraviolet (UV) or infrared (IR) radiation can reveal characteristics about subjects that otherwise would not be visible when using white or, as it is more commonly described, visible light. Revealing unique characteristics of a sample using invisible spectrums might be described as the "fingerprinting" of an object. Using photography to reveal behaviors unique to that sample can be a tool useful for identification or classification of samples. Fluorescence is one of these behaviors as well.

The invisible spectrum can be used to create images that share unique characteristics or behaviors of samples. Specifically, UV photography can be used when photographing skin for dermatological assessments or when evaluating botanical samples, for example. When photographing skin, UV energy can penetrate the epidermis to a range of 1–3 mm. It can also be useful for bite mark photography. It is used in the material sciences or when evaluating printed materials for authenticity. IR photography is useful for agricultural photography and aerial mapping, biology, and botanical applications, and for the photographing of human veins (and sometime arteries) since IR can penetrate epidermis. Infrared photography reveals structures that are several millimeters deep in tissue. Infrared has also found uses in glamour and landscape photography because of the aesthetic of the images from this spectrum. There is a characteristic appearance of an infrared image when compared to a photograph made using visible light.

Basic Problems

There are unique equipment requirements to make photographs using invisible spectrums. Choosing an appropriate energy source (light or not) that emits ultraviolet and/or infrared radiation is fundamental. Special filters are also required. Without an UV or IR spectral emission, no UV or IR exposures can be made. Another challenge is to create a repeatable

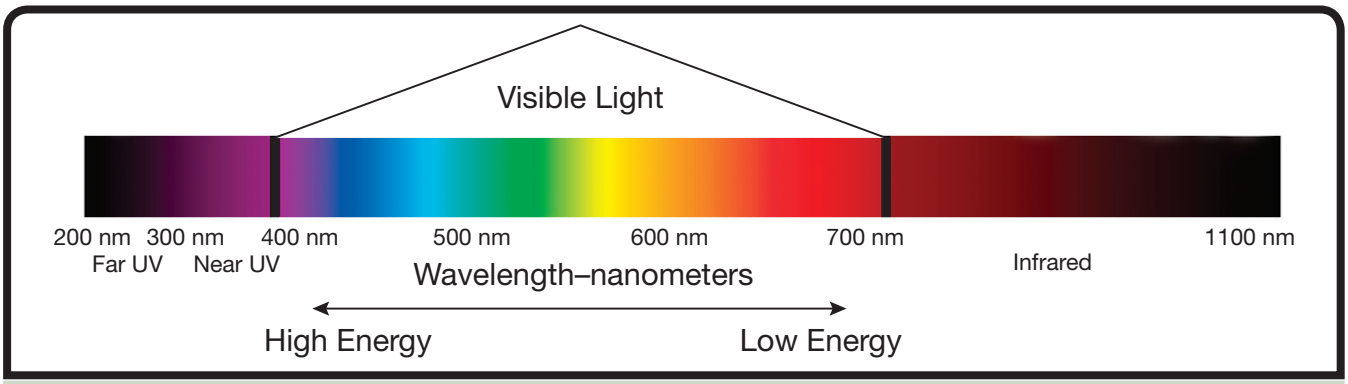


Figure 7.4 Ultraviolet (UV) radiation contains 200–400 nm energy. Energy that is 200–300 nm is characterized as far UV and wavelengths of 300–400 nm are described as near UV. UV is characterized as short wave energy with a higher energy than visible light or infrared. The visible spectrum includes 400–700 nm. The infrared region contains 700 nm–1,000,000 (1 mm) wavelengths. Imaging is typically accomplished from 700 to 1000 nm. A sensor's sensitivity range will dictate what is possible.

method for exposure determination. Tools designed for measuring visible light may not work in the same way when using UV and IR. Because we cannot see UV or IR, creating a method that achieves a proper focus is also a very significant challenge. These are but a few of the problems that will be encountered when photographing with these two spectrums. The following pages will share practical methods helpful for creating successful imaging outcomes.

Energy Sources

Identifying an effective source that generates both UV and IR photography is possible but not required. It may be good to have one source for UV and another for IR applications. An electronic flash or the sun would be a good choice for practical reasons but there are other contributing factors that will lead to the selection of one light or another radiation source for this work. Portability, subject movement, and size of the source are considerations for the selection of one tool over another. The decision about what source is chosen will play a role in an imaging outcome. Sunlight might seem like an obvious choice since it emits the entire spectrum including UV and IR. Using sunlight creates challenges for standardized applications, though. As the angle of the sun in the sky changes, so does the brightness and percentage of various spectral components in the atmosphere. More importantly, there are influences to the spectral composition of the energy by weather and specifically cloud cover. When there is a lot of cloud cover, the amount of UV and/or IR will be variable based on filtering phenomena from the clouds. This makes standardizing and creating repeatability difficult. For these two reasons, the sun can be a fickle light/radiation source to rely on for scientific imaging applications. Being available only during the daytime hours can also limit its practicality. It is interesting to note that while tungsten bulbs are not a good source of UV, quartz halogen lights are.

It is possible to buy a pure UV source but they can be expensive. Often these products are designed for specific applications (energy output) and use a fiber optic light guide to deliver the energy. An Arrowhead BIB-150P–365 nm is an excellent tool and produces a nominal steady state 365 nm intensity UV beam. The bulb produces an intensity of 4000 uw/cm^2 at 15 inches and has an average rated life of 5000 hours. For more broadly based UV applications, an electronic flash system is a good source of UV radiation and IR work. It is practical to use a high powered studio electronic flash system. The higher the power output, the more versatile the system will be for imaging a range of subjects. It is important that the flash tube does not have a UV removing filter located on the tube or protective housing. See Figure 7.5. Small portable electronic flash units—sometimes called speedlights—may not be powerful enough for this work unless the objects are very small. In a pinch, they will work when used at short working distances to a subject. Almost any electronic flash can be used but, based on practicalities, greater power output from professional studio equipment will provide great advantages. Specific spectral information about UV and IR emissions from various products such as Profoto, Bron or other electronic flash units, as well as features such as variable output, recycle times, and ability to be used in a battery mode, should be assessed before deciding on a purchase. Acquiring this type of lighting equipment can be expensive.

Not all flashes are effective for UV photography but all will work for IR imaging. Some flash tubes or chambers have been outfitted with a UV removal filter. This filter might be

characterized as being a yellowish haze or warming filter. It might be a Kodak #2 filter. Because studio lighting was often directed to the portrait market, a UV filter on the flash tube was used to warm up the color of the light. When film was used for photography, removing the UV emission led to photographs that were warmer in color. A light source that has an UV emission creates more cool colors. The UV removing filter often is located directly on the flash tube or in the diffusion chamber housing. All electronic flashes will have a useful IR emission, which is more than adequate. Both UV and IR photography will require the use of an increased ISO.

While electronic flash and the sun have significant advantages, other sources may also work. A tungsten source will be an excellent source for IR photography because it has a high percentage of IR emission when compared to flash. Unfortunately, the output of UV is relatively non-existent in most tungsten sources. There is a minor emission but the need for long exposure times would create significant noise in an image, which might mask the image data. Similarly "black" light fluorescent or germicidal fluorescent light sources will not work well for similar reasons. They do not emit enough radiation outside of the visible spectrum to allow for important imaging work to be undertaken with them. A quick inspection of the spectral emission for an LED light reveals it does not produce any useful UV radiation.

Figure 7.5 Electronic flash units are excellent choices to use for UV and IR photography. Not all flash equipment is effective to use in UV applications. The Vivitar 283® flash unit will not be effective. It has a yellowish/UV removing filter directly on the flash unit that will remove nearly all UV from its discharge. The yellow color of the flash diffuser is the telltale sign of the presence of this filter. Electronic flash units can either be portable such as these, a built-in camera flash, or studio electronic flash equipment. It is important to check for the presence of the filter. The Sunpak unit included in this photograph does not have a UV removing filter.

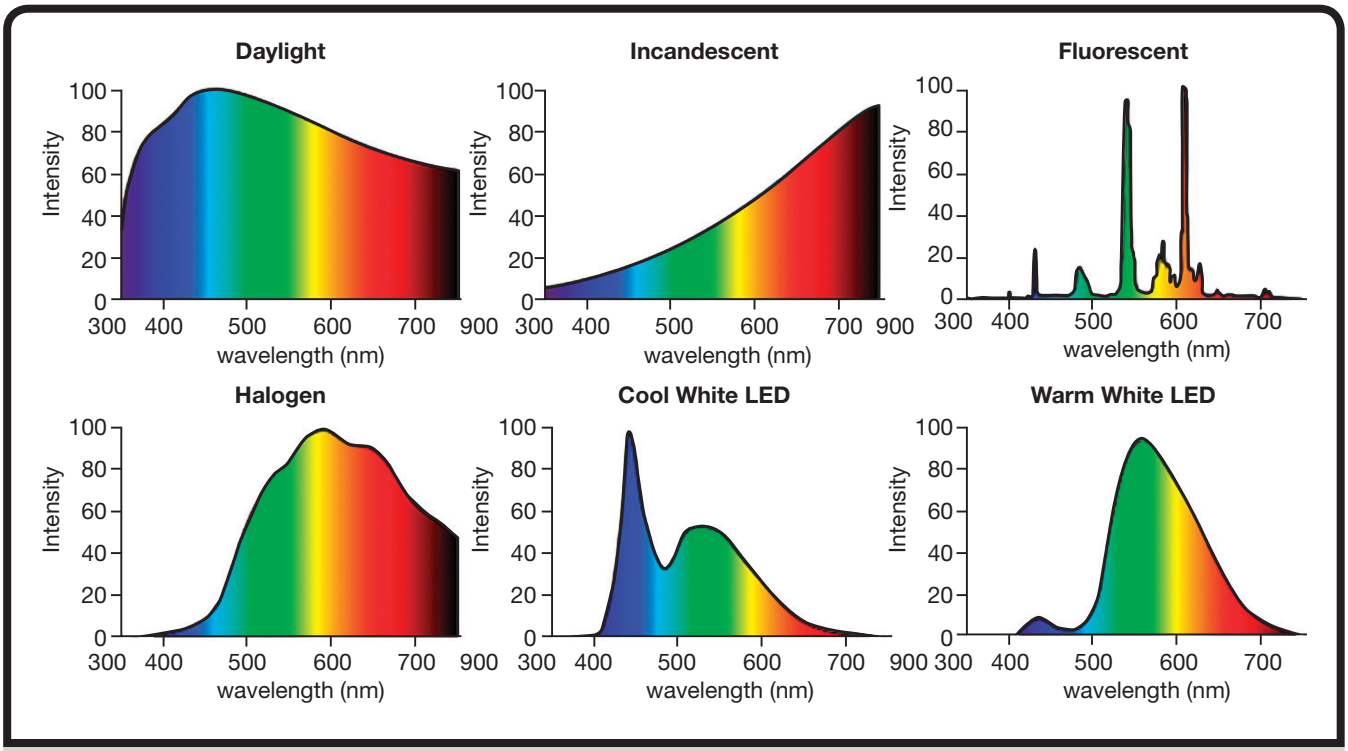


Figure 7.6 This graphic reveals the various spectral emissions from various lights. It is evident that only daylight sources and electronic flash have any significant emission in the UV spectrum. An incandescent source emits significant IR but little UV. LED has no UV or IR. As lighting technology advances, this data may change.

Cameras

Surprisingly, there are many cameras that might be suitable for invisible spectrum photography but few are manufactured specifically for this work. In 2006, Fujifilm released the FinePix S3 Pro UVIR, which is no longer commercially available. This camera was directed to the forensic market but never really found a large adoption. Fujifilm has recently introduced a new camera, the Fujifilm X-T1 IR (Infrared). This camera builds on the performance and capabilities of the original X-T1. It has new infrared technology that produces good results in a wide range of applications, including: crime scene investigation, fine art photography, healthcare diagnostics and observation, and other scientific and technical applications. There are also companies that will remove the IR blocking filters from a sensor and this is popular for IR enthusiasts. Removing the filter allows the camera to be used more effectively for IR photography but will not directly play a role in performance for UV applications.

Cameras used for invisible spectrum work will need adjustable and higher ISO capabilities, a high pixel count, and noise reduction capabilities as the primary features needed for use with this work. Being tethered to a computer can also be helpful. Live view may be another advantage but it will work only in certain situations where there is adequate signal (brightness). Truthfully, almost any camera will work but some may be better than others based on the energy source, sensor sensitivity to the spectrum, and the filters used.

Figure 7.7 The image on the left is from a thermal camera. It reveals temperature differences and displays them as colors. A thermal image (left) is very different than a reflected light IR image, reproduced on the right. Reflected light IR objects must be 500 ºF in order to register on a sensor. Thermal image courtesy of Ted Kinsman.

When doing research, you may find an interesting IR imaging camera designed for invisible spectrum applications sold by FLIR. Their cameras are designed for long wave IR imaging and would be characterized as thermal cameras. Thermal cameras are used to measure temperatures and not to record reflected IR radiation. This chapter has been written to cover methods used for reflected light near IR photography. Using a camera that records only grayscale might also be practical. Color is not a critical element in the majority of invisible spectrum work and a camera without a Bayer filter on the sensor will increase the chip's ability to work in low brightness. Monochromatic cameras are more sensitive to low light by a factor of two.

Lenses

Most types of glass will transmit near UV 300–400 nm radiation but glass will absorb and not transmit far UV 200–300 nm. To photograph using far UV, special lenses will be required. Nikon and Canon sell lenses that are made of quartz or fluorite for this application. There will be a distinct focus shift between the visible, UV and IR image plane regardless of the lens unless it has been otherwise corrected.

When photographing with near UV, a high quality macro lens will have advantages but almost any fast lens will work. It is important to work with a fast lens because of the low

brightness associated with most UV emissions and camera sensitivities in this region of the spectrum. This may or may not be true for IR applications. Many macro lenses will have an $f/2.8$ opening. These are preferable to lenses with a maximum aperture of $f/4.0$. Faster is better and highly desirable for work in the invisible spectrum using a camera that has a Bayer filter on the sensor. The UV and other visible light blocking filters will have very large filter factors and remove significant amounts of energy brightness.

Jenoptik makes a very special lens, the CoastalOpt® UV-VIS-IR 60 mm Apo Macro, for invisible spectrum work. It is corrected for application from 310 to 1100 nm and in 2015 had a list price of $5800. This lens can focus both UV, visible, and IR at the same focal point, which makes it ideal for invisible spectrum work.

Filters

There are a variety of filters used for invisible imaging work. Each has advantages and disadvantages. If the imaging will be accomplished in a darkened room, the filters can be located either on the source (if large enough) or at the lens, which is most common. UV filters specifically are made from special glasses and may be coated or sandwiched with other filters useful for blocking other wavelengths. A very good filter for UV photography is the B+W UV Black (403). It is rather expensive as filters go and costs approximately $90. The Baader Company also makes a very high quality UV transmitting filter as well. Kodak produced an excellent line of filters in their Wratten filter series. The #18A was the UV filter. The #18A filter transmitted 300–400 nm energy and also had a small amount of transmission between 700 and 740 nm. It is not an ideal filter for digital cameras for this reason, because many sensors remain highly sensitive to IR. A UV filter will be opaque to visible light. Schott, too, is a high quality optical glass and scientific filter company that sells excellent products for these applications. Their UG-11 glass filters are excellent for UV photography. It is important to keep in mind that some of the UV filters will leak IR into an image. Depending on the radiation source, this IR element may mask the UV in the image. It may be necessary to use an IR blocking filter on the lens, called a hot mirror, as well. Check various products' specifications to learn about transmission and other spectral characteristics.

For IR applications, an #87 filter is a good choice for putting onto the lens. There are other filters that can be chosen for this work. Kodak produced many Wratten filters used for film photography, including the #87C, which does not transmit the 700–800 nm region of the IR spectrum. Both the UV and IR filters are available in glass and acetate products. Acetate creates lower resolution but is cheaper to make, while glass is a better optical material but more expensive to make. Filters can be threaded for coupling to a specific lens or they can be square. When buying a threaded glass filter, you must size the filter to the lens's threaded outer diameter. A Nikon 60 mm macro lens, for example, will require a 62 mm diameter thread on the filter to be screwed in. A square, acetate, and a non-threaded type can be used with various sized lenses and adapters, or can be held or taped in place. This makes a square acetate filter a more flexible choice, but the filter remains vulnerable to tears or other damage.

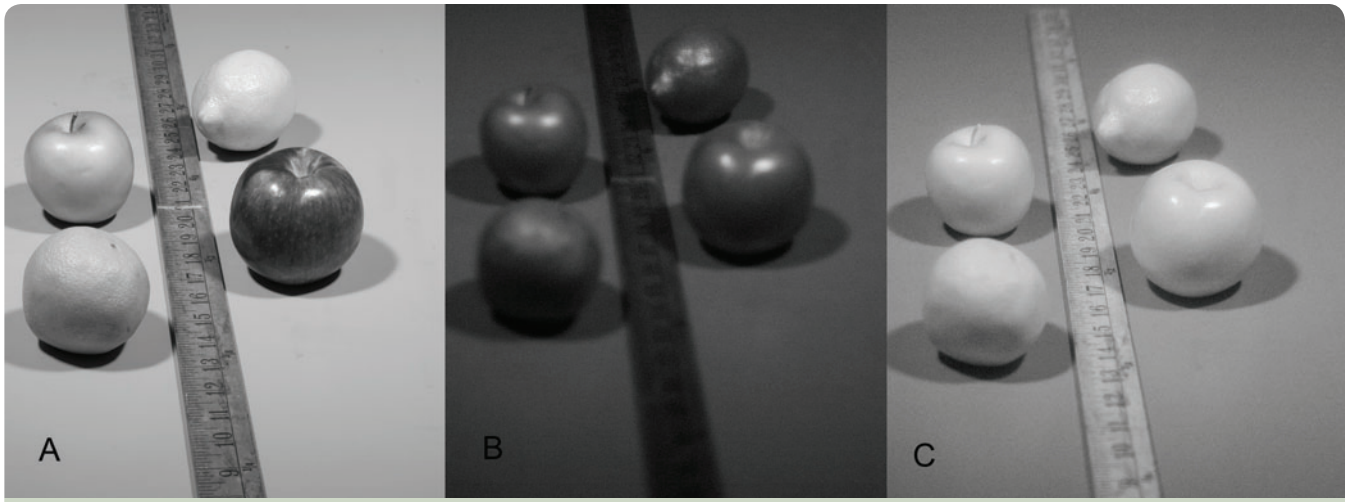


Figure 7.8 Image A reveals how the visible light image is reproduced. Focus and DOF are normal for what is expected. The ruler has been included to demonstrate where the focus will shift. Image B shares where the focus shifts (to the rear) when using UV. This image has no focus correction. Image C demonstrates how a corrected focus has been achieved when using the IR filter.

Figure 7.9 A Micro Nikkor 105 mm and a Canon 50 mm lens have infrared and UV focusing correction dots on the lens. The working distance (focus) can be migrated to the IR dot after focusing to the visible location. Only Canon continues to create a lens with this correction visible to a user.

Focusing

Focusing can be a significant challenge when photographing using invisible radiation. Since no image is visible in the viewfinder, photographers must create a method to produce a reliable way to achieve sharp focus. One slow—but effective—method is to place a ruler in the scene and then evaluate where the focus shifts when photographing with either UV or IR radiation and compare it to the visible light photograph. It is possible to evaluate the shift by first placing the focus normally in the region of interest using white light and no filter. Make an exposure confirming everything is working correctly and produces a good exposure. Next, adjust the exposure for use with the UV filter (more will be shared about exposure changes) and make a new exposure without changing the lens's focus. The exposures made both with the visible light and using either UV or IR will be dramatically different in their focus. Mark the focus point for the visible and then assess where the focus of the invisible spectrum has shifted to. Readjust the focus, place the filter in front of the lens and make an exposure to confirm success.

Some macro lenses have been engineered with an infrared focusing dot on the barrel. It is possible to focus the image using the visible light and then re-focus the lens adjustment dot. See Figure 7.9. Lenses that have been corrected to work this way can be adjusted in the same way for both UV and IR applications, since this correction interestingly works the same for both spectrums.

Live View and Autofocus

A camera with live view capabilities may display the UV and IR image or it may not. Live view might also be used with autofocus mode if there is enough brightness available. Some cameras will have multiple AF modes when using live view. Since there is significantly less radiation in the invisible spectrum for imaging, it is best to select the slower focus module instead of the fast mode if autofocus is selected. It is not recommended to use autofocus in scientific applications, but sometimes there are situations that can benefit from its use when nothing else is working. Normally, because there are various focal planes in a scene, the autofocus feature may not create the best focus placement for a given sample.

Exposure Calculations for Reflected Light Ultraviolet and Infrared Photography

When a subject is going to be photographed using the invisible spectrum, there is a good chance that the available light/radiation sources will emit various spectrums and in differing amounts. There will be exceptions, but frequently sources will emit multiple spectrums in differing amounts or percentages. Strong sunlight, for example, can be used for UV and IR work; however, this can produce variability because of time of day and cloud cover influences.

When determining an exposure, it is easy to use a light meter that converts brightness to shutter speed and aperture combinations. Unfortunately, very few meters can measure UV or IR, so it is important to create a way to correlate a visible light measurement into information useful for UV or IR exposures. For example, in UV work I have found that if I am photographing using ISO 200 for visible light it is best to increase the sensor's sensitivity to ISO 1200 or higher. Because of the sync speed requirement for flash, no shutter speed changes should be considered. Opening the aperture to its most open setting is the next adjustment to make. I have found there are approximately six stops less brightness of UV radiation compared to the exposure readings of the visible light as a rule of thumb, but not always. This relation for visible light to IR will be different source to source. An absolute conversion will need to be created for each situation. This conversion factor is dependent on the camera/sensor sensitivity to these spectrums, the type and spectral emission of the energy/light source, and the filter that will be used

Practical Suggestions for Reflected Light Ultraviolet and Infrared Photography

The following are useful strategies that can assist in making UV and IR photographs.

Increase the Camera's ISO

Since typically there will not be large amounts of UV or IR energy available for imaging, raising the ISO makes sense as step one. The challenge is compounded by the low sensitivity of the sensor to UV or IR radiation. Increasing the ISO does come with a downside: noise. Some cameras will be more vulnerable to noise than others. Noise is the byproduct of long exposures or can be formed when increasing the ISO of the sensor. It might be practical to evaluate what ISO setting will balance the need to increase the sensor's sensitivity and not

create noise. It is useful to consider the balance of signal to noise when choosing an ISO setting. For very difficult circumstances keep in mind that getting any result will always trump the negative of having noise in an image.

Use Noise Reduction Filters

Many DSLR cameras come with the ability to reduce noise. Noise reduction moderates the pixels in a sensor being stimulated by electronic sources rather than from photons when a sensor is operated for prolonged periods of time. Noise reduction blends or smooths random pixel differences affected by unwanted exposure within a pixel. A side effect of noise reduction will be the subtle softening of image sharpness. Noise filters create blur to blend the noise. Conducting a noise reduction evaluation prior to using these filters with important work can be beneficial to anticipate how to best achieve a result in a challenging and important experiment.

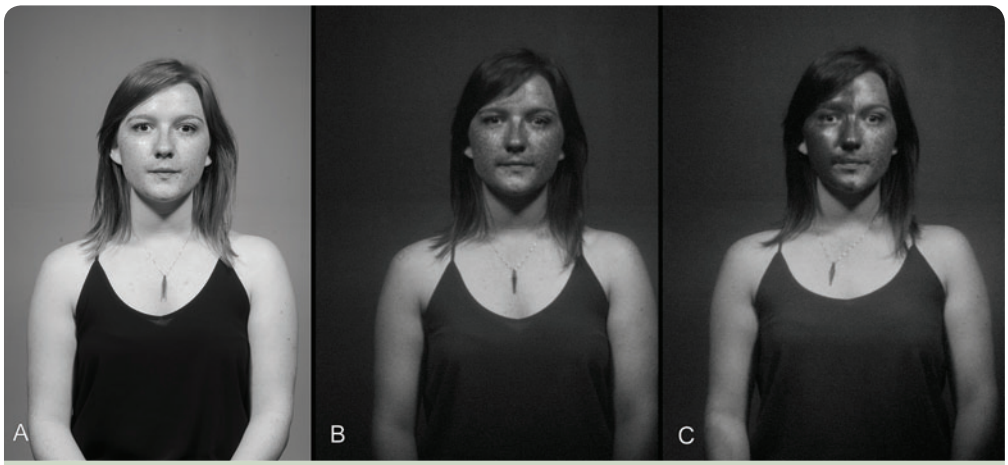


Figure 7.10 This series of pictures was made using a Nikon D300s camera, a 105 mm Micro Nikkor® lens, and Broncolor® Pulso G electronic flash lighting. The image on the left was made with a lighting ratio of 1:1 at ƒ/22 at ISO 200 with no filters and represents the "normal" black and white view (A) of the subject. The center image (B) was made using ƒ/8 at ISO 1250 and a black and white 403 UV transmitting filter. The image on the right (C) was made in same way as image B, but sunscreen was applied to the right side of the face.

Work Tethered

It can be challenging to evaluate an exposure using only the camera's preview screen and histogram because of its size. Tethering the camera to a computer when possible can be useful for displaying a larger image. Tethering will unfortunately cause camera batteries to be consumed more quickly. Using an AC adapter can be helpful. Using Adobe Lightroom software or other software such as Nikon Camera Controller Pro or Canon EOS Utility will effectively operate the camera remotely.

If there is not enough adequate energy to create an exposure, it can be helpful to move the radiation sources nearer to the sample, increasing signal strength. This step is easy and can be accomplished quickly. It might be useful to put marks on the studio floor showing where

to place the lights when used for visible light photography and where they are positioned for UV/IR exposures. This can make adjusting the equipment's placement repeatable and fast.

Multiple Discharges

For subjects that require more DOF a smaller aperture setting is needed, but sometimes this is not a possible choice. To use a smaller aperture, more energy is needed, and since the shutter speed cannot be changed to create more exposure in this instance, other things must be considered. A single discharge from an electronic flash sometimes does not have enough energy to produce a useable exposure in this situation. However, it is possible to discharge an electronic flash system multiple times—useful for creating more energy—using a small aperture and more DOF. This technique can be used in situations where nothing moves during the exposure. Samples that move—people or living subjects—cannot be photographed in this way because image registration errors will occur across the multiple discharges.

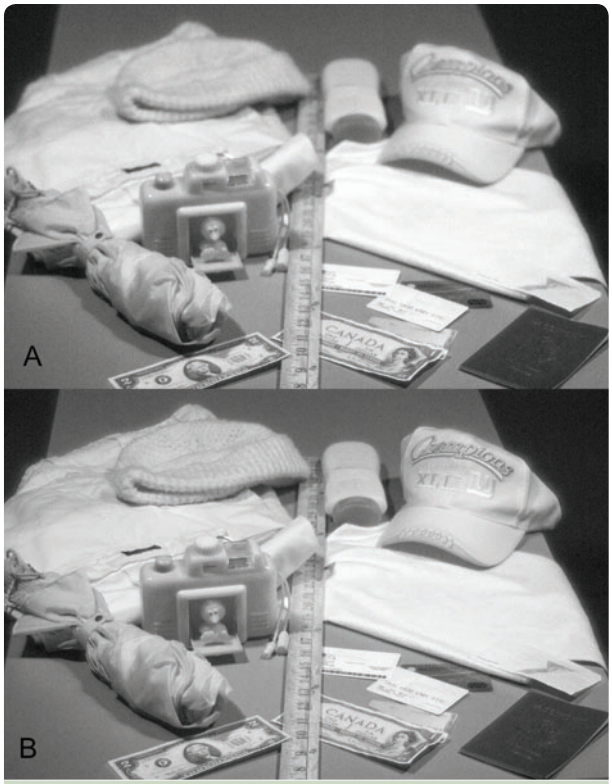


Figure 7.11 There will often be a limited range of focus contained within an invisible spectrum photograph. The image at the top was made using a single discharge using an aperture of ƒ/2.8 and an ISO at 1280. The bottom image was made using ten discharges from the flash, which allowed an aperture of ƒ/16 to be used, creating increased DOF.

Strategies

Apply Even Radiation/Lighting Across a Sample It is difficult to know how an object will behave when exposed to invisible radiation. Creating a raking illumination or an imbalanced lighting useful to reveal texture with visible light may mask important details on the sample made visible by the spectrum. For this reason alone, it is imperative to create uniform and equal amounts of light/radiation across the surface of a sample. While not overly dramatic lighting, it will correctly demonstrate how an object will present itself when exposed to either UV or IR radiation.

Do Not Diffuse or Modify the Light/Radiation Bouncing radiation into—or diffusing radiation with—various materials may actually remove some portions of the invisible spectrum or, worse yet, all of it. Paints, papers, and plastics may modify or remove UV or IR radiation from the energy source to varying degrees. For this reason, shine the light directly from the source onto a subject.

Bracket Focus and Not Exposure When working through the process of creating a good result in these challenging situations, it is natural to consider bracketing or changing the exposures during the session to ensure the best result. While image processing has become powerful, for this application it is best to make only small variations to focus placement and not to change the exposures at all. Minor errors in exposure can be corrected easily, incorrect focus cannot.

Use RAW File Format A RAW file is a powerful tool and useful for the optimization of recording sample data. Set the camera to its highest bit depth capability. RAW files will provide a wide tonal range and allow exposures in the highlights and shadows to be effectively managed

without changing the captured data. Image processing the RAW file does not change the data but rather only the data's display. This makes RAW files an invaluable tool for the scientist photographer. The file can be reverted to its capture appearance by discarding the sidecar .XMP file that accompanies a RAW file throughout its digital life.

Make the Image Size Smaller Since the amount of UV or IR energy is often low and requires using a wide open aperture such as $f/2.8$, the zone of focus will also be shallow. To insure the zone of focus is optimized, it is possible and practical to decrease the image size. This can be accomplished by moving the camera further from the subject. This will create a wider zone of focus using the same aperture setting. Most contemporary cameras have enough pixels to enable users to later crop into file and increase image size in the frame. This strategy might be called digital or "sneaker" zooming. While not ideal, it can be a useful "work around" when other imaging solutions conspire against success. Digital zooming is different than an optical zoom. Adjusting the number of pixels in a file reduces digital resolution, optical zooms do not.

Use a Monochromatic Camera Cameras with a Bayer filter will subtract energy before the energy interacts with the sensor. In fact, any filter will remove energy brightness from a system. Cameras without a Bayer filter will have a higher sensitivity to energy than cameras with filters that are located on a sensor. There are relatively few cameras that are monochrome for this application. The SONY XCD-MV6 might be useful for this application. Many instrument or industrial cameras, and not DSLR cameras, might be useful for this application. Instrument cameras will also require a C mount lens, and lenses with bayonet mounts cannot be used.

De-saturate Color or Convert to Grayscale Color is not an important element in an UV or IR image. What is important is how the sample absorbs or reflects these spectrums. To insure the maximum information is visible, de-saturating the color or converting the image to grayscale can be very helpful. More image processing strategies for RGB files to grayscale are shared in Chapter 13. The elimination of color as an element of an image can make it possible to see behaviors that might be masked behind the presence of color.

Polarized Light

Using polarized light for examining semi-transparent samples that may contain internal characteristics or to manage surface reflections (glare) that mask information can be helpful. Polarized light photography is a versatile method that can reveal the birefringence (colors) within materials that contain multiple refractive indices, sometimes called photoelasticity. Polarized lighting can also be used to control specular reflections on the surface of a material and is routinely used in the forensic and geological sciences, in light microscopy, and applied across many disciplines, including the material sciences.

Polarized light is light that oscillates in only one direction. For ease of understanding, consider that light emitted from a source can be oscillating both in the north–south direction and the east–west direction simultaneously. While this is a gross oversimplification, it may be useful for gaining an understanding of polarized light's behavior. Light waves are produced by a radiated energy source and they have both brightness (amplitude) and a

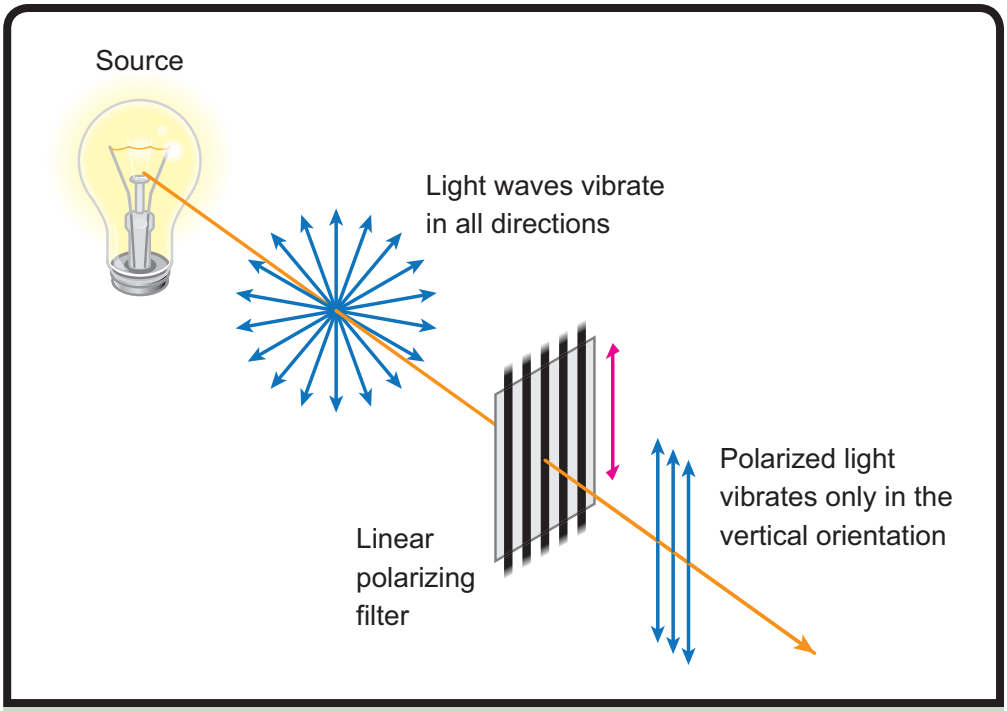


Figure 7.12 Light leaves a source oscillating in all directions. When the energy traveling in waves interacts with a plane or linear polarizing filter, energy oscillating in the same orientation to the filter's axis will pass. Light that is oscillating perpendicular to the filter's axis will be blocked by the filter. A polarizing filter might be considered to be a Venetian blind to light.

magnetic component. These components have orientations that are at 90 degrees to one another. A special polarizing filter or a flat surface can polarize light naturally depending the direction, size, and angle of the light source to the surface.

Polarized light can be produced naturally when light interacts with the surface of a flat material that is not an absorber of energy. Water, leaves, paintings, polished lacquers, windshields or the atmosphere can act as natural polarizers of light. The angle at which light interacts with the surface of the material and the corresponding angle of reflection from the material will affect the degree of polarization that can be observed. Of importance, too, is the location of the light source relative to the surface. Metallic surfaces will reflect light in a variety of directions because the light incident to its surface is non-polarized. Viewing objects with non-metallic surfaces lit by light that is not polarized may create glare light. A polarizing filter or polarizing sunglasses will remove (polarize) this diffuse reflection. This may also be evidenced with reflections from water that prevents seeing into the water. Depending on the location of the sun in the sky, a lake's surface, for example, will reflect partially polarized light from the atmosphere. Wearing polarizing sunglasses will remove some or all of the partially polarized light created by the water. By removing this plane polarized light, glare will be reduced and visibility increased.

Seeing Structure within Semi-transparent Objects

Polarization also occurs when light is refracted as it travels within or between materials. This may occur when light passes from one material into another with the same or a different refractive index. Refractive indices are indicators of a material's density to energy travel. The slowing down or speeding up of the energy occurs where the two materials share a border with different refractive indices. When the energy leaves the first material and enters the second, the refracted beam may experience some degree of polarization. Certain minerals are unique in their ability to facilitate polarization effects. Icelandic spar and quartz are two such minerals.

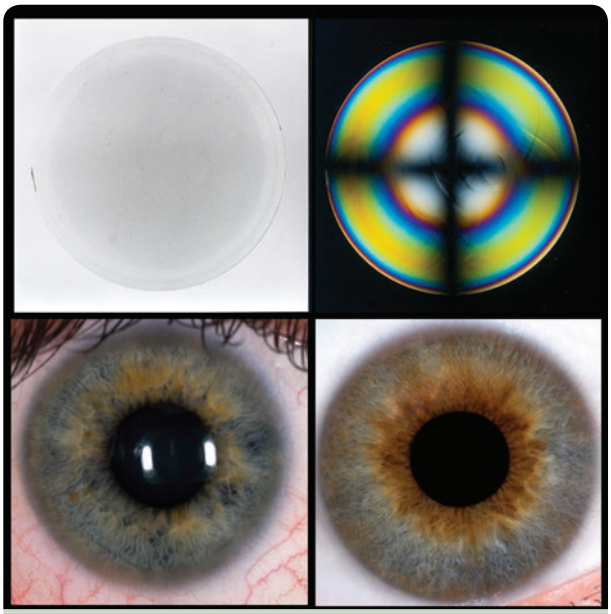


Figure 7.13 This illustration reveals the basic function of polarizing filters for transparent and opaque objects. In the top row, Image A is a large optical glass lens located on a large flat light. Image B is the polarized photographic result, revealing photoelastic stress in the lens. In the bottom row, human irises were photographed, on the left without polarization and on the right with polarization. Iris photographs (left) courtesy of Geoffrey Stein, (right) courtesy of Laine Maier, www.scientificshutter.com.

Once light has been polarized, it can be polarized again or controlled. Polarizing filters are required for this work. Filters can be used to manage either natural or man-made polarized light. Polarizing filters are made of a special material that transmits light vibrating in only one direction. This could be north–south or east–west in orientation, but not both. This is described as linear polarization. Another type of polarization is created through the use of a circular polarizing filter.

When non-polarized light goes through a polarizing filter, it emerges with half the brightness it had and vibrates in only one orientation. When the light leaves the filter, it is now described as polarized. Light entering the filter that is oscillating perpendicular to the axis of the filter will be absorbed. Light that is traveling parallel to the filter's orientation will be transmitted. If a polarizing filter is represented as a picket fence or a Venetian blind to light it will have an orientation to light travel based on its slats. The light vibrating in the orientation of the slats will be transmitted. Light that is traveling against the grain of the slats will be blocked.

The System

Polarizing light photography systems are really quite simple to create and use. One polarizing filter is used to polarize the light and a second polarizer is located on the camera lens. The second polarizer may also be called an analyzer and needs to be placed between the sample and the lens. It should be of the highest optical quality. When the filters are situated with the same orientation, light will travel through them. When the filter's transmission axes are perpendicular to each other, light transmission will be extinguished and only polarized light oscillating in the proper orientation will be transmitted through the analyzer.

Polarized light applications require the creation of flat and uniform light. This is important and is required to achieve a neutral and honest presentation of the results from a sample. Lighting that is not uniform can lead to false data or unevenness of the data that is revealed.

The lights can be polarized using a large sheet of polarizing material. Various sizes of both gray and brown sheets of polarizing material can be purchased from optical supply companies such as Edmund Optics. When using tungsten halogen sources, be careful to locate the filter far enough from the light itself. Melting the filter is a relatively easy thing to accomplish when it is situated too close to the light. Transmitted light and reflected light systems, while different, have the same requirement for uniform and flat lighting. A transmitted light system might also be described as a plane polariscope.

Photographing with polarized light systems requires no special photographic procedures. Most applications will include samples that are stationary and the use of higher ISO settings is not needed. Use of a camera stand or tripod will greatly enhance the imaging outcomes and is recommended. The use of the two filters will remove significant amounts of brightness. Each filter will have an optical density of 0.6 or equal to two stops of light loss. As an example, if an exposure value of 12 was measured, for example using an ISO 200, this brightness would become 8 with the filters in the system. This will create longer exposure times when using a continuous light source or require a more open aperture setting when using electronic flash.

When using reflected light, there are no major challenges for set-up or imaging. Locate and align the polarizer at the light source. If two lights are used, be sure to have the axes of the both filters located on the lights set to be the same. The lighting should be flat and uniform across the surface of the object. Locate the analyzer filter on the lens. Based on the alignment of the polarizer filters located on the lights, the filters should be oriented on the lens to extinguish stray reflections. Working with tungsten lighting will allow this adjustment to be easily seen. Working with electronic flash, this will be a bit more difficult but is required for the extinction of surface reflections. Images produced from this system will be rich with color and there will be very few specular reflections.

Schlieren Photography

Schlieren is a useful method of visualizing refractive differences in gases, such as warm air mixing with cold air. It is particularly useful for aeronautical evaluation of materials and airflow. Schlieren was first observed by the German physicist August Toepler in 1864. The German word *schlieren* means streaks or optical inhomogeneities. Schlieren methods are particularly useful for the study of ballistics, visualizing shockwaves in air, or creating visibility revealing what happens to air passing over a hot surface.

The simplest schlieren system uses light from a point light source shined into a convex mirror. The light from the source is focused at the front of the camera's lens or at one focal length from the light source. The convex mirror serves two roles in the system. One is to focus the light and the other is to provide an action field of where to locate a sample. A razor blade or other opaque object with a sharply defined edge is required and needs to be placed where the point light source is focused on the front of the lens. Once those conditions have been met, an object is placed in front of the field mirror lens. As the refractive difference of the sample moves the air or other gas, it splits the focused image of the point light to the dark or light side of the aperture or razor blade.

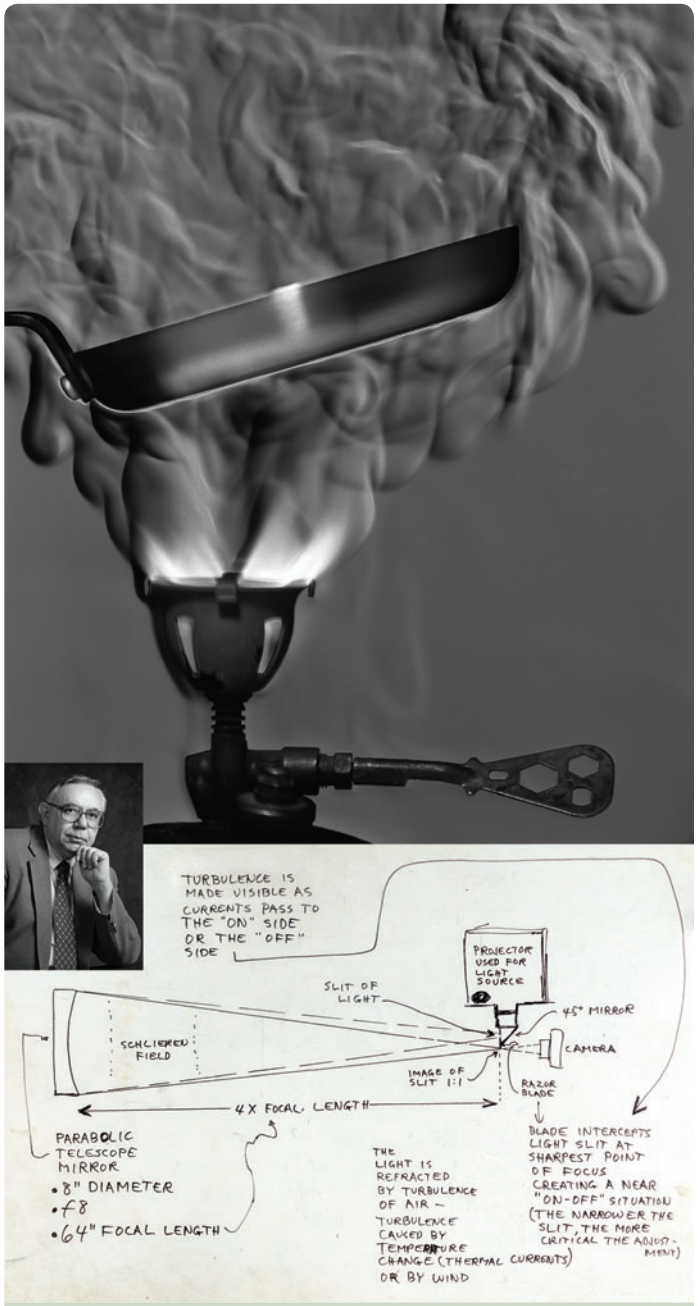


Figure 7.14 The upper photograph reveals heat produced from a gas-fueled backpacking stove around a small cooking pan. A slit light source was used with a Z-type schlieren arrangement that incorporated two 300 mm $f/10$ mirrors to make this photograph. This is a more sensitive system when compared to the schlieren system from this drawing. A stop was used to the control sensitivity of the system used for this picture. The image was recorded at lifesize because the system used no lens. Both the subject and the image plane were located 6 meters from the second schlieren mirror. An electronic flash having a flash duration of approximately 250 microseconds was used to freeze the high-speed event. Image courtesy of Phred Peterson. The inset portrait features Professor Emeritus Nile Root. The portrait was made by Nancy Stuart PhD in 1985. The drawing, a simple schlieren system, was drawn by Professor Emeritus Nile Root, c. 1975, Rochester Institute of Technology.

Schlieren images are often made using a system that creates light traveling in parallel rays. A light source is focused onto a slit or aperture that is placed just off-axis and exactly one focal length from a lens or mirror. The size of the imaging field is defined by the size of the mirror. A larger mirror will allow larger fields to be examined. The focused light containing the information about the specimen image is focused on the razor blade where half of the information is transmitted and the other half is removed. Any changes in the refractive indices in the action field will cause some part of the light to be bent or refracted into or out of the beam that passes the aperture or razor blade. This will amplify some regions of brightness and darken other regions. The image is focused into the camera using the camera's lens. Variations in the refractive indices of the various temperatures or densities of the sample will appear as a density gradient around the turbulence. It is common to use a continuous light source but it is possible to visualize a short duration to image high-speed events. Objects should be located in front of the mirror or action field.

Photographing Schlieren Images

There are no real photographic challenges in making schlieren images. There is adequate brightness, and the challenge comes from critically focusing the light and mirror's image of the system where it is bisected at the linear aperture. It is most useful when creating a system as described above to photograph using a lens aperture set to its maximum opening. This might be $f/2.8$ or $f/4.0$, for example. Closing the aperture to a smaller setting will create a larger depth of field, which is not useful for schlieren and creates other artifacts. Using a long focal length macro lens is ideal. A 200 mm is an excellent choice, but which lens is chosen will ultimately be influenced by the focal length of the mirror. The two must complement each other. Using a teleconvertor on a telephoto lens can also be useful in enlarging the image size.

Scanners as Cameras

Scanners remain an important tool for digitizing physical materials. In the earliest times of digital images, scanners provided an important method for producing very good high resolution digital files from analog materials. Direct digital capture using cameras at that time resulted in low resolution files. The need for higher resolution files used in publishing was far ahead of the digital camera's ability to create resolution. Many early high-end digital cameras were modeled after scanners; however, only stationary objects could be photographed. Leaf, an early innovator of scanning cameras, produced the Leaf® Micro Lumina scanner, designed specifically for the microscopy market and based on their successful line of scanners and scanning back cameras. The Micro Lumina used an ultra-fine stepping motor required to make high resolution files without vibration, which is a big problem when producing magnified images.

There are a number of reasons why scanners can also be used for more than simply scanning documents. Some of their unique features allow them to be more effective than cameras for certain things. One of the most useful and limiting aspects of a scanner is its linear array, sometimes called the scanning wand. Cameras that use an area array are capable of instantaneous capture but creating pixels can be expensive, and a scanning camera or scanner builds images by using a scanning pixel wand line by line. In this fashion, the price per Mb is much lower. Linear array devices are less expensive to build than array area cameras and require only one working line of pixels. In some high-end scanners, this leads to capture settings of 9600 pixels per inch, a very high digital resolution. This number—9600 pixels—is actually not meaningful except for use in setting the scanner's resolution. The 9600 pixels are spread over the subject width. For example, if a subject was 10 inches or 250 mm, the 9600 would be spread across the distance and would yield a scanned resolution of 960 ppi. This scanning resolution implies there will be many pixels in the scanned file. Taking a quick look at the file size in Mb will provide more information about a file's true dimensions.

There are two basic modes for operating a scanner. There is a mode for opaque objects such as documents and photographic prints, often referred to as the flatbed mode, and a mode for transparent objects such as film. The film mode allows for the selection of positive or negative film. Not all scanners are capable of scanning film.

When considering that a scanner is a type of camera, many more things can be photographed than simply scanning documents. Scanning objects will not be a fast activity. In fact it is actually very slow to use a scanner as a camera, but scanners frequently provide greater bit depth than cameras. Bit depth is a vitally important tool in collecting object data. The higher the bit depth, the more tonal detail can be seen within the file. Producing more data leads to more information useful for image analysis or image processing. Many scanners are capable of creating 14–16 bits per channel, which has also become the norm of some high-end DSLR cameras such as the Nikon D810 camera (14 bits per channel), the Canon 5D Mark III (14 bits per channel), and the Hasselblad® camera (16 bits per channel).

Scanner Settings

Selecting the best settings for scanning and achieving optimization of an image is the most important first step in using a scanner. One critical decision focuses on the selection of a resolution that supports future image usage. Dots per inch (DPI) is the most common but not the most accurate term used when selecting a scanner resolution. DPI has become more or less the de facto setting term embedded in scanner software. During the earliest beginnings of digitizing equipment, DPI as a term was used in the graphic arts industry to describe images based on half-tone dots per inch. DPI was the term used in pre-press and publishing industries that described the screening of images used in plate production needed for image reproduction in magazines and newspapers. This relationship played a role in early terminology. Actually pixels and not dots are used for calculating digital file sizes and more correctly should be described as pixels per inch or PPI. Dots are round and pixels are square. The resolution of an image is described by the number of pixels in the vertical and horizontal dimension. This might be 2400 x 3000 pixels, for example. DPI and PPI can be truly confusing. In the end, the terms mean more or less the same thing. What remains critical is the need to produce the correct number of pixels for immediate image use or possible use at some future date. This can be tricky and a best guess is fundamental. It is always better to have too many pixels than too few. Digital storage continues to fall in price per Mb and the time required to re-image a subject may be more valuable than having too many pixels in a file.

Scanning is slow and sometimes operators select a capture resolution based on how long it will take to scan a subject. The higher the DPI of the scanner, the longer it will take for the scanner to write an image. This is a predictable relationship. To the extent possible, it is important to know how many pixels are needed for the file's output requirements at some future time before selecting the final scan resolution.

Once the scanner resolution has been determined, the type of document and color information can be selected. Most frequently RGB, black and white, or line art are the available choices. Scanning using RGB color—while sometimes creating larger files—will record the most data about an object. Sometimes invisible to the eye, a sensor can see things in the R, G or B spectrum that is suppressed in human vision. For this reason, it is suggested to scan in the color mode always unless there are compelling reasons to scan in one of the other two modes. Black and white is most useful when scanning continuous tone black and white images, whether film or print. Line art is most useful for objects that have two tones, a pure black tone and paper white. It is frequently used for graphic arts applications of line art. Some scanner software will also allow the selection of a preferred color space to work in. Adobe RGB 1998 will provide an effective color space using a 16-bit file that will satisfy most of the difficult samples that will be encountered.

Most scanners have a feature that allows a scanner's contrast or tonal capture range to be optimized for different objects. The histogram from a scanner shares with a user the initial capture tonal range of the scan. Often this data can be adjusted prior to making a final scan during what might be called the pre-scan. This information is frequently displayed as a histogram in the scanning software display window. It is possible to adjust the black and white point setting using this feature. Optimizing the sensor's capture of the precise tonal value requirement of a sample can make this tool very powerful. Using the sensor's

entire recording range is an important step in creating data-rich images. If the sample's data is compressed or not recorded at all, the ability to make image data more visible will be lessened during image processing.

Descreen Function

Scanners can absolutely do some things cameras cannot. Images reproduced in books and other printed media will have been line screened. A line screen is a method used in the graphic arts to reproduce an image and includes how many dots were contained within an inch. Newspapers, for example, use a line screen of 66 lines/inch screen. A reasonable quality magazine such as *Time*® would use a 133 lines/inch screen, and a high quality publication such as a fine art photography book might use a 266 line/inch screen. The higher the line screen, the finer the detail that can be reproduced and the greater the number of dots that will make up the printed image. This allows the image to appear to be a continuous tone image rather than a bunch of dots. Once an image has been converted to a half-tone pattern and a scanned image is created, the dots become quite visible. Many scanners come with a descreen feature. Within this feature, there are algorithms to smooth out—or remove, in many cases—the result of the line screen, the half-tone dots. It is important when using descreen to correctly match the publication's dots per inch to the corresponding software setting. Incorrect settings will create other digital artifacts in the file, such as a moiré pattern.

Sometimes when scanning documents there is printing on both sides of the document. When looking at the page you can see the back image from the following page. This is sometimes called back printing and in the right conditions is highly visible through the page. Often this back printing is also enhanced and made more visible when scanning. Using a sheet of black paper behind the page will make the back printing disappear.

Figure 7.15 On the top row are two scans of a printed page from a university magazine. The image on the left reveals the back printing as an artifact of scanning/photographing these types of subjects. Placing black paper behind the page will absorb the back printing. In the lower row, a photograph printed in a magazine that had a 133-line screen was scanned. On the left is the half-tone pattern. On the right is the result when using a descreen setting.

Unsharp Mask at Capture

Sharpening a digital file is a common and expected step in a digital workflow. Because of the way the digital file is created, there is an inherit softness in the capture file. There are three places where softness occurs and where sharpening might be chosen for use. These locations would be at capture; after capture; or for use in creative images during their preparation for output. There are a variety of reasons sharpening is important, and more about sharpening can be read in Chapter 13.

Sharpening at capture comes with a risk because sharpening can add digital noise or artifacts along edges of samples. If important image processing will be part of the workflow of a file, sharpening is not recommended at capture using a scanner. When selecting to sharpen at capture with a digital scanner, the objective of the tool would be to minimize image blurring. Image blurring can be scanner induced, operator induced or the byproduct of the sensor. Capture sharpening algorithms take into account image noise when they are written. Additional loss of critical sharpness can occur when the RGB image is subsequently built from the pixel data into the spectral components, a process called de-mosaicing.

Capture sharpening is a choice given to users in most software and is frequently applied automatically by the device. Sharpening at capture can create digital artifacts that in subsequent image processing exercises will become more amplified and become digital noise or non-image data over time. For this reason, I am not a fan of sharpening at capture. It is most important to have total control of what is done to pixels before modifying the data. Capture sharpening software uses an algorithm called an unsharp mask. While the name implies the software is softening the file, the process is actually sharpening an image. An unsharp mask works by increasing the brightness difference along light and dark edges of structures within an image. By increasing image contrast at the structural edges of the image, there is an apparent gain in sharpness. This is all perceptual. At capture this process can often lead to false data and may create challenges in the production of clean files useful for image analysis.

I find it preferable to first sharpen during what is called the pre-processing stage of image processing after capture. While most scanners do not have the ability to make RAW files, there is a feature in most image processing software used to open many file types in the RAW file convertor. This sharpening feature in the pre-processing stage is often called clarity. A small amount of clarifying can be very useful at this stage of the image processing. It should be noted that JPEG file formats are not recommended for use when scanning objects and certainly not when data collection is a high priority. By selecting JPEG files, compression and data loss will always occur at capture and may lead to the introduction of noise in a file at capture. If scanned documents are being scanned for email use, JPEG is the recommended file format.

Imaging Objects on a Scanner

Scanning a 3D object on a scanner can sometimes provide unique and highly accurate images. Objects with a large Z dimension (height) may not be ideal; however, it can be interesting to try to scan objects if time permits, learning what is possible. Scanned images provide unique image attributes that are challenging to create using traditional camera and lighting equipment.

A flatbed scanner has a unique and short focal length lens that does not have an adjustable aperture or focus adjustment capabilities. I am not aware of any flatbed scanners that have a focusable lens system at this time. Early models of film scanners were focusable. Because of the fashion in which the optics of a scanner are engineered and created, some images may exhibit a larger zone of focus than might initially be imagined. It goes without saying that objects whose characteristics are more 2D than 3D will lead to images that are more effectively managed. Several practicing artists have developed methods for making what RIT Associate Professor Patti Russotti calls scan-o-grams. Russotti's work is rich with texture, color, design and information about an object or objects.

Figure 7.16 This beautiful photograph is from a body of work called scan-o-grams. Is the photograph science or art? The scan-o-gram features the seedpod of the common milkweed, *Asclepias L.* Image courtesy of Patricia Russotti.

The quality of lighting a scanner makes is unique. A long strip of lighting travels along with the pixel wand as it scans the subject. This washes the subject in a soft and non-directional diffuse light. Because the light is on axis with the sample, it is hard to easily re-create this same quality without a high degree of effort in a laboratory of studio. Objects will often appear to glow. Shadows and highlights will be rich and sample data can be easily recorded. Contrast and texture contained in scanned images comes from the object and not the lighting.

Subjects should be placed onto the scanner's glass platen. If the object has unique characteristics, it may be practical to locate a sheet of thin window-pane glass or other

optically good material between the sample and the glass platen. This may change the system's focus but it will protect the scanner. I have used Saran Wrap® for wet objects. It should be mentioned that the surface of the glass platen should be carefully cleaned and prepared before placing the sample there. Cleanliness can be next to godliness in digital photography. Dust can find its way into images in a million different ways. In many cases the location of an object on the platen will not allow the cover of the scanner to be closed. For this reason, scanning in a dark room is suggested. Additionally, a piece of black velvet can be draped over the sample to create a black background and is a useful technique for isolation. Pre-scanning the object will allow a user to assess exposure and other image attributes. By adjusting the exposure and contrast adjustment features in the scanner software, it is possible to isolate the ideal settings for this and subsequent samples. It can be very powerful to optimize the sensor's tonal reproduction settings to the inherent contrast needs of the sample.

Images created on a scanner will be without a strong perspective since the lens and all parts of the object will be captured at relatively the same magnification. This type of an image might be described as having no perspective. Image shape and magnification are important clues for viewers of images to assess foreground and background elements of a scene or the sample. Image perspective is a key element of an image and useful to create understanding of size and relationships. Images without perspective often cause viewers to pause when trying to gain an understanding of a sample.

Figure 7.17 Next to the black arrow is an image artifact called Newton rings. Newton rings are the result of interference. They are produced in much the same way as color rainbows become visible where oil and water are situated in puddles. This image artifact is the result of parallel surfaces in very close proximity to one another, causing light to become trapped between the layers and resulting in interference from the interaction. See Figure 3.10 for another example of interference.

Scanning semi-transparent objects can be practical and fun. Striking images can be produced when using the film mode. Large electrophoresis gels, some Petri dishes and of course large histology slides can be scanned. Scanning flat transparent samples mounted in glass can come with challenges. If it seems too good to be true, it probably is. Locating many parallel surfaces in close proximity to one another can create an interference outcome. Interference in this instance will look like rainbow rings called moiré patterns.

When scanning semi-transparent objects such as a leaf, it is very useful to use an area mask to hold back non-critical light and exposure. By repressing unwanted light that is not relevant to the sample, a much better exposure of the sample's edges can be obtained. Managing random and unwanted light that creeps into an image exposure helps to maintain image contrast and prevent blooming and adjacency affects. Edge definition can be lost when subjects are more opaque and require more exposure to achieve detail in the dark and shadow regions. This high dynamic range can be more effectively managed by blocking light from traveling near and around the subject. This is called masking and will require some image retouching to manage the irregular edges of the mask that are irrelevant to the sample. When the illumination field is masked, the exposure through the subject can be precisely managed.

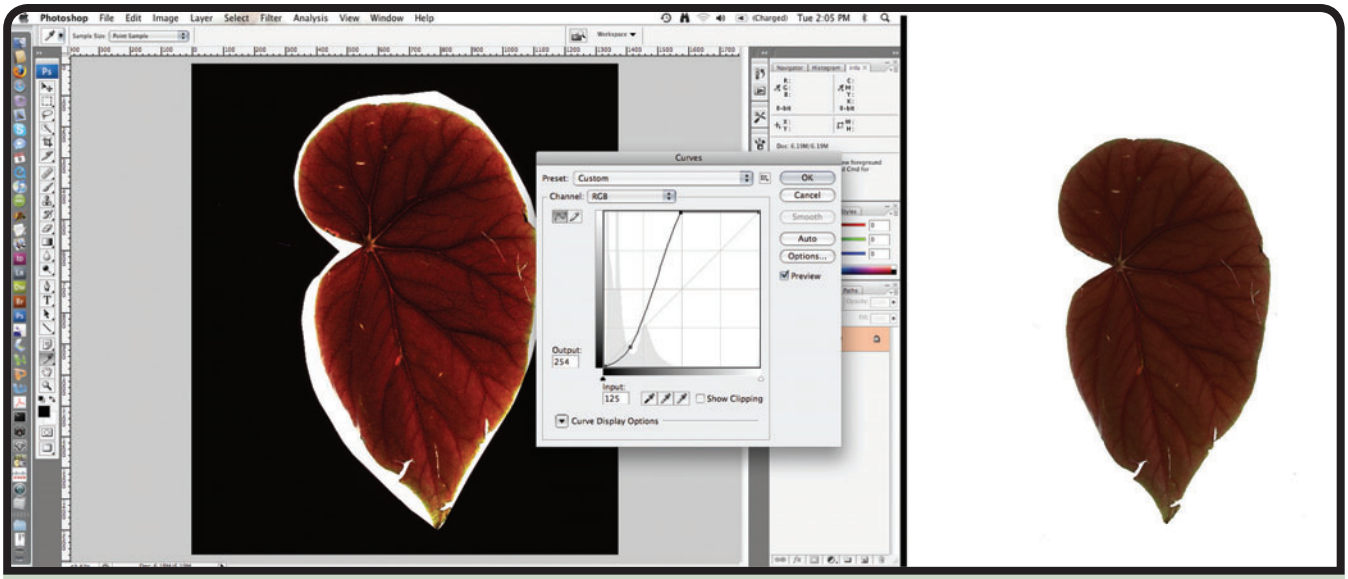


Figure 7.18 Using a black mask around a subject that is being scanned can control flare created when using backlighting using a large source. The mask will better manage image contrast by making the light smaller. The leaf on the left includes more structural detail and localized contrast than the image on the right where no mask was used to form the initial image. Consequently the image on the right reveals less internal contrast and appears flat or muddy in its tonal range.

Peripheral Photographs

Something useful but challenging is the scanning of 360-degree objects on a flatbed scanner. A 360-degree object can have only one side photographed at any time using conventional methods. This could be the front, left, right or the back. Scanning the object on the scanner can provide some fascinating images when imaging the periphery of the subject. The process will require some creative solutions to insure the object rolls smoothly, straight, and in sync with the scanning wand as it travels across the platen. Only objects that are uniform and smooth can be imaged this way. Rough and not smooth surfaces will make this impossible. Images that are made using this approach might create what is called a peripheral photograph. A peripheral photograph records the 360 degrees outside of a subject. This requires the sensor wand to travel under the subject while it is constantly rotating in front of the wand. This technique would be similar to strip photography used in aerial surveillance film photography. In aerial strip photography, a camera mounted in an airplane that was flying at a low altitude and flying at high speeds would be aimed downward towards the earth's surface. Film in the camera would be moved at the same speed as the moving image projected on the film was traveling on the ground. This was synchronized with the airplane's speed. Equipment used for making peripheral photographs can be found in computer tomography and a Panorex® X-ray imaging system (dentistry).

There are some challenges in making precise images this way. It is imperative to have the object rotate in a straight line down the platen. Using a guide prepared from cardboard or a small piece of wood taped in place could be an effective method to roll objects in a uniform

Figure 7.19 The type of photography that records the entire 360 degrees of a subject's outside is called peripheral photography. In this photograph, a prescription bottle was synchronously rolled with the scanning wand. Where the speed was consistent, the tone remains the same. Where the tone becomes darker or lighter, the bottle was rolled at a different speed from the scanning wand. When the bottle did not move in a precise straight line, the peripheral photograph does not create smooth edges but rather irregular ones where the travel path departed from straight. On the right, the bottle as a single view.

and straight fashion. The object must also travel at the same speed as the scanner wand. If one or both change speeds during the scan, brightness changes will be evident in the image. It might be practical to create a black box cover to be situated over the scanner platen when imaging this way, to control ambient exposures.

Stereo Photography and Anaglyphs

Seeing the world in stereo or with binocular vision provides vital clues for humans and other animals to function in the world they live in. Seeing in stereo allows the identification of a foreground and background elements, which enables images and scenes to be better understood, both in static and dynamic situations. Stereovision also provides clues to how far something is from the viewer and the speed of moving objects. It is a very powerful sense.

Making a Stereo Pair

It is quite easy to make stereo photographs. In its most basic application, two images are made of a subject using the same working distance at approximately a distance of 2 inches apart. This is all that is required. This can easily be accomplished by moving the camera the distance from one pupil to the other pupil as precisely as possible. A focusing rail is a great tool for this and allows precise distances to be easily managed as required to make a stereo pair. The distance between pictures is in part influenced by image magnification. Objects at infinity will require a different camera separation than images made at higher magnifications. To maximize how the stereo effect of the image(s) will appear to the viewer, it is recommended to compose the photograph where elements in the foreground and background are carefully placed. The settings on the camera and lens should not be adjusted between the two views. It can be helpful to use a small aperture setting that will lead to

images with a large DOF. Similarly, image processing should be duplicated consistently across the two views to minimize any differences within the pair.

Stereo images can be effective for learning about an object or scene. Shared is an example of a stereo pair. Seeing paired images as a stereo image requires the fusing of the images by looking at them cross-eyed or looking at the pair using a stereo viewer, commonly referred to as a stereoscope. See Figure 7.20.

Making Anaglyphs

Anaglyphs are also stereo images, but rather than being looked at using a stereo viewer, anaglyphs are viewed as one image. In an anaglyph, the red channel of the left image and the red channel of the right image have been swapped out with one another. To see the stereo in the image, a viewer needs to wear special glasses with cyan (right) and red (left) lenses for viewing. To create a stereo anaglyph, two RGB images are made using the above-mentioned technique where the camera is moved approximately 2 inches. To make the new anaglyph stereo file, highlight the red channel in the left image. Once highlighted, select the red channel's data and copy the red channel. Once copied, paste the red channel on top of the red channel in the right image's red channel. It is that easy. I have found it useful to experiment to determine how far to shift the camera between views to obtain the best effect. Deciding the correct image separation distance and placement of the subject requires a bit of practice and pre-planning. For magnified images, less movement is needed, and for images that are smaller, the distance will need to be more exaggerated.

Stroboscopy

Objects are sometimes moving and sometimes the movement of the subject is the subject. Creating a still image of movement that effectively communicates a change in location over time can be challenging for the scientist photographer. The use of a long shutter speed with a continuous light will lead to images that are blurred, and the use of a single-discharge short burst of light from an electronic flash can only serve to freeze the motion of the subject at one location from what might be a range of locations. Stroboscopic photography allows images to be made of a subject that is moving and changes location, or both. This type of photograph is made using a special light called a stroboscope. A stroboscopic light source can be

Figure 7.20 The photographs on the bottom of this illustration are retinal images and feature a stereo photograph of the condition papilledema. Image courtesy of Joseph Territo. Stereo images require the use of a stereo viewer, although some people can fuse images without the assistance of an optical system.

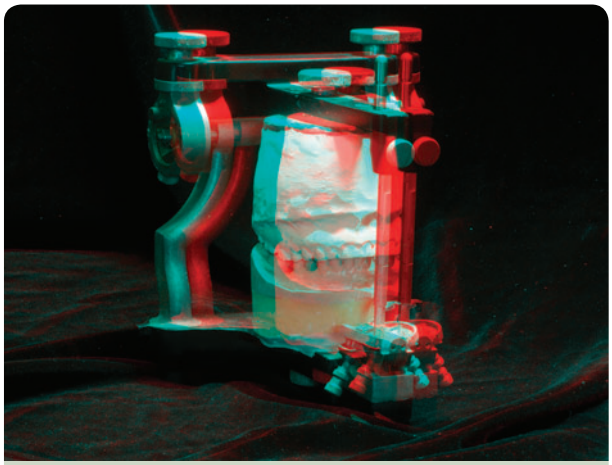


Figure 7.21 This photograph features a dental articulator shown in stereo. Red–blue glasses are required to see the stereo image effect. The camera was shifted 1.5 inches between the two views. How much separation is created between the two camera placements is a function of the magnification/reduction of the scene. Higher magnification photographs will require less camera shift than smaller subjects located far from the camera.

discharged multiple times during a short period of time using short flash durations. This allows the light to be discharged during a pre-determined time while the subject moves. Because discharges are created at different instances, they create exposures that track a subject's position over time. Making a stroboscopic image is accomplished by creating an event with its movement component in front of a camera. Using a darkened room, the camera's shutter is opened for a specified period of time, and while the subject moves, a stroboscopic light source is discharged during the event. When the event has concluded, the shutter is closed.

The subject is lit only by the discharges of the electronic flash in a darkened room. All ambient room lights will need to be off, including the model lights of the electronic flash. The ability to control the number of exposures in the final photograph is influenced by several aspects of the event itself. How fast the event is moving/occurring, what is actually moving, and how far the subject travels during the pre-determined time of the open shutter will all play a role. The frequency of a stroboscope's discharge is sometimes adjustable. For example, if one discharge occurs per second, the subject may travel too great a distance in the second to be useful. Discharging the stroboscope five times during the one second will create more locations and shorter distances between exposures. There is no absolute method for determining how many discharges are needed. Stroboscopes require special engineering needed to produce a frequency of discharges that have useable power and are delivered during a very short time increment. An electronic flash's light comes from a charged gas held in a glass tube. When electricity is passed around the charged tube, a controlled discharge of light is produced. This discharge has power and duration. It can be difficult to create a lot of light and delivery in in a very short time. So, more light creates longer "burn times" or duration. Additionally, to recharge the system, there is time required to refill the power for the triggering system (capacitor). This is called the recycle time. Stroboscopes have been engineered to be effective for the three performance considerations in their capabilities of speed of recycling, frequency of discharge and duration or exposure time.

To manage the event, it may be possible to control or slow down the speed of an event. Sometimes, but not always, an event cannot be speeded up or slowed down. These are the two characteristics of an event that influence how many images should be recorded during the open shutter. This in turn will dictate the image distance traveled between exposures. Stroboscopic images reveal characteristics of a subject's motion, subject speed, or range of motion of the device.

The ideal light source for this type of work is a photographic stroboscope. This is expensive, but there are other methods that can be used to create localized exposures if a stroboscope is not available. I have known photographers to use a novelty store "disco type" light (stroboscope) with a high ISO on the camera to make some acceptable photographs. This light source can be adjusted for the number of discharges per minute but, because of size and power, this light will only be practical for events or objects approximately the size of a basketball. To use this light for photography, locate the event/subject in front of a black background or wall. The camera's ISO will also need to be increased. A very dark night sky can also work. Position the light near to the action field and create a hard sidelight. Using a hard sidelight will be most effective for creating easily identifiable locations of the

Figure 7.22 This image reveals the range of motion of the subject's arms. To create this stroboscopic image, the arms were moved through their range from top to bottom in approximately two seconds. Using an open shutter in a blackened room, the stroboscope was discharged eight times when the arms were at eight different locations going from the apex to the bottom. It is important to minimize lighting that falls on the center of rotation to the extent possible. The torso in this image received eight exposures and the individual locations of the arms only one exposure. Wearing dark clothing can help. The final exposure's brightness was determined by comparing the brightness required for the arms' exposure balanced to the exposure that would occur on the torso. Image courtesy of Nathan Pallace.

event in the cumulative exposure that is made over time. Be careful not to shine light onto the background and select an aperture that provides the correct amount of DOF for the need. Increasing the ISO or adjusting the light source to the event distance are the two most useful tools for achieving a useable exposure.

Another method is to use DSLR electronic flashes that have a feature called burst. The burst mode in modern high-end speed lights is a built-in feature of some models. Canon calls burst multi mode and Nikon calls it repeating flash (RPT). Using the burst mode controls the flash's power output, frequency, and number unit of time delivered. To start, set the power of the flash using the manual mode. This setting will influence the number of discharges that are possible and is described using frequency expressed in Hz or the number of flashes per second. The lower the power setting, the more flashes can be produced in a shorter period of time. It is important to locate the light very near to the event and use a hard sidelight from an oblique angle. A flash meter or estimating can be used to determine the correct aperture setting while operating the camera using a timed or bulb exposure. Also it is possible to use through-the-lens (TTL) if the flash and camera are a dedicated system.

A primitive way to create multiple bursts is to use a small hand-held electronic flash unit with the unit's power dialed down. When the power of the flash units is reduced, the recycle

time becomes very short. I have used electronic flash equipment that can be operated at 1/128 power producing flash durations of 1/50,000 sec. with a nearly instant recycling time. It is quite easy to build or purchase an inexpensive timing circuit that can discharge the flash unit over the course of several seconds in this way. The light will need to be located at a very short distance to the event because it will produce a very low brightness when operated in this way.

It is interesting to note that a black disk containing an aperture, placed near one of the edges, can be rotated in front of the lens, and when using a bulb exposure in a black room with a light shining on the subject can create a series of exposures. The event should be set up in front of a black background and use a very bright continuous light shined from a strong angle from the side. Using an open shutter and a darkened room, spin the disk while the event occurs. Surprisingly it is possible to emulate what an expensive stroboscope can produce to some extent. It is recommended to use a high ISO for this type of work. It also goes without saying that all stroboscopic work should be accomplished using a tripod.

To gain additional separation of images of an event such as a tennis player swinging a racket, the camera can be panned during the burst. By panning, the individual locations of the body and torso will be separated from one another along with the specific locations of the racket and arm as it moves through its range.

SUGGESTED READING

Duncan, Christopher D., *Advanced Crime Scene Photography*, second edition. Boca Raton, FL: CRC Press, 2015; ISBN 1482211874.

Chapter 8 A Primer for Lighting Small Laboratory Subjects

The Da Vinci Bat. People initially thought that bats were closely related to rodents until science proved otherwise. This photograph clearly shows why bats are more related to humans and primates than rodents. London-based photographer Tim Flach titled this photograph *The Da Vinci Bat* because of the bat's resemblance to Leonardo Da Vinci's flying machine illustration. The bat in this photograph is an Egyptian fruit bat, *Rousettus aegyptiacus*. The photograph was taken in 2008 in the United Kingdom. Flach's excellent lighting shares the bone structure of the wing in a new way. Photograph courtesy of Tim Flach, www.timflach.com.

Light and Lighting

Technical elements of radiated energy behaviors—and light—have been covered in Chapter 3. Brightness, color temperature, and other characteristics of energy travel were shared using a scientific point of view. Creating effective lighting for science is more than simply having a technical knowledge of radiated energy and related behaviors. There is light, and then there is lighting. How to create effective light for scientific photographic documentation does not have to be an overly complicated challenge. Using basic equipment in conjunction with some practical strategies can lead to surprisingly good outcomes.

It is possible without great skills in photographic lighting to produce adequate results, but very often artificial lighting photographs will look more like amateurish snapshots for the uninitiated. Many times the on-camera flash will be part of the solution or problem. The short working distances associated with close-up photography in conjunction with electronic flash lighting leads to the creation of very direct and harsh (contrast) light. The subjects in pictures illuminated with direct flash at a short working distance will display very "white" whites that have no detail and black harsh shadows, also without detail. Ambient room lighting can also play a role in creating "not so effective" lighting for some subjects. Bad lighting leads to bad pictures. It's just that simple. Good lighting makes the characteristics of subjects visible and can make the nearly invisible visible. This is the ultimate goal of good lighting required for and used in scientific photography.

Photography by definition is writing with light and describes one of the most fundamental components needed to make a good photograph. Effective lighting reveals the characteristics of a subject. In the fine and applied arts, effective lighting might reveal a mood or a concept. It may emphasize a time of day, or sometimes the light itself might become the subject of the photography.

For science images, though, using artificial light needs to be carefully considered. Science photographs need to be first and foremost about scientific data and not about an interpretation or bias. They need to exhibit neutral points of view. Because photography is a continuum of events that leads to the production of images, what type of lighting is created is one important element of that process. Good lighting should not be a random accident or an afterthought. Scientific photographs cannot embellish, amplify or distort characteristics of subjects. The lighting that is created must reveal the characteristics of the subject and not imply a bias or lead to a false perception about the subject that compromises truth and scientific veracity. Science pictures must be about science facts and not science fiction.

Sometimes creating factual pictures can be more challenging than the process would be presumed to be. How lighting is used can be one element for changing the visual data revealed from a sample. Photography is an interpretive process. Just as a lens forms and shapes an image and an aperture influences the range of focus in a picture, lighting defines and reveals facts about an object. This outcome is based on decisions and knowledge used by the scientist photographer. Lighting can change the emphasis and make common subjects look dramatic. It can distort relationships of elements contained within the frame or make the subject appear normally, as a viewer might expect it to be. Where the brightest light is shined or how the other qualities of the lighting are used to create emphasis might be considered to be similar to the way adjectives are used in writing. The elements and

importance of the elements can be diminished or embellished simply by where and how a light is shined onto and at a subject. Producing effective lighting is a process that starts with an analysis of the sample and what needs to be revealed, recorded, and why.

The subject will always play a fundamental role in the development of effective lighting. A subject can be opaque, such as a rock, or shiny, like a coin. Subjects can be translucent, like a fish embryo, or can be pretty much anything. They can be relatively flat or very 3D. A sample's contrast or surface characteristics will dictate where to place a light. The subject dimensionality or shine will influence how diffuse or broad the light should be. There are a myriad of variables to consider. Rarely will one lighting method work for all things in the laboratory.

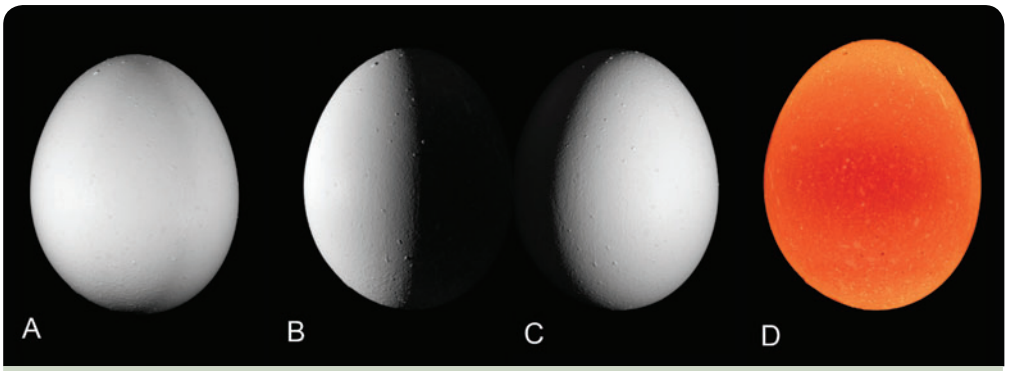


Figure 8.1 A chicken egg can have its appearance changed when using different lighting approaches. Image A is an example of flat frontal lighting. Images B and C demonstrate the influences of directional and medium contrast lighting with some fill. Image D is demonstrative of backlighting. In each view, the egg reveals different aspects of the shell and its characteristics. Which view is best? It depends. Photographs courtesy of Jace Artichoker.

Light sources come in a variety of sizes, shapes, and types. Continuous or short duration discharge is but one of many features to consider when selecting lighting equipment for a particular subject. Each feature of a light can play a role in how the creation of lighting outcome will work. In Chapter 7, fluorescence photography, imaging with the invisible spectrum, polarized light, and schlieren imaging were all covered. These methods are very special applications of lighting. The emphasis of this chapter will be how to create effective lighting for laboratory subjects that might be the size of a grapefruit or smaller and for use of visible or white light.

Fundamental methods for lighting and modifying lights used for artificial lighting will be explored in this chapter. For the more interested reader, I suggest you seek out other more detailed books about methods useful for artificial light and studio photography techniques. For all artificial light photography, the concept of a main light and a fill light is fundamental. Because people live in a world with only one light—the sun—there is a subliminal expectation by a viewer to see only one shadow. The main light will make a shadow and the fill light will make the shadow more or less gray. A direct small light will make a very black and sharply defined shadow and a diffuse large light will make a less defined and gray shadow.

Fiber optic lights are special lights. An important part of the fiber optic light will be its light guide. Fiber optic lights deliver light to a very small and precise location using a pipe or light guide. This is accomplished through the use of fiber optics. Light guides or pipes are composed of hundreds—if not thousands—of microscopic glass fibers that begin at one end of the light guide where the light enters the fibers and then terminate where the light leaves the fibers at the other end of the guide. The fibers, on average, are slightly larger than a human hair. They are fairly robust but remain vulnerable to breakage when used without concern for their care. These fibers cannot be bent into a 90-degree angle or dropped on hard concrete floors. Dropping a light guide will certainly break fibers. As fibers become damaged, their ability to transmit light will become diminished, and its brightness will go down.

Fiber optic lights are ideal light sources for laboratory photography and their use will be the emphasis of this chapter. There are other lights that can work, but for the sake of efficiency and the ability to offer the most focused suggestions, the fiber optic light source will be the one featured. A fiber optic light is comprised of a light housing or lamp box that usually will be compact and contain a very bright light. The light is directed by fibers into a bundle of light that forms a small circle of light. The light's brightness will be adjustable and there may be a fuse on the light box as well. These lights can be outfitted with a variety of light guides that will be further discussed in the next paragraphs. Fiber optic lights are sold in a variety of models ranging from inexpensive to very expensive. The cost for a unit will be influenced by how robust it is and whether the brightness can be reduced by voltage or through the use of a graduated neutral density filter (tungsten halogen models). LED light models will use pre-stepped brightness range settings. More expensive units will also come equipped with fans that are dampened for vibration and may have the ability to operate brighter bulbs. The heat and air currents from a lamp and its housing can affect delicate biological subjects by causing them to move or dehydrate. Illuminators with robust fans will not be ideal for photography because of the heightened vibration they create that can affect image sharpness.

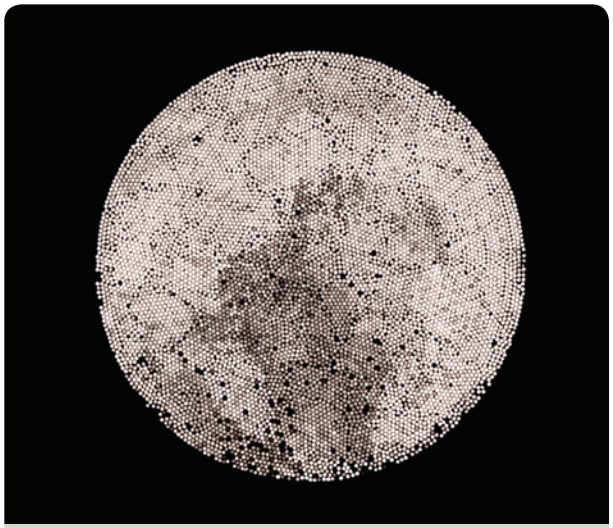


Figure 8.2 A fiber optic light is a very important tool for scientist photographers. It can produce highly precise lighting to specific locations on a sample. The nearly microscopic fibers carry light and behave much like a pipe used for water. When the fibers break, though, they do not transmit light. In this photograph, broken fibers are evidenced as black dots. The white dots represent fibers that are capable of carrying 100 percent of the available light. The diameter of the light guide was 5 mm. The gray dots represent fibers that are having problems.

It is quite simple to check a light guide for damage by removing it first from the lamp housing. By pointing one end of the guide towards a light, and using a finger as a shutter, it is possible to peer into the guide to determine the percentage of fibers that are not transmitting light. Put a finger over the end of the guide near to the light and open and close the finger over the end of the guide when pointing it at a light. You will be able to see what fibers are broken. Broken fibers will appear as black dots within the guide and fibers that are transmitting will be visible as white dots.

Fiber optic light guides come in a variety of types and sizes. They are unique to specific lamp housings and the types and size of the coupling port. Common light guide styles might

include a single or bifurcated rigid gooseneck. The light guides can be encased in rigid coaxial cable or flexible casing, which allows the light to be positioned and remain in that location. Rigid guides that allow positioning can be very practical. The following is a short list of other types of light guides, and each light guide has typically been optimized for a specific type of subject or how the light might be used: straight and single fiber optic light guides; a dual branch bifurcated fiber optic light guide; multiple gooseneck light guides; quartz light guides; ring lights; line lights; and backlights. I find the rigid bifurcated gooseneck model to be most useful across many diverse applications. A ring light and line light can be useful for lighting when the working distances are small and access to a sample can be a challenge. Ring lights make omnidirectional lighting that is low contrast. Ring lights are useful for non-shiny objectives where color and textural surface information is the subject. When used with reflective objects, a circular reflection of the light will be evident in the photograph. Line lights are great for showing texture when placed at an acute angle to the subject's surface. This light will not produce as much contrast as a direct or point light from a gooseneck light guide.

Making Good Light

Good light is the product of knowledge, skills, a good sample, and using the right light source and/or modifier. Depending on the subject, effective lighting can be pretty easy to achieve or really challenging. Having photographed laboratory subjects for more than forty years, I subscribe to the KIS method—"keep it simple" whenever possible. It is easy to make things complicated and the lighting should work without becoming the subject of the photograph. I believe it is very useful to use only one light. In addition to a light's brightness and color temperature, the physical size of the light guide is an important characteristic for artificial light photography. Purchasing the right light guide is an important decision. As an accessory, a light guide will have costs, and acquiring a few may be cost prohibitive. Picking the right sized light guide matters. I have seen fiber optic light guides that may be as small as 4 mm in diameter or as large as 15 mm. The smaller the light source, the more contrast and directionality will be possible from that light. The larger the light source, the less contrast the light will make. A light should be proportionally sized to an object's size. As a rule of thumb, small subjects benefit from using small lights and larger subjects may benefit from larger lights.

The term "quality of light" describes how the light achieves, or does not achieve, effective lighting for a subject. Quality refers to the characteristics of the light such as direct or diffused illumination. Direct light is very harsh and creates hard shadows. Harsh light can come from the sun or from the raw light that leaves the light guide. It can come from the small on-camera flash or even from a candle. The size of the light has a direct relationship to the harshness of the light. The quality of light is one measure of the type of the light and the other is the direction of the light.

When making lighting decisions, it is important to consider what you are trying to show and how the light interacts with the sample or surface of the sample. Color, texture, shape, topography, cracks, or any other characteristics of the sample that are relevant need to be made more visible in the viewfinder. Lighting can come from above the sample, called reflected

light, or can come from below a translucent or transparent sample, called transmitted light. Some samples will require both transmitted and reflected light components to show surface and internal structure characteristics.

White and Neutral Backgrounds

Subjects can sometimes be transparent and benefit from using transmitted light. Producing uniform transmitted light when not using a light microscope is not overly complicated but there are challenges to create a uniformity of brightness across the field of view. A light box or panel if available will be the most effective solution for making even lighting, but shining lights from the light guides onto a white piece of board can also work. Using ping pong balls on the ends of the light guides can be helpful to spread light out more evenly. Creating uniformity across the entire field of view of the lens is very important. If the light is not uniform, false data can be created in the image as well as the creation of other image artifacts. While the background is not the subject in science pictures, it will play a role in the image's chances of success. A well-managed background is a fundamental expectation in science pictures. Irregular tones and textures will compete with the sample for the viewer's attention.

I have on occasion used my laptop's screen as a white background to create uniform backlighting since it is so neutral and uniform. For this method, I create an image file that has 255 RGB brightness values. I make the file size the number of pixels used in the laptop display. A file of 3000 x 2000 pixels will be perfect. This file is then displayed as a white image on the screen, eliminating all docks and sidebar menus. You can then locate a subject in front of the white screen using some sort of an improvised holder. The computer screen works best for samples that can be suspended vertically in front of it. It is possible to redirect the light using a mirror, or laying the laptop screen on its side can also work. Care must be taken when working around liquids, for obvious reasons. I have also used an iPad® for backlighting, which is easier than a laptop. The size of the screen will dictate how large an object can be transilluminated. Using a Petri dish filled with milk can also be a terrific white background. When lit from below, whole milk will act like a diffusion filter and is surprisingly neutral. Depending on the sample, the milk will also remove the infrared or heat from the light.

There is an important ratio required for backlighting when some front light is also needed. The amount of light shining onto the sample when compared to the amount of light shining onto the background should be a 1.5 f-stop in difference. If the background is less than this amount it will photograph as a gray, and if the background is brighter than this amount it will be too bright, causing the background exposure to bleed into the subject located in front of it.

Making Contrast

Contrast is an important element in a photograph. Increased contrast will make pictures look sharper even though they may not be. Creating more contrast can be accomplished in

several ways. One of the quickest ways to increase contrast is to move the light further from the subject. While the brightness goes down, the size of the light will become proportionately smaller relative to the object's size. As a smaller light, it will create more contrast on the subject. The location of the light relative to the object can also modify the contrast the light produces. Having a light proportionally sized that is brought closer to the subject will make less contrast when located at a 90-degree angle to the surface of the subject. This technique might be called edge or texture lighting. If light is raked across the surface of an object it makes more surface contrast than if the light comes from the same axis as the point of view of the camera. A small light used from an acute angle, it will make strong raking light and produce shadows that will be very black. The reason to make this lighting would be to show surface structures and textures.

The brightness ratio of the brightest elements in a photograph or highlights when compared to the darkest shadows of the scene is called the luminance ratio. When light is added to the shadow side of the scene, this light is called fill light. Adding fill light will lower the brightness ratio and contrast. The brightest light used in a photograph is called the main light. It is the dominant light for the photograph, and any other lights would be considered fill lights. The main and fill lights are described as having a ratio. A fill light is most frequently located at or near to the camera but not always. A lighting ratio of 1:2 means the brightest part of the picture will be one f-stop brighter than the darkest region. A ratio of 1:3 will make the brightest part of the scene two stops brighter, and so on. The higher the lighting ratio, the higher the contrast will be visible in the image. A lighting ratio of 1:1 describes lighting with an equal brightness across the entire field of view. Digital cameras can typically record a 6+ f-stop range from the darkest tones to the brightest of tones. Once the brightness range exceeds this f-stop range, detail will not be recorded either in the darkest or brightest regions depending on how the exposure is adjusted. HDR or high dynamic range methods are required for creating a file with detail in both the brightest and darkest parts of the picture.

Fill light can be added to scenes produced from fiber optic lights by using another light source of similar color temperature or simply by using a small white card or a small piece of aluminum foil to reflect light back into the scene. By varying the angle of the reflector card to the camera and the distance to the subject, the amount of brightness reflected into the shadow can be adjusted. Fill cards for small objects can be held in place using many strategies including modeling clay, or a third arm—a soldering tool accessory—or a lab stand with black tape. I have used a piece of an aluminum soda can as well. The physical

Figure 8.3 This composite photograph reveals how the seed of an *Acer rubrum*, red maple, will appear with minor changes to its lighting. The subject has characteristics that are opaque, textural, elevated, and semi-transparent. In each photograph, slightly different characteristics of the seed were made visible, or made less visible, as a consequence of the main light's positioning (lighting direction) and use of fill lighting. The color temperature of the light was not adjusted and the changes to the seed wing—or key—color is a by-product of where the light was located, the amount and direction of the fill light, and the localized contrast created from the sample's characteristics made visible from the light's contrast and directionality.

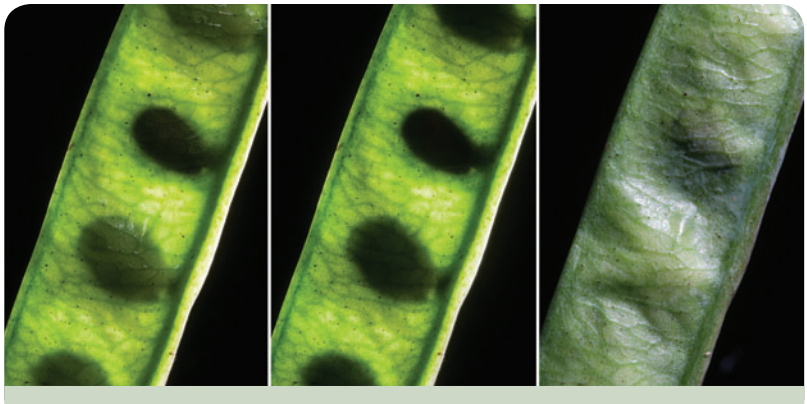


Figure 8.4 This composite photograph features the seed pod of a *Cercis canadensis* or Eastern Red Bud tree. In the right view, the light was from the top and left raking across the pod. The middle view used light coming from below the pod, and in the left view a combination of both methods was used, revealing the maximum information about this object.

space under the camera will typically be small. Creating the correct lighting ratio is a very important feature in a photograph and needs to be carefully managed in a small space. Adding fill is not an overly difficult adjustment to make. It is easy to add contrast to a picture in the captured file during image post-processing in software. This being the case, I often create a relatively low contrast lighting ratio, which is sample dependent. Subjects that are already high contrast will benefit by photographing them using flat or low contrast light, and subjects that are low contrast will benefit from using lighting that has more contrast.

Reducing Contrast

To reduce the contrast of a light, the light's surface must be made larger or moved closer. Lights contain both a bulb and sometimes a lamp-housing reflector that affects the size and subsequently softness or harshness of the light. Some lights may also use a convex-like mirror behind the lamp, making the light larger. Moving the light closer to a subject can make the light larger proportionally to the sample. This method will have limitations to its effectiveness and should be explored as a possible first attempt solution. Moving the light closer to a subject will increase the light's brightness and an associated exposure correction will be required. A better approach is to broaden the light by shining the light through a piece of diffusion material such as tissue or by shining the light directly onto a semi-reflective surface such as piece of white cardboard of appropriate size located near to the sample. The cardboard will reflect the light back onto the subject. Making the light source larger can have a very strong influence in managing the light's contrast. Shining low contrast light using a low lighting angle to the subject's surface can be very effective in revealing a sample's characteristics and maintaining details in both the highlight and shadow regions of the scene.

When considering how to light, a few things come to mind:

1. Analyze the subject.
2. Determine what is to be emphasized.
3. Choose the appropriate lighting strategy.

A favorite trick for enlarging the surface of a fiber optic light guide is by using 3M Magic® Tape or milky plastic bottles originally used for packaging products such as milk or ammonia (here in the US).When using a ping pong ball as a light diffuser, it is useful to poke a small hole into the ball where the logo has been printed. Keep the hole smaller than the diameter of the light guide. Once the hole has been established, the ball can be slid over the end of the light guide. Relative to an object's size, this approach can produce excellent and well-managed contrast and uniformity of the light. Highlights or bright reflections can be easily

managed in this way. This method is practical for semi-reflective metal objects such as coins, circuits, and many other things. The quality of the light's contrast when using this method will be affected by the subject's size, surface characteristics, the sample's texture, and the placement of the light. Similar to any of the other light modification methods, the light-to-subject distance will continue to play a role in the outcome.

Another way to diffuse the light that will produce a more specular light, but one that is still effective, is to put 3M Magic Tape loops over the ends of the light guides. The loops should use a generous amount of tape to make the loop stand up and away from the end of the guide. The idea is to create some space between the tape and the end of the light guide. The tape will diffuse the light and hopefully become larger than the end of the light guide. Using Magic Tape will not be as effective as ping pong balls in making diffuse light, but it will work well for objects that are not shiny or mirror-like. It can be helpful to use two or three loops stacked on top of each other to increase the material's ability to diffuse the light.

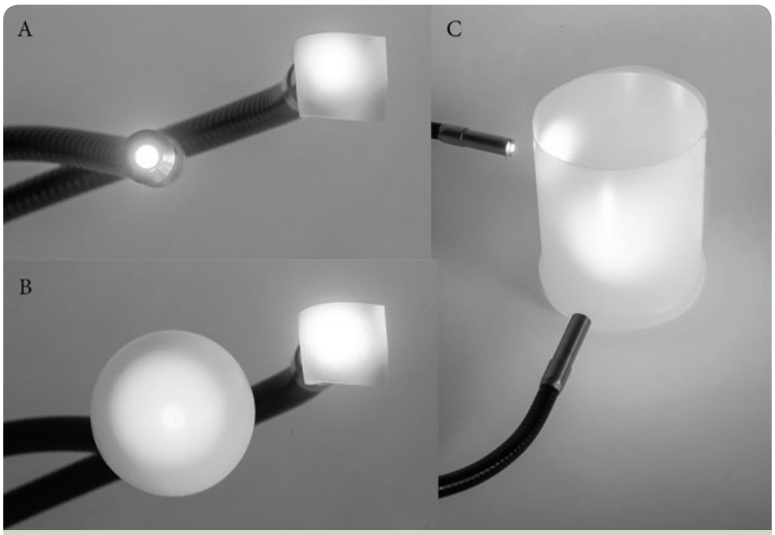


Figure 8.5 Light guides can be small lights and create high contrast or specular light. Sometimes less contrast is needed and a flat lighting is needed. It is relatively easy to create diffused light using cellophane Magic Tape, ping pong balls, or recycled plastic bottles that are used for liquid products such a windshield washer solution or milk. In each case, making the light larger reduces the light's contrast.

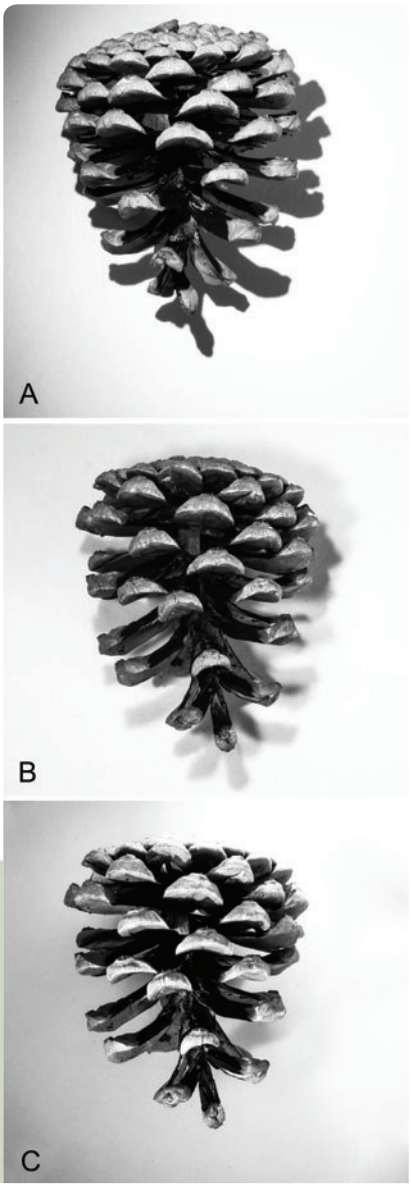


Figure 8.6 A *Pinus* cone is a useful subject to demonstrate challenges in managing the tone reproduction of a subject and the role lighting plays in that outcome. These pictures reveal how the size of the light used to photograph a pine cone creates a range of outcomes. In this series of pictures, image A was made using raw direct light from a medium contrast direction. The associated shadow bears testament to the size and location of the light. Image B has been lit using the same light—located at the same distance—but using several loops of matte cellophane Magic Tape on the light guide. Image C is an image created using a medium sized milky white plastic bottle to create a very large and non-directional light. It is useful to compare the shadows in the three photographs.

Shining light through a milky plastic bottle is the most effective approach used to make a broadly diffused light. There are a few commercial products that can be purchased for this task but I find homemade light modifiers work quite well. It is best to cut the bottom and top of the bottle off, leaving only the cylinder of the vessel for use as the diffusion material. The height of the tube will be determined by the focal length of the lens. Once positioned over the subject, the light guide or guides can then be positioned at short distances away from the diffusion cylinder. This type of lighting behaves much like a light tent and subjects will be surrounded and bathed in diffuse and 360-degree light. It is possible to locate ping pong balls over the ends of the light guides to further diffuse the light before it enters the plastic, creating the maximum effect.

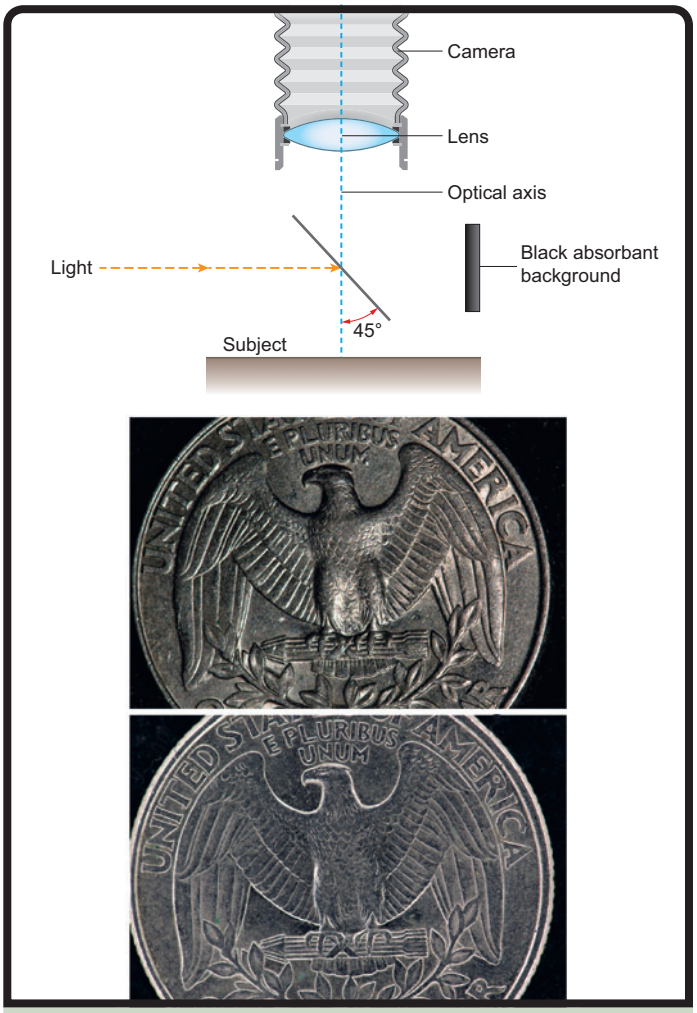


Figure 8.7 Axial and diffused lighting can create very different results. In this composite, the middle image is a US quarter illuminated using axial lighting. The bottom image was created using diffused lighting. More surface characteristics are revealed in the axial and higher contrast lighting. The image's structure exhibits more crispness and the image appears to have some relief when compared to the flatter and more diffused non-directional lighting used for the bottom image. Images courtesy of Torey Miller.

Axial Lighting

Axial lighting is a unique type of light that makes pictures that look as though the light came from the camera's lens itself or is on an axis with the camera. To achieve axial illumination, a beam splitter is located at a 45-degree angle to the lens between the lens and the subject. When using axial lighting, 50 percent of the light that is shined onto the beam splitter will be reflected down to the subject and 50 percent will be transmitted through the glass and away from the subject. Axial illumination produces lighting that is nearly shadowless and exhibits a high degree of contrast on the surface of the subject. It is very useful for shiny objects such as coins and circuits. It reveals surface texture and structure without the creation of directional shadows that obscure detail.

Axial lighting can be a little tricky to set up but once established can be a valuable strategy when used with the right subject. I frequently use pre-cleaned microscope slides or cover slips, depending on the size of the subject, as beam splitters. I also use a point light source from a small fiber optic light guide. The glass beam splitter is located above the sample and positioned at a 45-degree angle to the surface of the subject. The camera lens is located directly above the beam splitter. There are no photographic challenges when using axial lighting but there are a few things to keep in mind about lens and aperture settings. Because of where the light strikes the sample and the fact that it comes from the same pathway where image is formed, it is crucial to photograph using a nearly wide-open aperture. When an aperture is closed down, it will make more DOF. If the DOF of the system is large, the

"light" reflecting off the beam splitter might become defined and create flare in the system. The diameter of the lighting coverage is also affected negatively. The circle of illumination will get smaller as the aperture is closed. It has already been shared but bears repeating—never use autofocus for technical photographic applications, especially in axial lighting set-ups. It is also very important to ensure the beam splitter's alignment achieves the same height across the sample from left to right. It is practical to use modeling clay or a third arm soldering tool to position the beam splitter. It can also be helpful to locate a small piece of black velvet on the side opposite the light itself. The surface of the beam splitter that is away from the light may reflect the ceiling or other things back into the lens. It is also better to work with the room lights dimmed or off.

Laboratory Glassware

Glass is an interesting problem to photograph because it has 360 degrees of reflective surfaces. It can be colored or neutral. It can also be holding liquids, such as might be the case of a test tube or other flask, for example. Because of the importance of controlling specular reflections, it can be very useful to really broaden a light or to light from behind the subject. Previously discussed broadening of a light source was accomplished using a white cardboard. That is still an excellent strategy to consider; however, it will create a large and soft white reflection on the surface of the glassware. A better way to light glass is using white line and dark line lighting. When making dark line lighting, first place a white or light gray background behind the subject. It does not need to be huge but simply should fill the camera's viewfinder. Place the glassware on a sheet of glass that is elevated above the table. Allowing light to enter the glassware from the bottom will produce better lighting. Once this is composed and focused, shine a small spot of light directly behind the subject. This will make the subject appear backlit and display dark edges. It is important to attempt to keep the raw lighting from striking the glassware. Place the light in the front

Figure 8.8 Laboratory glassware can be a challenge to light effectively. Managing stray reflections is one of the primary challenges. The image on the left was made using dark line glassware lighting and the image in the middle was made using white line lighting. It is very similar to how darkfield lighting used in microscopy is created.

of the camera and use light baffles or cardboard to block any direct light from striking the glassware.

There is also "darkfield" lighting, which is used for glassware; it is called white line glassware lighting. It is useful to reveal glass inclusions and edge definition. In white line lighting, a piece of black material is located behind the subject and needs to be large enough to cover the field of view of the camera's lens. Two lights located outside the field of view are shined through the subject from behind it using an oblique of view to the camera lens. It is best to locate pieces of black cardboard or other black material to act as a curtain or baffle to the lights. In this fashion, there is the creation of slits of light that comes from behind the glass subject. Light coming from this direction will become trapped within the vessel and cause the edges of the glass to glow. When permissible, it is important to clean the glassware carefully using lint-free materials. It might also be advisable to use a dusting brush as a final treatment prior to depressing the shutter. Fingerprints and other particulate material can quickly become the focal point of a photograph if prominent in the composition and made visible by this type of lighting.

Metal and Tent Lighting

Shiny objects like glassware can be difficult to light. Similar to glass, highly polished metal or mirrors have entirely reflective surfaces reflecting anything that is in front of them. A shiny surface can be a very tough subject to light and photograph, for sure. Lights, cameras, photographers, and anything else will be reflected back to the camera. Depending on the size or prominence of the reflections, they can be terribly distracting and certainly are not a positive element in the photograph.

Better lighting can be achieved by making a very broad light, which also will reflect itself in the subject but will be more pleasing and less random across the highly reflective surface. A sometimes better approach is to light the mirror-like surface using what is called a tent. A tent used for photography is almost always going to be white. A large and semi-rigid system is ideal—white cardboard, for example—or something such as a white shower curtain or liner might work equally as well. The subject's size will influence how large a tent is needed. A light or lights can then be shined through or inside the tent, which will be reflected in the highly polished and reflective surfaces of the subject. On occasion, it can be effective to locate rectangles of black paper or other appropriate materials to create desired reflections in strategic places. These other tones may be useful to break up the possibly monotone tent reflections. Black reflection may also create the perception of shape in the subject.

Immersion

Wet samples can often benefit by being photographed in liquids. In Chapter 5, strategies were shared for using immersion methods in general. The objective of keeping a sample wet is to manage surface specular reflections. When a light is shined onto a wet object, the various topographical structures will produce many reflections. These reflections

will be random and may possibly obscure important details. It is possible to place a sample under water or another appropriate liquid and manage these reflections using one light. Use distilled water for this or, better yet, water that has been de-oxygenated by evaporation over time. It may be necessary to hold the sample below the surface of the liquid through the used of some type of a weight. Many objects will float. Using one small light, the surface of the subject can be illuminated without the presence of any specular highlights to compete with any surface details. Immersion management and lighting will create low contrast lighting. I have found it useful to add contrast back into the picture during the image processing.

Figure 8.9 A crab apple was cut longitudinally and would be categorized as a wet object. The image on the left reveals countless specular reflections on the fruit's surface. "Speculars" are the by-product of a wet surface when photographed using a small light source. In the image on the right, the crab apple was submerged in a water bath and the specular reflections have disappeared. Only one reflection is produced and is on the surface of the water. It can be easily cropped out or the light located in a better spot for a particular situation.

A Working Summary

The lighting of laboratory objects does not have to be overly difficult. Using some practical and proven strategies it is possible to produce significantly better pictures. The subject of the photograph will play a role in determining the quality of light that is required. Effective lighting is one of the foundations required to make an effective photograph. Controlling light and creating managed highlights is part of the task. Careful observation of, and the placement of, the highlights is one of the ways to make effective light. To the extent possible, it is useful to create a collection of lighting accessories too. This would include small pieces of clay, white cardboard, black cardboard, modeling clay, pieces of aluminum and small mirrors. As experience is gained, better skills will be developed.

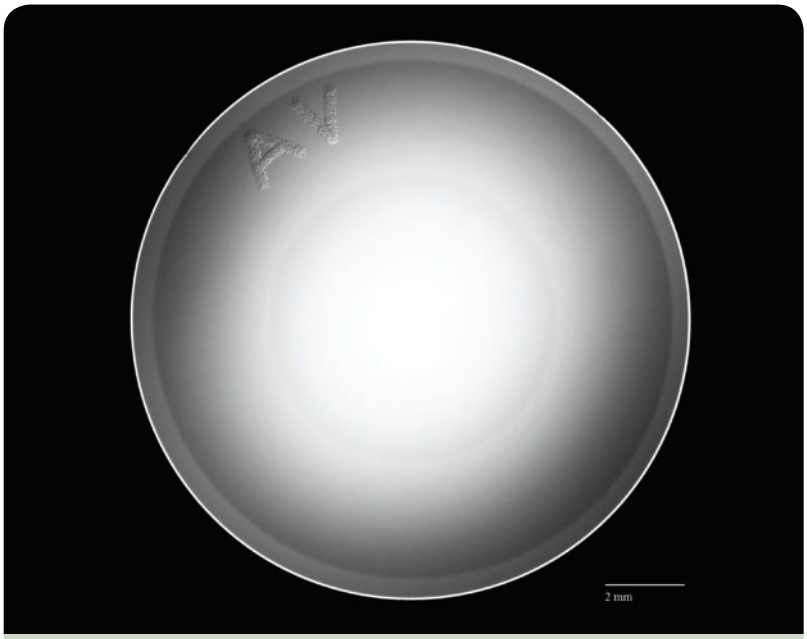


Figure 8.10 This is a photograph of a contact lens. AV stands for Acuvue®, a Johnson and Johnson company. The lens was photographed in a small aquarium using distilled water. Dirt and air bubbles are a constant challenge for photographing in this way. Image courtesy of Lindsay Quandt.

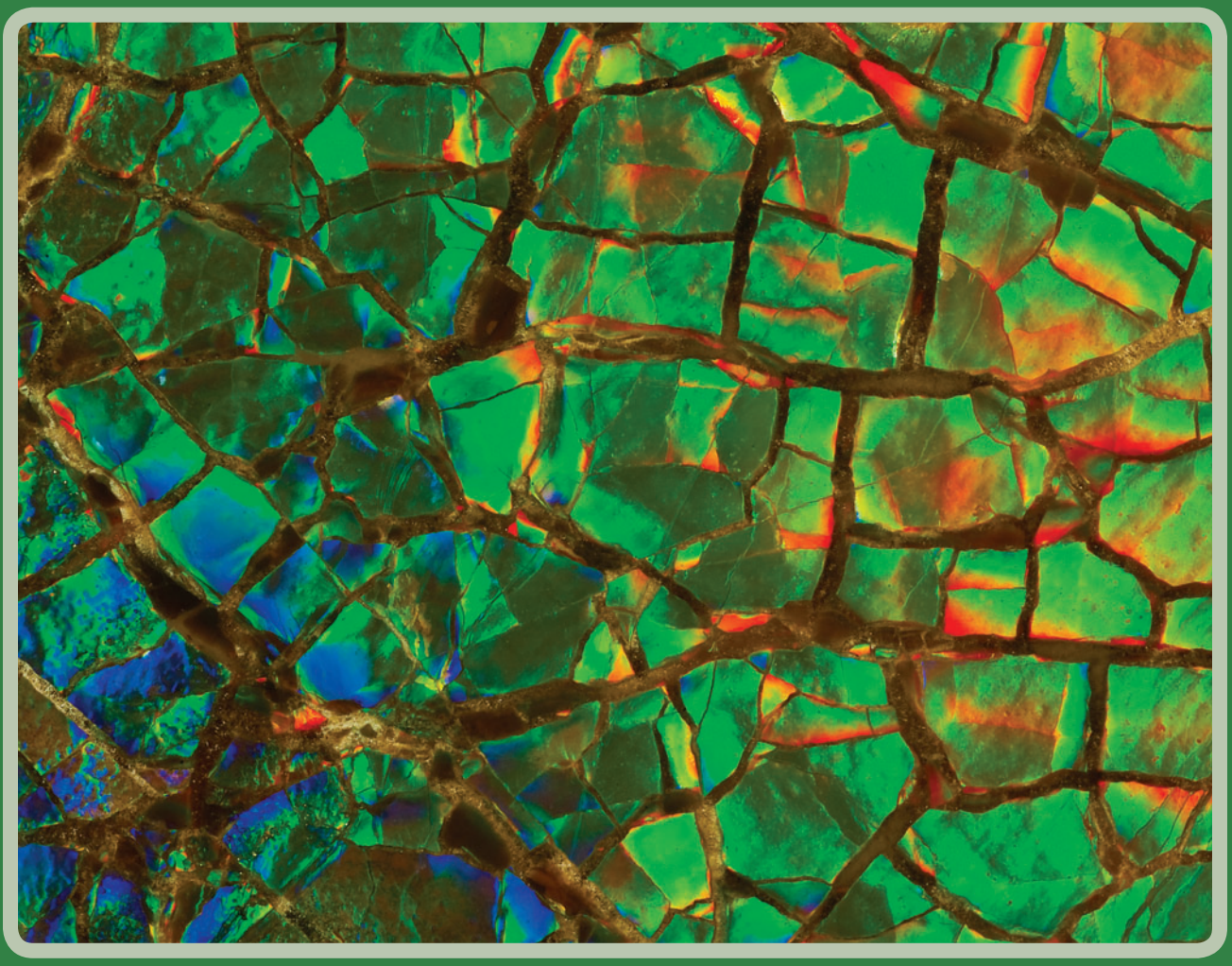
SUGGESTED READING

Prakel, David, *Basics Photography: 02 Lighting*. London: Bloomsbury Academia, 2007; ISBN-10: 2940373035.

Reznicki, Jack, *Studio and Commercial Photography* (Kodak Pro Workshop Series). St Louis: Pixel Press, 1999; ISBN-10: 0879857838.

Stoppee, Brian, and Stoppee, Janet, *Stoppees' Guide to Photography and Light: What Digital Photographers, Illustrators, and Creative Professionals Must Know*. New York: Taylor & Francis, 2012; ISBN-10: 1136100539.

Chapter 9 Light Microscopy



This photograph features the shell of a fossilized ammonite that was hundreds of millions of years old. The iridescent colors are produced by alternating layers of aragonite and conchiolin, or from minerals that have replaced them over the millions of years during the fossilization process. The shell was photographed with a simple microscope. Axial illumination was used to show the iridescent colors. The specimen was coated with mineral oil to control specular highlights. Creating effective focus at high magnifications is always difficult. This photo was originally published in *Ancient Microworlds*, published by Customs and Limited Editions, coauthored with Giraud Foster. Image courtesy of Norman J. Barker.

Foundations and Brightfield Methods

Photographing using a light microscope can be challenging. There are numerous problems that need to be resolved and managed to produce effective results. Some of the challenges include:

- working with nearly invisible and very tiny 2D or 3D subjects;
- very small working distances;
- physical and optical behaviors affecting energy traveling in and out of glass or other materials that affect image formation;
- lenses that have a finite and small range of focus and the presence of optical aberrations;
- the constant presence of dirt.

Forming highly resolved images of infinitely small objects requires a working knowledge of applied physics and careful attention to the influences of the sample itself on the image. This can be more complicated than it seems because of the Heisenberg Uncertainty Principle. This theory describes how it is not possible to know all things about a small structure or particle at the same time because it is not possible to measure multiple quantum behaviors concurrently. This chapter will focus on core principles and other fundamental approaches needed to successfully form and capture images produced by a compound light microscope. There is not enough space in this book to develop all of the complex laws of physics that play a role in image formation, so the chapter will take a rather holistic view of the subject. The Internet also continues to be an ever increasing resource and this chapter has been produced to share information in a complementary way to the numerous and ever expanding web resources.

The interest in seeing more than the human eye can resolve has inspired discovery and innovation in science, optics, and photography for centuries. Curiosity when coupled with the invention of new tools has accelerated the ways scientist photographers can make images. The rate of release of new products does not show any signs of slowing, and as such, light microscopy will continue to benefit from new and better products from innovations in optics, imaging, and computing. Chapter 10 explores confocal microscopy and also shares content, exploring some new technologies such as PALM, FRET, and STORM.

In the mid 1980s, there was something of a renaissance for light microscopy as a consequence of the release of several new technologies simultaneously. Computing had improved enough that it could be coupled to newly redesigned optical research-grade microscopes that were being outfitted at the time with digital cameras and software. As a consequence, light microscopy and imaging began an expansion of new discovery and probing the boundaries of what was possible. This expansion has not slowed down in more than twenty years.

The early period of digital technology was soon followed by the commercialization of the first confocal microscope in the late 1980s by the company Bio-Rad. Confocal microscopy produces images with infinite focus. Concurrently with these technology advances, there was also an explosion in the invention of new fluorescent probes including green fluorescent protein, GFP, which was first used for scientific discovery in 1994. Never before in the history of microscopy could so much be accomplished using the instrument. More about GFP and confocal methods is shared in Chapter 10.

Fundamentals of Magnified Images

Users of a microscope rely upon direct observation, whether using the microscope's eyepieces or viewing the image on a computer monitor. Microscopists are frequently reminded about how the human vision system functions and how it influences the process of observing magnified images which are sometimes very dim or poorly formed. More aspects of human vision and perception can be explored in Chapter 2. A working knowledge about how people see and understand what is seen can be an important advantage when looking at nearly invisible subjects and determining what is real and what is perceptual—a sort of illusion, similar to how a star seems to blink in the sky.

A compound microscope is a complex optical instrument designed to magnify very small objects. It forms images that can delineate fine structures contained within a larger sample. The ability to delineate structures is called resolution (optical). To see structures within an image, a microscope must create three outcomes: it must be able to magnify the sample; it must be able to discriminate fine details within the sample (resolution); and it must be able to create/maintain sample contrast necessary to delineate structures within the sample. These three outcomes assist in making things visible to the eye, camera, or other imaging devices.

A compound microscope produces an aerial image (see Figure 9.2). An aerial image is formed by a positive lens and is located in space. Aerial images can be focused onto a screen (or in the eye), if placed in the optical path and at the right location. The microscope's aerial image is relayed to the retina by the operator's cornea/lens, or to the camera's sensor by a lens called an eyepiece. It is important to keep in mind that the human lens and the eye's refractive properties are influenced by age and become part of the optical elements of the microscope. Younger eyes are more elastic and can see more easily at shorter distances. As the human eye ages, it becomes less elastic and near vision may be difficult. Another component of seeing in challenging situations may be the presence of cataracts that accompany age. Because images formed by microscopes are aerial and interpreted by the influences of the brain (perception), having distinct vision of images produced by microscopes can be challenging or easy to create. Sometimes aerial images can create illusions or the perception that something was seen.

Microscopes form images that are larger than the subject. Magnified images may be produced by a simple microscope such as a pocket magnifier or a scanner, or as the result of more complicated optical instruments such as compound microscopes. A single stage of magnification is called a simple microscope. Decades ago, slide projectors and photographic enlargers were examples of simple microscopes. Photomacrographic instruments such as the Nikon Multi-Phot® were also sold as microscopes and used a single stage of magnification. This chapter will share principles used in brightfield, now more commonly referred to as widefield, microscopy. Brightfield methods might be considered the most simple of all the microscopy techniques. White light is shined through the sample in the brightfield method. The chapter will also feature other microscopy methods including darkfield, fluorescence, phase contrast, polarized light, and Rheinberg illumination.

Optical Magnification

A compound microscope uses two lenses to create magnification. The initial stage of magnification is produced by the primary lens or objective. Once formed, the primary image is further magnified by a second lens, the eyepiece, which relays the primary image to the eye or a camera. The first lens produces a real image while the second lens may produce either a real or a virtual image. A real image is an image that can be focused onto a screen and a virtual image is one that can be seen but without additional lenses cannot be focused onto a screen. Together they form a real and inverted image that is larger than the subject. For example, if a 10x objective were used with a 5x eyepiece, the combined image will be magnified fifty times or x50. When referring to the lens magnification, the number typically goes before the "x", e.g. 10x, but when referring to the total image magnification, the "x" precedes the number, e.g. x100. The proper way to indicate image magnification is x50 and 50x is the proper way to indicate an objective.

Estimated magnifications of a system can be determined by multiplying the magnification of the objective and eyepiece. This calculation provides an approximate magnification of the images that are seen; however, photographing a stage micrometer—a microscope ruler—is required to determine the exact magnification for a microscope's imaging system. Stage micrometers come in different calibrations and a 1 mm scale often is subdivided into units of 1000 um. An imaging system (where the camera system is located) will often have a different magnification than the images that are seen by the user in the eyepieces. This is due to variances in the image projection distance between the photo/imaging eyepieces and magnification of the two eyepiece types. Many research-grade microscopes will have software that can also create a measurement system for magnified images once calibrated. Calibration requires the use of the stage micrometer to teach the imaging software's scale. Imaging software can count pixels but not measure directly without being calibrated. It is also possible to calculate the system's magnification if the image projection distance from the eyepoint to the sensor can be measured.

System magnification = eyepiece (mag) x objective (mag) x projection distance/ 250 mm

This equation describes how the magnification is determined by using a object of known size, and then measuring the size of its image when projected 250 mm from the exit pupil of the instrument or, in the case of the microscope, 250 mm from the exit pupil of the eyepiece. In some literature the eyepoint is called a Ramsden disk.

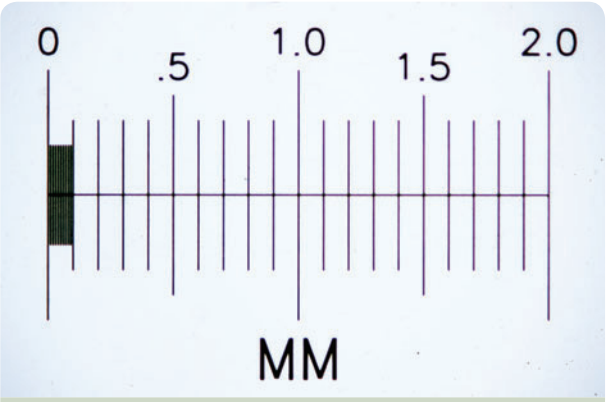


Figure 9.1 A stage micrometer. The micrometer scale is 2 mm, divided into 0.1 mm units. The first 0.1 mm on the left is further calibrated into 100 um increments.

The microscope's objective produces the primary image. The quality of this image is influenced by the objective's characteristics and focuses its image into the microscope's body tube. A body tube is the long tube that holds the eyepiece and connects it to the objectives (see Figure 9.2). The image is actually formed at the intermediate image plane, a location that is situated within the body tube. Another important optical location visible within the body tube is the exit pupil of the objective. The exit pupil can be observed in the microscope's

body tube when an eyepiece has been removed from the instrument. The body tube is the tube-like structure that is located between where the objectives are mounted onto the nosepiece (turret) and where the eyepieces are located on the microscope. The primary image comes to focus at the intermediate image plane in the body tube. The intermediate image is also located at the front focal point of the secondary magnifier, the eyepiece. Being aware of these locations can be useful when troubleshooting when things are not working properly. More than twenty years ago, microscopes were built to have specific tube lengths, e.g. 160 mm. Today contemporary instruments are designed to use infinity-corrected ∞ objectives. Exit pupil locations and intermediate image planes are useful for alignment of phase contrast optics and play a somewhat lesser role for other applications.

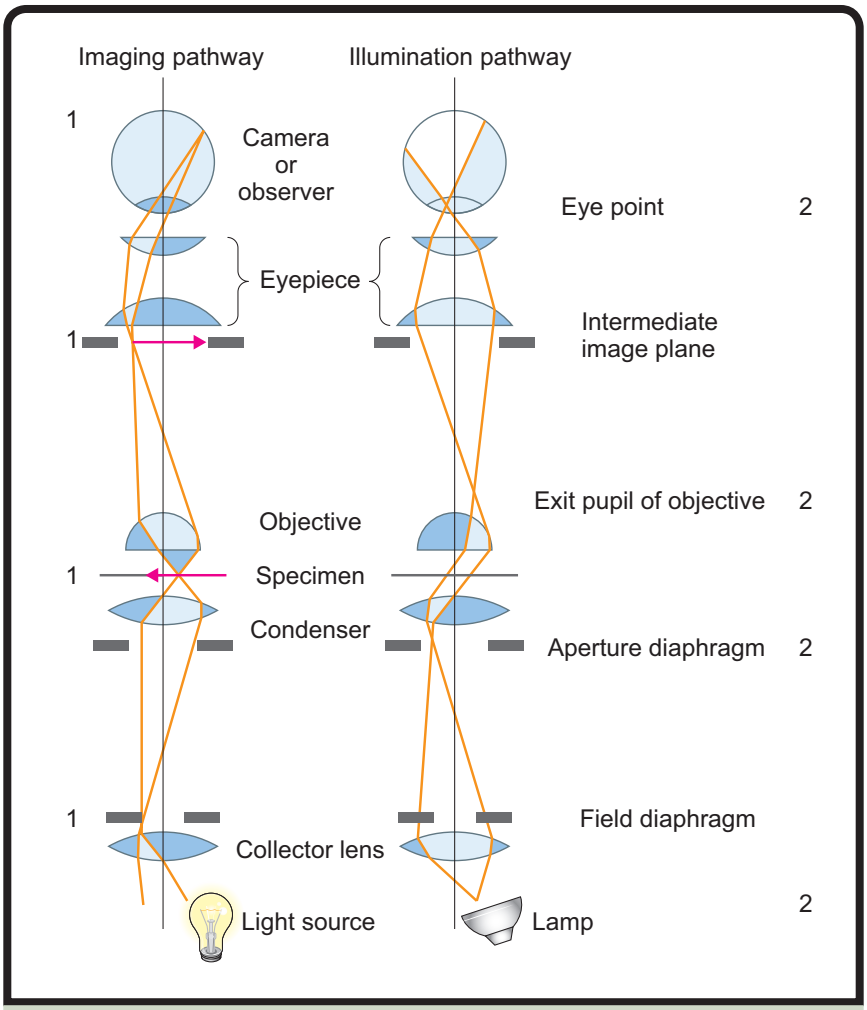


Figure 9.2 This illustration reveals the illumination and imaging pathways used when working with a brightfield compound microscope.

Once the primary image has been formed and relayed through the eyepiece, the image will exit this secondary magnifying lens at a location called the eyepoint. The eyepoint can be observed by holding a piece of paper near to the lens and will be visible as a tiny dot of focused light situated very near to the eyepiece itself. If the small piece of paper is held approximately 1 cm from this lens, a very bright small dot will be visible on the paper. Moving the paper closer or further from the eyepiece will allow exact determination of this location. The eyepoint can be a useful location for imaging. A smartphone's lens, for example, can be placed at the eyepoint, which will allow the smartphone to make photographs of the image from the microscope. When photographing using a fixed lens camera such as a compact digital camera, the front of the camera lens must also be placed at this location. In today's era of compact digital cameras, it is a relatively simple to make "adequate" photomicrographs using this method. A fixed lens camera can also be used this way. A camera stand or tripod will be required for best results. With surprisingly improved sensor sizes, quite good photomicrographs can be produced with care and patience.

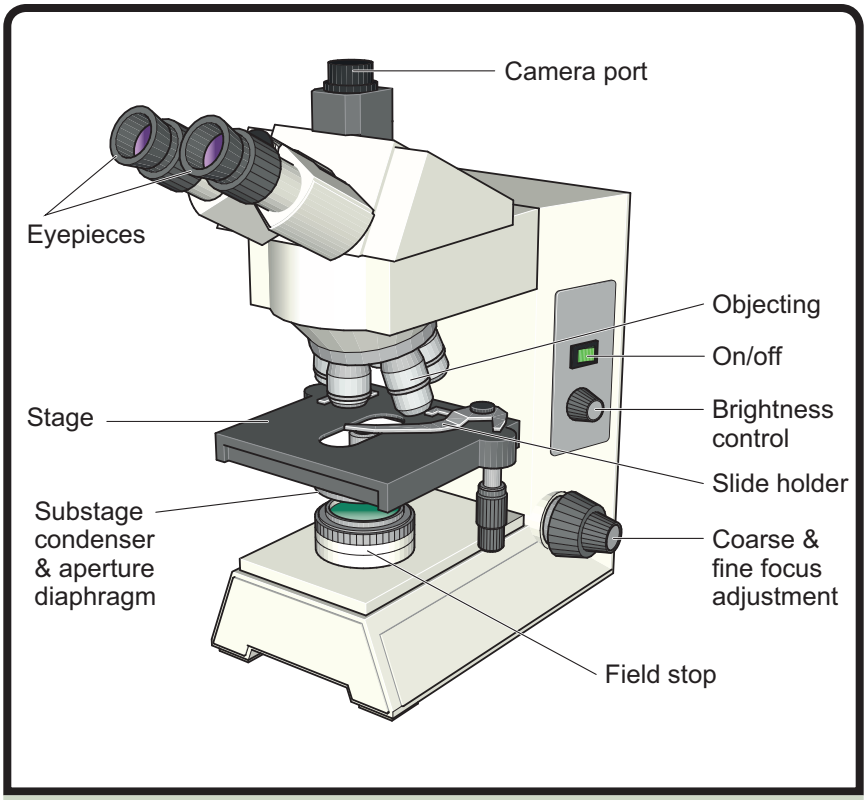


Figure 9.3 This illustration shares the key parts of a light microscope. Knowing where each of the controls is situated on the microscope can enable an operator to improve imaging outcomes in much the same way as drivers must know where all the controls for operating an automobile are located. This knowledge might be called "knobology." Never looking away from the sample during evaluation can allow very subtle differences in an image to be evaluated. This might include not blinking.

Optical Elements in a Light Microscope

Eyepieces

Observation of the image occurs at the microscope's viewing eyepieces, sometimes called oculars, or on the monitor. There are different eyepiece lenses used for imaging systems than for viewing. Typically the imaging system's field of view will be less than the viewing system's. Although a viewer may see two images in the binoculars and some may consider a microscope's image to be stereo, it is not. The primary image is formed from a single objective having a single point of view. A microscope with only one eyepiece is called a monocular microscope and a microscope with two eyepieces is called a binocular microscope. A microscope with three eyepieces is referred to as a trinocular microscope, which has two viewing ports and one imaging port and is often called a photomicroscope. Trinocular microscopes are typically designed for imaging and there may be other imaging ports added as needed.

One or both viewing oculars will come with adjustable diopter correction(s) for each eye. There is a neutral position (o) as well as + and – settings. Each eye will require a different diopter setting to create the best focus for the unique vision for each eye. Users should be aware which is their dominant eye when establishing the correct diopter setting. Not adjusting the eyepieces properly for each eye will lead to eyestrain and headaches. Both lead to the formation of inferior images. I have seen students become nauseous when not properly establishing these diopter settings.

There are two main types of traditional viewing eyepieces, Ramsden and Huygens. There have been improvements to their design over the years; however, they are basically the same as when they were invented and comprise the majority of basic eyepieces used today. Depending on the design of the lenses and the budget of the operator, Huygens negative lenses are most frequently used with most achromatic objectives and positive highly corrected eyepieces, while Ramsden are used with the finest apochromatic objectives. A good strategy when considering what eyepieces to select is to consult with a knowledgeable

instrument vendor. The selection of the microscope's objectives will influence the choice of eyepieces, which leads to the optimization of the optical performance and improved imaging. The ability to see an image produced from a microscope is influenced by the environment where the instrument is located, as well.

Proper viewing conditions are required to look at magnified and sometimes very dim and low contrast images. Gas-lift chairs also are recommended for extended shifts at a microscope and should be adjusted each time an operator sits down to work. It is important for an instrument's eyepieces to be situated at the height of the viewer's eyes. The best practice when sitting at a microscope requires an operator to raise or lower the chair to locate the eyes at the height of the eyepieces. Viewing oculars should be located at eye level. This will minimize body strain such as stretching of the neck or simply having poor posture. All of these conditions, including poorly adjusted eyepiece diopters, can lead to headaches and ineffective results. Being in an uncomfortable position will produce inadequate work. The lights in the room where the microscope is located should also be lowered or turned off completely.

The Prism

All instruments use prisms to direct light into different optical pathways within a microscope. Once an image has been formed by the objective, it must be relayed to the viewing ports, to the imaging system, or to a video port. Once at the prism, the real image can be directed either to the viewing eyepieces or to the camera by changing the setting of the prism/mirror. This change of light pathway can be accomplished either manually or by automation in research-grade microscopes. When photographing, the prism will need to redirect the light to the camera's imaging lens and away from one or both the viewing eyepieces. The prism is located within the head of the microscope and may be rotated using a plunger or other methods used for the redirection of the light. Adjustments to the prism will cause the light to be redirected either partially or completely. Frequently there will be a minor loss of brightness in the prism. Prism heads can influence the light redirection in various specific percentages. This could be 20 percent to the viewing eyepieces and 80 percent to the camera, 50–50, or 0–100, for example. If the instrument's prism is of low quality, or a first-surface mirror, optical degradation can occur to the image as a consequence of poor optical glass and correction of the prism. Flare and dispersion might be introduced at this location. When using fluorescence technique, a 100 percent beam splitter is the ideal choice because a fluorescence image will exhibit a very low image brightness to start with. It should be noted that one of the most common things photographed in a microscope is dirt. Dirt can find its way to the prism and may be recorded as dark spots on the image. Dirt will also image as a shadow or dark spot by casting a shadow on the sensor.

Photo or Imaging System Lenses

When using a trinocular microscope, there is a third tube that contains the eyepiece lens called the photo or projection lens (eyepiece). It is so named because it relays the magnified image to the camera system. The eyepiece features will be inscribed on the eyepiece barrel. This will list the lens's magnification and degree of correction. Sometimes it will also share

the field of view of the lens. The diameter of the eyepiece aperture (in mm) is called the field of view number, or field number (FN).

With the FN it is possible to calculate the diameter of the sample's imaging field. An eyepiece with a larger field of view is useful and practical and allows for more ease in scanning a sample to locate an area of interest.

Field size = field number/objective magnification (Mo)

All microscope eyepieces do the same thing: they magnify the primary image and relay it for viewing or for imaging. The photo eyepiece will usually be of higher optical quality than the viewing eyepieces, and in many cases the photo eyepiece may be of lower magnification than the viewing eyepieces. In fact, the photo/optical relay system may have a magnification that is adjustable when different specific magnifications are required. Some photomicroscopes may also have an additional optical zoom lens that is highly corrected and can be used to achieve just the right magnification based on object size. Many older imaging lenses will have the letter P or PHOTO inscribed on the lens. This information means that the lens has been specifically designed for picture-making applications and has flatfield correction. Photo relay lenses are made and manufactured to the highest degree of correction that manages the presence of aberrations. Contemporary imaging eyepieces, because sensors are smaller than film, may be 0.5x or 0.7x, for example, while the viewing eyepieces on that same microscope are 10x.

Substage Condensers

Located beneath the stage of a biological microscope is a lens assembly called the substage condenser. The substage condenser is primarily responsible for creating the cone of illumination used to illuminate the specimen, necessary to create and maintain image contrast and definition. The substage might be the least understood optical element in a microscope. Often this control is completely neglected or used incorrectly. Similar to objectives, substage condensers have numerical apertures (NA), which describe their resolution potentials as well as their degree of correction. The concept of numerical aperture will be explored in great detail in the upcoming pages. The substage condenser contains a diaphragm called the aperture diaphragm. This diaphragm will affect the resolution potential of the objective. The condenser's NA should be at least equal to that of the objective for optimal performance. Substage condensers play an important role in creating and managing image visibility by improving the use of illumination.

There are several types of substage condensers used in a brightfield microscope and there are condensers used for contrast-producing techniques. The three common types of brightfield condensers are an Abbe condenser, a flip-top or swing-out condenser, and an achro-aplanatic condenser.

The Abbe condenser is named for Ernst Abbe. The Abbe condenser, like any other condenser, focuses light that will pass through the specimen prior to entering the objective. Condenser assemblies will have several controls to properly operate them. One control adjusts the distance of the condenser to the sample and the other the aperture diaphragm. Adjusting the height of the condenser affects the diameter of the beam of light and the aperture diaphragm controls the brightness, contrast, resolution and DOF. Abbe condensers

typically have an NA of 1.25 and are useful for all types of microscopy and all magnifications. As a general purpose condenser, they are quite inexpensive and are useful with 4x or 100x objectives. As a consequence of their average quality and degree of correction, images will be adequate. They are not fully corrected for chromatic aberrations and have a comparatively high dispersion number.

Swing-out—sometimes called flip-top—condensers are designed for use primarily with low magnification objectives such as a 2x or 4x objective. They have numerical apertures that are in the range of 0.65 to 0.95 since they are used primarily in air. They are quite versatile and are adequately corrected. The degree of correction is typically on par with achromatic optics.

An achro-aplanatic condenser is the most highly corrected condenser and is corrected for spherical aberration and chromatic aberration. It is quite expensive and typically has an NA of 1.35. There is also an aplanatic condenser with an NA of 1.4, which was excellent for use in black and white film applications many years ago.

Figure 9.4 Left to right an Abbe, a swing-out (sometimes called flip-top), and an achro-aplanatic condenser. Many contemporary microscopes can be outfitted with what is called a universal condenser. Each of these substage condensers will produce different cones of illumination. See Figure 9.10 to see the angle of cones of illumination from these condensers.

Objective Lenses

The single most important optical element in a microscope is the objective. The objective lens produces the primary image and creates the first stage of magnification in the microscope. The objective lens influences the image quality, contrast, and magnification as well as all other characteristics in the primary image, such as depth of field. There are several inscriptions engraved on the barrel of the objective lens. These inscriptions share the

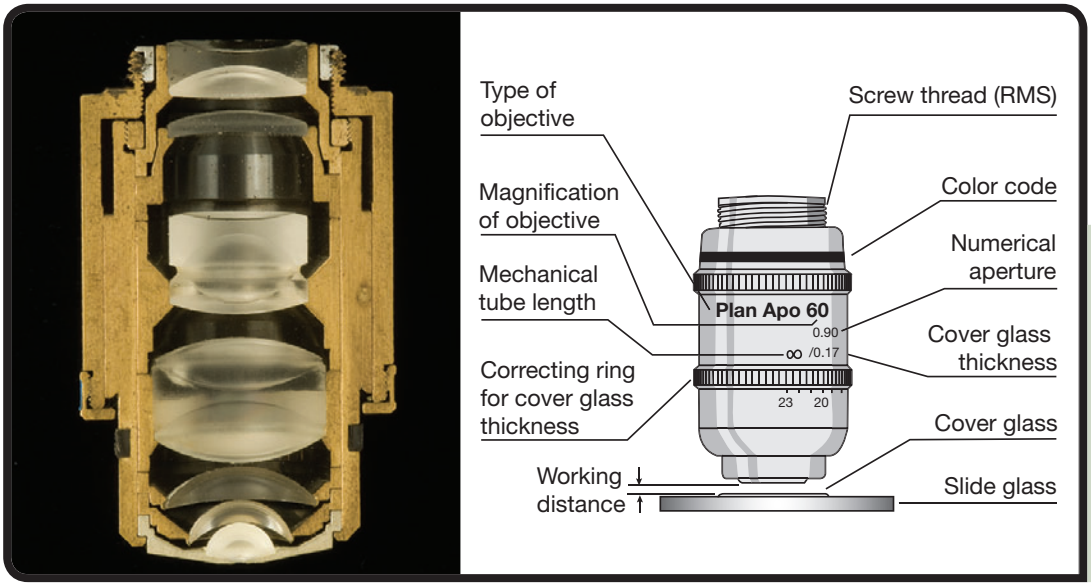


Figure 9.5 This illustration shares the inside of a 100x APO Zeiss objective. On the right is a figure on the outside barrel of a 60x objective identifying what all the nomenclature describes.

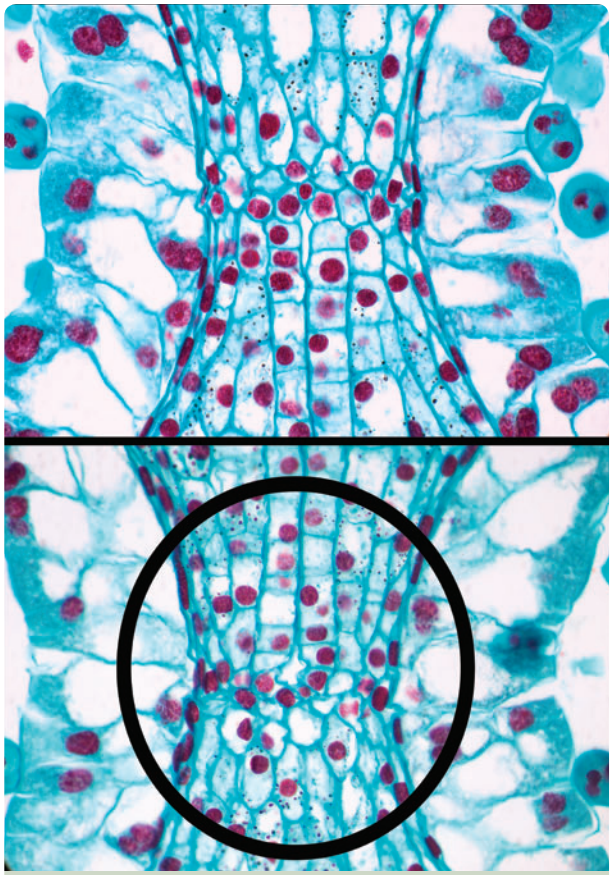


Figure 9.6 This composite shows the effect of two different objective lenses, one corrected for flatfield and the other not. The top image was produced from a PLAN objective, the bottom image from a non-PLAN objective. Notice the image area outside of the circle. It is not sharp.

lens's features. It can be very useful when using a microscope for the first time to review the objectives and become familiar with what types are on the instrument. Objectives are complex and have a number of internal lenses that affect the image attributes that the less complex can produce.

An important inscription visible on the objective's barrel will be PLAN. PLAN refers to the objective's correction for flatfield. Traditionally, a biological microscope is used for subjects that are flat and for thinly cut sections that are placed on glass slides. Since a subject will be situated very near to the front of the objective, small differences in working distances will be amplified by the optical elements in the system. Consequently, small differences in the sample distances and proximity to the front of the object can make focusing difficult across the entire field of view. The correction for this problem is called PLAN or a PLANO lens. If the lens is not corrected for this, curvature of the field will occur and be observed. A lens not corrected for flatfield will demonstrate focus in the center or on the outside regions of the image but not in both regions at the same time. This outcome is called curvature of the field. See Figure 9.6.

Another inscription on the lens barrel will be the objective's magnifying power. The magnifying power will be indicated using a number and may be followed by x, e.g. 10x. Each objective on the turret will have its own magnifying power. For those who have worked with photography, an objective lens has a focal length similar to a traditional photographic lenses used on DSLR cameras. The 10x objective has an equivalent focal length of a16 mm lens. This can be calculated by dividing the objective's magnification into the mechanical tube length. Samples are typically placed at one focal length away from the front of the objective. A typical range of objective magnifications for a light microscope might include a 2x, 4x, 10x, 20x, 40x, 60x or 100x. These represent common magnifications but are not the only choices. There are hundreds of types of objectives designed for many unique applications.

Numerical Aperture

The quality of an objective lens is indicated by its numerical aperture (NA). Often NA is shared as a decimal such as 0.45 or greater than 1.00 for higher magnification objectives. When the objective is used in air, the NA will be less than 1.00. When it is used with immersion oils, the NA will be greater than 1.00 (see Figure 3.8). The NA is a ratio of the light available for image formation measured against what is actually gathered up by the lens. The higher the NA of the objective, the better the image definition. The higher the NA, the more the objective will cost. NA is also related to magnification. Typically lower magnification lenses will have lower numerical apertures and higher magnification

objectives will have higher NAs. Higher NAs will create more optical resolution but possess a lesser DOF (see Figure 9.7).

Forming Images: Diffraction and Resolution

Before going into more detail about an objective's numerical aperture (NA), it is useful to understand how the objective actually forms an image. In microscopy, many aspects of image formation are described using diffraction theory. This idea was initially proposed by Ernst Abbe in the late 1880s while working at Zeiss. Refraction is the bending of light as a consequence of a speed change to light when entering a new medium and plays an important role in image formation. Diffraction plays an even more important role because it influences the objective's optical resolution. It is unfortunate that as the magnification of an image is increased, the resolution of an image often becomes less defined. This can be caused by diffraction. When radiated energy encounters an opening (aperture), the interaction may cause several things to occur. The number of the openings and the physical size of the apertures (openings) will determine how small or large the effects of diffraction may be.

An aperture in the pathway of traveling light might behave like an energy source itself. The edges of small openings cause some energy to be redirected into new directions. The new directions will contain less energy but provide information about the structures with which it interacted. The size and direction of this behavior will be dependent on the diameter of the aperture. If the aperture is wide, most of the energy will travel through the aperture unaffected. In this situation, most of the peripheral energy will be bent slightly outward away from the original travel direction and having a minimal effect on an image (see Figure 9.11). If the aperture is small, the bending of light away from the original travel direction will be greater. This becomes important because the sample itself is often a porous grating and will behave like many small and separate apertures. Objects that are porous are sometimes referred to as diffraction gradients. The smaller the fine structural details, the more the light will be affected upon exiting the sample. The ability to delineate small details in a sample is described as resolution. The smaller the structural details, the more difficult it becomes for the objective to resolve these details. For a microscope objective to resolve detail, it must be able to collect all the radiated energy that interacts with the sample. This of course is impossible. The challenge arises when the energy leaves the sample after going through the aperture diaphragm in the microscope. As the energy interacts with various waves from across the sample, interference occurs. Interference can either lead to more visibility by creating contrast or produce a data loss and poor resolution. This outcome is a consequence of the objective being unable to gather all the now diffracted rays required to form resolved images. There are a number of figures in the book that share that outcome; see Figures 3.19, 6.9, and 9.10.

More on Numerical Aperture

When characterizing a traditional photographic lens, a useful indicator to a user is the maximum aperture or f-stop of the lens, as f-stops can serve as indicators of light transmission for users. A lens's maximum aperture can be determined by dividing the focal length of the lens by the maximum clear diameter of that same lens. Microscope lenses

are also described by their light-gathering potential. The factors which influence their performance would be the medium in which they are operated and the acceptance angle of the diffracted energy into the objective.

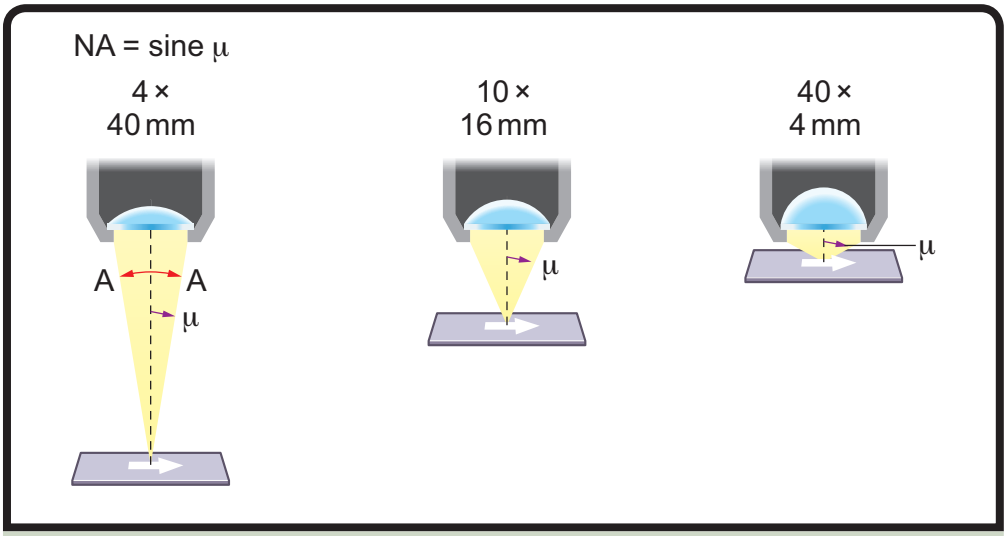


Figure 9.7 In this figure, the relationships between working distance, magnification, and numerical aperture (NA) are shown.

The objective's NA can be mathematically calculated. The following equation is provided to users for theoretical reasons and will rarely be calculated by a user. In forty years, I have never calculated an NA but I am always aware of how light travels in the microscope and how the aperture diaphragm allows me to direct the light needed to form resolution of contrast.

$$\text{NA} = n \sin \mu$$

where n is the refractive index of the medium that the objective will be used in and sine μ is the acceptance angle of that specific objective lens. Air has a refractive index of 1.00, water 1.33, and immersion oil 1.53.

The NA of the objective is influenced by the focal length of the objective as well. Shorter focal length lenses will have a shorter working distance and consequently a greater angle of acceptance creating more resolution. As the magnifications go up, so will the corresponding numerical apertures. The objective's NA will dictate the smallest of structural details that can be resolved. This smallest distance an objective lens can discriminate can be determined using the following formula

$$D = \lambda/\text{NA}_{\text{obj}} + \text{NA}_{\text{cond}}$$

where D = minimum resolvable distance between two points and λ = the wavelength of light in the system. White light is often represented in this equation by 550 nm.

Airy Disk

An instrument's optical performance can be predicted considering the Airy disk produced by a lens and the diffraction the system creates. An Airy disk is defined as the best-focused spot of light that comes from a point of light formed by a perfect lens having a circular aperture. An Airy disk will only be limited by the system's diffraction. The diffraction pattern resulting from a uniformly illuminated circular aperture is characterized as having a bright region in the center, the Airy disk, surrounded by concentric and rings of diminishing brightness. The Airy disk plays a central role in indicating a microscope's resolution. When the Airy disk spreads, the outcome is called a point spread function and plays a significant role in image clarity and whether independent points remain separated. Many confocal microscopes have controls to manage and influence the point spread function of an image.

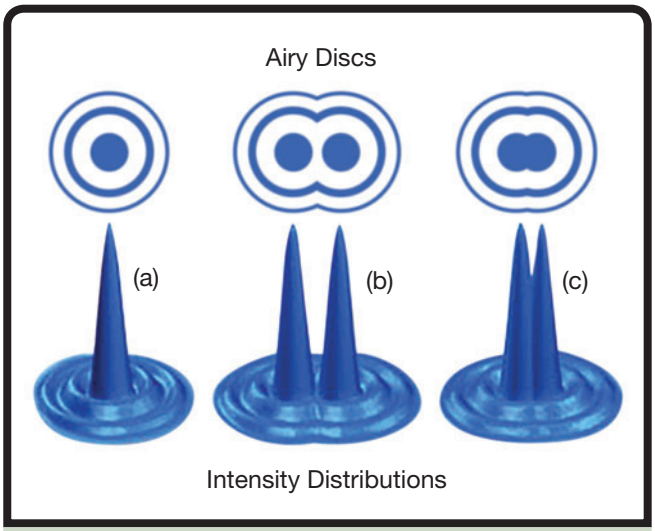


Figure 9.8 An Airy disk is a useful measure to predict how a location (point) will be modified when diffraction affects its sharpness. When two Airy disks are adjacent to one another, sometimes diffraction will cause the two points to become one. Higher numerical apertures produce better resolution.

A sample's diffraction potential is a significant element of a system's performance when coupled with the objective's numerical aperture and defines its resolving power. Part of this theory can be understood when considering how light interacts with a diffraction grating. Light travels through the grating, an optical component with repeating structures that splits and diffracts light into several beams travelling in different directions. What is not bent would be characterized as the light's zero order. To the outside of the zero order would be the first order of diffracted rays. The angle and departure of this deviation is a function of the size of the aperture. Adjacent to the first-order rays and further from the zero order would be the second order of diffracted rays. The zero order is principally responsible for providing the illumination used for the background in brightfield applications. The diffracted rays carry the information about the sample's structure. It becomes the job of a microscopist to direct as many of the diffracted rays into the objective as possible. The greater the acceptance angle of the lens, the easier it becomes to collect the diffracted rays. It is possible to see the diffracted rays at the exit pupil of the objective in the body tube. As the aperture diaphragm (AD) is closed, evidence of the diffracted rays will be observed as a sort of cloudy veil of light that surrounds the image of the AD in the tube.

NA defines the highest image magnification that an objective can produce before an image will begin to lose visual crispness and the magnification where only space and not structure is enlarged. The magnification where detail starts to diminish is called empty magnification. Predicting where empty magnification begins is accomplished by multiplying the NA of the objective by 1000. Magnification is a multiplicative process and empty magnification should take into account the final publication magnification. It is not simply a number when considering the best magnification at capture.

Empty magnification (x) = NA x 1000

Objective Corrections

Another inscription found on an objective's barrel—an indicator of optical quality—is the degree of color and spherical aberration for which the objective has been corrected. Common objectives or general purpose objectives would be characterized as achromatic or achro objectives. A higher quality objective would be a fluorite (fl) objective, while apochromatic (APO) objectives would be considered the highest quality objectives available. Achromatic objectives are the most common objectives and represent the highest percentage of all objectives sold. Achro objectives will not have ACHRO inscribed on the lens barrel. Achro lenses are spherically corrected for green and chromatically for blue and red. Additional improvements can be made to achromatic lenses by adding fluorite to the glass. A fluorite objective will optically perform better than the achromatic lens but not as well as an apochromatic objective. APO objectives will have a noticeably higher NA. The costs of fluor and APO objectives will be higher than ACHRO lenses. Having a higher NA leads to more optical resolution and, in the case of an APO objective, better visual crispness displaying a high degree of color accuracy, too. Apochromatic objectives are spherically corrected for two colors and chromatically for all three colors. Another color correction that has found its way into objectives is VC or violet corrections. A typical NA of an achro 10x would be 0.25; a similar fluor lens would have an NA of 0.30 and an APO objective would be 0.45.

Other numbers found on the objective will refer to the instrument's optical body tube length requirement and whether or not the specimen should have a cover glass between the objective and sample. Most contemporary instruments use infinity-corrected objectives; however, in the not so distant past, 160 mm was the standard. The Royal Microscopical Society proposed a tube length standard of 160 mm and this lasted for more than a hundred years. Infinity objectives have advantages because the same objective can be used for many applications such as phase contrast, brightfield, and DIC. Infinity-corrected objectives focus at infinity. They use longer working distances and allow the insertion of optical components such as lenses or filters into the optical path at many locations.

Using an objective incorrectly can affect the quality of the characteristics of the images that are produced and often leads to the creation of aberrations and other visibility issues. If the objective has been designed to examine subjects using a cover slip, it will have 0.17 on the barrel. If the lens works best without a cover slip, it will have (–) or 0 inscribed on it. Looking at samples not matched to its design will produce soft and low contrast images. In this application, the coverslip and sample all become part of the optical system in total.

Spherical aberration is a condition where light is refracted differently in the middle of the lens than in the outside areas, resulting in low contrast and haze that comes from a single location in the sample. At lower magnifications, this can be easily managed by creating more contrast by using the aperture diaphragm. At higher magnifications, the problem is more severe and may not be managed. Photographing using incorrect coverslips will cause spherical aberration (see Figure 3.16). Cheating when using a lower magnification is possible and the loss of image contrast can be reconciled in image processing software. Coverslips come in many thicknesses and materials. Plastic cover slips will absolutely degrade an objective's performance. The component of least optical

quality will have the most negative influences on image characteristics from the system. A helpful illustration sharing how spherical and chromatic aberrations are formed is evidenced in Figure 3.17.

Fundamentals of Operating a Light Microscope

Operating a microscope is not overly complicated but there are a few practical approaches that can assist in gaining more efficiency and image quality. When first turning on a microscope, select a brightness that is comfortable for viewing. It is easy to change the brightness of the lamp and not affect the optical performance of the microscope. The controls to adjust the microscope's brightness will be specific to each microscope. Adjusting the brightness is an acceptable practice for viewing; however, it is not suggested when imaging. Brightness can be reduced using the microscope's voltage or by using of neutral density or polarizing filters. Gray polarizing filters are preferable to brown for this application. Some photomicroscopes may have a symbol that can be used as the brightness setting for imaging. This setting was most useful for film photography but can play an effective role in standardizing the bulb's color temperature for imaging. Once established, it remains important not to change the lamp brightness if you are using tungsten halogen lamps during the imaging session. Consistent and more easy-to-manage color will be produced when not changing the color temperature of a tungsten halogen lamp.

If your microscope has LED bulbs, they are a different technology and can have their brightness changed. These changes lead to only small variances of colors. LED sources are of two types, continuous and pulsed. If they are of a pulsed type, their brightness can be changed and there will be no color change. If they are of the continuous type when they are dimmed, their color will change slightly, but only by a small amount.

Setting the Eyepieces

When looking through the eyepieces, it is useful to focus the microscope using your dominant eye and set the eyepiece diopter to the 0 position. The image visible to the dominant eye needs to be brought into sharp focus using the microscope's focusing knob. Once focused for the dominant eye, focus the image for the non-dominant eyepiece using the eyepiece diopter lens. By focusing and adjusting things in this fashion, both eyes will have the same focus. Viewing eyepieces are not usually designed to be of the highest imaging quality and should not be used for imaging unless there is no other option. The eyepieces may degrade certain aspects of an image because they have not been corrected for aberrations or flatness of field. Never assume the eyepieces to be properly adjusted when sitting down at the microscope.

Focusing

Once the brightness has been adjusted for comfortable viewing, locate the microscope's focusing controls on the side of the microscope. They will be near to the rear of the microscope and below the stage. There are typically two large knobs on the chassis of the instrument used for focusing; however, many research microscopes can be focused using the computer or auxiliary control panel. One of the knobs controls the coarse focus and

the other—usually smaller—knob will control the fine focus. Using either hand (whichever is more comfortable), rotate the coarse focus knob and observe what happens to the stage. This adjustment will make the stage go up and down. It can be very useful to know the travel direction up or down when the focus knob is rotated clockwise or counter-clockwise. The stage is the large rectangular plate where the slide is located. By rotating the focus knob, the distance between the front of the lens or objective to the subject is changed. This distance is called the working distance. The fine focus is capable of moving the stage in single micron increments with the smaller knob and can be useful in measuring thickness of samples. By focusing on the top of the sample and referencing the number on the fine focus knob and focusing to the bottom of the sample and referencing that number, the difference can be determined in millimeters. The difference between the two values can be multiplied by the refractive index of the material in which the sample is located. A common mounting media would have a refractive index of 1.53.

Very Small Working Distances

It can be very practical to know the stage travel directions without having to look. This can be learned by moving the front lens as close to the glass slide as possible by adjusting the coarse focus and watching the objective move towards the sample from the side of the microscope. Rotate the focusing knob to make the subject move away from the objective lens. Perform this task several times while looking at the stage, not into the binoculars, to become familiar with your instrument's travel directions.

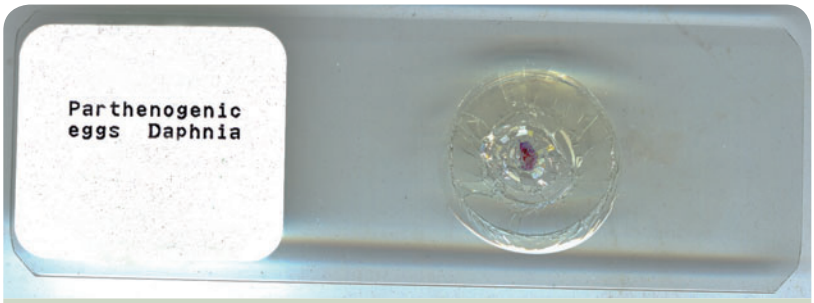


Figure 9.9 When focusing a microscope, it is easy to become disoriented. Not paying attention to how close or far from a slide the objective is can sometimes lead to driving the objective into the slide when trying to focus. Inadvertently placing a slide upside down on the stage can also lead to this outcome. Image courtesy of Lynne Tseng.

Knowing which way the stage moves when the focus knob is rotated can allow users to focus without the fear of driving the objective lens into the sample! Focus the image by rotating the focus knob in the proper direction, which will increase the working distance! This will move the subject further from the lens. It may require moving through several rotations of the knob to become familiar with this process and travel directions.

Interpupillary Distance

The distance between pupils is unique to each person and good microscopes allow this distance to be adjusted for an operator. The name given to this is the interpupillary distance. It is easy to measure this distance using a ruler, but a microscope is equipped to easily determine this number for an operator as well. Using both hands, grasp the tubes where the eyepieces are located. These tubes move inwards or outwards. Look into the microscope and move the tubes until both images are seen as one. You may have to do this a few times. A normal distance is somewhere between 59 and 69.

Looking into the Body Tube

Grasp one of the eyepieces and gently pull it out from the microscope. This will allow the eyepiece to become disconnected from the microscope. With the eyepiece removed it is possible to see into the body tube. The microscope must be turned on. Looking inside the microscope tube where the eyepiece was located when the microscope is on will reveal light within the tube. The body tube is the pathway where light travels from the objective to the eyepiece. The microscope's body tube acts to block stray and ambient light from entering the microscope. This allows the image characteristics to be well defined and maintains good contrast. The aperture diaphragm will also be visible at the exit pupil of the objective and visible at the bottom of the body tube.

The length of the tube was initially fixed and necessary for correct image formation before infinity objectives became the universal technology used in contemporary microscopes. Specifically, the mechanical tube length was very important in older instruments and 160 mm was a typical distance. Contemporary objective lenses have been designed to work at specific distances from the microscope's eyepieces. Most contemporary transmission light microscopes use objectives requiring a mechanical tube length that is now infinity corrected.

Nosepiece or Turret

The location where the objective lenses join the microscope is called the nosepiece or turret. Often there are four or five objective lenses mounted there. The nosepiece rotates in a circular direction. Each objective is properly engaged when the objective clicks or locks into place below the prism. Microscopes can have either a rear-loading nosepiece or a front-loading nosepiece. Front-loading turrets allow users to see the objective at the front of the instrument rather than in the rear of the turret, which can be more difficult to access. A few companies still produce front-loading nosepieces; however, rear-loading nosepieces are more common. Turrets can be rotated from the computer on advanced photomicroscopes and can also be very simple and removable from the microscope for cleaning and service.

Substage Condenser (Height) Adjustment

On the side of the microscope adjacent to the substage condenser there will be located a small knob similar to the focus knob. This knob allows the condenser to be moved up and down. It is important to locate this knob and understand how it operates. The substage condenser should move up and down, and it is critical to adjust the height of the condenser when establishing proper illumination. This adjustment allows an operator to move the substage condenser at the right height for each objective and its particular magnification requirements. Setting the proper height controls the cone of illumination the condenser produces. For larger opaque samples, it is possible to remove the condenser and locate the sample there. Using the condenser height adjustment to focus can be very helpful in working with oversized and irregular samples.

Setting the Field Diaphragm or Field Stop

An important diaphragm located in a microscope is the field stop or field diaphragm. Either term is acceptable. The field stop is situated in the base of a biological instrument and directly below the substage condenser. The field stop controls the diameter of the illumination beam and it is safe to say it creates the width of the beam of illumination that enters the substage condenser. There is an adjustment mechanism located near to the field stop that changes the field stop's size. The proper diameter is a function of the objective and related magnification. The field stop only controls the diameter of the illumination and not image brightness. Opening or closing the diaphragm causes the diameter of the illumination to change. If the field stop is too small, the aperture will encroach into the image. If the field stop is set too large, this can lead to the creation of flare light. Flare may have influences on image contrast.

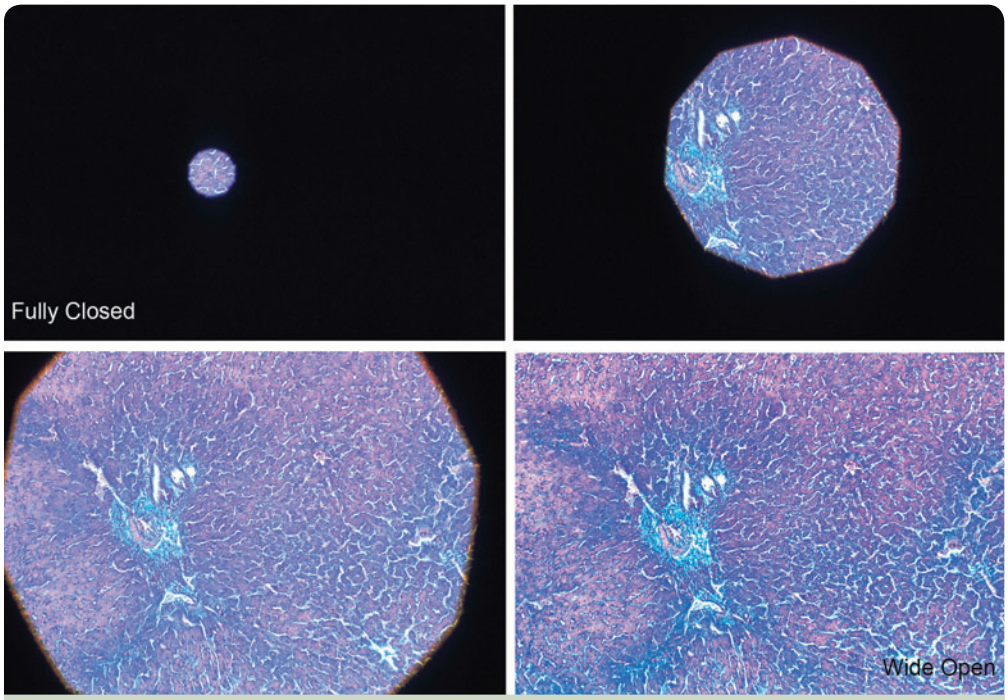


Figure 9.10 The field stop controls the diameter of illumination but not its brightness. In the top left image, the field stop was completely closed and exhibits the same brightness that the image situated in the bottom right includes.

Lamp

Located typically in the back of a microscope is the light source or lamp. It will probably be a tungsten halogen lamp although LED bulbs are becoming increasingly common. An advantage of a tungsten halogen bulb was that it maintains its color temperature for life when compared to a tungsten bulb. A halogen bulb has a life expectancy of several hundred hours. Precise and standardized color temperature was more important when scientist photographers used silver halide films. This type of bulb produces a very bright

and continuous spectrum over the life of the lamp. Newer microscopes can be equipped with tungsten bulbs or with LED technology. LED lamps can last for years and they are more expensive. Their color is more constant than tungsten halogen lamps.

It is always useful to find where the lamp is located on the microscope and determine how it is attached. Take note of where the screws are located on this housing. Bulbs will have a long life expectancy but may need to be changed at one time or another. A bulb may be characterized by its brightness and the spectrum that it emits. Bulbs also have a color temperature. The color temperature is shared using Kelvin degrees. The ideal color temperature for silver halide photography was 3200K. Color temperature does not play as critical a role in digital photography. The bulb brightness can be controlled by a switch called a rheostat. When adjusted, changes in bulb color temperatures occur. Operators should not directly handle the tungsten halogen bulbs directly because it will leave finger oils on the lamp, which can lead to problems over time. The glass chamber can become etched from high temperatures. It is useful to wear gloves or handle the bulb using tissue paper.

Fluorescent illuminators are different and may use a xenon, sodium or mercury vapor lamp. These bulbs are very expensive and have a recommended length of usage. LED light sources are making inroads into the fluorescent lamp product lines too. They are, in some ways, very practical because they can create customized excitation spectrums that can have real advantages for exciting specific fluorophores.

Aperture Diaphragm

The aperture diaphragm (AD) is the steering wheel for radiated energy in a microscope. Located in the substage condenser, this diaphragm controls the contrast, resolution, depth of field, and intensity of the formed image. Operating this diaphragm is crucial to forming the best image possible. Keeping the diaphragm wide open leads to an image with more resolution and less DOF and contrast. Closing the AD down too far produces an image that has a lot of contrast, significant DOF, and poor resolution. The diaphragm's setting for each sample will be different, and more about how to establish the best setting will be shared later in this chapter. The aperture diaphragm because of where it is located will also affect image brightness but should never be used to adjust image brightness.

Establishing Proper Brightfield or Köhler Illumination

Creating illumination that is uniform and creates/maintains structural information about a sample was first proposed by August Köhler in 1893. Managing the widefield illumination in a transmitted light microscope is an important variable required for achieving high quality images. Variances in illumination can be a problem when photographing because any differences in illumination will be amplified by the camera and image. Sensors and the way a pixel discriminates variances in brightness can embellish lighting problems. Since a pixel records data either as off or on, illumination errors will be exaggerated by a sensor. Achieving Köhler with lower power magnification is more challenging than for higher magnification applications. Some imaging software can correct for shading or illumination errors. The feature is called shading correction.

Steps required for Köhler illumination:

1. Turn on the microscope and set the interpupillary distance.
2. Adjust each eyepiece diopter as required, starting with the dominant eye.
3. Focus the image by changing the working distance using the dominant eye. Next focus the eyepiece diopter for the non-dominant eye to bring images to the same focus.
4. Close the field stop fully.
5. Using the substage condenser, focus the image of the field stop until the blades are well defined and at the same plane as the focused image.
6. Center the image of the field stop using the substage condenser centration screws. The centration should be visible in the field of view
7. Open the field stop until it just leaves the field of view.
8. Set the aperture diaphragm to the correct setting.

When changing objectives, it is important to re-establish Köhler. Typically, once established, changing of magnifications will require only a minor correction.

How Köhler Illumination Works

Step 1: Set the Interpupillary Distance First, establish a comfortable viewing brightness. Once this has been accomplished, look into the microscope and set the interpupillary distance by adjusting the eyepieces. It is important to have the two concentric images visible at each eyepiece to appear as a single image. Adjusting the height of the chair and locating your eyes directly across from the eyepieces without effort is also a component of this step. The eyepiece diopters must also be properly set at this step, by making the eyepiece adjustment using your dominant eye.

Step 2: Focus the Image of the Subject Bring the image into rough focus. Once the rough focus has been achieved, using the fine control, critically focus the microscope. At this time make sure the substage condenser is set to the highest setting or most near to the sample. Once you have established these conditions, it may be necessary to tweak the eyepiece diopter settings.

Step 3: Close the Field Stop (FS) Operate the field stop control and close the stop until it is at its smallest size. This should be done while looking into the microscope; however, looking into the microscope is not essential. Depending on the state of the microscope's alignment, you may not be able to see anything, or the image of the FS may be clear and visible. In either situation, operators may need to close the diaphragm slowly and continue to make minor adjustments while proceeding. These adjustments include centering the image of the FS using the substage condenser. At this time, it is required to keep the image of the field stop visible at all times.

Step 4: Focus the Image of the Field Stop Grasp the substage condenser focus knob and rack the condenser up and down until an image of the field stop is sharply defined in the oculars. The image will be focused when the edges look very crisp and black when focused by condenser. The sample's image must also remain sharply defined when the field stop image is sharply defined. You may notice color fringes (red or blue) at its edges. This indicates the presence of some chromatic aberration. Chromatic aberration is a lens defect where the various spectral components RGB are all brought to focus in different locations. Depending

on how out of alignment the field stop is, this step of focusing the field stop image could be easy or challenging. See Figure 9.9.

Achieving the parfocality of the specimen and the image of the field stop may be a bit more difficult in some situations depending on the state of the alignment or the sample thickness. Parfocal describes a condition where all the images are in the same plan of focus. It may require that you move very slowly making small adjustments, e.g. the closing of the FS or minor height adjustments to the substage condenser. Do not be in a hurry, but rather study the field of view to determine the location of the field stop relative to the edges of the field of view. Once the image has been successfully located, it may be advisable to begin to center the substage condenser, which moves the image of the field stop. Move the substage condenser using the centration tool typically located just below and on the bracket that holds the substage condenser under the stage. Move them gradually and observe what happens to the image of the field stop. Move the image of the field stop roughly into the center of the field of view.

Step 5: Center the Image of the Field Stop Locate the centration screws on the front of the condenser assembly and look into the microscope. Adjust each of these screws individually or together. Move the image of the field stop into the center of the field of view. If the image of the diaphragm is crisp, this condition will have been established; if not, you may have to go back through each of the steps prior to this point to accomplish this. The goal is to achieve a focused and centered image of the field stop that is superimposed over the focused image of the specimen when looking into the eyepieces.

Step 6: Open the Field Stop Until It Just Leaves the Field of View Once the image of the field stop is focused and centered, the final adjustment can be a bit finicky. Looking into the microscope and grasping the field stop, begin to open it. Bring the field stop to the edges of the field of view and, at this location, assess the centration of the image of the field stop to the edge of the field of view. If additional centering is needed, using the substage condenser assembly knobs to finalize the adjustment. Once the image is truly centered, open the field stop until it is just outside the field of view and is not encroaching into the field of view. Sometimes this setting will be different for photography and for viewing. It is best to adjust the field stop for the imaging system requirements. You may also notice that the image of the field stop will get less defined as it is opened. Focus should be assessed based on the central region of the field of view and not the periphery.

Step 7: Adjust the Aperture Diaphragm Establishing the aperture diaphragm setting is probably the most important adjustment an microscopist can make. The next few paragraphs will share how to create the ideal setting. The aperture diaphragm is located in the substage condenser but visible in the body tube when an eyepiece has been removed. By moving the aperture diaphragm (opening and closing), it can be observed as it moves in the tube. It can be very useful to move the diaphragm through its entire range and observe the brightness and location changes.

The goal is to have the diaphragm just interject itself into the body tube. When the diaphragm is all the way open, the diaphragm is not visible in the body tube, and when the diaphragm is all the way closed, it is may cover more than 50 percent of the tube. A general starting point for the diaphragm location would be where the AD is visible in the body tube, and where 80 percent of the tube is open and 20 percent of the tube is covered by the aperture

diaphragm. If the AD is outside of the tube, it will have no effect on image formation, and if the AD is closed to its smallest size, it will create significant contrast, increasing the image's DOF and diminishing image resolution. See Figure 9.10.

Establishing Optimal Diaphragm Settings

There are two apertures on a microscope, the field stop and the aperture diaphragm. Using them properly will play an important role when optimizing images. The field stop controls the diameter of the illumination beam, which can have an influence on contrast. The aperture diaphragm controls image DOF, intensity, resolution, and contrast. The proper use of the aperture diaphragm will significantly influence the photographic result.

To observe how the aperture diaphragm controls resolution, contrast, and DOF, set up a microscope using proper Köhler illumination and a 10x objective. Select a microscope slide of a subject that has easy-to-see structural details. It is important not to use a weakly stained sample. Pull out an eyepiece and observe the aperture diaphragm moving in the tube at the exit pupil of the objective. An image of a bright disk of light and an image of the aperture diaphragm at the periphery of the tube should be visible when moving the AD though its range. Grasp the aperture diaphragm, look into the body tube and rotate the diaphragm and observe the operation of the diaphragm in the microscope.

Substage condensers will also have a numerical aperture. When the image of the aperture diaphragm in the tube is set so that its edge is at the edge of the body tube, its numerical aperture is matched to the objective. This is not always desirable, but can be a useful starting point for the creation of contrast, resolution and DOF. The diaphragm will have minimal if any effect on image formation when its NA matches the NA of the objective. Only when the diaphragm interjects itself into the illumination pathway will image enhancement or degradation occur. Put the eyepiece back into the microscope.

The aperture diaphragm plays a critical role in the characteristics of an image. These four image attributes are controlled by the aperture diaphragm:

- Image contrast
- Resolution
- Depth of field
- Intensity.

The aperture controls these four outcomes by controlling the microscope's diffraction. The degree of diffraction required to optimize an image will be influenced by a subject's characteristics. Blood cells, for example, do not experience any change to the above mentioned image attributes from the use of the AD; however, other samples are noticeably degraded.

The proper setting for the aperture diaphragm will be subject-dependent and based on visual judgment and personal taste. A person just learning about microscopy will need to take the time and practice at seeing diffraction to become proficient in mastering the power of observation. It is very easy to see contrast and it is not so easy to see resolution.

Relocate the eyepieces in the body tube and grasp the aperture diaphragm when looking into a microscope. Rotate the diaphragm through its entire range, refocusing as necessary. Study the effects of the diaphragm very closely by examining an edge of the sample.

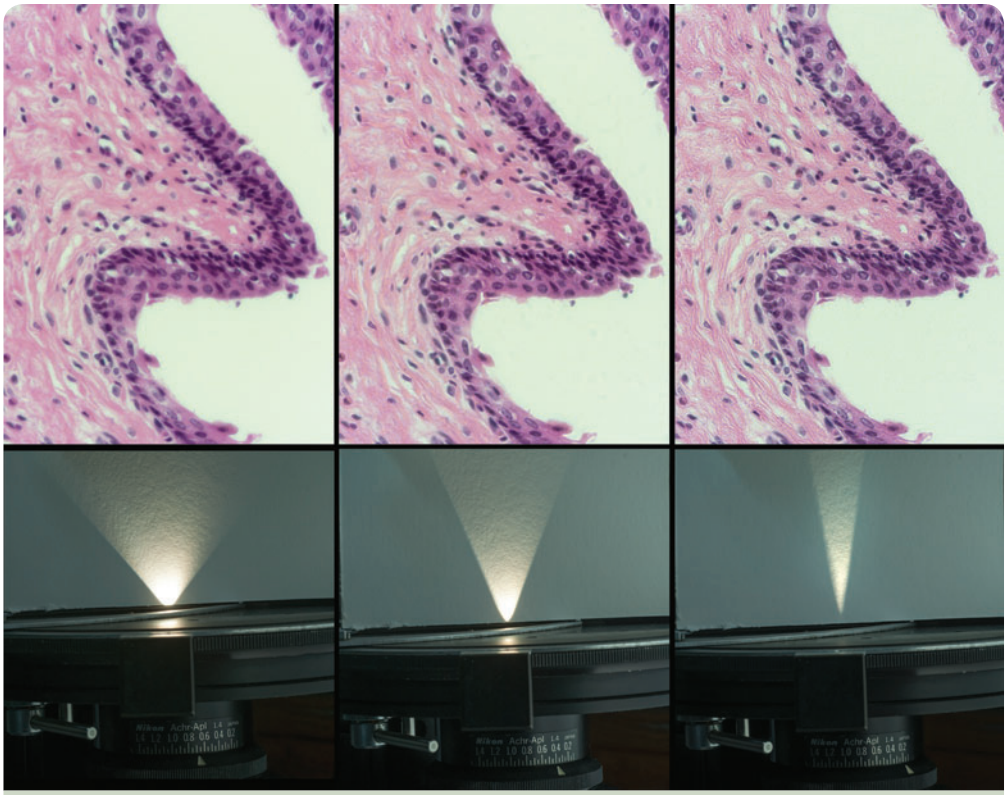


Figure 9.11 In these three photographs, the aperture diaphragm has been set to three different locations. The photographs were each made using a 10x FI—NA 0.45 objective and an achro-aplanatic substage condenser, NA 1.40. The photograph on the left was made with the aperture in its maximum opening for the 10x lens (NA 0.65), creating the maximum resolution and the least amount of contrast. Notice the angle of light leaving the condenser. The middle image was made with the NA set to approximately 0.3, which created the optimum balance between the contrast and resolution potential for the objective. Notice the angle of the light becomes smaller when the aperture diaphragm is closed. The image on the right has the most contrast and least amount of resolution as a consequence, the aperture being nearly closed to its smallest setting at an NA of 0.15. The angle of light is also the smallest when operated in this position.

Critically focus. You may need to continue to refocus throughout the evaluation of how much contrast, resolution, and DOF the sample requires.

As the diaphragm is closed, the image will get darker, show more contrast, and present more depth of field. As the diaphragm is more open, the image will get brighter and exhibit less contrast and less depth of field, but it will have more fine detail. Somewhere in this range will be the ideal setting for the specimen. Every subject will have a different setting needed for the creation of optimal results. Go through the diaphragm range again and observe the fine detail. Be careful not to perceive contrast as resolution and detail. It is quite easy to add contrast to an image during image processing and it is much more difficult to form optical resolution.

Open the diaphragm and begin to study the effect of the diaphragm as it is opened. Locate a setting that seems to balance the sample's contrast requirement and fine detail requirement.

Once achieved, imaging can begin. It might be practical to make a series of photographs using numerous aperture diaphragm settings until you get more proficient.

Photographing Using a Light Microscope

Making photographs using a light microscope is not overly complicated as a subject. The formation of highly resolved images comprises more than half of the challenge when trying to make publication-quality photomicrographs. The next few pages will address the use of dedicated instrument cameras and DSLR cameras. It is very easy to use a smartphone for quick-and-dirty snapshots and the particulars of using this type of camera were discussed earlier in the chapter. There are actually a few advantages to the use of a smartphones, including their terrific simplicity. The challenge of creating high quality and repeatable results will be the constant problem when trying to use them. Getting repeatedly good color results and consistent exposures is hard to achieve. It can also be tricky to create the proper alignment of the eyepoint to the camera lens. There are a few companies that produce brackets that are for coupling a smartphone to a microscope and for astronomy/telescopes as well.

Instrument Cameras

These are ideal for use on a photomicroscope. Designed specifically for a microscope, they are sold with camera controller software such as Zen® from Carl Zeiss Microscopy or Elements® from Nikon. A camera and its corresponding software can be a powerful tool, useful in the recording of more data than the eye can see. Any camera used on a microscope must be able collect image photons, record light as variances of brightness, and record the image as grayscale needed to create color images based on information collected at each pixel (RGB). The camera will have a shutter that may be a source of vibration, and no matter how basic, the camera will need to be able to measure the light needed to create the correct exposure.

Instrument cameras offer some advantages when properly matched to the output needs of the users. They are very compact and typically offer various features across different models ranging from very basic cameras, useful for common brightfield applications, or very sensitive low light high resolution cameras useful in fluorescence and other challenging applications. The basic features of these cameras include preview or live view, exposure modes of manual or automatic, exposure adjustment controls, sensor sensitivity adjustment, gain, binning, digital resolution pixel (megapixels), white balance controls, image contrast capture mode or input dynamic range. Some cameras may have a spectral response or color adjustment features, bit depth setting, and other image-adjustable features such as sharpening. Higher-end cameras can measure and perform basic image analysis functions. Chapter 4 goes into great detail about these attributes and functions.

Once an image has been properly formed, it can be captured. All camera controller software must be launched prior to using the camera. Given the wide range of products and operating systems, it is impossible to share any particulars for cameras sold by Zeiss, Olympus, Leica®, Nikon, Hamamatsu®, or Spot Instruments®. Only the fundamental and universal controls can be discussed.

Once the image has been directed to the camera and camera controller software is initialized, it is time to focus using the live view or preview. The camera can be operated in fast focus or slow focus mode. When used in fast focus, typically half the camera's pixels are used for the focusing. The still image will not be adversely affected using either the fast or slow focus modes. It may be impossible to reshoot a sample so creating the proper pixel resolution is a important decision the scientist photographer must make, separate from the pixels used for the focus mode. When time is crucial, fast focus makes sense. When time is not a factor, slow focus—while a bit less responsive—will create images that will most resemble the recorded file. Microscope cameras come with many resolutions and an average sensor creates a 13 Mb file that has 14 bits per channel. Some cameras are equipped with micro-stepping motors, allowing them to make multiple recordings creating more highly resolved digital files. DSLR cameras will frequently be able to create higher resolution files when compared to instrument cameras. A full sensor camera such as the Nikon D810 creates more than 70 mb and a 12 or 14-bit file.

Once the focusing mode has been selected, the exposure mode must be chosen. There are only two modes of operation for instrument cameras: auto and manual. A DSLR will have an additional setting for program mode. In the manual mode, the operator selects the proper time increment needed to create a correct exposure. The time will depend on the sample's brightness and other factors that affect the microscope brightness. Seeing the image and histogram in the camera preview mode will provide the operator with information about the accuracy of the time. In the manual mode, shorter times will make the image darker and longer times will create images that are brighter. Manual mode can be very effective when

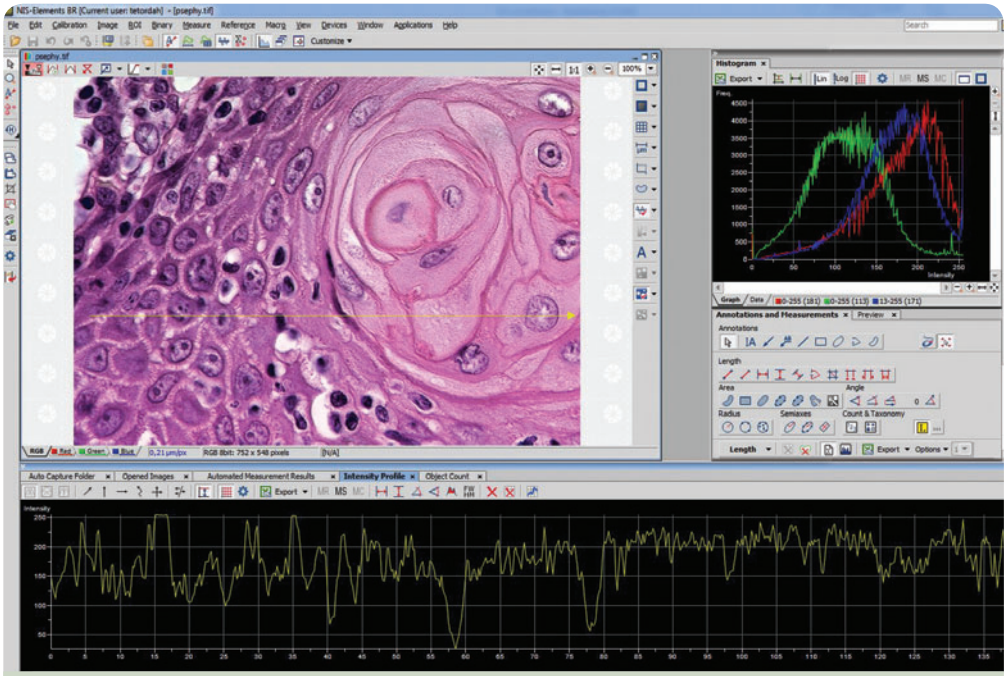


Figure 9.12 A photomicroscope's camera software is noticeably different than the menus used in a DSLR camera. This illustration reveals the appearance of a screen from the Nikon Elements software.

working in fluorescence applications for tighter controls. There is more about cameras, shutters, and exposure in Chapter 4.

When the camera is operated in the automatic mode, the basic exposure that the camera makes will be determined using a brightness reading that creates an 18 percent gray tone. The information provided to the user for this average exposure would be indicated by N, normal, or 0 setting. For brightfield microscopy it is suggested to increase the auto exposure using the software's exposure compensation control. A normal adjustment would be +1 or +1.5 EV (exposure value) for brightfield microscopy using a sample with average thickness and contrast (see Figure 4.3). When working in darkfield illumination, the exposure compensation would be –2.0. Using the preview mode will be a useful tool for identifying the best exposure. It is important to base exposure judgments on the brightest region of the sample. Viewers will be more accepting of regions that are too dark in a picture than regions that are too bright and overexposed. An ideal exposure has detail in the bright and dark tones. Sometimes these adjustments are accessible by moving regions of the image displayed in the camera's software.

Sample contrast will play a role in the creation of the correct exposure. If the sample is very thin or weakly stained, a good exposure will use different times than if the sample is thick and densely stained, even when using the same microscope brightness settings. The sample's internal visibility or contrast is a function of the sample's thickness, the stain darkness/concentration and the type of stain. To make better images it is helpful to profile the capture contrast or to modify the gamma of the sensor in the camera to the sample characteristics. Some cameras will allow contrast to be changed in different ways. Samples that are thin and weakly stained will benefit from a sensor setting having an increased contrast or gamma. For samples that are densely stained and thick, it is useful to select a low contrast setting. This feature will be located in the camera's software and will be described in different ways. Modifying the shape of the image's histogram in the look-up table (LUT) is one common method to do this. Scene choices provide another way and might include fluorescence, brightfield, darkfield, H & E, or high, medium or low. Carefully selecting exposure and contrast settings is an enormously important step needed to record the maximum data about a sample.

It might be useful to create an imaging test to evaluate how software tools operate. When learning new software for a new microscope or camera, I often go back to the basics and, using the automated metering method, I create an exposure series going from –2, –1, 0, +1, and +2 using a typical sample and changing nothing else. It can then be useful to make a series of exposures using the various contrast settings with the optimal time determined from the exposure series. The more precision in establishing the camera's settings, the more information will be recorded. This in turn makes more information available for image processing as needed. The better the recorded image, the lower the noise and digital artifacts that will be recorded in that file as well.

A camera's color reproduction is a separate characteristic than a camera's white balance. Both can be changed. White balance describes the process of equalizing the data in a white target from the scene that has equal units of RGB information. When considering a brightfield microscope, the clearfield or background is an excellent and uniform target to use for white balancing. Measuring and calibrating the sensor to this value can be useful

for the creation of standardized views over time. It is possible to apply white balance by averaging the whole scene or measuring a specific number of pixels in a region using the software eyedropper. For brightfield images, a white point brightness of 240 in the RGB pixels is ideal. When averaging, no part of the stained tissue can be located in the illumination pathway. The tissue must be moved back and forth to determine the best white balance. Whatever method is selected it is operator preference and in the end, so long as the process is managed and not random, effective photomicrographs can easily be reproduced time and time again.

The amount and purity of color that is reproduced is often equated with image saturation. Every sensor sees color in slightly different ways and, based on the characteristics of the filters used on the pixels, there will be variances across brands and models. Certain colors will reproduce with increased saturation or purity and other colors will be more correctly reproduced. When a sensor is more responsive to certain spectrums, adjacent pixels may be involved during exposures or capture. Decreasing color contrast or saturation is also an important tool for the recording of useful and precise image data. Some cameras will characterize saturation as color contrast. Proper selection of this capture attribute can assist in the purity of the image signal that is recorded. Sometimes when adjacent pixels are affected by minor exposure errors or increased color saturation, the image outcome is called blooming. Blooming is characterized by pixels sharing exposure information with neighbors. De-saturating or diminishing exposure at capture can best manage this outcome and control how the volume of color contributes to seeing more.

In certain situations, there will not be adequate brightness to make a good exposure. Unlike DSLR cameras that can have their sensitivity adjusted using the ISO setting, instrument cameras are not adjustable in the same way. To change a sensor's sensitivity, the gain tool is used. Increasing gain will cause the sensor to become more sensitive to light, but using too much gain will cause the production of digital noise in the image. Because of the potential to create this image artifact, increasing the camera's sensitivity should be chosen when other considerations for making a brighter image are not possible. Sometimes there can be other ways to manage microscope brightness once there can be no more increases in the brightness of the lamp. If neutral density (ND) filters or internal polarizing filters are in the optical path, they might be removed. If gain is required, select the lowest possible gain setting that creates a useable result. It is best to inch towards a solution. Experience will be a great teacher.

Another way to increase the camera's sensitivity is through the use of binning. Binning is the grouping of pixels together to act as one. By grouping pixels together, the individual pixels act like one large image sensor. Larger pixels collect more light and create exposures needing less light or signal. The disadvantage of binning is that the number of pixels that makes up the final file will be lessened by the factor of the binning that was selected. For example, if a sensor has 1800 pixels in the long dimension and the camera is set to the 2 x 2 binning selection, or four pixels, the binned file will be 450 pixels, not 1800. More about gain and binning is shared in Chapter 10.

Many instrument cameras offer some basic but useful features that include the ability to annotate images, add magnification bar scales, and perform basic image processing. There are also many filters that can be selected for use at capture, such as sharpening.

Annotating can be very useful for later use and so can the addition of the bar scales placed into image files. It is important to keep in mind that any image processing work done on a file using proprietary camera software will not be always compatible with other image processing software such as Adobe Photoshop or GIMP® (GNU Image Manipulation Program). Image J software may be able to open some proprietary file formats but determining this with unimportant files is suggested before finding out at a critical time. As such, saving recorded images as TIF or PNG should be a first choice. JPEG, while a universal format, will subtract data from pixels that contain similar data. Data will be lost when making JPEG files, and over time compression artifacts will be created, too. Image processing software will be more effective and precise than camera capture software used for image processing.

DSLR Cameras

Digital single lens reflex cameras can easily be used for photomicrography and there are advantages and disadvantages for use with this type of a camera for microscopy. Mirrorless cameras are also a popular type of camera and will have slightly different considerations for their use in the laboratory environment. Mirrorless cameras function in much of the same way as a DSLR but do not use a reflex viewing system and are more compact. For this reason, they have become very popular for travel and applications where the size of the camera matters. It does not seem that they have found many unique applications in the laboratory environment, and DSLR cameras seem to be more prevalent.

DSLR Camera Advantages

There are many camera settings that can be used for optimizing the sensor's recording features. These include:

- Variable ISO and noise management choices
- High resolution pixel counts for lower costs
- Portability and ability to use the camera for other imaging applications
- Color reproduction and color management choices
- Various file format choices including RAW
- Live view and video capabilities of high-end cameras
- Bit depth.

DSLR Camera Disadvantages

- Source of vibration when used in magnified imaging applications
- Coupling to a microscope
- Alignment and setting up/tear down
- Focusing in the viewfinder
- Changing image features using menus and not on the bigger screen of a computer
- Battery consumption
- Delayed shutter activation when mirror lock-up mode is on.

Attaching and Operating the Camera

How to couple, or not couple a DSLR camera to a microscope is one the first considerations when moving forward with using this camera for photomicrography. A DSLR camera can be simply hung over the microscope's photo eyepiece using a vertical copy stand or tripod. It is very practical to use extension tubes or bellows on the camera to act as a light baffle, which is required to manage ambient light. Ambient light in the lab can create flare and other image defects in science images. Flare will lower image contrast and create poor definition or visibility. If extension tubes or bellows are not available, it is possible use a toilet or paper towel tube lined with black tape as a substitute. I have also used black construction paper as a light baffle. The sensor will need to be carefully aligned over the eyepiece and the sensor will need to be perpendicular to the optical axis. Using a spirit level or smartphone level app can be useful as an aid in making a good alignment (see Figure 6.6). The sensor needs to be parallel with the stage. The camera should be at a distance from the sensor where the circle of illumination produced by the eyepiece is large enough to adequately cover the sensor without producing a circular image. The formation of circular images is not desirable for research but can be fun when used for artistic work. Moving the camera further from the eyepoint will increase the circle and subsequent image size. When moving the camera further from the eyepiece, the image will become dimmer and the circle of good definition will become larger than the sensor, allowing it to cover the sensor. See Figure 3.6.

If a more structured system is desired, coupling the camera to the microscope will require an optical and/or mechanical couple. Optem Thales or Spot Imaging sells couplers for any camera and microscope. Many manufacturers of microscopes such as Nikon or Zeiss also sell adapters for DSLR cameras. There are many configurations, as you might imagine, and it is necessary to correctly select a coupler for a specific type of camera as well as the microscope and type. C-mounts, bayonet mounts, or other particular mounts can be suggested by a vendor. Coupling might be considered ideal; however, ensuring the optical elements in the optical coupler are of the right quality should be considered before purchasing. "You get what you pay for" will define the characteristics of many optical elements. Inferior optical elements will diminish image quality significantly. A full sensor digital camera will require a different magnification than a cropped sensor camera, and some couplers will have lenses while others will be without glass lenses.

Focusing the magnified image using a DSLR camera can be accomplished in several ways. It is possible to simply project the microscope's image directly into the DSLR's viewfinder without a lens for focusing. Seeing a crisp image can be challenging. The coarseness of the viewfinder's surface is rougher than the fine detail of the magnified image. As such, the image never appears sharp or crisp in the viewfinder because its elements are spread across a diffuse textured screen designed for different applications. It might be useful to focus using the coarse focus. It is also very important to properly set the camera's viewfinder reticle. Most brands of DSLRs have a diopter setting control near to the eyepiece. Nikon has large and small controls. Canon's is more discreet. The diopter adjustment knob works as a lens switching from near to far focus. It does not affect the focus circuitry of the camera as it is after the autofocus mechanism. It only affects how you see the image in the viewfinder.

It is possible with some cameras to focus the image using live view. This feature projects the image from the sensor directly to the computer. This allows precise viewing, focusing,

and composing on the computer monitor. In a dimly lit room, this can be very helpful for precise control and location of critical definition within the sample. A small disadvantage might be that the preview window for live viewing might be smaller than desirable or, when photographing dynamic events, there is a lag in the shutter release as live view shuts down. Live view can be very helpful although the mirror and other mechanics of the shutter system when depressed can be a source of vibration to the image if not carefully managed.

It is also possible to parfocalize the camera's viewfinder to the viewing eyepieces. This would create the same focus in the camera and eyepieces. This can easily be done by first focusing the image in the camera's viewfinder. Once the image has been focused in the camera, redirect the image back to the microscope's eyepieces. Using the eyepieces' diopters only, refocus the image without changing the working distance. If done properly, both images should be in the same plane of focus.

Vibration is a very real problem in microscopy. Vibration can be produced by many sources both within a camera and caused by environmental sources. Vibration will make images appear not sharp. These images can be well focused and blurry because of image movement during exposure. Computers and internal fans in light sources can create vibration if they are located on the microscopy table. Fiber optic lights can also have fans built into them for cooling and can be a source of vibration. There can also be environmental sources of vibration including elevators within buildings, subways that go under buildings in major cities and sometimes the powerful HVAC systems located on the roof of a building. Anti-vibration tables are ideal solutions to isolate vibration but are very expensive. There are vibration reduction settings in some cameras and VR lenses, which are more common. Image processing can be somewhat effective in removing some of the artifact of image shake but is not an ideal solution. See Figure 4.8.

DSLR cameras themselves can be the source of vibration. Simply depressing the shutter can jar the camera and create image shake. A DSLR camera uses a mirror that allows for the viewing of the image in the reflex viewer. When the shutter is depressed, the mirror moves out of the way and the focal plane shutter opens and closes, allowing the light to strike the sensor. All of these mechanical processes can have significant influences on image stability and can produce vibration. The mirror will create camera shake and the movement of the focal plane shutter also can make vibration. The effects of vibration in the image can be controlled through the use of slower shutter speeds rather than using shorter times. By using a longer time, the effect of the vibration exposure will be absorbed into the longer and more stationary exposure.

There are a few things that can minimize the production of camera vibration. Triggering the camera from the computer and not using the shutter depress button directly is a good place to start. If tethered, the shutter can be activated using Adobe Lightroom or other camera-controlling software. It is also suggested to consider using the camera's self-timer. This will dampen any vibration caused by the operator. It can be practical to use an electric remote shutter release, too. Any or all of these three approaches will be helpful in controlling vibration that is produced when firing the camera.

Probably the most useful tool for vibration control using a DSLR camera is by selecting the camera's mirror lock-up feature. For mirror lock-up, focusing is accomplished first and then the mirror is locked up. Once the camera has settled down, the exposure can be made. An

image that is vibration-free is a great accomplishment using a DSLR camera. Sometimes it is suggested to make multiple photographs of a sample using the same settings, hoping that one of the files will be slightly sharper than the others.

More Advanced Methods that Produce Contrast

Darkfield illumination produces images with high contrast and results in objects appearing white against a field of almost black. Darkfield microscopy requires the use of a special condenser but no special objectives. Creating a dark background has some challenges and results are contingent on the sample and its preparation. Everything and anything that ends up in the preparation can glow white in this type of illumination.

A darkfield condenser illuminates samples using oblique angle lighting. The illumination comes from oblique angles to the system's optical axis. Light that normally would illuminate the background (zero order) is subtracted from the system using an opaque disk located where the aperture diaphragm would normally be located. The diameter of the background illumination should be equal to or greater than the NA of the objective. Anything in the system that is transparent will refract light and will be illuminated, especially dirt. Cleanliness in sample prep is an absolute requirement.

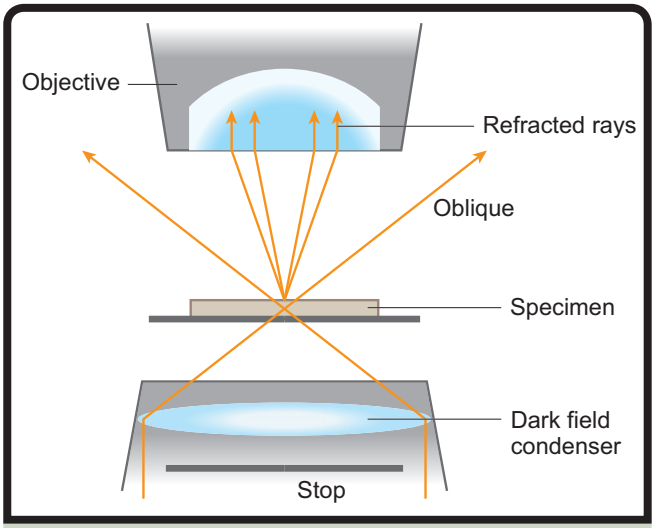


Figure 9.13 This figure shares the basic lighting pathways used from a darkfield condenser.

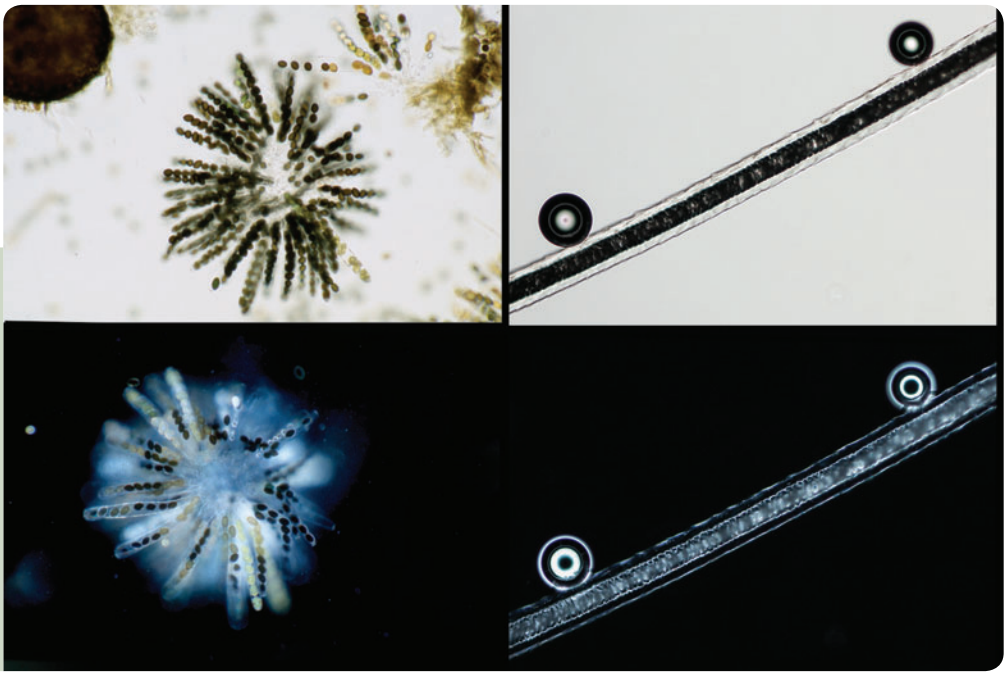


Figure 9.14 The figure on the left shares how *Sordaria fimicola* will appear in bright-field illumination (BF) on the top and in darkfield (DF) on the bottom. The image on the right features a dog hair and air bubbles examined with BF on the top and DF on the bottom. A 10x objective was used to photograph both samples.

Oblique angle lighting is produced and shined through the sample. Because of the oblique angle of the lighting, illumination becomes trapped within the subject and light is refracted into the objective.

There is significant light loss in a darkfield system. The zero order of illumination is the brightest and strongest part of the illumination. The disk stop, if properly sized, removes the zero order illumination and, as a result of low light, exposure times may be longer than expected when compared to brightfield methods. Contrast is generated in this technique; however, live and unstained materials are a challenge to photograph because of subject movements and long exposures.

Setting Up Darkfield

Köhler illumination is not used in darkfield technique. The brightfield condenser should be replaced with a darkfield condenser and the field stop opened to its maximum size. Diffused light is required. The condenser must be centered and adjusted to its correct and proper height in the system. This can be achieved by placing a small piece of white paper where the sample would normally be located. Adjust the condenser's height to the position to where the "circle or donut of light" produced by the condenser becomes a dot of black or a point directly under the objective lens. Look into the binoculars and center the illumination covering the entire field of view evenly. The dry darkfield condenser works best with 10x and 20x objectives. For lower magnification work, a typical darkfield condenser may not be able to create a large enough illumination to cover the field of view of the objective. For applications that require more magnification, e.g. 40x objective, an oil darkfield condenser will be required. There is the requirement in darkfield technique when compared to brightfield that the darkfield condenser's numerical aperture must be higher than the numerical aperture of the objective lens. This is the exact opposite for brightfield methods.

Photography Using Darkfield

When photographing using darkfield illumination, exposure times should be shortened from the suggested meter readings when using automatic metering. Reducing exposure times by a factor of –1 or –2 using the camera's exposure value compensation system will be helpful. If using manual exposure controls, it is ideal to establish a time based on highlight detail. White balance is also not a significant concern since the background will be black. If it is possible to set a black point, an RGB value of 15 can be an effective calibration setting.

Modifying a Brightfield Condenser for Darkfield Illumination

If a darkfield condenser is not available, there may be ways to create oblique angle illumination. It might be possible to use a coin or another opaque material that can be located in the center of a clear plastic disk and inserted into an Abbe condenser An achro-aplanatic or flip-top condenser cannot be modified for this. A central disk stop can be created by using a circle of black construction paper mounted in the condenser

using friction or some other non-permanent adhesive. The improvised disk stop will need to be located in the center of the condenser where the aperture diaphragm (AD) would be located and perpendicular to the objective's axis. It is possible to determine precisely how large the disk stop should be by pulling out an eyepiece and establishing the aperture diaphragm setting to match the body tube's diameter. The substage condenser can be removed and the physical size of the AD blades can be measured. A disk stop can then be cut and located where the AD would be located. The aperture will need to be fully opened when inserting the disk.

Challenges in Darkfield Microscopy

Darkfield microscopy creates a high contrast image by using oblique angle lighting and the refractive properties of the sample. This method can become a challenge because not only does the specimen refract light, but internal particles and bubbles in the preparation will also be illuminated by the lighting. Time and attention should be paid during sample preparation, leading to cleanliness. Glass slides will need to be carefully cleaned, paying attention to dust and particulate materials. When photographing live aquatic materials using darkfield, it may be necessary to use distilled water and allow the air bubbles to evaporate from the water before imaging. Samples should be prepared as thinly as possible because too much sample will produce a DOF challenge as the object behaves like a light itself. When the entire sample is out of focus, delineating focused sample from out-of-focus sample can lead to unusable images.

Differential Interference Contrast

Making contrast means that structures that are barely visible will become visible. An effective but expensive microscopy technique that accomplishes this would be differential interference contrast or DIC. DIC is an interference technique that is very useful for examining unstained and living materials for different reasons than darkfield. DIC images will have topography or relief. DIC is a very complex technique that uses interference to create images containing contrast and structural data that is viewed as "an almost topographical map" of an object. DIC images may appear monochromatic but colors can be created using an additional quartz wedge. A quartz wedge is special optical element used to create multiple beams of light from one.

For DIC methods, specialized optical glass and quartz components are required. A special DIC objective is required and the method requires stress-free APO objectives. A DIC condenser is also needed, along with two Wollaston prisms and two polarizing filters that are strategically located. These elements work together to form images that have what is often called pseudo-relief.

Creating a DIC image requires the use of a prism that creates two illumination beams. One beam is called the sample beam and the other is called the reference beam. These beams use plane-polarized light. These two beams strike different regions of the sample and surrounding material that creates an out-of-phase relationship of the two beams. When the beams come together at the intermediate image plane, interference occurs. Chapter 3 describes in great deal how interference operates.

Setting up a DIC microscope is relatively straightforward. For transmitted light, there are specific steps that are necessary.

1. Focus the sample and then focus an image of the field stop at the same plane as the sample.
2. Using an Abbe condenser located in the universal condenser, it is important establish cross-polarized light. This is accomplished by removing the quartz wedge and rotating the polarizer located on the field stop until the light is extinguished or the background goes black using the 10x DIC objective.
3. Once this has been accomplished, the matched DIC condenser must be selected and rotated into place and the quartz wedge needs to be reinserted. Once that has been accomplished, the system is ready for use. The effects of DIC can be changed by adjusting the position of the quartz wedge located on or in the optical pathway. More or less contrast can be created, as well the inversion of the image from black and white to white and black. The proper location of the wedge is based on the creation of visibility.

There are no real photographic challenges since a DIC system has adequate brightness and the image contrast does not require any special approaches to manage. Sometimes it is

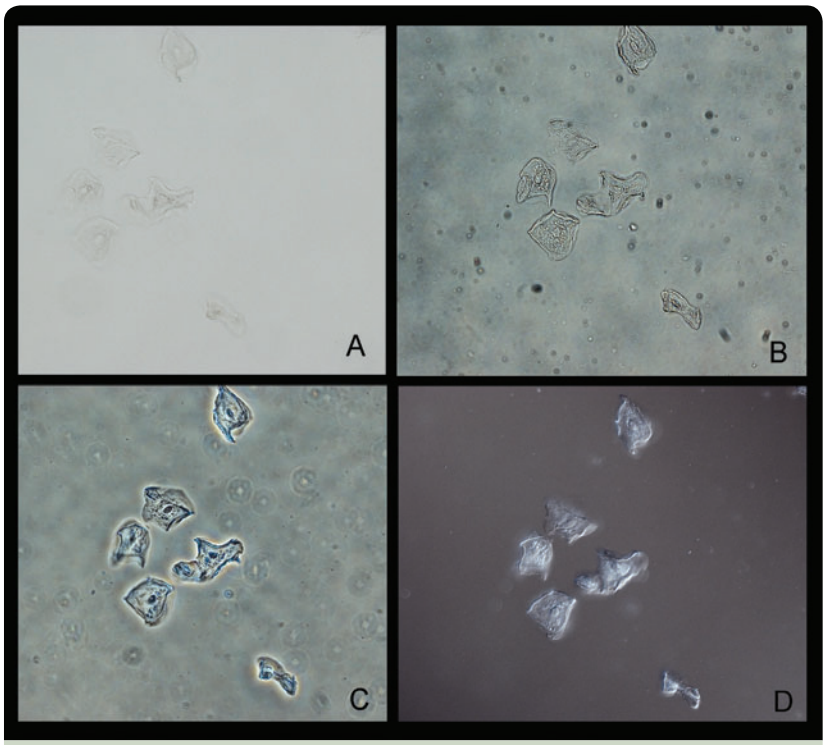


Figure 9.15 This illustration reveals how one subject—cheek cells—will appear when examined using various methods. Image A shows unstained cheek cells photographed using brightfield microscope, 10x objective and with the aperture wide open. In image B the aperture diaphragm was completely closed down. Image C shows phase contrast revealing internal structure, and in image D, differential interference contrast (DIC) reveals sample topographical information.

useful to combine DIC images with fluorescence images, though, and this requires careful set-ups for both techniques and achieving excellent exposure and highlight detail. This combination provides viewers some context to the sample and its characteristics in addition to the presence of the fluorescence phenomenon from within.

Fluorescence

Methods used for close-up fluorescence photography were shared in Chapter 8. When fluorescence methods are used with microscopy, the challenges become amplified by the problems found in creating magnified images of low brightness. Fluorescence microscopy uses epi-illumination, where light comes from above the sample. There are a number of important reasons for illumination to come from above. In fluorescence, when the light passes through tissue it can be scattered and absorbed by the tissue. When the illumination comes from above, this surface absorption and scatter is reduced because there are shorter distances to travel. This shorter distance leads to images with better delineation and brightness. Much more about fluorescence, a method integral to confocal methods, is shared in Chapter 10.

In fluorescence work, a sample is infused with a liquid stain that fluoresces (glows) when bombarded with high, short wave energy. These dyes may be called fluorophores, probes, markers or fluorochromes. There are a number of common stains, such as GFP (green fluorescent protein, DAPI (4',6-diamidino-2-phenylindole), and Alexa fluor. Fluorescence by definition is a process where a material absorbs short wave energy and re-emits it as longer wave energy or commonly light.

In a microscope, there often is a special illuminator that uses a xenon or metal halide light source since brightness is a big problem in fluorescence work. Tungsten halogen sources are not very effective because of the brightness requirements of a fluorescence system. At this time, LED lights are also finding adoption as fluorescence illuminators. Lasers are used for confocal microscopes. Xenon and mercury short arc plasma lamps have the highest brightness of any continuously operating light source.

In a photomicroscope, fluorescence requires the use of filters, which are situated in what is called a cube. See Figure 9.16. There are two filters and a dichroic mirror in a cube. The filters must be mutually exclusive, which means the excitation transmission must be discrete from the emission filter characteristics. For example, if excitation is from 420 to 450 nm, the emission filter must transmit waves that are 460 nm or longer. The excitation filters can be either narrow or broadband. Emission filters function to remove the excitation energy. These filters are highly engineered and will have a specific energy travel direction; they often come with a small arrow etched on the side of the filter providing information

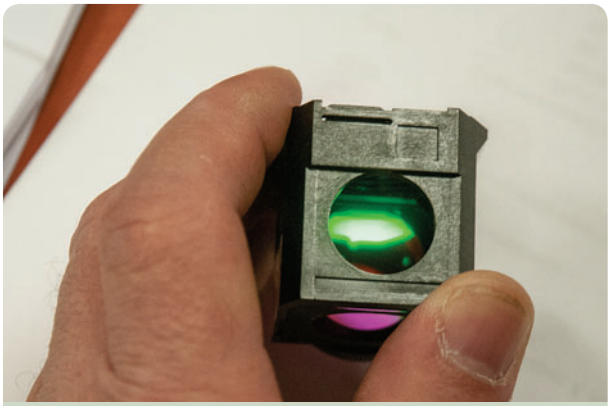


Figure 9.16 Fluorescent cubes have three components: an exciter filter, a dichroic mirror and an emission or barrier filter. Seen in this figure is the appearance of the effect of the filter. The color of the lights that leaves the cube will be the complement to the opposite color of the filters based on travel direction. When looking at the cube in one direction, green will be observed, while when looking at the cube from the opposite direction, magenta will be seen.

about orientation in the cube. Once the light has left the excitation filter it strikes the dichroic mirror and is directed to the objective and subsequently the sample.

Dichroic filters are very thin pieces of glass and play an important role in fluorescence microscopy. Dichroic glass or an interference filter is designed to very accurately transmit light of a specific spectral composition and reflect wavelengths of other colors. There are many applications of dichroic materials in various lighting applications where control of the color composition of the light requires control. Dichroic filters/mirrors in the cube act as a beam splitter as well and only 50 percent of the light that strikes its surface is transmitted.

Images with Low Brightness

Because of the manner in which fluorescence images are formed, not very much image brightness is present at the image plane. When considering the initial brightness of the lamp and the subsequent locations where brightness is removed, there is actually very little light available to produce an image. Pretend for a moment that a light source has 100 watts of brightness in the bulb. Where this light enters the cube, it will be fitted with a narrow-band filter such as 420–450 nm blue. After interacting with the filter, 66 percent of the light is removed by the excitation filter. When this blue light strikes the dichoric mirror, 50 percent is reflected down to the sample, where it interacts with the markers and other components of the sample, and 50 percent goes through the mirror and is forever lost. The fluorescent image is next formed by the objective and transmitted back through the cube in the opposite direction. Now 50 percent of the brightness is again removed prior to engaging the emission filter by the mirror. The cube by design only transmits a fraction of what was present to start. It is possible to speculate that a mere 1–5 percent of the initial available brightness might be present in a fluorescent image. For this reason, the signal-to-noise ratio of fluorescence images might be high because of the requirement for long exposure times. Many microscopists use noise-reduction filters or cameras with cooled chips that are excellent for low light imaging that require long exposure times.

Even Illumination

Fluorescence investigations evaluate the presence of a compound of varying strength or no reaction at all. To ensure these reactions are most effectively recorded, the illumination that is shined onto the sample must be uniform and of equal brightness across the entire field of view. A lack of uniformity of illumination can lead to false data being created by brightness differences from the lighting and not the reaction. There are several ways to create uniform and even excitation lighting, dependent on the type of illuminator. Traditional xenon or metal halide sources can be housed in two types of cases. Older lamp housings used a convex mirror behind the bulb. Using adjustment screws in the lamp housing, an image of the lamp filament needs to be focused and positioned adjacent to a focused image of the filament produced by the mirror. The mirror and the bulb would each have several adjustment screws to assist in the management of this important step. Metal halide and xenon bulbs have a useful and safe operating life that is clearly indicated to users. Almost all power supplies for these types of lights will have a clock timer.

More contemporary is the modernization of the housing that uses a mixing box rather than a mirror to condense the illumination into a small area. The condensed light is delivered to

the microscope using a fiber optic cable rather than having the hot and large bulb housing on the microscope. LED sources that are tunable are also finding their place in this area of imaging. Using several color LED bulbs, almost any desired color can be mixed and used for excitation. Because the excitation and brightness are controlled in this fashion, exciter filters are not required. Modern fluorescent illuminators use fiber optics to move the light from the source to the microscope. The fiber optic cable is vulnerable to damage/breakage. Placement of the microscope and illuminator needs to be considered so as not to pinch or bend the cable in a harsh 90-degree angle.

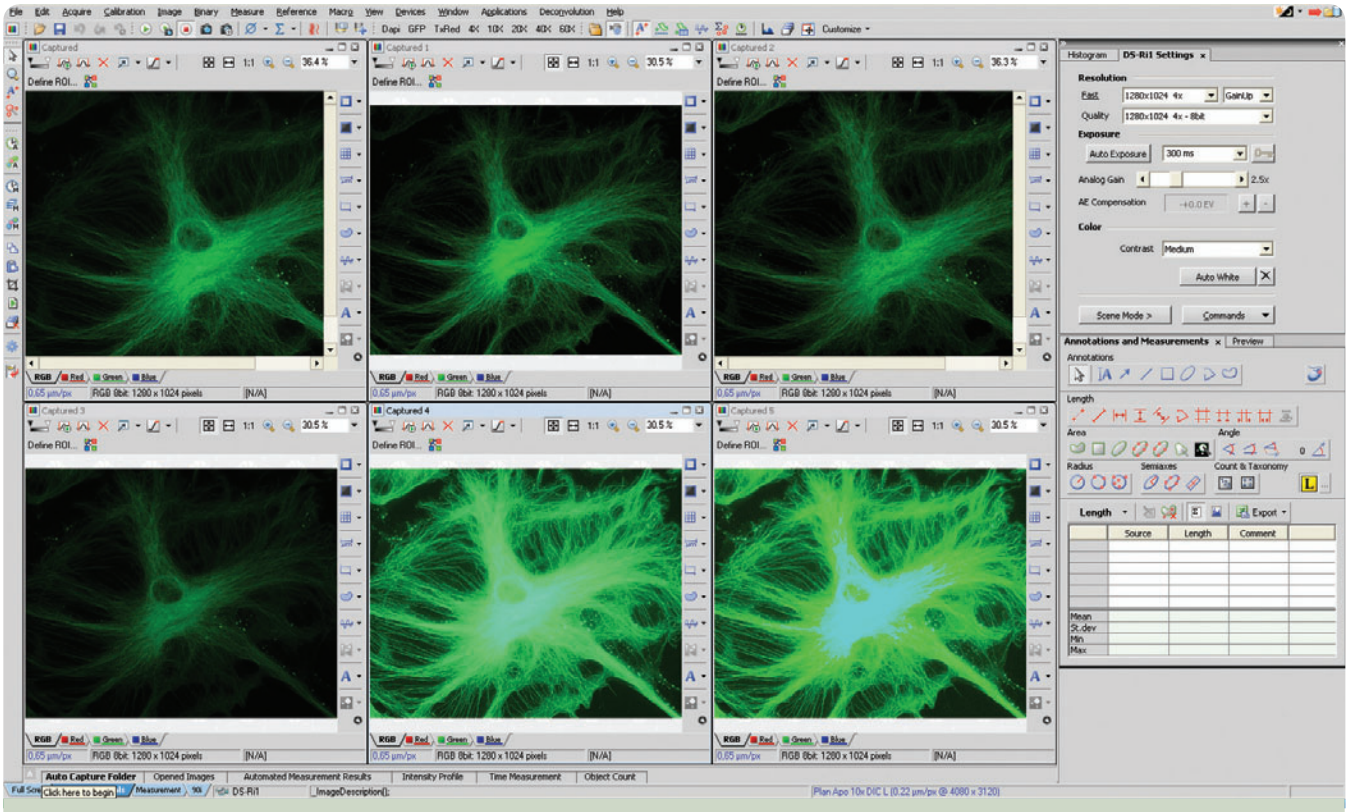


Figure 9.17 This series of photographs demonstrates what happens to light brightness and photographic exposures when fibers used to transport the high brightness light used in fluorescence in light guides that become broken. Each image in this composite was exposed using the same time. Evidenced in the results, the brightness from each cable will vary cable to cable because of damage to a cable over time. Image courtesy of Torgeir Dahlen.

Photographing fluorescence images can be tricky for other reasons simply beyond low image brightness. The sample and stain itself can be a real challenge for photography. The various stains (fluorphores) have a timed element to emission. The duration of time these chemicals can be excited may vary from very short to longer. The exhaustion of the chemical reaction has been given several names. It might be called quenching, burn out, extinguish, bleaching, or photo-bleaching. Each description means the same to a scientist photographer—the reaction is over and no longer able to generate light. In some cases, the imaging experiment may not be completed before extinction occurs. A few dyes can

be recharged but not all. Taking the slide out of the excitation beam and then rechilling it in a refrigerator might be helpful; however, most often they do not come back to their peak brightness. When considering this challenge compounded with a subject with low brightness, once a scientist is committed to imaging, the clock is ticking. Attempting to locate the field of interest, focus the sample, and making an image of an event that begins to fade the moment the fluorophores are exposed to excitation energy is full of challenge and anxiety.

Imaging Problems

There will always be imaging challenges, and probably more in fluorescence work than any other type of microscopy. The following list shares expected challenges found in fluorescence applications.

- Samples with a high dynamic range/contrast
- Samples with low brightness
- A short duration chemical event; this can tax even the most experienced photomicrographers
- Critically focusing; a challenge because of the scatter and diffusion of light within tissue
- Possible autofluorescence from the sample and system from the specific fluorescence of the desired fluorescence
- Camera sensitivity and performance in low light.

Suggested Imaging Solutions

- Creating a precise highlight exposure—detail in the highlights, not shadows
- Establishing a low gamma capture setting
- Closing the field stop more than normal, creating a smaller circle of illumination
- Turning the room lights off to increase operator low light vision
- Removing polarizing filters from the light path when not in use
- No white balance setting is needed for fluorescence images.

There are a number of other things such as using:

- Gain
- Binning (which comes from the word "combining")
- High NA objectives with low dispersion (APO)
- Low brightness for viewing and increasing brightness for imaging
- Closing the fluorescence shutter when not evaluating or imaging
- A grayscale camera without a Bayer filter
- A low monitor brightness ensuring a large pupil
- A 100 percent beam splitter
- ND filters for viewing, removed for imaging
- Manual exposure modes rather than automatic
- The numerous features in the camera's software to optimize the data recording.

Phase Contrast

Phase contrast microscopy was invented by Frits Zernike in 1953 and he was awarded a Nobel Prize for the discovery. Phase technique is used to look at unstained subjects such as live nerve cell cultures or other semi-transparent samples with refractive differences. Phase contrast microscopy requires special objectives and condensers. Samples examined using phase will exhibit a white halo that surrounds the cell(s) against a gray background when using bright phase optics.

In phase technique, the condenser separately illuminates the sample from the background using energy that has experienced a phase change. Located in the condenser is the phase annulus, a structure that is similar in function and size to a darkfield disk stop. Located in the objective is the phase ring that shifts the light, which goes through it at a quarter wavelength. The subject is illuminated by the diffracted rays while the central rays illuminate the background using light that has been shifted slightly, now having a different phase. Structures within the sample slow down and refract the diffracted light to a different point of focus, which generates constructive and destructive interference. The interference is caused by the sample and occurs at the intermediate image plane. It is a rather complex process, and what is most relevant is not the physics of the process but how to use the optical components to their maximum.

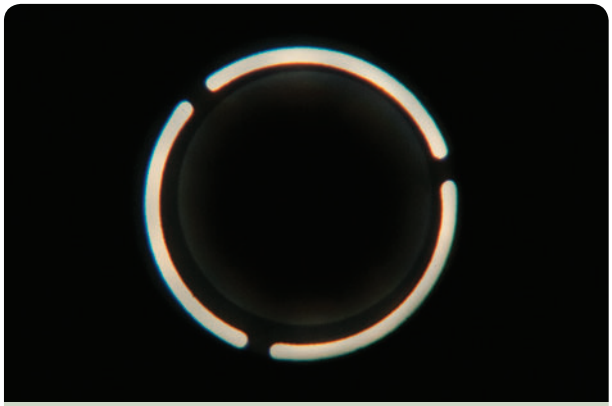


Figure 9.18 In this figure, phase annulus and ring alignment are visible and are superimposed on top of one another. This photograph was made looking into the body tube at the exit pupil of the objective where the components should be aligned. This photograph was made using a phase telescope.

Setting Up Phase Contrast

Using an Abbe or brightfield condenser to begin, insert a sample into the microscope and focus the image. Some samples will be difficult to see. If needed, close the aperture diaphragm completely to create some focus and composition. Replace the brightfield condenser with the phase or universal condenser. Phase objectives will also be required. These lenses will have Ph inscribed on the objective barrel. It is important to match the condenser with the objective, e.g. Ph 1 or Ph 2. Once the sample has been located and focused, replace the Abbe condenser with the phase condenser and select the correct position to match with the objective. Close the field stop and, using the substage condenser, bring the blades of the field stop into sharp focus. Performing a sort of partial Köhler is required for phase technique.

With the image now focused, and when the correct combination of objective and condenser have been put in place, remove an eyepiece and insert a phase telescope, which is useful for making the final alignment of the phase annulus—located in the condenser and the phase ring—in the objective. A phase telescope is an important accessory that allows a microscopist to see the exit pupil of the objective where the ring and annulus are visible. The telescope may need to be focused. Using the phase annulus adjustment screws, superimpose the phase ring over the annulus. When this condition is achieved, the technique has been successfully established.

There is nothing particularly difficult about making photographs of monochromatic images produced from a phase contrast microscopy.

Polarized Light

Some aspects of polarized light methods were discussed in Chapter 7; however, it was not specific to microscopy. It is possible to use any microscope as a polarized light by placing a linear polarizing filter on the field stop and another polarizing filter, which is called an analyzer when located behind the objective or in front of the eyepiece. The images from this type of microscope conversion would be qualitative but not quantitative. For true analytical work using a true polarizing light microscope, objectives with POL or that are stress-free are required. This instrument would also have a nosepiece that can be centered and have a circular or rotating stage. See Figure 7.14.

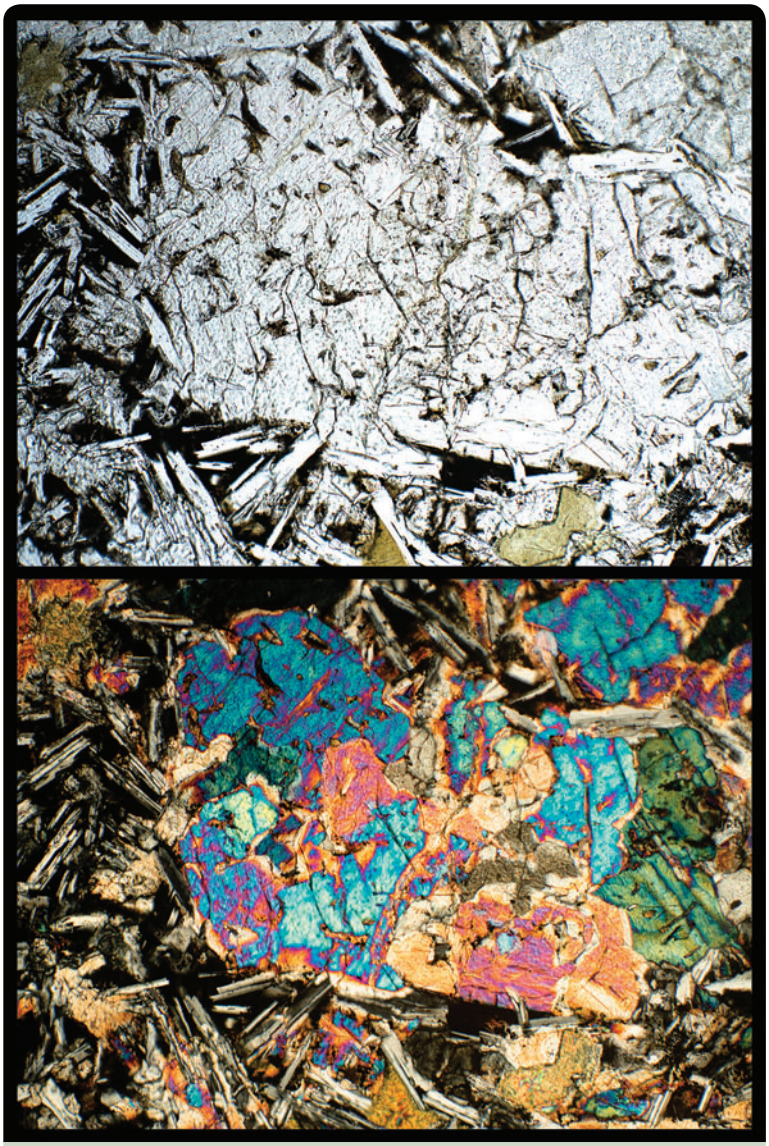


Figure 9.19 This composite photograph shows how the mineral olivine basalt porphyry appears with and without polarization. The non-polarized view is at the top. The magnification at capture was x20.

The underlying advantage for using polarizing light is to evaluate for the presence of birefringence in materials. Birefringence is a behavior whereby a material that is composed of multiple refractive indices will reveal colors when examined using polarized light. A material with one refractive index would be characterized as isotropic and a material with multiple refractive indices is said to be anisotropic. Glass, water, and air are isotropic. When an object of unknown refractive index is placed in an optical system where two linear polarizing filters are oriented at a 90-degree angle to one another or crossed, the presence of refractive properties can be observed and photographed. Substances that reveal colors and are lit against the black or extinguished background are anisotropic and objects that are black or do not glow are isotropic materials. The field of chemical microscopy and forensic microscopy relies heavily on polarized light methods. The McCrone Institute in Chicago, Illinois, is well known for its polarized light microscopy short courses. The colors that are produced in polarized light can be used to identify materials of objective based on refractive properties. Geologists also use a polarized light microscope to identify minerals. Crystals are popular subjects for photographing using polarized lighting.

Rheinberg Differential Colorization

In 1896, Julius Rheinberg published a paper in the *Journal of the Royal Microscopical Society* describing a method for optically coloring a subject's features using a microscope. This method, now commonly referred to as Rheinberg differential color illumination, uses concepts derived from darkfield technique and also uses some of Ernst Abbe's original theories.

Among the more difficult samples to photograph are those with little or no internal contrast. These might include live materials or very thin and nearly transparent subjects. Common techniques that are useful for nearly invisible samples might include darkfield, differential interference contrast, or phase contrast methods. These techniques require modifying the illumination that is produced in the microscope. In these methods, the sample can be made to exhibit more contrast but will often be rendered in monochromatic tones.

Using the Rheinberg method can also lead to increased visibility. The premise of the technique is simple. The disk stop normally used in darkfield to subtract the zero order of illumination is replaced by a dense and highly colored disk stop. The clear region of the darkfield condenser that surrounds the disk stop, or annulus, is covered by a lightly colored material.

The Rheinberg effect is very attractive and can lead to images that are more interesting than otherwise monochromatic images from the previously mentioned methods. Early methods for creating filters required for this technique were slow and laborious. As new technology has evolved, there are now simple ways to produce Rheinberg filters comprised of a large range of colors that can be overly saturated. These filters produce elegant results because of the precise image editing software.

Producing Rheinberg Filters

Adobe Photoshop or any other image processing software can easily be used to produce Rheinberg filters. Producing custom-made Rheinberg filters can be a very fast and easy process. Before the filters can be made, precise measurements determining their size requirements must be obtained from the microscope's substage condenser. The aperture diaphragm settings in the substage condenser must be determined for each objective where Rheinberg illumination will be used. The higher the NA of the objective, the larger the colored disk stop that will be required. Abbe condensers are the ideal because they provide easy access to the location where the aperture diaphragm would be situated. This is the location where the Rheinberg filters will need to be placed. The higher the quality of the condenser, the more difficult it will be to access this location in the condenser. The following steps will work when using an Abbe condenser. Establishing a Rheinberg system may not be possible with universal or phase type condensers.

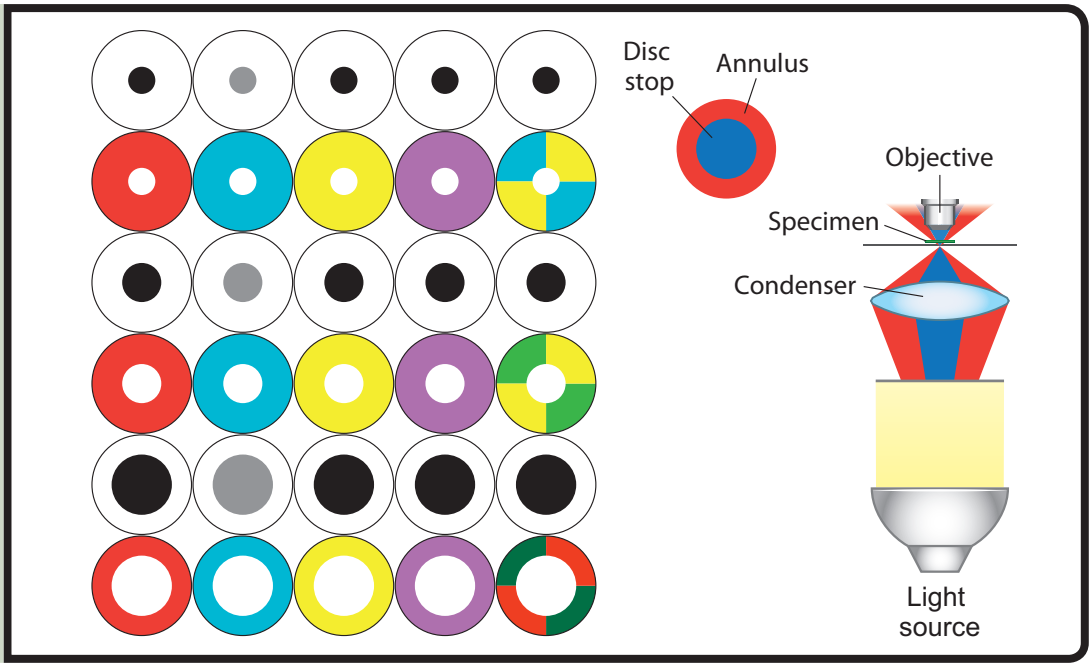


Figure 9.20 This figure shares a series of Rheinberg filters. They could be printed onto transparent materials and then cut out for use. On the right is a graphical representation of how the optical staining is achieved when locating the central stop and colored annulus in the substage condenser, where normally the aperture diaphragm is located.

Determining the Disk Stop Size

1. First establish Köhler using any slide. It is best to start by using a 10x objective. The sample is not important. It is important to use an Abbe condenser for this. Other condenser types will not work well.
2. With the objective in place, establish Köhler illumination for that objective, and close the aperture diaphragm down completely.
3. Remove one of the eyepieces.
4. Looking down the body tube of the microscope, open the aperture diaphragm until it just leaves the field of view.
5. Carefully, without disturbing the aperture diaphragm setting, remove the substage condenser.
6. Turn the condenser over and, using a precise measuring tool such as a vernier caliper, measure the diameter of the opening of the blades of the aperture diaphragm.
7. Record the number. This diameter will be used for the disk stop for that objective.
8. Replace the condenser and repeat this process for all of the objectives where Rheinberg illumination will be used.

To find the outside diameter of the annulus filter, either measure the filter size for the holder that will be used or open the blades of the aperture diaphragm completely and measure the total diameter of AD in the condenser at its widest setting.

The actual production of the filters is somewhat involved, but once the steps are determined it is a very fast and reliable way to make a variety of filters.

1. Open Photoshop software or any image processing software and create a new file.
   a. Set the height and width of the document to be equal to the total diameter of the desired filter.

   b. Set the resolution to the output resolution to 300 ppi.
   c. Set the background to transparent.
2. Select the elliptical marquee circle tool.
   a. Make sure the anti-alias box is unchecked.
   b. Select a fixed size under the style menu.
   c. Enter the total diameter of the filter in its millimeter or other distance measurement using the height and width boxes.
      i. Click the cursor in the work area using the shift key to make a circle.
      ii. Reposition the circle so it is where desired.
      iii. Select the color that is desired and fill the circle or wait to fill later.
3. Under the edit pull down menu, select the stroke command.
   a. Make the width one pixel.
   b. Set the location to center by using the shift/alt and option keys.
   c. Make the opacity 100 percent.
4. Reselect the elliptical marquee (M on the keyboard).
   a. In the marquee options window set the height and width to the diameter of the disk stop.
   b. Click inside the first circle.
   c. Move the new circle to the center of the first circle. The circle will snap into the exact center because of the registration lines.
   d. Under the edit pull down window select the stroke command.
   e. Make the width one pixel.
   f. Set the location to center.
   g. Make the opacity 100 percent.
5. Repeat this process for each objective using the correct diameter of the disk stop and annulus size.

Once the templates for each objective have been made, it is very easy to make a sheet containing multiple filters. An example is shared in Figure 9.20. There are two basic ways the filters can be designed. The first method is to fill both the disk stop and the annulus with different colors on the same filter. The advantages to this would be the ease of use at the microscope and improved optical quality because the light will not have to pass through more than one filter. The other way to make the filters is to produce the colored disk stops and annulus separately. The advantage to this approach is a higher degree of variability because they can be mix and matched.

The filters can be printed either using a color ink jet printer or a color laser jet that can print directly onto overhead or transparency material. They can also be printed on transparency materials using various devices. The filters may not be dark enough because of the quality of the printer, so the filter sheet may need to be double-printed, or two stacked together, or both. The ideal way to have these filters printed is to have a local high-end imaging center print the files onto a sheet of transparency (slide) film. This process is considerably more expensive; however, it provides the best darkness in the colors and the least amount of distortion for the light passing through the filters.

The only requirement for the choice and use of the colors is that the central stop needs to be considerably darker than the color of the outer annulus.

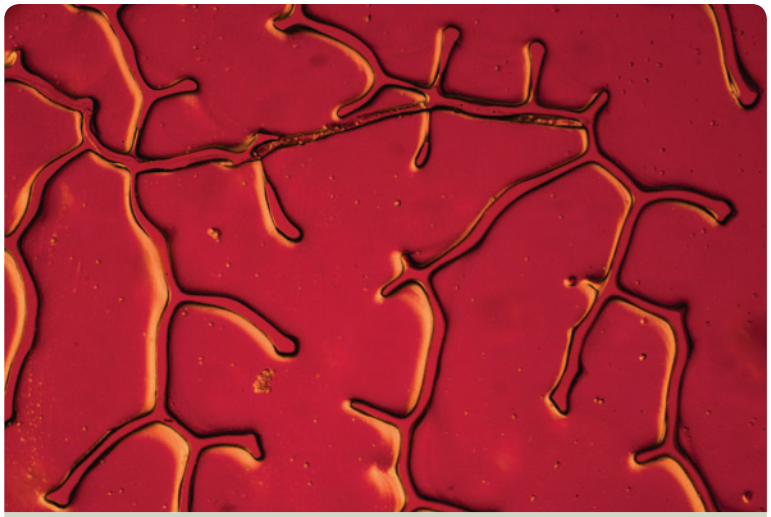


Figure 9.21 This photomicrograph features cracked mounting media. It was lit using Rheinberg methods. The annulus lit the media and the central stop the background. Image courtesy of Sarah Alharbi.

To place the filters in the correct spot within the substage condenser, the ideal might be to fabricate a custom-made holder designed for a particular condenser. With the rise of 3D printing, it does not seem like an overly complicated problem to create a plastic filter holder. If creating a holder is too complex, it might be possible to fabricate something from a plastic milk jug that is more than adequate to hold nearly weightless plastic filters at the correct height within the condenser. When the filters have been inserted into the condenser, put a piece of white paper where the sample will be located. Raise and lower the condenser until the color from the disk stop disappears. Replace the paper with the sample and "refine" the condenser's height, based on how the sample looks.

SUGGESTED READING

Foster, Barbara, *Optimizing Light Microscopy for Biological and Clinical Labs*, first edition. Dubuque, IA: Kendall Hunt, 1997; ISBN-13: 978-0787235383.

Herman, Brian, *Fluorescence Microscopy* (Microscopy Handbooks), second edition. New York: Springer, 1998; ISBN-10: 0387915516.

Murphy, Douglas B., *Fundamentals of Light Microscopy and Electronic Imaging*, first edition. Chichester, UK: Wiley-Liss, December 2001; ISBN-10: 047125391X.

Wayne, Randy O., *Light and Video Microscopy*, second edition. Cambridge, MA: Academic Press, 2013; ISBN 0124115365.

http://micro.magnet.fsu.edu/primer/index.html

http://www.olympusmicro.com

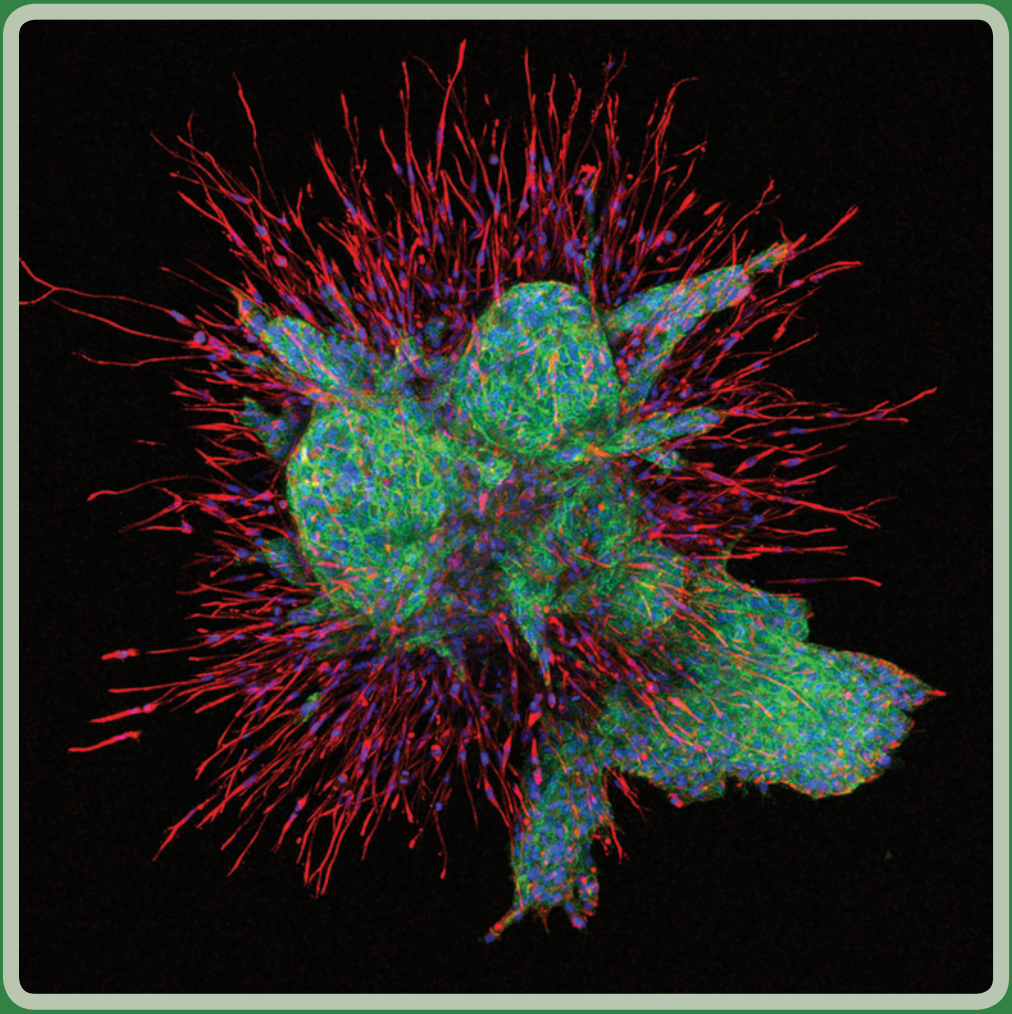
http://www.microscopyu.com

http://www.leica-microsystems.com/home/

http://zeiss-campus.magnet.fsu.edu/index.html

Chapter 10 Confocal Microscopy

JAMES HAYDEN



This photomicrograph features a human oral cancer cell line growing in the form of a spheroid. This culture technique avoids artificial adhesion of cells to a culture dish and promotes the cell–cell interactions that naturally occur among tumor cells. The spheroid is embedded in a matrix of type I collagen, which models the normal barriers encountered by oral cancer cells as they invade destructively into adjacent tissues. Immuno-fluorescent staining was used to show E-cadherin (alexafluor 488, green) and vimentin (alexafluor 568, red). Cell nuclei are imaged in blue (DAPI). The image was captured with a Leica TCS SP5 II confocal laser scanning microscope in twenty sections with a 10x objective and presented as a maximum projection. Excitation wavelengths at 405 nm, 488 nm and 561 nm. Image created by James Hayden (The Wistar Institute) and Dr. Dev Basu (University of Pennsylvania).

Introduction

The basic light microscope—familiar to every biology student—is the result of many technological advances that have occurred over several hundred years. Emerging insights into physics and optics have honed development of its major features. Innovations in the material sciences and engineering led to more accurate and efficient designs found in modern microscopes. The driving force behind this continued advancement, however, was much less about "how" the construction of an instrument has changed but rather has much more to do with "why" the specimens are observed. As biological concepts have advanced, better instruments have been needed to observe the theoretical concepts. As new ways of working with light or new ways of staining cells have developed, those approaches have been incorporated into modern microscopes almost as fast as they have been imagined. At many periods of time, a new technology has been developed to specifically address a particular issue found in microscopic imaging. Today's advanced microscopes are no longer simple instruments that fit in your hand or on a small table, but they are an amalgam of technologies, definitely larger than a breadbox, and allow imaging to be accomplished at the cutting edge of scientific advancements. The microscope has continued to evolve as the needs of its users evolve. Those needs have allowed the best of optics, advances in computing, new ideas in molecular biology and chemistry, the newly understood physics of lasers and light detectors, all to have been integrated into the creation of modern microscopy. This microscope is called a confocal microscope.

Why Confocal?

It might be useful to think of a confocal microscope as a regular—also called "widefield"—fluorescence microscope on steroids. Often taking up a small room and costing as much as a house or two, these microscopes have become the workhorses in many biological research labs around the world. There are simplified versions that can be smaller, with lots of automation, but on the whole they are larger, complicated instruments and require much practice to master. These microscopes, however, deliver enhanced resolution, sharper images and 3D capabilities, making the results well worth the effort (Figure 10.1).

The overall sharpness shown in Figures 10.1B and D is primarily the result of using a confocal microscope, and the comparison of images taken of the same sample with both widefield and confocal is striking. Notice the blurred-out details in the widefield image (Figure 10.1C) compared to the complete sharpness evident in the confocal image (Figure 10.1D). The main feature, common to all confocal microscopes, that makes it possible to have focus throughout an image, is its ability to optically (non-destructively) section through a sample and record what is happening inside. A confocal image's resolution is defined by only the diffraction limit created from Ernst's Abbe's equations first developed in 1872. By recording many images while focusing through the specimen, a stack of thin, highly resolved image slices is acquired. This stack can then be used in a variety of ways to visualize the subject as either a very clear two-dimensional image or a three-dimensional reconstruction image. Confocal microscopes remove the out-of-focus light from the image

plane, so that everything that is observed and recorded includes only the in-focus light. The resolution of a standard confocal microscope is actually no different than the resolution of a standard light microscope. The images from a confocal microscope appear as they do because the system removes all of the unwanted light and out-of-focus image light leaving only a focused and highly defined image.

Confocal microscopy has other advantages when compared to standard fluorescence microscopy as well. Many models use an advanced beam splitter system that provides very precise spectral separation of light. All microscopes use a sensor technology that is usually much more sensitive and advanced than that found in widefield systems. Using lasers as light sources provides a highly efficient and exact fluorescence excitation as well as the ability to define small areas of interest within the specimen. This level of precise control allows the scientist to perform exacting, quantitative experiments like FRAP (Fluorescence Resonance After Photobleaching) or FRET (Forster Resonant Energy Transfer) or FLIM (Fluorescence Lifetime Intensity Measurement) in sharply defined areas that can be as small as a portion of a nucleus or even a single mitochondrion. The software that runs a confocal microscope is also very flexible and provides a variety of balanced options to create an exposure that takes advantage of both the digital imaging system and the specific requirements of the specimen. In fact, these options are what make learning how to operate a confocal microscope so challenging. It is not enough to simply know that the light can be made brighter to get a better exposure. Operators must understand the hardware options for specific needs and then balance those needs with the consequences each will have on a specimen or affecting the image quality. In the end, every image is a compromise that works to bridge the gap between an aesthetically pleasing image and one that merely recorded all the vital data. The confocal microscope is a powerful instrument that takes advantage of modern technology enabling the formation of highly beautiful and data-rich images of the microscopic world.

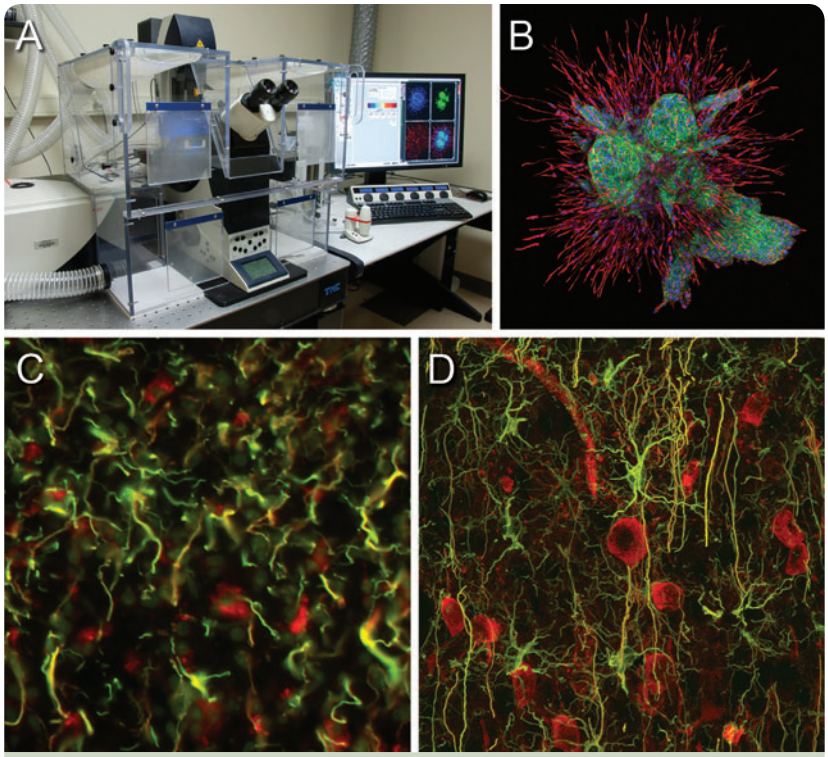


Figure 10.1 There are advantages and reasons for using a confocal microscope. The author's Leica SP5 II Confocal Laser Scanning Microscope (A) was used to create many of the images included in this chapter. Image B features a triple-labeled (DAPI, Alexa 488, and Alexa 594) oral cancer spheroid and is the final image created from the data visible on the monitor. Image C is a dual-labeled (Alexa 488 and Alexa 568) section of mouse brain taken with a widefield fluorescence microscope, and Image D, on the SP5, demonstrates the enhanced sharpness possible with confocal systems.

Types of Confocal Microscopes

There are essentially two types of confocal microscopes, the spinning disk confocal and the confocal laser scanning microscope (CLSM). Historically, the spinning disk microscope was the first microscope released and was outfitted with a Nipkow disk, created by Paul Nipkow in Berlin in 1884. The most important element of any confocal system is the pinhole that restricts the light reaching the image plane, and the pinhole allows only the in-focus light to reach the detector. The spinning disk system incorporates paired disks with thousands of tiny pinholes that rotate very fast, sweeping the fluorescence excitation light onto the specimen with the first disk and passing the emission light back through a second set of pinholes to be recorded by the camera. The greatest advantage to the spinning disk system is the speed at which the images are created, so its most common application is to image live cells and visualize very fast biological processes in the micro and millisecond range. The microscope's greatest disadvantage is a result of that speed, and the amount of light that actually reaches the imaging detectors can be as low as 1 or 2 percent of the light emitted from the source. Weak, transient signals can be very difficult to capture, and factors such as electronic noise, low resolution, and large pixel sizes can limit the information being collected. The continual development of the technology has helped, especially now with more sensitive detectors, brighter light sources, more light efficient optics, and brighter fluorophores. Much effort has also gone into upgrading the function of the pinhole, the reason that images demonstrate a continuous focus. The Yokagawa disk added microlenses to every pinhole, thus concentrating the amount of light that gets through, and slit disk technology changed the singular holes into larger slits that deliver the same resolution. For confocal applications that will not look at live specimens, there are even better options available.

A confocal laser scanning microscope (CLSM) is generally a more versatile instrument and a more common system than spinning disk microscopes. CLSM will be featured in this chapter. A scanning system incorporates two pinholes to remove out-of-focus light. Each is a single, adjustable pinhole; one is scanned over a specimen using galvanometers, much like a traditional analog television set, and the other is placed into the imaging pathway to restrict the passing of out-of-focus light. Scanning confocal microscopes generally use laser excitation, which provides extremely bright light at a very specific wavelength(s) and adjustable detectors that can be tuned to record very specific wavelengths. Scanning confocal microscopes also have other adjustable elements that can be selected for particular conditions, and this is what makes them so versatile. On the down side, they are slower than a spinning disk microscope (but still fast enough to image cellular processes) and tend to be much more expensive. A spinning disk microscope can be purchased for approximately $150,000. A high-end scanning confocal system can cost upwards of $500,000 and can top more than $1 million, depending on hardware options.

Fluorescence Microscopy and Confocal Methods

Confocal microscopy primarily is an advanced fluorescence microscope that captures imaging data from the emissions of fluorescent probes (fluorophores). As such, understanding confocal techniques requires a thorough grasp of the fundamental of fluorescence microscopy, not only from the perspective of the detection hardware and software, but also from the more basic idea of experimental design. There is additional information shared about fluorescence microscopy in Chapter 9. Understanding fluorescence starts with looking at emission curves and the electromagnetic spectrum (Figure 10.2).

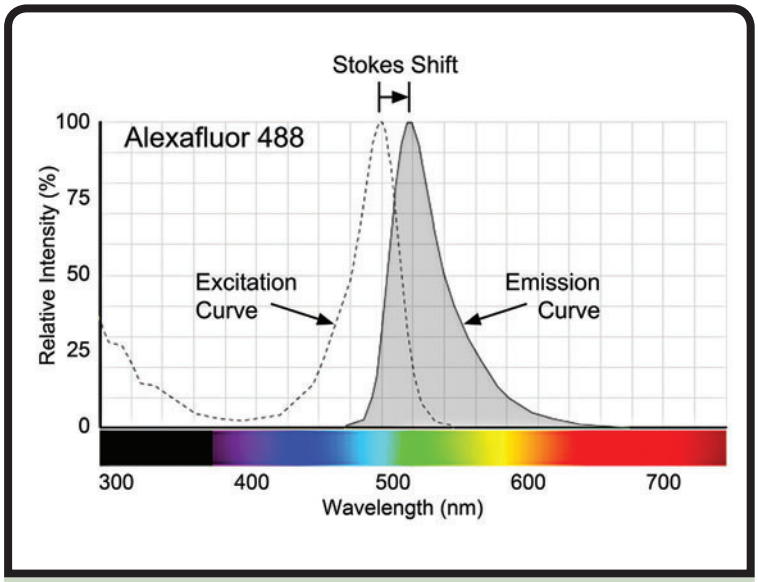


Figure 10.2 This figure shares the fluorescence curves for Alexafluor 488. Every fluorophore has a unique set of characteristic curves showing the excitation wavelengths (dotted line) and emission wavelengths (solid line). The fluorophore can be excited at any wavelength along the dotted curve, but the higher the intensity value, the more efficient the excitation. Once excited, the fluorophore emits light at varying levels of intensity within the range of the emission curve.

When fluorescent molecules are incorporated into a specimen, it is visualized in a fairly straightforward way. Generally, high energy, low wavelength photons (colors toward the blue end of the spectrum) are used to excite a fluorophore. In the process, some energy is lost and the rest is emitted as a lower energy, longer wavelength energy (colors shifting to the right, or red direction, of the spectrum). A fluorophore can be excited to a greater or lesser extent with wavelengths that fall anywhere along its particular excitation curve (dotted line) and it emits light everywhere along its emission curve (solid line); see Figure 10.2. The peak of each curve represents the wavelengths of light that are most efficient in the overall process. The excitation peak (or maxima) is generally the single best wavelength to use for exciting a particular fluorophore and the emission peak is the single wavelength that will emit at the brightest intensity. The difference between these two peaks is referred to as a Stokes shift. The wider the shift of the curves, the better the signal's separation will be. Every fluorophore will have a unique plot and these would be available from their manufacturers. There are also excellent resources available that greatly help to bring this information all together in one place. ThermoFisher Scientific makes an interactive spectral viewer that was first developed by Molecular Probes (https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html). This is an excellent resource and is frequently updated to include new fluorescent markers available from their extensive portfolio, as well as sharing the outcomes for other major fluorescent proteins.

When considering individual wavelengths, it can be useful to take into account the specific colors they represent. What is most important, though, are the wavelengths that are in question. This is because object data is defined by the numerical value of the wavelengths, not the appearance of the colors or how they look. The general rule is that a wavelength

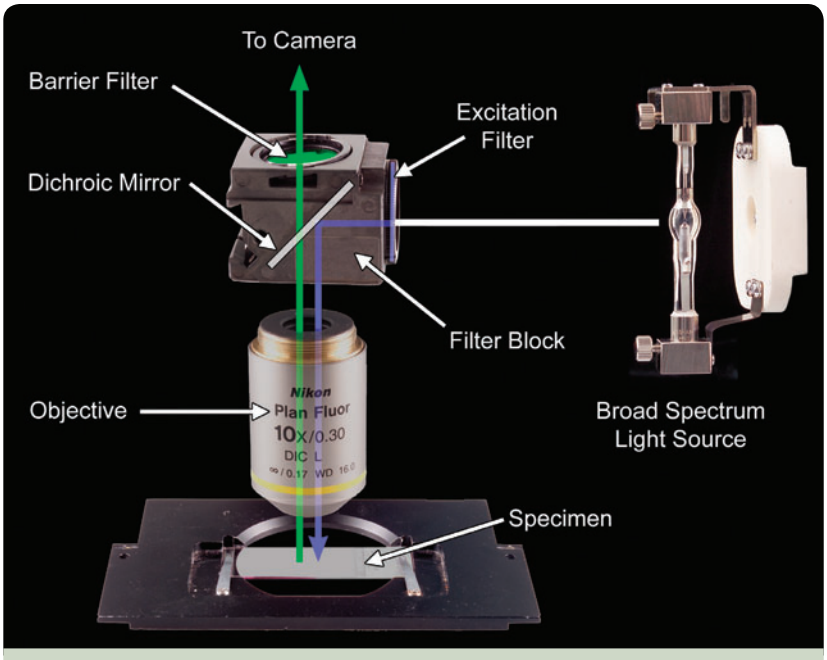


Figure 10.3 This figure shares the basic illumination and imaging pathway for fluorescence microscopy. Imaging should take place in a darkened room. White light from an illuminator is directed into a fluorescence filter block, sometimes called a cube, which is placed in a microscope's imaging pathway. The excitation light (blue line) is reflected by a dichroic mirror down to the objective, and then to the specimen, which emits a fluorescent signal (green line). The emitted fluorescence light passes back through the dichroic and is attenuated by a barrier filter. The remaining light continues through the microscope to be recorded by the imaging system.

of a lower number (such as 488 nm) will create excitation with a higher wavelength (such as 525 nm). From a practical point of view, though, it can be useful to consider the general colors in question. When light of a particular color is observed striking the sample, anticipating what color will be produced and what will be seen can help in interpreting the results. Violet or ultraviolet light will create blue fluorescence, blue light creates green emissions, green excitation will result in red emissions, and red light will fluoresce in the far-red range. This shift in wavelengths and colors separates the light used to excite the molecule from the emission or fluorescence used for imaging. Only the light coming from the fluorescence emission is actually detected. Often multiple fluorophores are used simultaneously in an experiment, and keeping the wavelengths separated is one of many challenges in any type of fluorescence experiment. A typical widefield fluorescence system will use filter blocks to separate the excitation light and the emission signal (Figure 10.3).

Light from a continuous white-light source, such as a traditional mercury or xenon arc lamp or more modern LED options, is focused in the microscope to a filter block designed for use with a specific fluorophore. The white light first passes through an excitation filter, which removes all wavelengths except those of specific interest to be used for excitation. The example in Figure 10.3 illustrates the filter set for a green fluorophore such as Alexa 488, FITC or Green Fluorescent Protein. In this example, a blue excitation filter transmits wavelengths between 450 and 490 nm. All other wavelengths are absorbed by the filter, which effectively reduces the original intensity significantly. The excitation light then interacts with a dichroic mirror designed to reflect certain wavelengths but pass others. In this example, it reflects light below 490–500 nm so the blue light is reflected down through the objective and used to illuminate the entire cell. Light will be absorbed in this process. Within the cell, all of the fluorophores of interest are excited by the blue light and emit (in this case) green light. That light is emitted in all directions and only the light that is directed toward the objective is actually collected (again, more light is lost). The emitted light travels back through the objective and encounters the dichroic mirror again, but in this travel direction, it will pass wavelengths above 500 nm and the green light from the signal continues through (again, there is energy loss at this stage). Any reflected blue light bouncing around in the system is blocked by the dichroic mirror, which reflects any blue

wavelengths traveling in this direction. The emitted light continues up through the filter block to the barrier or emission filter, which allows only certain wavelengths of green light to pass. In this case, between 500 and 550 nm is transmitted to the eye or the imaging system. In a widefield system, both focused and out-of-focus light is transmitted to the eyepieces or the camera. Some areas of the image will be sharply focused and some will be blurry and it will all be blended together.

Some confocal systems use a basic filter cube technology, but most use monochromatic laser lines instead of white-light and excitation filters, and the dichroic filters that are used have been designed to provide a much more efficient use of light. Many incorporate an electronic device called an AOBS (acousto-optical beam splitter) instead of a dichroic mirror, which uses an electronic signal through a crystal to precisely isolate wavelengths for better spectral separation. Finally, instead of a barrier filter, tunable detectors can be set to record signals from specific wavelengths and are adjustable in one-nanometer increments.

Fluorescent Markers

Fluorescent molecules are integrated into an experiment in a variety of ways, including direct and indirect staining with fluorescent dyes as well as genetic modification with fluorescent protein markers. There are common dyes that provide a direct conjugation to specific biological parts such as DAPI (4',6-diamidino-2-phenylindole). This is a direct stain that binds strongly to DNA and is excited with ultraviolet radiation in the 350–405 nm range. It emits blue light that peaks around 456 nm. Other dyes have also been developed that incorporate directly into the normal function of cells, such as lipophillic stains that label the cell membrane and others that target the mitochondria, lysosomes, or the endoplasmic reticulum. Most of these dyes are available with various fluorescence spectra, meaning there are versions that can be chosen and based on the light that they will emit, such as green, red, and far red. By mixing and matching different probes, cells can be labeled with multiple markers simultaneously, making it possible to explore the spatial relationships between intercellular organelles.

When direct staining is not possible, indirect approaches must be used. Indirect labeling is most commonly done with immunological staining. With this approach, a specific part of a cell is targeted with an antibody, which is called the primary antibody. A secondary antibody is then created which recognizes the primary antibody and attaches to it. The other end of the secondary includes the fluorescent marker. In this way, specific parts of a cell can be indirectly labeled with specific fluorophores. As was the case with direct labeling, there are a variety of color combinations available, making it possible to label more than one chosen target at a time. Most of the constructs for indirect labeling are standardized and available commercially. Molecular Probes, originally a small company in Oregon and now part of ThermoFisher Scientific, has been instrumental in developing these reagents and labeling techniques, which allow a scientist to simply mix and match primaries and secondary dyes for specific needs. ThermoFisher® has made the Molecular Probes handbook available online at https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook.html. This is an excellent resource for understanding and planning fluorescence experiments.

A third method for integrating fluorescent markers into cells is by using fluorescent proteins. One of the disadvantages of attaching dyes to cells with either direct or indirect techniques is that the process is often toxic to the cell being labeled. Studying real-time processes in live cells requires that those cells not be harmed or even slightly modified in their normal reactions, or the results would be useless. With fluorescent proteins, the signal is essentially built into the DNA of the cell in question, making it possible to visualize a fluorescent signal from a known location just by exciting with the correct wavelength. The DNA sequence for GFP (green fluorescent protein) can be temporarily introduced using a plasmid construct, or permanently incorporated into an organism's DNA using transgenic techniques. The discovery and application of GFP in the early 1990s was such a major discovery in microscopy techniques that it changed the playing field. Three scientists, Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien, were awarded the Nobel Prize for Chemistry in 2008 in recognition of their work and the impact that GFP had on scientific research.

Choosing and Working with Fluorophores

Every fluorophore—whether a secondary dye or an integrated protein—is excited by specific wavelengths of light and will emit—as fluorescence—other specific wavelengths. However, these emissions are not limited to single wavelengths. Excitation and emission will generally occur over a wide range of wavelengths, with greater efficiencies exhibited at specific peaks. Because of these general ranges, one of the first issues to address when designing and implementing a fluorescence experiment is whether the wavelengths of a chosen fluorophores might somehow overlap and interfere with each other. To assist in such determinations, it is important to understand how to interpret overlapping excitation and emission curves (Figure 10.4).

Figure 10.4A illustrates the relationship of three common probes used in confocal experiments, DAPI, Alexa 488, and Alexa 568. These three are often chosen because they exhibit good separation of their signals. Notice the three distinct peaks of the well-separated excitation curves and the three separated peaks of the emission curves as well. Unfortunately, also notice the regions near to the bases of the curves where there are overlapping areas. Under the DAPI excitation curve, there is a good range for both the Alexa 488 and 568 excitations, and under the Alexa 488 excitation curve, there is an area that overlaps with Alexa 568 as well. For excitation, this means that it is important to find precise laser lines that will excite only one fluorophore at a time, if possible. If more than one is excited, care must be taken to only record the part of the signal known to come from each dye separately. Looking at the overlapping emission curves, it can be seen that the DAPI signal emits across the entire curve of Alexa 488 and even into the Alexa 568, just at a lower intensity. For imaging, this means that these dyes must be imaged separately because any photons that come from the overlapping regions cannot be easily separated. There is a small amount of overlap between the Alexa 488 and 568 as well, but it is more manageable than the DAPI.

Efficiency of the excitation wavelengths will also play a role in how much a fluorophore will emit. Scanning confocal microscopes generally use laser light sources that typically have a limited number of laser lines available. The excitation curves in Figure 10.4B show how strongly a particular fluorescent probe will be excited using a specific laser line. The line

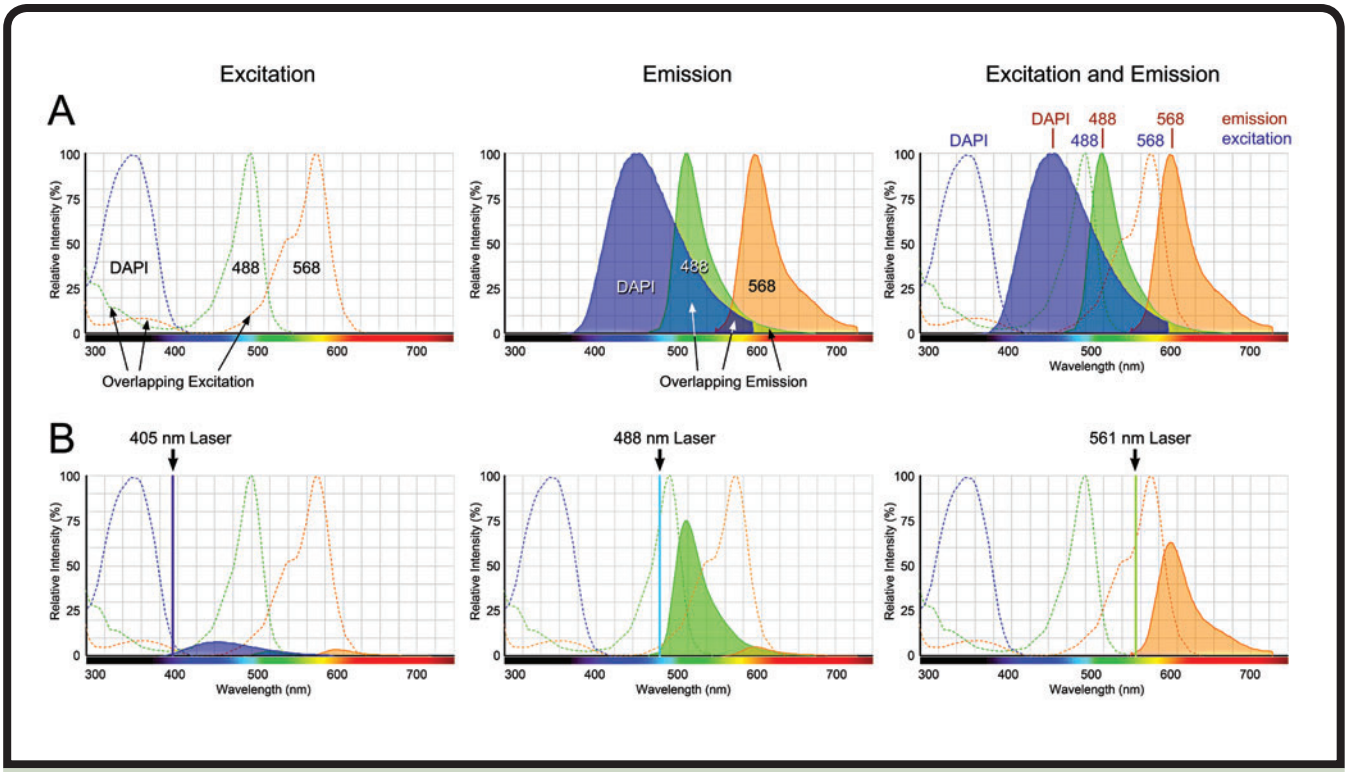


Figure 10.4 This illustration shares examples of interacting fluorescence curves. Excitation and emission curves for a combination of DAPI, Alexa 488, and Alexa 568 (A) demonstrate the range of wavelengths at which specific fluorophores can be excited (dotted lines), and then the range they will emit a signal (solid lines and color blocks). Areas of overlap (arrows) must be considered when using such combinations. The choice of excitation laser will greatly influence the efficiency and overall brightness of the emission (B) as demonstrated by the lower emission peaks shown when the signal is normalized to a specific laser line.

that comes closest to the peak of an excitation curve is generally used. In the case of DAPI, the peak excitation is around 350 nm, which is in the ultraviolet range. Unfortunately, most microscope optical systems will have difficulty with these wavelengths and most optical elements in an instrument will not transmit these wavelengths. It was found that DAPI creates a very bright fluorescence so, even if it is excited with low efficiency, it will still be sufficient to create enough signal to work with. For this reason, a 405 nm laser is used. Notice, however, that the 405 wavelength is extremely low on the DAPI excitation curve and is about the same as the overlapping Alexa curves, so a 405 nm laser will excite all three probes, but at a low level. The overall emission of each is shown in the solid color curves. Examining the relative emission intensities of each probe, it can be seen that they are all about the same, so if you can see one, you can see them all.

Excitation with the 488 laser is shown in the second part of Figure 10.4B. Notice that the peak for Alexa 488 is actually around 498 nm, so the 488 nm laser does not create the most efficient emission. However, the emission level is about 75 percent and Alexa 488 is a very bright fluorophore, so this is sufficient. The 488 laser also excites Alexa 568 to a smaller degree, but because it is of such a low brightness, it can effectively be removed by using the instrument's threshold control. Finally, in this example, there is no 568 laser available, so a

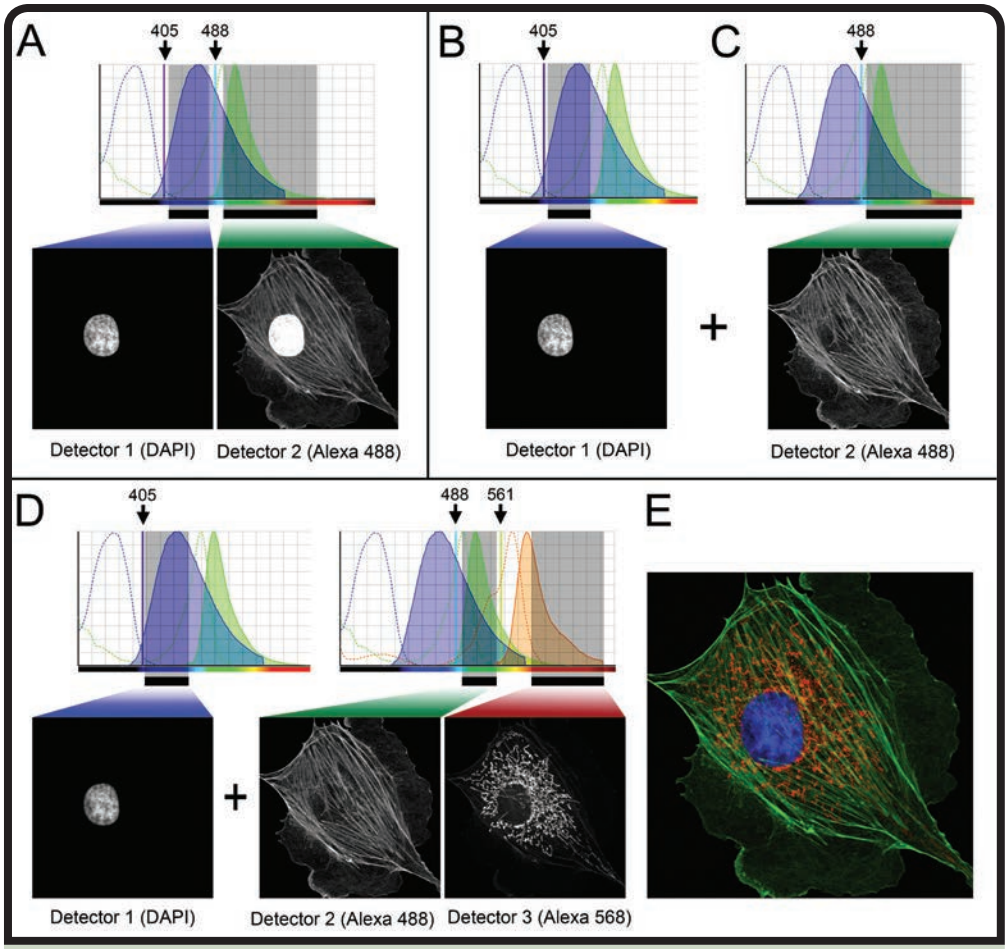


Figure 10.5 In this illustration, simultaneous versus sequential scanning in a triple-labeled cell is evident. Interference from overlapping signals creates mixed signals in Detector 2 (A) when DAPI and Alexa 488 signals are captured simultaneously. When the same sample is scanned for each channel separately in sequence (B and C), the overlapping DAPI signal from the nucleus (B) no longer appears with the Alexa 488 signal from microtubules (C). A combination sequence which includes DAPI by itself and Alexa 488 and Alexa 568 simultaneously (D) provides sufficient separation of the three signals to create the final color composite (E).

561 line is used instead. The 561 laser creates emission in only the Alexa 568 signal, even though it is also less than ideal.

Figure 10.5 adds an actual imaging situation to the numbers discussed in Figure 10.4. Figure 10.5A shows the effect of setting the DAPI and Alexa 488 detection together using both a 405 nm laser and a 488 nm laser line. Detector 1 (the thick black line under the curve) is set to read emissions from a range just past the 405 laser line to just before the start of the green emission curve, so the only signal it will detect comes from the DAPI-labeled nucleus. Detector 2 is set to read emissions from just past the 488 laser line, up to the end of the green emission curve. The area under Detector 2, however, shows that it falls within the emission range of both the DAPI and Alexa 488 emission curves so, because both signals are emitting at the same time, the resulting image is composed of

signals from both the green Alexa 488 as well as the blue DAPI fluorophores. The detector only sees light intensity, not colors, so it all blends together and shows an overexposed oval in the middle of the cell, which is actually the DAPI signal bleeding through. If the scans are done in sequence, exciting only DAPI with the 405 laser and reading only DAPI with Detector 1 (Figure 10.5B), then exciting only Alexa 488 with the 488 laser and reading only the green emissions (Figure 10.5C), there is no interference and the Alexa 488 signal from the microtubules will be seen without the interference from the DAPI signal. Having determined that the DAPI channel must be imaged by itself, attention can then be given to the relationship between Alexa 488 and Alexa 568 (Figure 10.5D). These two fluorophores show much better separation of their emission signals and can be imaged simultaneously. The 488 laser excites Alexa 488 efficiently and Detector 2 can be placed just past the laser line and set up to the beginning of the Alexa 568 emission curve, assuring that the only signal Detector 2 will image will be coming from Alexa 488. There is a bit of overlap where the 488 and 568 curves meet, however, so Detector 3 is placed a little further away, starting at a point where there is very low 488 signal but still high 568 signal. Detector 3 then sees mostly the Alexa 568 signal of mitochondria with a very low but acceptable bleed-through from the 488 line. The final part of the process is to add a color look-up table (LUT) to each of the monochrome channels to create the three-color composite image (Figure 10.5E).

With a thorough understanding of how the curves work, it will be easier to choose the most effective fluorophores for an experiment. Probes with the best separation should be chosen to avoid signal bleed-through, using the curves as a guide. There are other factors to consider as well. For instance, longer wavelengths (toward red) will penetrate deeper into tissue and impart less damaging energy on live cells, so fluorophores that emit around 647 nm can be useful for those purposes. Also, fluorophores should be chosen that have minimal bleaching characteristics, like the Alexa series of dyes from Molecular Probes or the ATTO dyes from Sigma-Aldrich. These probes were designed for excitation specifically by lasers and the number in the name indicates the excitation maxima.

How a Confocal Microscope Works

When first looking at a scanning confocal system, it doesn't appear all that different from a widefield microscope. There is a regular upright or inverted microscope at the core of the system, but what fills up the rest of the room are the lasers, the scanners, the detectors, environmental controls, and the large computer/monitor combination that runs it all. Many confocal microscopes are built using an inverted platform that provides the most flexible imaging options. With an inverted system, typical glass slides can be used (mounted upside down), or any type of plate, flask or dish where cells are grown. One of the primary reasons for using a confocal microscope, however, is to obtain high magnification/high resolution images. This requires the use of high numerical aperture objectives. Immersion optics (oil or glycerin) are commonly used and the working distance to the specimen requires special dishes with coverslip bottoms when imaging live cells at high magnifications. Once the specimen is placed on the microscope stage, the operator first uses the base microscope as a regular widefield system, with the white-light excitation source and fluorescent filter blocks, to look around and find any areas of interest. Once a site has been selected, the lasers

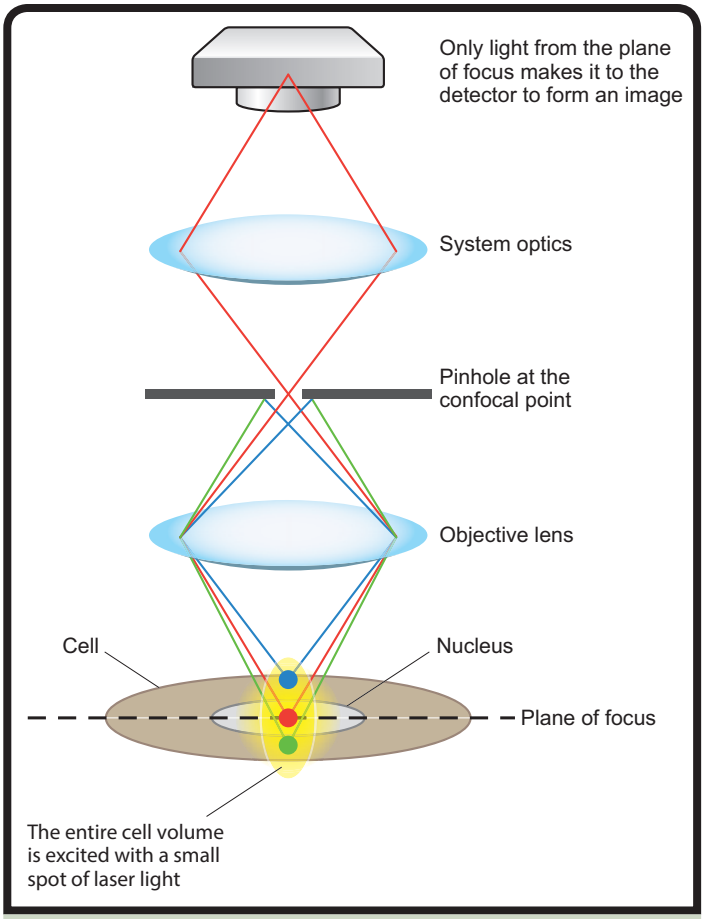


Figure 10.6 In this illustration, a simple ray diagram shares the basic confocal principles. A singular spot of light is scanned across a cell, bathing the entire volume in excitation light and creating fluorescence emissions from the labeled parts of the sample. In a confocal microscope, only light from the plane of focus passes through a pinhole and is recorded (red line). The pinhole is placed at a nodal point that is confocal to the plane of focus. Light that starts from a point above the plane of focus (blue line) focuses below the plane of the pinhole, crosses over, and is removed from the imaging path. Likewise, light emanating from below the plane of focus (green line) impacts the pinhole before it comes together to form an image and is also removed from the imaging pathway.

are used to create the image. Often the light cannot be viewed directly, so the eyepieces are ignored and the microscope is run remotely using the computer interface. From this point forward, all decisions about the imaging parameters of the experiment are decided by and controlled using the instrument's software.

The real trick for the operation of a confocal microscope is to eliminate the out-of-focus light from an image in order to create a very sharply focused image plane. Only light coming from the plane of focus should make it to the detectors. The beating heart or the core of every confocal microscope which makes the formation of an image with continuous focus is the pinhole. Light emanating from the specimen is essentially passed through the pinhole and only the light in sharp focus actually passes through the pinhole and is recorded as an image. Any out-of-focus light is essentially removed from the imaging pathway and isn't observed or recorded. Figure 10.6 is a very simplified diagram illustrating how this happens. A common misconception when first working with a confocal microscope is that only the part of the cell being visualized is being illuminated and affected by the excitation light. In reality, the entire volume of the cell is being illuminated but only part of the resulting emission is being seen.

In Figure 10.6, the light paths from three different locations in the cell are color-coded red, green, and blue. Consider that all three of these locations would actually be emitting the same wavelengths of light, depending on the fluorophore being excited. In this example, the microscope is focused on a point that passes through the middle of the cell, through the middle of the nucleus. This illustration shows a point of light directly above the nuclear spot, located on the cell membrane (the blue path) and a second location directly below the nuclear spot in the middle of the cytoplasm (the green path). When observed from above with a widefield system, the light from all three locations would overlap and be imaged as one blurry spot because the out-of-focus parts would be the widest part of the signal. In confocal microscopes, however, the pinhole is placed at a nodal point in the optical pathway that is confocal to the plane of focus—that is, the sharp plane of focus on the nuclear spot is brought together and focused at that particular nodal point. Following the ray diagram, it can be seen that the light from the nuclear spot (the red path) makes it through the pinhole unimpeded and continues to the detector to become part of the image. The light from the blue ray, however, actually

focuses to a point below the pinhole and begins to spread again as it approaches the pinhole. This light is then blocked by the structure and never makes it the rest of the way through the microscope. Likewise, the green ray from the spot below the nucleus would actually come together to focus on the far side of the pinhole, but the light is blocked before it ever gets there. In this fashion, the pinhole only allows the light from the plane of focus to pass and creates an image with the out-of-focus light removed.

Balance and Compromises Required for Forming a Good 2D Image

Making good images with a confocal microscope is best accomplished by understanding all of the available tools and balancing the settings, providing the best compromise between a large, high quality, smooth, noise-free image, which takes a long time to scan or a smaller, noisier, weaker image, which retains all of the necessary scientific data. Between the hardware options and software selections, choices must be made necessary to arrive at the image that provides the best result with the least amount of problems.

When a scientist photographer sets up a microscope and chooses specific settings for an exposure, they make choices using the hardware that will affect how the final image will look. If the exposure is too dark or light, changes must be made to improve the exposure. For example, if an image is too dark and needs to be brightened, there are multiple ways to do this. However, each of the choices comes with advantages and disadvantages that affect the final result. If the exposure time is lengthened, then sample movement can become a problem by causing blurring. If the aperture is opened wider, it changes the desired depth of field. If the ISO is increased to make the camera more sensitive, the image can get noisier. If more light is added to a studio set-up, it may cook the sample. A final image will always be a balance of many factors that go into making that image. Compromises must be evaluated and decisions made about what provides the best approach needed to achieve the desired result.

Working with a confocal microscope can be much like this example shared above, except that many of the starting parts of the experiment are initially unknown and their effects are not always fully understood until the experiment has begun. With complex systems, decisions about the choice of fluorescent markers is one of the first decisions that needs to be made and based on factors described above. Even those decisions must be balanced with the knowledge of how a microscope works. For example, if a system has a 561 laser and the choice of fluorophores includes Alexa 555, or 568, then the best choice would probably be 568 because both fluorophores fall at about the same intensity levels on the emission curves, but the 568 would be better separated from any green fluorophores. There are many other decisions to be made; however, not every system will have all of the choices. Hardware choices are generally determined at purchase and cannot always be changed later. For this reason, understanding the choices that are available is vitally important. Some instruments may have other settings as well. Often, the best options become mutually exclusive to one another and the best compromises must be determined. The main lesson here is to focus on how each option affects the end result and make the best compromise possible that is useful to acquire the most accurate data.

Hardware Choices

Upright or Inverted?

A scanning confocal system can be attached to either an upright or inverted microscope, depending on the needs of the user. If a fixed specimen is located on glass slide and the sample is to be studied, an upright configuration will be fine. However, if ever the specimens of interest are live cells labeled with fluorescent proteins and are growing in a dish of some kind, then an inverted microscope is required. Most spinning disk systems are built on inverted bases because their primary advantage is found with imaging of live cells. The advantage of an inverted system is that glass slides can also be viewed using it, as long as they are turned upside down where the coverslip is located closest to the objective under the stage. This flexibility means that almost any sample can somehow be imaged. There are disadvantages to an inverted microscope as well. Because one of the main purposes of confocal is to image at high magnification, immersion lenses are usually required. Performing oil immersion work on an inverted base is challenging. The dishes used for live cells must have coverslips in their bottoms so that working distances are matched for objective lenses. This can make it tricky to set the dish before the oil, water, or glycerin flows away. Live cells also generally require some kind of environmental chamber to maintain biologic viability while imaging, especially for long-term time-lapse studies. A chamber can encase the entire microscope and bathe the sample in 37-degree Celsius, 5 percent CO^2 humidified air, or it can be a small stage-top system that does the same thing on a smaller scale. If fast live cell imaging is required, a scanning system is still desired and there is also the option of a much faster resonant scanner that can be added. Although fast, these scanners tend to be very noisy as well, so they come with some practical limitations.

Lasers

One fixed aspect of a confocal microscope that cannot be easily changed is the selection and purchase of the laser lines. There are many choices available, but the microscope generally has a limited number of available ports to use, so care must be taken when purchasing a new instrument to choose an adequate grouping. Lasers come in a variety of types, like large gas lasers—especially an Argon laser—that can often incorporate multiple lines from one source. Other lasers, such as helium–neon or diode lasers, can be smaller and cheaper, but only offer one specific line. A good selection would generally include excitation with a 405 nm laser for DAPI excitation, as this is specifically used to label nuclei in many experiments. An Argon laser will provide five lines to excite in the blue range for green fluorophores, including 458, 476, 488, 496, and 514 nm lines. Adjacent to the blue laser would be a green laser used to support red fluorophores as needed, which could be either a 555, 561, 568, or 594 laser. Finally, a far-red choice is also practical, which could be a 633 or 647 nm laser. If money is no object, a white-light laser is a good option. This is a continuously adjustable laser source that allows the operator to tune it to any wavelength within its working range. The super-continuum white-light laser from Leica Microsystems allows the user to choose from 470 to 670 nm in 1 nm increments with up to eight lines employed simultaneously. The choice of lasers will boil down to cost and available ports, but the more that can be had, the more flexible the system.

Detectors

Imaging detectors in a confocal microscope are also an important option to consider when first setting up an instrument. For as much as the parts of this chapter have mentioned specific colors, confocal detectors are highly sensitive black and white devices designed to be sensitive to specific wavelengths of light. It is the wavelength that defines the signal, not the color, because the wavelength is more quantitative. Color in a confocal image is displayed as a chosen color from a look-up table (LUT) applied to a grayscale original. For spinning disk systems that require fast exposures and high sensitivity, there are a variety of CCD cameras that are used. Electron multiplying charged couple device (EMCCD) cameras are a current high-end favorite, but scientific CMOS cameras are becoming more sensitive as the technology continues to develop. In scanning confocal systems, the default detectors are usually photomultiplier tubes (PMTs), which do an adequate job of recording the signal, but there are better, more expensive options available as well. Gallium arsenide photomultipliers (GaAsP) detectors are more sensitive and have better contrast, but come with higher prices. Some confocal microscopes use linear arrays that split an incoming signal into discrete chunks of wavelengths to provide spectral separation of the signal. Others employ an adjustable detector that can be set to any range of wavelengths desired. Understanding how the detectors work in a specific system will be important to get the most signal out of any instrument.

Unlike when photographing a color sample with a one-shot color camera, fluorescence signals are captured in one wavelength range at a time when using a single monochromatic detector. For this reason, it would actually be possible to get by with only a single detector built into a system that could be adjusted for each condition. Each channel could be taken in sequence and then combined to form a final image. Usually, however, there are multiple detectors so that different signals can be acquired simultaneously and/or different types of detectors can be employed. A series of three PMT detectors combined with two interspersed GaAsP detectors is a common configuration in high-end systems.

Overview of Instrument Controls and Software

Laser Power

Overall light intensity is a large problem in any type of fluorescence imaging. If the light is too intense, it will damage the specimen in multiple ways. The most obvious result is bleaching of the fluorescent signal. More subtle effects include DNA damage in live cells or other phototoxic effects that will kill the very sample that is trying to be imaged. Sometimes, too much light will just affect the individual behavior of a cell for unknown reasons. In a confocal system, especially one that uses lasers, this problem is intensified because the lasers can be very strong. If a signal is weak, it can be tempting to just turn up the light intensity to make the exposure, but using this strategy can make the specimen unusable and so other approaches must be considered. The higher the power, the more light is available for imaging, which comes with the potential to damage the specimen. Microscope software will have option settings for laser intensity that uses a sliding scale from 0 to 100 percent. Different laser lines all come with different power levels, so it is important to know

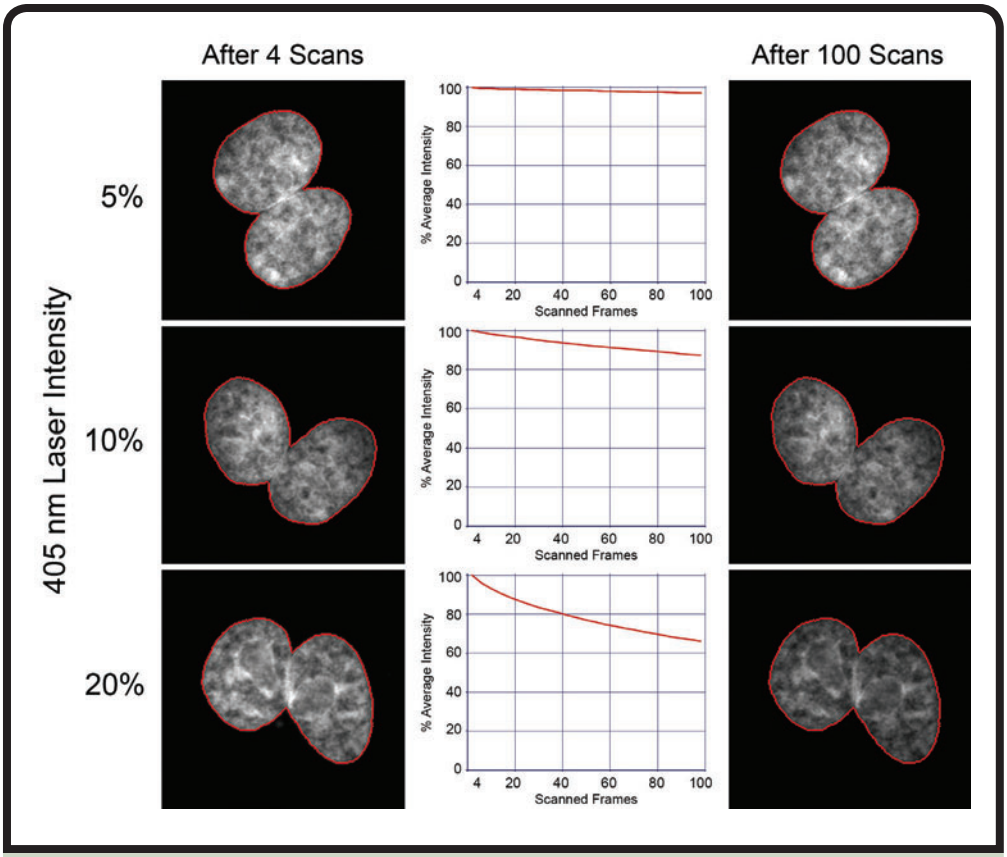


Figure 10.7 This figure shows how increased specimen bleaching can be caused by laser intensity. Three separate pairs of DAPI-labeled nuclei were imaged using 5, 10 or 20 percent laser intensity settings on a Leica SP5 II confocal microscope using a 50 milliwatt 405 nm violet laser. For each location, a series of twenty-five images was collected over time, and each image was created from an average of four scans. The data was collected from the average pixel intensity within the outlined nuclei. The graphs show the amount of signal bleaching as the number of scans increased. The first image in each series (after four scans) was used to normalize the data to 100 percent, with the final image in the series demonstrating the amount of signal degradation after a hundred scans.

the laser's strength as it enters a microscope. An intensity percentage should be chosen that keeps the total power at the specimen at less than 1 milliwatt if possible (and that is pushing it). Initial tests with a sample will determine how robust the signal is and whether even this level would be too much. Figure 10.7 illustrates the effect of increasing laser intensity on a signal. In the first example, a low 5 percent intensity level was used and the sample still faded by 3 percent after 100 scans. A 10 percent setting resulted in a 13 percent drop and a 20 percent laser level created a loss of 34 percent. Generally, once a fluorescent molecule has faded or quenched, it does not regain its intensity. Note that the results are not linear, and twice the power does not create twice the bleaching. Also realize that *any* scan will bleach the sample, whether imaging is actually taking place or not. Just looking at a sample and allowing the system to continuously scan will adversely affect the results and make quantitative measurements invalid.

Detector Settings

The first choice for detection is to choose which detector type to use. Photomultiplier tubes (PMTs) tend to be less sensitive and have less contrast. This performance is useful for brighter signals with a wide dynamic range, like DAPI stained nuclei. GaAsP detectors are much more sensitive and can be used for quantitative photon counting, but tend to be higher in contrast. They are particularly good for small, weak signals. Following the choice of which sensor will be used for viewing/imaging, the wavelength range for the detector must be set. Using the excitation and emission curves as a guide, the lower wavelength should be set so that it does not get too close to the laser line being used. Setting a detector to include the excitation laser wavelength would be like looking directly at the sun and could damage the sensitive electronics of the detector. The lower value should be set 5–10 nm above the laser line to avoid this problem. Ideally, the detector range should be spread out to cover the entire emission range of the fluorophore to collect every possible photon being emitted. In practice, however, "bleed-through" from one signal into another is a constant problem and the range should be restricted to avoid this (see Figure 10.5). The upper limit should be set to include the major portion of the emission curve for the fluorophore, but not extended into the emission range of another fluorophore that is being used. The detector sees only light intensity, so if set at a point where photons from more than one fluorophore could be emitted, it would display both as part of the same image, which causes the signals to overlap.

Simultaneous or Sequential Acquisition

Scanning confocal microscopes have the ability to capture individual channels either one at a time, all at once, or in combinations. Simultaneous acquisition can capture multiple channels faster, but detectors have to avoid bleed-through signals by restricting their detection range. Sometimes bleed-through (cross-talk) cannot be avoided and sequential capture must be used. Sequential imaging provides a cleaner and more pure signal with optimal detection, but takes longer (see Figure 10.5).

Gain and Offset

Setting gain is basically the same as changing the ISO when using a DSLR camera. Each detector has a range that can be set from low levels to high. The lower the gain value, the less sensitive the detection and the higher the image quality will remain. The higher the numerical value, the more sensitive the detection (weaker signals can be picked up with less intense light) but the more noise the image may display. The best setting is always a compromise between increased sensitivity and reduced noise and will vary depending on the sample and the other settings chosen in the software (Figure 10.8). Offset values set a black threshold level and help to adjust contrast levels with PMTs. While all of the referenced options will affect the image intensity, fine-tuning is often done using the gain. Achieving the overall proper intensity (exposure) can be determined by evaluating the entire image and avoiding the oversaturating of pixels.

Pinhole Size and Resolution

As previously mentioned, the pinhole is the main element for creating thin visual image slices through a sample in a confocal microscope. With scanning systems, the size of the pinhole is often adjustable. Referring to Figure 10.6, it can be observed that if the pinhole is made larger, it allows more light to pass through from areas above and below the plane of focus. This in turn would effectively increase the thickness of the slice being viewed and lead to a more blurry image that is produced from the inclusion of more out-of-focus light. More light would be useful for weak samples, but it would come at the cost of resolution. A smaller pinhole, on the other hand, would let through less light but it would also create a thinner slice, making an image with finer visible detail (Figure 10.8). The basic starting point for the pinhole size is 1 Airy unit (AU). This value is determined by the software and takes into account the objective used, the zoom settings, and some other factors built into the microscope. The pinhole also helps to define the visible resolution. Resolution is defined as the closest that two points of light can be measured and still be discerned as separate points of light. However, a point of light is actually a small circle of light called an Airy disk, and when two disks are so close that they intersect, the individual points effectively merge into a single, blurry dot. The best theoretical resolution is roughly 200 nm using 520 nm (green) light, based on Abbe's diffraction theory, but pushing the confocal to its limits can often reduce that level to 150–180 nm.

Spatial Resolution/Format

Spatial resolution is defined by the physical size of the image in pixels. A smaller image size will capture images faster but creates a more jagged or bitmapped result because it is composed of fewer pixels. Larger image sizes produce smoother, better-looking images but take longer to record. If the point of the image is data collection, then the pixel size is the most important point. The size needs to be only big enough to show the information. Generally, a pixel with an equivalence that is less than 80 nm is considered oversampling and is unnecessary. However, if the point of the image is to capture a beautiful image for display on a journal cover, then larger is better. From a practical point of view, it is often useful to use a preview scan at a lower value, such as 512 x 512, taking advantage of the faster scanning, but then switch to a higher value like 1024 x 1024 for capturing the image.

Scanning Speed

In a scanning system, a small dot of light is scanned across the sample and the fluorescence emission from that dot is recorded as it passes through the pinhole. The faster the scan goes, the faster the acquisition can be. The scan speed is usually displayed in Hz, with 400 Hz being a standard starting point. It is reasonable to think of 1 Hz as one line per second, so the higher the number, the more lines that are scanned in one second. An image that is made up of 400 lines would take one second to scan at 400 Hz, but only 0.5 seconds at 800 Hz. Typical scanners can be as fast as 1800 Hz, but to go higher requires a special resonant scanner. Slower scan speeds increase the dwell time (the amount of time the dot of light stays on a specific point), which can damage the sample, but collects more information from that spot in the time it is there. Generally, faster scans are more desirable, to speed up

the imaging process. Unfortunately, the faster scan creates more noise in an image and can reduce the size of the area that can be scanned. Faster scan speed will equal a faster acquisition but more noise. Slower scan speed will equal less noise but longer dwell time, which can affect quenching and cell viability.

Bidirectional Scanning

Normally, a scanner works in one direction. It turns on, reads left to right across the image, then turns off and returns to the starting point. Next, it drops down one line and scans left to right again. With bidirectional scanning, the scanner works in both directions, reading left to right across, then dropping down and reading right to left on the way back. This setting makes it possible to capture the same image in half the time. The only drawback is that the lines can drift out of sync and have to be corrected occasionally.

Digital Zoom

Optimal image resolution depends on the objective used, the spatial resolution, the digital zoom, the pinhole size, the wavelengths used, and several other factors. The primary resolution of the system will be determined by the numerical aperture of the objective and additional factors that are affected by that resolution as a starting point. Zoom settings will enlarge the image, but cannot increase the resolution (Figure 10.8). If you image a small fuzzy dot, the zoom will make it a big fuzzy dot without adding any more information. This point is considered empty magnification. However, the confocal software can show a value for the pixel size and that can objectively be used to determine the optimal zoom setting. If increasing the zoom decreases the pixel value below 80 nm, then any additional increase in zoom is essentially useless.

Bit Depth

The default value in a confocal is usually 8-bit imaging (256 levels of grey), but should only be used for qualitative imaging. For quantitative results, higher bit depths (12, 14 or 16 bits/pixel) should be chosen. Higher bit depths result in larger individual files, so larger stacked images and/or time-lapse studies can become very large when using files with greater bit depths.

Averaging/Signal to Noise

Digital imaging captures numerical readouts from the photoelectric effects of light falling on electronic devices. When that light is generated from the sample, the resulting values are part of the signal. When a pixel is triggered in a transient way because of the electronics in the system, it is considered noise. The goal of a good digital image is to attain a high ratio of signal to noise which results in high contrast, low background and good tonal range. Many of the choices explained in this chapter generate noise and reduce the overall quality of the images, so it is useful to have ways to compensate and remove as much noise as possible. Averaging is one good way to do that (Figure 10.8). When reading a pixel scanned multiple times, if it always generates an intensity value then it is part of the signal. If instead it fires off sometimes, but not always, it is probably noise. Using

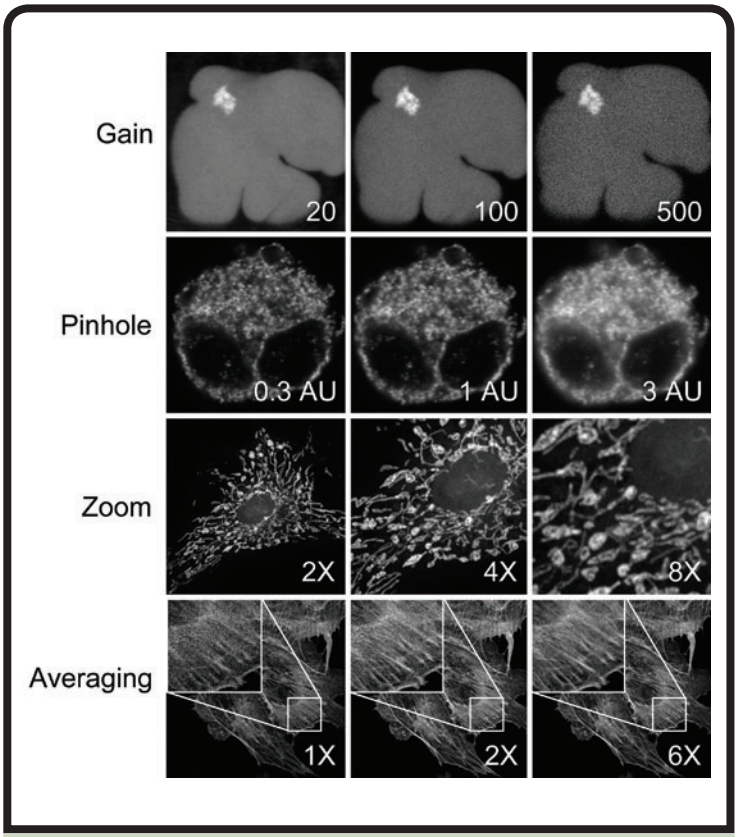


Figure 10.8 This figure shares how variable software settings can affect image quality. Every operator decision when establishing instrument settings requires that compromises will need to be made. In these examples, the gain settings on a hybrid GaAsP detector demonstrate increased noise with increasing gain. Likewise, increasing the size of the pinhole from 0.3 Airy units to 3 reduces overall resolution. Starting with a digital zoom of 2x and increasing to 4x provides additional information, but continuing to 8x just makes the small fuzzy dots into larger fuzzy dots—the definition of empty magnification. Scanning a frame 1x generally results in significant noise, but averaging more frames together enhances the signal and reduces the noise.

averaging, the more times the pixel is scanned, the more the noise value will be reduced, leaving the signal proportionally higher (higher S/N). In general, more averaging reduces more noise, but takes time.

There are two ways to average, either line by line or frame by frame. In line averaging, the microscope scans a line all the way across, and then again for as many times as selected, before dropping to the next line. Averaging then occurs in almost real time, so the image displayed on the screen is always the averaged view. In frame averaging, an entire image is scanned top to bottom, then scanned again and averaged along the way, then again for as many times as selected. Regardless of the approach, the total amount of time it takes is the same. Generally, every image should be averaged at least 2x to reduce any major noise. There is usually no additional benefit at 4x or 6x.

Accumulation

As with averaging, accumulation combines multiple scan passes together, but instead of averaging the numerical values of the pixels, it adds them together, increasing the total amount of intensity at each pixel as it does so. This results in an increased signal value, so a weak signal can be amplified, but it also increases background, so maintaining low background levels are important. Accumulation also works to reduce noise, but at the expense of an increased background level and the time it takes to do multiple scans.

3D Imaging

Once the skills are in place to capture good two-dimensional images, that knowledge can be applied to advantage, and the major reason for using a confocal microscope is to create three-dimensional data sets. The confocal has the unique ability to visually section, non-destructively, through a specimen taking clear, sharp slices along the way, much like an MRI can image through an entire human body. Those individual slices are then put together into stacks and displayed in a variety of ways to extract the three dimensional relationship of the fluorescent labels in the subject or just create sharper two-dimensional images. Single cells are often viewed in this way, but often larger tissues or cell aggregates are the subject of interest.

Imaging depth becomes part of the problem when trying to peer deeper into a specimen. The deeper the plane of focus goes, the harder it is to get a good image. First, the excitation light is diffracted and dispersed by the cellular components as it goes deeper into a tissue, resulting in less light for excitation farther down. The fluorescence signal then has just as hard of a time working its way out to be recorded by the detectors. Increasing the laser intensity will allow greater depth, but only to a point, and even then it will overexpose the fluorophores closer to the surface. Exactly how deep a confocal microscope can image depends in large part on the sample. A very dense sample, like bone, will be unforgiving and limit the depth to 50–100 microns (0.05–0.10 mm) at best. A much more transparent specimen, like brain tissue, might be penetrated 200–300 microns (0.2–0.3 mm). Longer wavelengths, like 647 nm, will penetrate the farthest, so fluorescent probes in this range should be used in experiments requiring significant depth.

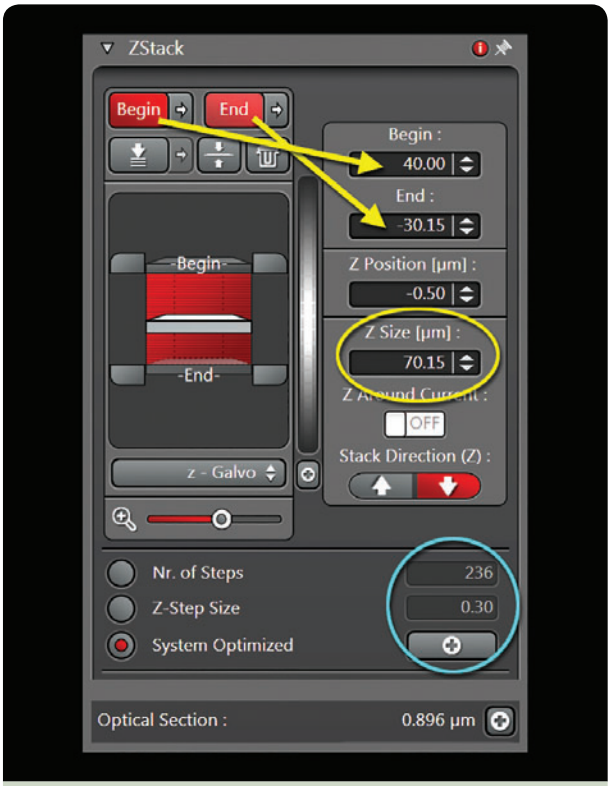


Figure 10.9 This figure shares the Z-stack settings panel from Leica LASX software. Basic controls for setting acquisition parameters in a Z-stack include first marking the top and bottom locations for the volume to be captured (arrows), which then displays the calculated depth of the stack (yellow circle). Next, choices about the slices, or steps, must be made (cyan circle). These values can be entered manually, based on the desired thickness, in a number of steps, or an optimized value can be chosen which is based on all current instrument settings.

To capture a Z-stack, it is a simple matter of using the software to set up a good set of imaging conditions for a single 2D image, then using the Z-stack controls to set the rest (Figure 10.9).

First, the starting point location is set in Z to begin the stack and a second Z location is chosen to end the stack. The software then calculates the total depth and provides options for the number and thickness of the desired slices that will make up the final stack (it is always a good idea to focus a little bit past both extremes to compensate for any vertical drift during imaging). Once the confocal microscope has calculated the optimum parameters, it can populate the controls with those values. This optimum is based on a complex formula that determines proper Nyquist sampling and factors in the NA of the objective, the wavelength being imaged, the Airy unit of the pinhole setting and the refractive index of the immersion fluid. The mathematical details of optimal sampling size can be a complex matter and go well beyond the scope of this chapter, but a good overall reference for confocal microscopy that discusses this idea and more of the complex statistical concepts is *The Handbook of Biological Confocal Microscopy* by James Pawley.

Having a button to set optimal parameters can be useful, but when clicking on the automatic, fill-in-the-blank buttons, care must be taken to make sure the theoretical aspects don't outweigh the practical applications. The example in Figure 10.9 shows that that the overall thickness of an area to image would be about 70 microns. An optimized stack would take 236 individual 0.30 micron slices. That is a lot of slices. Considering that it could take 30 seconds to image one good 2D slice, it would take 118 minutes to image the entire stack! Clearly, compromises have to be made or imaging sessions would be unreasonably long.

For quantitative methods, Nyquist is still the best, but for qualitative imaging, the 2.5x oversampling used by that approach could be overkill. In general, a stack with half of the suggested slices would be more than sufficient, especially if the end result will be a projected image. With half the slices, the example in Figure 10.9 would only need 118 sections and each would be 0.60 microns thick, which would cut the imaging time in half.

Considerations for Making a Good Z-Stack

A single 2D slice can be optimized to provide the highest resolution, the best noise reduction, the largest size, and the best spectral separation. To do all that, however, choices are made that favor quality imaging over speed. When the end result is a stack of images through a thick sample, speed becomes the overriding concern and it is easy to see how some small compromises can make big differences. For instance, if the decision was originally made to average each 2D image six times to get the best noise correction, then changing that to two times would cut the imaging time by two-thirds. The 118 minutes from this example for Figure 10.9 would become 40 minutes implementing that change alone. Combined with the decision to cut the time in half by making each slice 0.60 microns, the scanning time would be reduced to 20 minutes. Table 10.1 provides a guide to consider other starting parameters for 3D imaging.

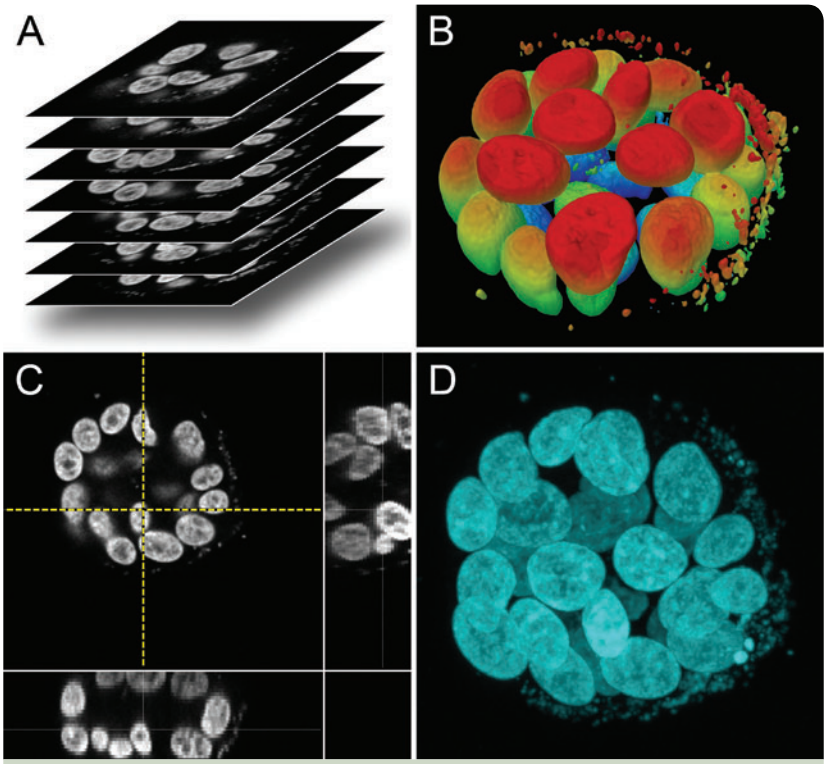


Figure 10.10 This figure shares the display of three-dimensional data. A three-dimensional data set is created from a series of individual 2D slices collected as a stack (A). The information can be parsed in various software and displayed as (B) a 3D reconstruction, including shadowing and depth-coded color, (C) an orthogonal projection showing 2D slices taken through the volume and viewed from three different directions, and (D) as a maximum projection image, where all of the 2D information is flattened down to one image.

Once all of the parameters are set, pushing the "Start" button will automatically begin the sequence, capture all the individual images, and save them into a single file. With no additional work, each image is still viewed as a single slice. There are multiple options available to display the information in a stack, and ultimately the choice will depend on the specific purpose of the images. However, the most common visualizations are 3D reconstructions, orthogonal views and maximum projections (Figure 10.10).

Additionally, the series of images can played as a video file, giving the visual effect of focusing through the specimen. The maximum projection (Figure 10.10D) is a 2D image created from the 3D stack. In the created image, the software polls each pixel location throughout the stack and uses the brightest pixel it finds. This is why it is so important to make sure that there are *no* saturated pixels in *any* of the image slices. The maximum projection provides

Table 10.1 Starting Parameters for 3D Imaging

Variable Option	Setting and Effect on 3D Data Set
Number of slices in the stack	Start with half of the optimized, Nyquist-based setting. The fewer slices, the faster it will be to acquire a stack, but the more jagged the vertical resolution in the Z dimension will be.
Thickness of a slice	Start with twice the optimized, Nyquist-based setting. If the software displays the thickness of an optical section, use that value as the upper limit. The stack slice should be smaller than the optical section or gaps will appear in the Z dimension.
Spatial resolution	Most of the time, an image at 512 x 512 pixels is sufficient to collect the 2D imaging data, so use this value or smaller with a stack.
Simultaneous/ sequential	Simultaneous scans are best if possible. When the software creates a sequence, it takes time to scan each sequence in turn and it takes time for the system to physically change the settings.
Averaging	Every image needs at least an average of two (either line or frame averaging can be used). When many images from a stack are combined, they have the effect of increasing the signal-to-noise ratio anyway, so two should be enough.
Accumulation	Avoid any accumulation—it adds time. Try to increase the light levels or detection efficiency instead.
Scanning speed	The faster the scan, the faster the stack can be taken. Try to start at 700 or 800 HZ if possible. Note that higher speeds will limit the lowest magnification levels. A zoom setting of 2 or higher may be required.
Bidirectional scanning	Always use this option to speed up scanning.
Pinhole	Start with 1 Airy unit, but if more light is needed, increasing it to even 1.25 can provide a bit more light without making the optical slice too much thicker.
Detector gain	Increasing the gain will increase the signal detection without adding additional light, but it will add more noise as well. Keep gain as low as practical.
Laser power	Increasing the laser is perhaps the last thing that should be done to increase the brightness of a sample. More intense light means more fluorescence fading and the sample being imaged will be scanned many, many times during a stack sequence. In a small stack of ten slices, with two frame averages, the sample is actually scanned twenty times during the imaging process. A stack of 100 would result in 200 scans. Every scan from an intense laser will fade the sample a bit more and there may be no signal left at the end.
Scan starting depth	If the software provides the option, start scanning a stack at the point deepest in the tissue. More light and/or sensitivity is needed the deeper the slice so, while each slice will be closer to the surface than the previous slice, the fading that may occur will counter the brighter intensity near the surface and make the overall stack more evenly illuminated.

the visual result of seeing everything in the stack at once in sharp focus. The orthogonal view (Figure 10.10C) is a multiple image option that displays a cutaway view with three orientations on the specimen: an individual 2D XY slice as well as a vertical Z slice of the sample taken from two directions. Three-dimensional reconstructions (Figure 10.10B) are rendered with an isosurface or "skin" and can be opaque, transparent, so that probes can be visualized inside the structure, or a combination of both. Because the original information is black and white, any color look-up can be applied to the image, including a color gradation based on the Z depth of the data. Once created, the reconstruction can be rotated and converted into a video file. Regardless of the method, 3D software can be tricky and third-party options are often better at specific functions, like 3D measurements, than the native acquisition software. To get started, the free image analysis software NIH Image has the ability to create 3D data sets from all of the major manufacturers' files when the FIJI version is used (FIJI is just ImageJ; http://fiji.sc/Fiji).

Considerations for Live Cell Imaging

Live cell imaging could comprise an entire book in itself, but there are some basic issues to share in this text. As with Z-stacks, working with live cells on the confocal has its own set of requirements. All parameters should be chosen to make sure that the cells are affected as little as possible by the imaging technique:

1. An incubation chamber must be used to maintain the viability of the cells.
2. Fluorescent proteins and vital dyes that do not affect cellular function should be incorporated into the experiment.
3. Longer wavelengths will do less damage to the cells, so fluorophores that emit in the red and far-red should be used when possible.
4. Live cells move, so imaging at fast speed is critical. Fast resonant scanners that scan at 8000 Hz are often used, but even a standard scanner at 800 or 1000 Hz will be useful.
5. Laser power should be kept as low as possible, so compromises in favor of low laser power are very important. Phototoxicity due to the excitation light can cause cell death, interrupt biological processes, or cause the cells to move out of the way.
6. Magnifications should be low so that the cells don't move out of the field of view. Lower magnifications will also spread out the light and make it less intense.
7. Many of the factors used to speed up stack acquisition should also be incorporated with live cells to speed up the process, avoid cellular movement artifacts, and avoid the phototoxic effects. Smaller spatial size, fewer slices, simultaneous acquisition, low averaging, no accumulation, bidirectional scanning, higher detector gains, and slightly larger pinholes will all help, as long as the information being gathered is still visible.
8. In a time sequence, keeping the number of time points to a minimum will reduce the cumulative effects of the acquisitions.

The Holy Grail of live cell imaging would be to capture fast events of weak samples in 3D, in multiple channels, with low light levels and minimum noise. All of these conditions are not always possible and it is the job of the confocal operator to understand the system well

enough to be able to compromise and find the optimum balance of settings that will provide the best data possible.

Advanced Techniques

This chapter has merely scratched the surface of the capabilities of a confocal microscope. There are many quantitative techniques that are performed on both fixed and live specimens that are part of the daily repertoire of many imaging labs. Capturing an image, even a good one, is usually just the start of the process and analysis of the images provides the real scientific data. Techniques like 3D co-localization and FRET provide information about how close fluorescent molecules are to each other and whether they may be interacting. FRAP experiments follow diffusion dynamics of molecules within a cell. FLIM provides information about the decay rates of the fluorophores. All of these techniques require the ultra-sensitive detectors and precise laser localization available with confocal and can be used to measure extremely small values in subcellular organelles.

Technology continues to advance and other techniques have been established based on confocal instrumentation. Now, 2-photon microscopes use special femto-second pulsed lasers and non-descanned detectors to image even deeper and more non-destructively than is possible with the standard confocal. New instruments for super-resolution include the STED system (STimulated Emission Depletion) that was designed as an extension of confocal microscopes. Super-resolution has broken the diffraction limit and made it possible to directly visualize features as close as 20 nm apart—ten times better resolution than the theoretical limit. Based on the evolving needs of the imaging community, future microscopes will allow us to see smaller, deeper, faster, weaker specimens in more dimensions and with less damage than anything available today, but even those systems will have their limits and microscopists will still need to make informed choices to get the optimal results.

SUGGESTED READING

Pawley, James, *The Handbook of Biological Confocal Microscopy*, third edition. New York: Springer Science & Business Media, 2010; ISBN-10: 0387455248.

http://micro.magnet.fsu.edu/primer/techniques/confocal/index.html

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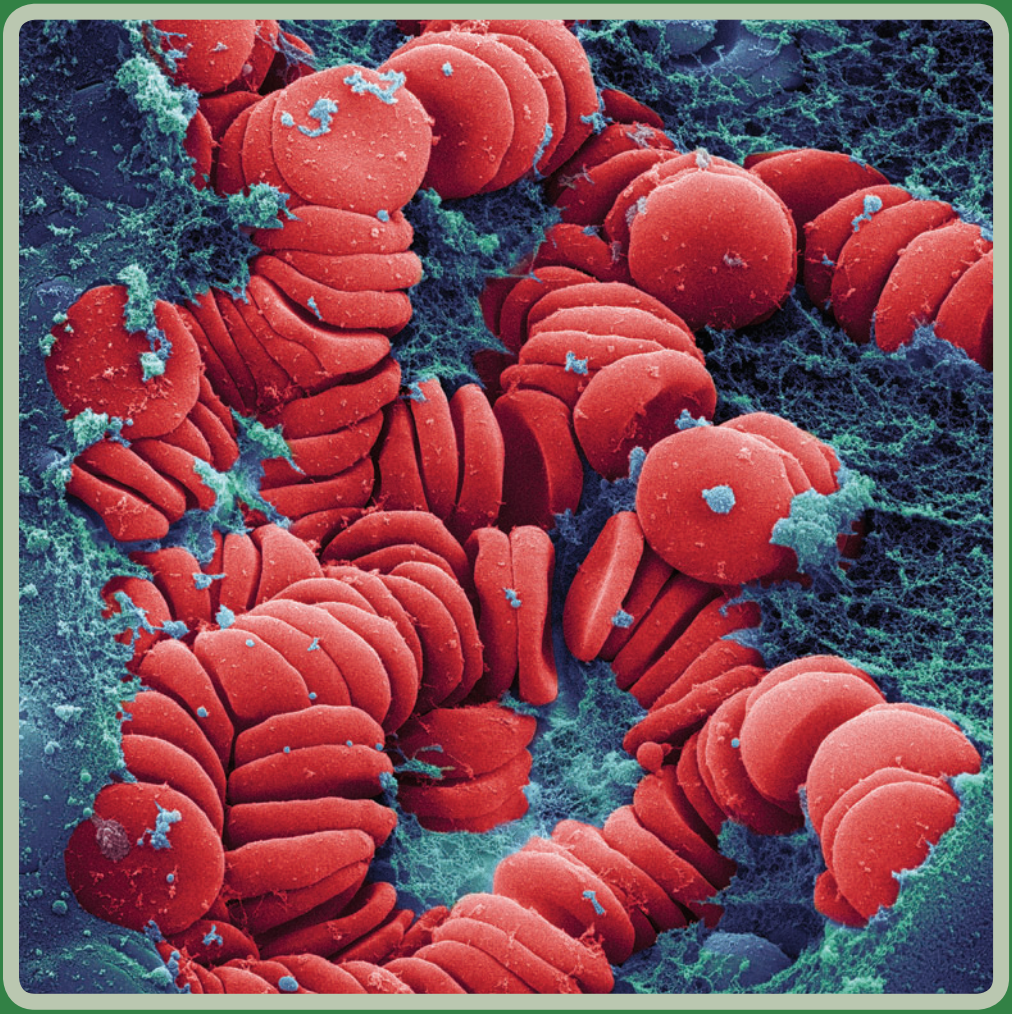
Chapter 11 Scanning Electron Microscopy

TED KINSMAN



This photomicrograph features a colored scanning electron micrograph (SEM) revealing the clotting of red blood cells collected from an eighteen-year-old patient. The red blood cells were just beginning to clot when this image was made. The image magnification was x2990 when printed at a distance of 10 cm wide. Image courtesy of Ted Kinsman.

Introduction

The primary use of scanning electron microscopy (SEM) is to observe topographical structures leading to the production of images with large depth of fields at relatively high magnifications. Current machines are now capable of performing a diverse set of imaging functions including material analysis accomplished by the use of scattered X-rays. This chapter will provide an overview of SEM and suggest strategies that can be used in the production of scanning electron photomicrographs. Contemporary detectors now can create images formed by a diverse range of electron and scattered X-ray energies. New electron sources can increase the strength of electron beams, which allow precise measurements down to the nanometer (nm) level. Modern instruments combined with novel sample preparation techniques allow scientists to visualize the micro-world with unprecedented resolution. New developments in the field of scanning electron microscopy continue to improve the capabilities of instruments at rapid rates.

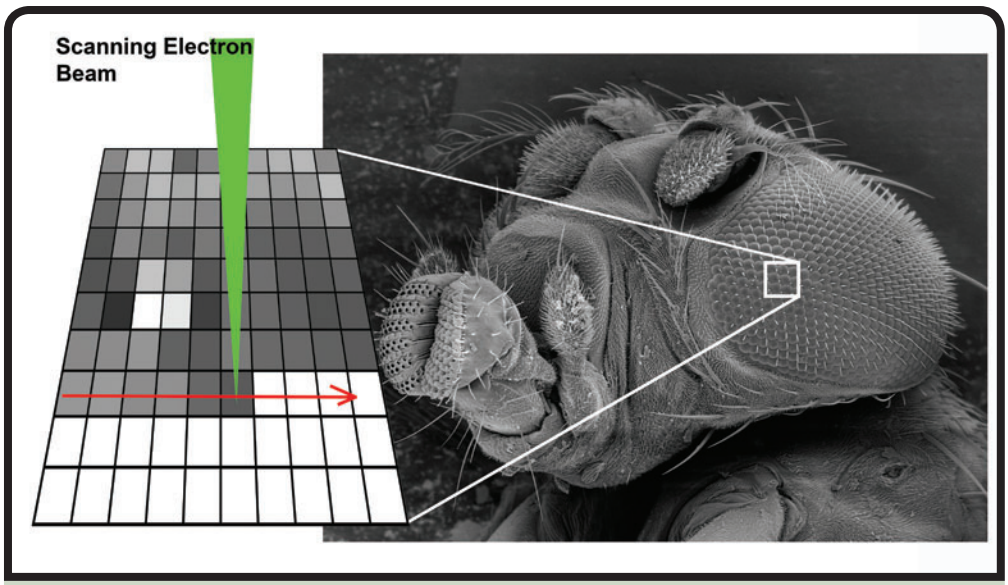


Figure 11.1 An accelerated beam of electrons is focused into a tight electron probe and then scanned across the sample. At each point or location on the sample, a number of electrons is collected by either a back scattered electron (BSE) detector or a secondary electron detector (SE). Point by point an image is created as the electron beam moves across the surface of the sample. Image courtesy of Ted Kinsman.

In an SEM, the electron beam is swept across the surface of the sample in the x and y directions. For each position that the beam interacts with the sample, emitted electrons form a signal that is collected by the electron detector. Pixel by pixel, an image is built up. The technique is similar to how a musician plays a song note by note, leading to a finished song. An observer listening to any note cannot identify the song, but the stream of notes builds to the full song. In an SEM, the image is recorded pixel by pixel and sometimes over the course of many seconds. High resolution and large images may take several minutes to

collect. This imaging technique is different than other forms of photographing where all the pixels' values are collected at the same time using simultaneous capture. Modern digital scanning electron microscopes can take up to several minutes to record a large image. Time is required to collect a signal from the electron detector and creating highly resolved images can take substantial amounts of time to collect.

To increase magnification, the size of the sweeping beam is reduced and it will cover a smaller area, producing a higher magnified image. Very finely controlled voltages and currents are required to produce a stable beam required to collect signals on these types of contemporary machines.

The stability of the electro-magnetic environment is crucial to achieving high resolution images collected over long periods of time. A slow scan is required for large images and may lead to sample charging. When there is a build-up of electrons unable to flow to ground, it is called sample charging. When this occurs, the sample may benefit from being sputter coated using gold to increase thicknesses. An increased coating will increase conductivity and allow the electrons to flow off the sample in time. It is quite common for experimenters to keep the scan speed relatively fast, and this will manage to keep the charging under control.

History

The scanning electron microscope was first proposed by Max Knoll in 1935, but the first actual machine— an experimental unit—was built in 1938 by Manfred von Ardenne. This first machine was a transmission electron type and not a scanning microscope. This microscope soon became a test bed for the theory of instrument designs and von Ardenne subsequently pioneered a number of features during that time that remain standard on modern machines. In 1942, the first SEM to examine bulk samples was constructed by Zworykin who recognized that not only are the back-scattered electrons (BSE) important, but he built a detector used to record images from secondary electrons. In time following the earliest years, a number of advances in electronics, transistors, and photomultiplier tubes allowed Pease to build a prototype for the first commercial machine in 1963. Cambridge Scientific Instruments Inc. released the Mark I scanning electron microscope in 1963. The most complete history of SEM can be found in many writings produced by Sir Charles Oatley in the 1970s.

Building on the early designs and technologies, advances have been made in electronics, detector design as well as the integration of computers into the design of modern SEMs. The size of the device has also been reduced and small tabletop SEMs have appeared in recent years. Originally designed to compete with the high magnification optical microscope market, the use of these small, simple-to-use machines continues to grow.

Modern Machines

The modern SEM consists of an electron source, a scan generator, magnetic lenses, electron detectors, and related electronics required to synchronize the position of the beam to

the electronic detector needed to create a digital image. Older machines—still found in a number of labs—will have an output and the signal that must be photographed from the microscope's cathode ray tube (CRT) using a DSLR camera outfitted with a macro lens. Many organizations maintain these older machines and they are still quite common among businesses and universities.

Theory and Design of the Instrument

The nature of an electron allows it to act as both a particle and a wave. Behaviors of electrons allow for the beam to be focused to an extremely small spot size. The design of electron optics combined with the small spot size of the electron beam can create an extremely deep depth of field. This deep depth of field makes the image very useful for understanding the topography of the micro-world. The extremely short wavelength of the electron used in combination with modern electron optics allows scanning electron microscopes to have resolutions of 1 nanometer. This resolution far exceeds what is possible using an optical light microscope where its resolution is limited by the wavelengths of visible light. Recent advances in electron sources have advanced from a single tungsten filament. Contemporary instruments use a crystal of electrically heated lanthanum barium oxide (LaB_6) as the electron source. The highest resolution instruments use a field emitter. These design improvements and technologies have their advantages and each has found uses in modern instruments.

The Nature of an Electron in a Vacuum

The electron beam that interacts with a sample's surface has to be operated in a good vacuum environment. If an electron collides with a molecule of air, it will not make contact with the sample. The probability of the electron traveling a distance without a gas interaction is called the mean free path of the electron. The mean free path of an electron is dependent on the pressure of the gas. The SEM design must have the total length of the microscope's lens column to be shorter than the mean free path of the electron. Simply stated, the majority of electrons emitted from the electron source (commonly called an electron gun) will travel to the specimen without colliding with any atoms of gas in the microscope's column, to a length of approximately 1 meter. To obtain a good vacuum of 1×10^{-5} Torr or better, a series of vacuum pumps is used to produce a gas-free environment to run the microscope.

A mechanical pump is the type of pump that lowers the column pressure to a few microns of mercury pressure. This allows a second pump called a high vacuum pump to work. The high vacuum pump can be an oil diffusion pump or of a turbo-molecular design. Each vacuum pump system has its own design parameters and consequently offers advantages and disadvantages. Oil diffusion pumps are very rugged, but require water-cooling, while turbo molecular pumps are very clean, but expensive.

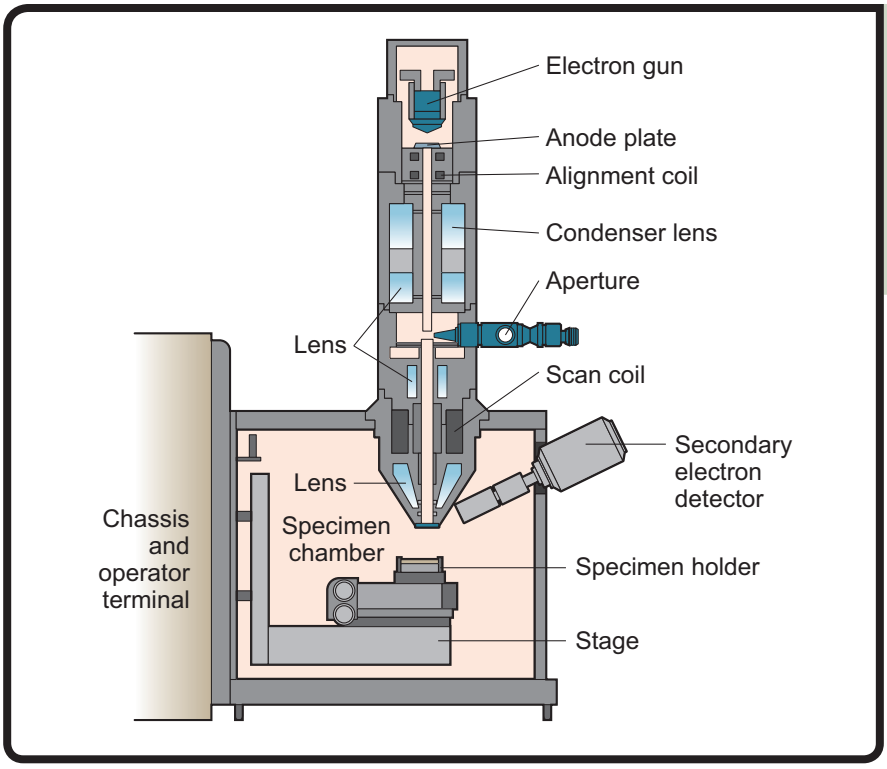


Figure 11.2 The major parts of the scanning electron microscope are shown in this figure. The electron source is at the top drawing and the specimen will be located at the bottom. Not shown is the extensive vacuum system required to remove atmospheric pressure in the column.

Electron Source Design

Electron sources can be characterized by the electron current that is created by the source and the amount of current that actually arrives at the sample. This electron current is called the beam current. Each style of an electron source will develop a unique beam current.

The tungsten filament source is by far the oldest design and is classified as a thermionic emitter. It emits electrons in response to the temperature of the filament. A tungsten filament is the most rugged of all of designs and has the ability to operate in a vacuum environment of 1 x 10^{-5} Torr. These filaments can also be cycled from vacuum to atmospheric pressure in a relatively short period of time and do not need their own dedicated vacuum environment. The replacement cost of a tungsten filament is relatively inexpensive and can be replaced without too much trouble by a skilled microscope operator. The lifetime of a filament in normal use would be approximately several hundred hours. The drawback of the tungsten filament as a source is that it does not generate as strong of a beam current as other types of electron sources are capable of. The tungsten filament source is

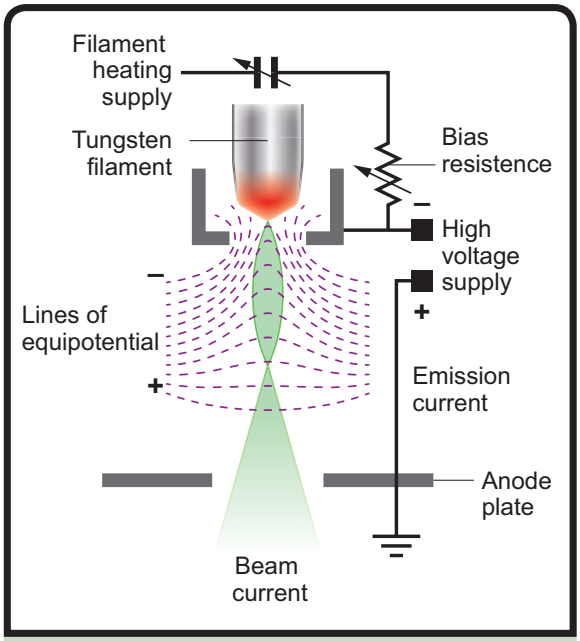


Figure 11.3 The tungsten filament is mounted in the Wehnelt cap and becomes the source of the electrons used in a scanning electron microscope. High voltage is applied to accelerate the electrons and the placement of the Wehnelt cap and anode plate creates a feedback loop that stabilizes the emission of electrons. With the placement of the cap and anode in this location, a resonate cavity is produced that maintains exceptional beam stability, somewhat similar to a vibrating string on a violin.

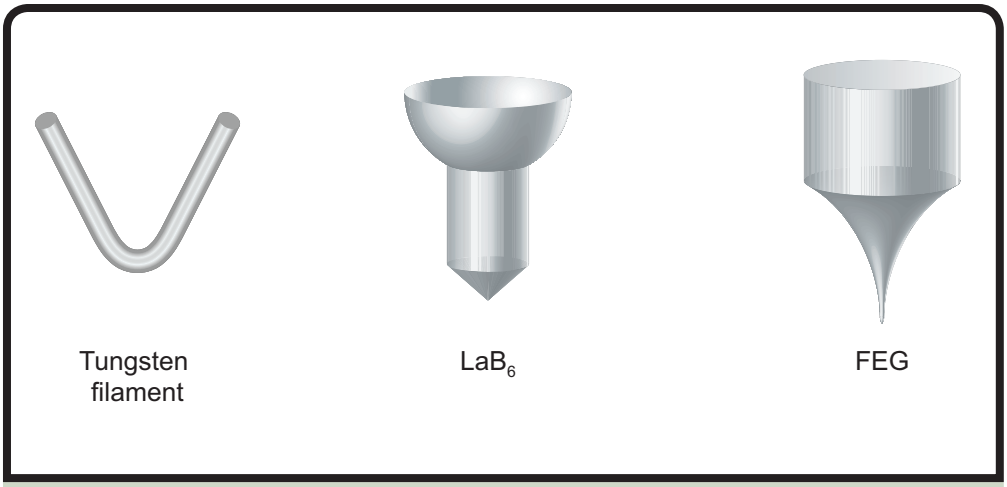


Figure 11.4 The three major types of electron sources are shown: (A) a tungsten filament, (B) a LaB_6 electron source, and (C) a field effect (emitter) source.

also limited in maximum resolution due to the physical size of the filament itself and due to the variations in the energy of the thermal electrons. The rugged tungsten source is still a common electron source found in many microscopes.

Brighter than a tungsten filament and having the highest beam current is the lanthanum hexaboride (LaB_6) source that uses an oriented crystal of lanthanum hexaboride. The crystal is used to generate a strong bright beam of electrons. Lanthanum hexaboride sources appeared on microscopes in the early 1980s and are still used on modern machines. The LaB_6 source must be kept under its own vacuum conditions, and for this reason it must be located close to a vacuum pump. The LaB_6 also requires less current to generate a brighter beam of electrons than a tungsten filament. As a consequence of this source requiring a high vacuum environment to operate, a dedicated pumping system using gate valves is required to cycle samples in and out of the SEM sample chamber. The LaB_6 source is substantially more expensive than a tungsten filament. The higher cost of this source and required vacuum environment components make using it a trade-off between the improved quality of its signal and the additional costs. Many machines that were designed for LaB_6 filaments are also designed to accept tungsten filaments.

The field effect emitter (FE) as a source creates the brightest electron current and is the standard in the highest resolution machines. The field emission source uses a uniquely shaped surface that is held at several thousand negative volts. It is located close to an electrode, which causes field electron emission. The field emission source is a fine point of tungsten that is 100 nm in diameter. This very small source can be focused to an extremely small point on a sample's surface. The field effect emission source can also create an electron source current that is much larger than any other sources are capable of. This high current leads to an unprecedented signal-to-noise performance. Since this source works at room temperatures, it is often classified as a cold cathode source. This type of a source is kept in its own very high vacuum environment and resides in a machine that is often fitted with a vacuum exchange chamber. This design keeps the microscope in a very high vacuum when in operation. To exchange a field effect emitter source requires a knowledgeable and

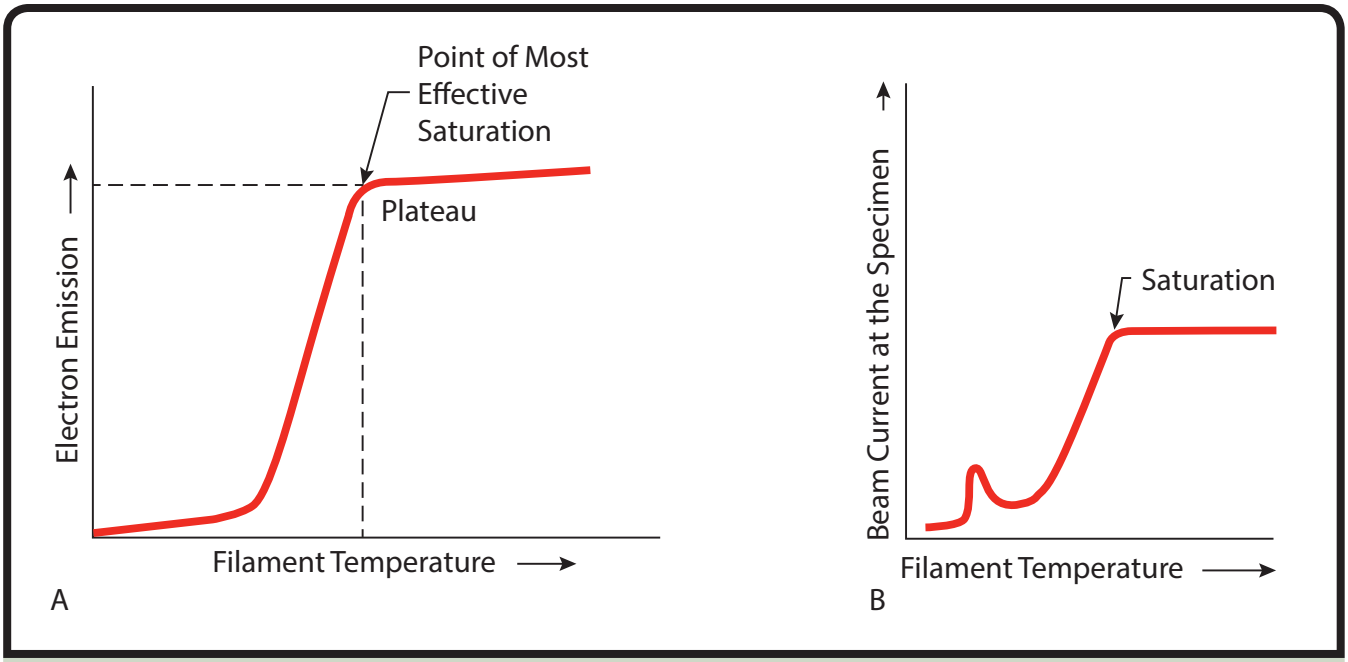


Figure 11.5 Adjusting the tungsten filament performance is improved by increasing the current passing through the filament causing the temperature to be raised (A). The condition of saturation (B) is achieved when there is no more change in the beam current as the filament current is changed. The best image is achieved when the filament is run at the saturation point.

trained technician. As a source, a field effect emitter costs about four times more than a Lab_6 filament. When a machine needs to operate at the highest magnifications that an SEM can produce with high resolution settings, the field effect emitter is the only choice. Low operating temperatures in combination with a high vacuum environment leads to a source with a long life when compared to other electron sources.

Adjusting the tungsten filament is crucial for achieving the maximum resolution from an instrument. The filament needs to have a current applied because the output is measured using the line scan mode. This mode allows the operator to determine the signal generated against the tungsten filament current. The current traveling through the filament will change and is represented as a characteristic curve in Figure 11.5. In this representation, the first crest in the graphic represents a false peak, and effective filament saturation is only achieved at the second peak. At saturation, the filament becomes part of a feedback loop between the filament and accelerating components of the gun, resulting in a very stable beam of electrons.

Electron Microscopy Optics

Modern scanning electron microscopes use an accelerating voltage of between –500 and –30,000 volts. The accelerating voltage changes the velocity of electrons and thus their wavelengths. Changes in acceleration will affect the resolution that an instrument can achieve. The accelerating voltage also affects the way the electron will interact with the

surface of the sample. The higher voltages will give electrons more kinetic energy, which allows electrons to interact with a material and produce a more significant electron sample signal. Higher energy electrons will interact more effectively with magnetic fields and can be focused to a tighter spot. This leads to an instrument with higher resolution. Higher energies also will interact with a larger volume of the sample, which may be a determining factor for resolution when imaging some materials.

How an electron behaves in a magnet lens is similar to how light interacts with a thin lens. The difference between light and electrons is that light experiences refraction when changing materials, leading to bending, while the electron is bent using magnetic fields. The electron travels in a corkscrew pattern. Coils of copper wire in the column of the microscope generate the magnetic fields used to bend the electrons. Each coil is called a magnetic lens. Microscopes typically have three magnetic lenses. The beam is collimated by the first condenser lens and then focused to a point by the second condenser lens before meeting the objective lens to be focused into a very fine point on the sample. The focused spot of electrons on the sample surface is now called an electron probe. The two magnetic deflection coils produce a scanning beam of electrons in the x and y directions. The goal of the electron optics is to make a real image of the electron source as small as possible on the sample's surface. This electron spot is what probes the surface, thus the name given to this spot is the electron probe. It is the scanning of the electron probe that is responsible for the creation of an image.

Astigmatization

Astigmatization of the horizontal and vertical directions causes the electron beam to fail to properly focus at the same location. To correct this problem the microscope is fitted with a control called a stigmator that will have an x and y direction adjustment. The stigmator is usually located between the last condensing lens and the objective magnetic lens. The difference between a novice and a skilled operator is their ability to achieve the highest maximum resolution using any sample through the elimination of astigmatism from the optics. To remove astigmatism from the electron pathway, the operator will first observe a circular structure using a magnification that is higher than the required image magnification. By adjusting the x and y stigmation controls and focusing at the same time, the circular structure is made as spherical as possible. The magnification is then lowered to the desired magnification and can be used to capture the image. When correct focus and astigmatism is achieved, the resulting image will show the maximum resolution that an instrument is capable of. The correction of astigmatization is often the biggest challenge needed to achieve the highest resolution that a microscope can generate.

The Electron Aperture

A critical part of the electron microscope's optics is an aperture placed after the second magnetic condenser lens and in front of the final objective lens. The aperture is a hole ranging in size from 5 um to 500 um. A small aperture leads to the creation of a small

electron probe on the sample. This condition leads to greater resolution, but also the lowering of the electron current, which leads to a lower signal for detection. A small aperture will also decrease charging on the sample. The correct adjustment of the aperture and the selection of the aperture size are dependent on the design of the microscope and the type of image or measurements being made. Samples that do not conduct well are prone to charging of the sample. This may require a small sized aperture to limit the number of electrons that hit the sample. This condition will also lead to a lesser signal that creates a noisy image. Many of the adjustments that can be found on an SEM can be used to improve an image. Each setting is used to optimize the signal-to-noise ratio. There will always be trade-offs across various image characteristics. Ultimately it is the skill of the operator to locate the balance of all the controls used to optimize the microscope, leading to the best possible image.

Apertures are made out of materials that can take the punishment of being bombarded by a beam of electrons. Tungsten, gold, and platinum are common materials used for apertures. Apertures need to be adjusted for optimal placement in the column for best resolution, and will also need to be cleaned periodically.

After electrons pass through the aperture, the electron path is bent by the last magnetic lens and in front of the objective lens. This lens brings the beam to a sharp focus on the sample. The distance between the sample and the specimen is also critical to achieving maximum resolution. A short distance of a few millimeters will lead to maximum resolution but a relatively shallow depth of field. A long working distance of 40 mm will lead to a deep depth of field and is ideal for low magnifications where the image does not require maximum resolution.

The operator needs to be aware of the location of the sample relative to the objective lens. Some microscopes will have an infrared video camera located inside the chamber so the operator can see the orientation and the position of the stage. The objective lens can be easily damaged if the sample stage comes into contact with it. Microscopes have a warning system that will go off if the distance between the two is too short. If electrical continuity between the stage and the objective lens is established, the stage will shut down and the system will warn the operator.

The column and the sample compartment of a scanning electron microscope are constructed using high-grade stainless steel. The solid chamber shields the sample compartment from external magnetic and electrical fields. Stray fields and vibrations will limit the microscope's resolution. The microscope should be mounted on a solid and stable platform that is isolated from building vibrations and is situated in a building that is suitable for stable measurements.

Tungsten atoms ejected from a filament over time can cause electrostatic problems if allowed to build up inside the column. Periodically, the column should be disassembled and cleaned to keep electrostatic build-up to a minimum. Scratches to the objective lens from a collision with the stage can also lead to electrostatic problems.

Resolution in an SEM

The diameter of the electrons that are focused onto the sample describes the electron probe's size. The smaller the spot size, the better the resolution of the image. However, the image quality is also linked to signal strength. The smaller the spot size, the lesser the signal and the lower the signal-to-noise ratio. The beam current is the amount of electrons that flow through the lenses to the sample. The beam current is proportional to the probe diameter, so small probe sizes represent high resolution, but a small probe current leads to a loss of signal. The resolution of the image is also dependent on the location of the sample with respect to the objective lens. The smaller the working distance, the smaller the electron probe can be focused. High accelerating voltages can also increase the electron probe interaction volume. In general, higher accelerating voltages will yield higher resolutions.

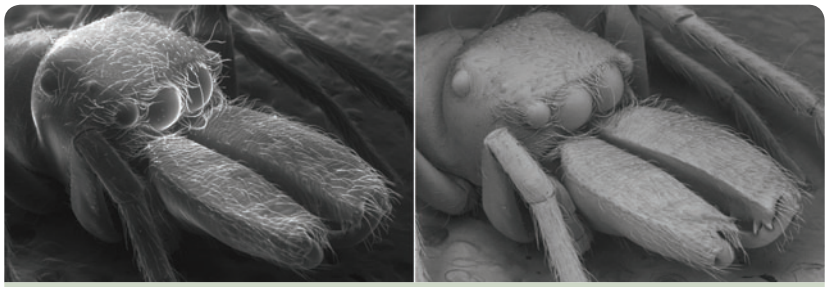


Figure 11.6 Electrons can be collected from backscattered or secondary electrons methods. The image on the left was made by collecting secondary electrons. The image on the right was made from backscattered electrons. Note the charging on the hairs on the left image; also note the edge effect taking place on the spider's eyes on the left image. Magnification was x30 when printed to a width of 10 cm. This reproduction is approximately the largest this particular instrument can produce. Image courtesy of Ted Kinsman.

When an electron beam hits a gold-coated or conductive sample, numerous sample beam interactions will take place. An individual electron from the beam can undergo an elastic or inelastic interaction that leads to small or large amounts of energy being lost. The depth to which an electron can penetrate a sample is dependent on both the accelerating voltage and the material of the specimen. Typical penetration would be in the 1–5 um range. The interaction of the sample and electron takes place in the interaction volume. Computer programs can model the penetration volume and various types of scattered electrons can be found that originated at different locations inside this penetration volume. When there are electron interactions with a sample, electrons can lose energy (secondary electrons), they can be scattered (backscattered electrons), they can produce characteristic X-rays, or light can be emitted. Each of these occurrences is measured using different types of detectors and yields different information about the sample.

Elastic scattering is caused by an electron bouncing off an atomic nucleus and this produces a small loss of energy to the electron. These reflected electrons are called backscattered electrons (BSE) and have energies very similar to the incident electrons, if not identical. How many backscattered electrons are produced is a function of the mass of the atomic nucleus and the distance from the detector. Materials of higher masses will yield more backscattered electrons. These electrons are detected using a solid-state detector that is located very close to the objective lens. These very high energy electrons are relatively unaffected by sample charging and can often be collected and used to form a good image of a sample that exhibits electron charging.

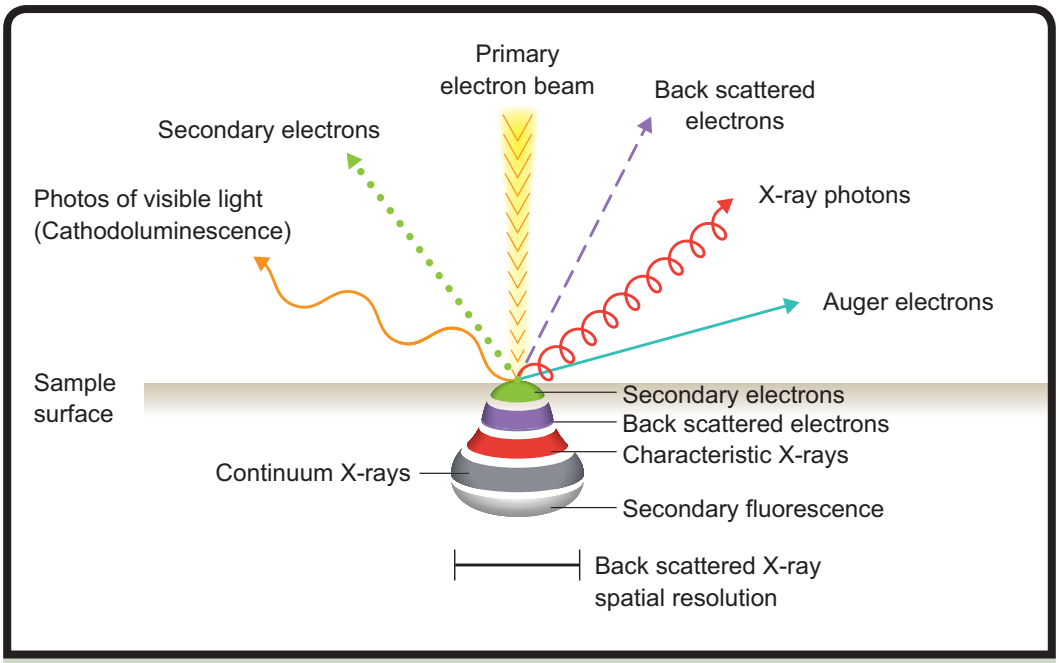


Figure 11.7 This illustration shows the possible interactions and outcomes from an electron and a sample. Each of these effects can be measured and used to generate a unique image of the specimen.

The most common type of electron that is detected in an SEM is the secondary electron (SE) that is emitted from the incident electrons on the sample. These electrons are primarily emitted from a relatively small volume of the sample and are often found to originate no deeper than 5–50 nm in depth on the sample. These electrons are collected by a positive electric field and attracted to the secondary electron detector.

An operator can maximize a signal by adjusting the position of the sample relative to the SE detector. The detector is a thin layer of scintillating material. The scintillator converts incident electrons to photons of light. The scintillated light travels through a plastic light pipe to a photomultiplier tube that takes a single photon and converts it into a large electrical signal that can be amplified. To help collect electrons in the sample chamber, a metal grid is placed over the end of the light pipe and held at a positive voltage of several hundred volts. This voltage is called the secondary electron detector bias voltage. The positive grid attracts the negative electrons from the sample chamber to the scintillating material. By changing this bias voltage the operator can control the sensitivity of the detector.

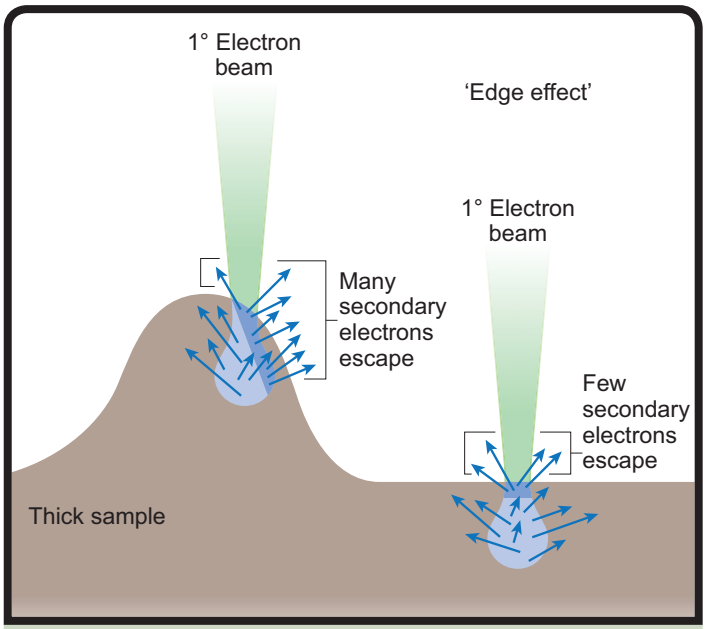


Figure 11.8 The electron edge effect takes place whenever an electron beam interacts with a slanted surface. The number of emitted electrons increases when the surface of the sample is closer to the interaction volume of the electron. This effect often causes the edges of fine structures to glow because it emits many more electrons than the other regions of the surface.

The creation of secondary electrons is also affected by the angle of the beam that hits the sample. When a beam hits the sample at an angle, the electron interaction volume remains unchanged with respect to the incoming beam; however, if the angle of the specimen allows the sample's surface to be closer to the parts of the interaction volume, this allows more secondary electrons to escape. The tilt or angle of the specimen is a powerful technique used to control the number of secondary electrons produced on a sample. The edge effect is commonly observed on edges of structures located in a sample. Any edge will also be represented as an angle to the incoming beam and generate more secondary electrons—this is known as the edge effect.

Signal-to-Noise Ratio

The desired signal produced by the SEM compared to the random noise collected by the detector creates the instrument's signal-to-noise ratio. A large signal-to-noise ratio is desired and required to achieve a good image. Operators will need to use all of their knowledge about a machine to maximize this ratio. Noise can be introduced from many sources including a requirement for high image resolution (too small a electron spot size and thus too small an electron current). Noise can also be minimized by aiming the sample towards the detector, and this can increase signal strength.

Even when a poor signal-to-noise ratio exists, a good image can be formed by creating a number of digital images that can be later recombined. Since the noise is random on an image, a user can integrate or average a number of images ranging from two to sixty-four. When utilized, the noise can be mostly eliminated from the final image in the blending of the image data and in particular the noise. Images formed this way are called integrated images. When working with integrated images, it is important that the sample is not charging and that the electron beam is very stable. Modern digital machines have been

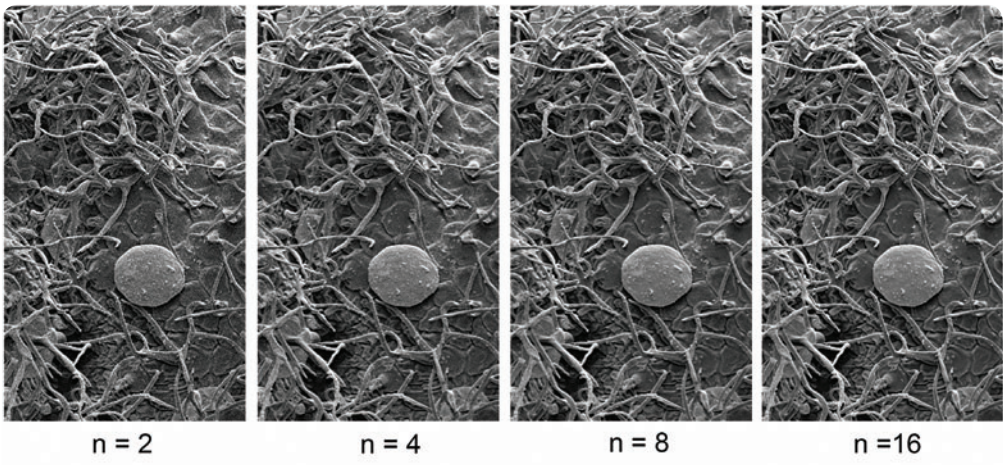


Figure 11.9 A fast electron beam is needed to collect a signal and signal integration is used to remove noise from files. The images shared here are the result a number of images being integrated together, ranging from two files to sixteen. The images have been enlarged by a factor of two, revealing noise reduction, a byproduct of the integration. The photograph features a lavender leaf at a magnification of x500. Image courtesy of Ted Kinsman.

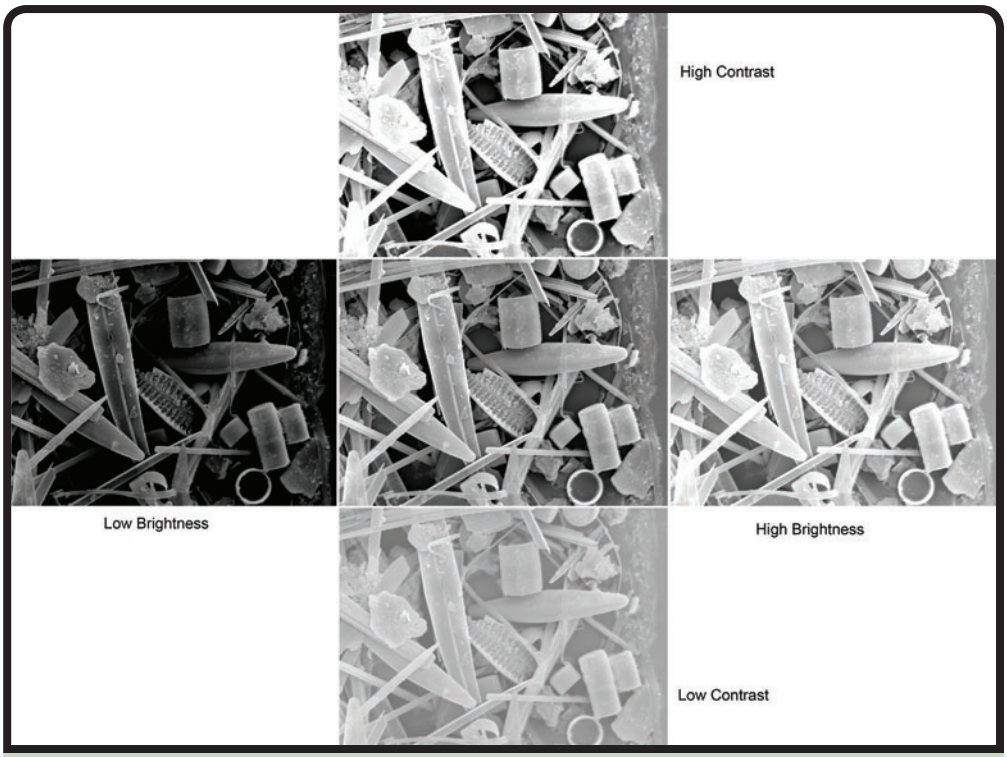


Figure 11.10 This chart shows the effect of changing the brightness and contrast settings. A freshwater diatom sample was imaged at a magnification of x600. The images included in this composite were produced by changing the brightness and contrast of the microscope. The correct exposure requires an adjustment of both the brightness and the contrast. Image courtesy of Ted Kinsman.

optimized to take advantage of this noise reduction technique. The trade-off when making integrated images is that extended times are required to collect a good image.

The amplitude of the detector signal defines the brightness of an instrument's output and the contrast of an image is described as the difference between the brightest and the darkest areas of an image. An SEM monitor has both brightness and contrast adjustments. Obtaining the correct image appearance on a calibrated monitor is very important. An image display that has too much contrast will form images that contain poor surface details, while a display that is too bright will produce images with areas that have less or no detail in the brightest areas.

Scan Rotation

The scan rotation feature for a microscope allows the physical beam to be rotated relative to the sample. This allows the operator to position a sample in the beam that produces an image with the best composition and that shows the desired topography in the best way. This is a very convenient adjustment tool. In some instances, the electron charging that occurs can be controlled using the scan rotation. The sample can be rotated to place the charging object near or at the end of a scan line. Using this type of image composition, the

charged problem area will have some time to discharge the excess electrons before the next pass of the scan line.

It can be useful to finely position a specimen without moving the specimen. For this outcome, a small angle beam deflector is used. When operating at high magnifications the mechanical stage often does not have the fine position required to always center an area of interest in an image composition. In these situations, the beam deflector can be used. The deflector uses two small magnetic fields that nudge the electron beam a relatively small distance on the sample's surface.

Specimen Charging

Specimen charging is one of the most common problems that occurs when making precise observations of a specimen. Charging is the outcome of electrons that are incidental on the sample but do not leave the sample as backscattered or secondary electrons do. If these stray electrons do not find an efficient path to a ground using the sample stage, they build up on the sample. This build-up of charging electrons will have adverse effects on the incident beam of electrons and will be observed as bright spots or high contrast areas in an image, or they will create a shift in the beam's position.

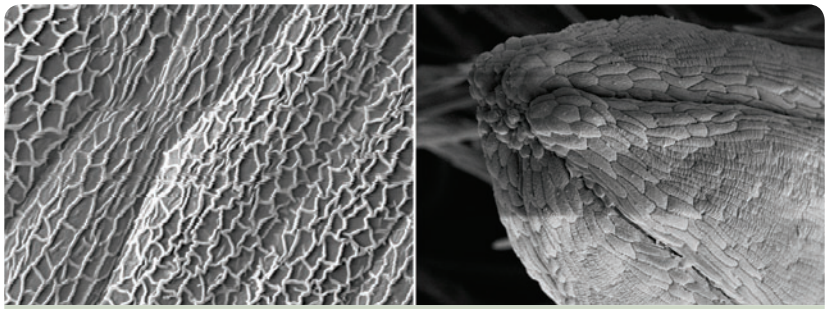


Figure 11.11 This composite shows how two biological samples may reveal the effects of electron charging. The image on the left shows the effects of charging on an unidentified leaf sample. Charging causes the electron beam to be displaced in numerous locations in the image. The image on the right shows the effects of charging by a change in brightness on the image. Image courtesy of Ted Kinsman.

Any loose material on the sample that does not have a good conductive path to a ground can lead to charging. Common examples of materials that cause specimen charging would include insect hairs, blood cells, and dust that is on a sample. These charging areas deflect the incident beam slightly so there is a reduced emission of electrons around the charging areas.

In samples that have large degrees of charging, the beam will appear to jump all around on the sample. The resulting image will have a number of wiggles and bright spots randomly found across the image. A skilled operator will need to find ways to reduce electron charging when making images of a sample.

Lower acceleration beams are more affected by a charging sample, but typically lead to less charging. There are some adjustments that can be used for the removal of charging from a sample.

- Coat the sample with a thicker layer of conductive material in the sputter machine. This will increase conductivity of the electron path to ground, allowing electrons to flow to ground with less resistance.
- Make the aperture smaller.
- Reduce the accelerating voltage to a lower value.

- Shorten the scan time.
- Change the detector to backscattered electrons.
- Reduce the detector bias, or turn it off.

If an electron beam does not move and stays directed onto one particular region of a specimen that does not have a good electrical conduction, the specimen can become degraded. Typically, this outcome is evidenced in an image as large square block areas that are burned into the sample. This frequently happens when looking at delicate plant materials. As a consequence of the drying process used for sample preparations, the plant's material does not possess good bulk conductivity. The electrons can only escape from the sample by using the conductive layer on the top of the sample. Areas that are far from the conductive sample stub containing delicate biological structures are vulnerable to electron beam damage. The sample damage can come from heat generated by the electron beam or chemical change of the specimen.

To minimize specimen damage, using the following adjustments will be helpful:

- Decrease electron beam power by decreasing the acceleration voltage.
- Increase the scan area. By using a lower magnification, the beam's power will be dissipated over a larger area, minimizing surface heating.
- Decrease the scan time.
- Use a smaller aperture.
- Use a faster scan rate.
- Increase conductive coatings on the sample.

Maximizing Resolution

Each sample has a sweet spot for the best outcomes and it will be the result of optimizing various instrument settings. A skilled operator will realize that there is a balance that is gained to results produced using high accelerating voltages and beam interactions on the sample. Finding the right balance will be heavily dependent on the sample type. By using accumulated experiences, operators will find imaging solutions more quickly. The more time an operator spends using an instrument, the more quickly they will be able to problem solve when imaging difficult samples. In general, high accelerating voltage will yield higher resolution images but these images will demonstrate the adverse effects of having an increasing surface charging, characteristic of samples that are not very conductive. Lower accelerating voltages will give more surface details than higher accelerating voltages. This is due to the deeper penetration of the high energy electrons and the increased interaction volume of the electron beam.

In general, the use of the following steps will maximize an instrument's resolution:

- Insure the filament is correctly saturated.
- Use a higher acceleration voltage for a higher resolution because of the smaller electron probe.
- Use a smaller aperture diameter. The smaller the aperture the finer the resolution, but this leads to a loss of signal.
- Use a small working distance. Get the sample at close as possible to the objective lens.

- Remove all aberrations—particularly stigmatizations.
- Optimize the electron gun alignment.
- Insure the optimal positioning of the aperture.
- Select the best detector type.
- Establish the best specimen tilt and direction.
- Image using a proper scan rate.
- Insure there is removal of vibration and stray electric and magnetic fields.

Interaction of the Electron on the Sample

The interaction of incident electrons and the sample will produce characteristic X-rays that can be detected to determine the exact composition of a sample. Although the topic of X-ray analysis is beyond the scope of this chapter, the reader should be aware that many older SEMs are updated with modern X-ray detectors. They can be used for material analysis.

In special materials, the electron will not only yield the above shared interactions, but electrons will also liberate light from some samples. The measurement of light from the sample is called cathodeluminescence. Recently there has been a resurgence of interest in cathodeluminescence due to the development of new and improved detectors. The incident electron replaces a low orbital electron that causes the molecule to emit light in a process almost identical to fluorescence. The emitted light is measured in response to the position of the beam and generates an image.

Recommended Standard Process for Achieving an Image

- Place a sample in the chamber.
- Set the high voltage to the desired level.
- Turn on filament.
- Use the line scan mode to look for false peak on filament signal.
- Focus the image.
- Adjust electron gun's alignment.
- Adjust the SEM astigmatism and use the focus wobble if required.
- Set the aperture to desired size.
- Adjust the aperture position.
- Refocus and adjust astigmatism again.
- Focus system at a higher magnification than the required image.
- Reduce the magnification.
- Adjust the brightness and contrast.
- Collect the image.

Sample Preparation

The specimen needs to be physically secure when placed into the SEM vacuum tube and it is often taped or glued onto what is called a specimen stub. These stubs are unique to each microscope.

Once the sample has been adhered to a stub it must be prepared for examination. The role of sample preparation is to insure that a sample is clean and will not outgas any

materials in the vacuum. If outgases are produced, they will contaminate the high vacuum environment inside the microscope. To be able to observe fine specimen detail, all liquids will need to be removed from the sample before placing the sample into the high vacuum environment. Failure to remove liquids will cause the sample to change shape and will not accurately represent the correct morphology or topography of the sample.

Samples can be conductive or non-conductive. The easiest preparation can be accomplished using a conductive sample, where the sample is placed on a suitable stub. Next, make sure there is a low resistance conductive path to the sample stage. Conductive samples are often mounted onto carbon sticky tape or by using conductive silver glue. Conductive samples are simple to prepare and are recommended for new operators when learning how to use a scanning electron microscope.

Non-conductive samples are much harder to prepare and consequently to work with. They fall into two categories—solid materials such as glass and plastics, and biological materials. This section will focus on biological materials. The goal of preparing organic material is to remove all the moisture from the material while maintaining its surface topography as much as possible. Liquids will need to be removed from a sample so that it does not change shape in the vacuum of the SEM. Dehydration also insures that the venting liquids do not contaminate the clean environment inside the SEM chamber.

There are a number of techniques that can be used to prepare an organic sample for study using an SEM. A quick literature search sometimes will reveal a technique that works better for dehydration, but the serious experimenter should evaluate various sample techniques for their experiments. Significant improvements have been made in methods used for sample preparation in the last few years. Standard techniques include critical point drying, freeze-drying, and the use of hexamethyldisilazane (HMDS). Identifying what is suitable for a particular sample and what method will lead to the best outcome can only be accomplished by evaluating the image quality that each method produces and which method is most suitable for a particular experiment.

Biological specimens are non-conductive samples, which require the most care in preparation for observation in the SEM. The first step in preparing a biological specimen is to place it in a preservative such as glutaraldehyde in a 2.5 to 5 percent water solution. Glutaraldehyde is an excellent preservative and keeps a biological specimen inert for a very long time. Since glutaraldehyde is toxic, proper eyewear and gloves must be worn when working with this chemical. The chemical is capable of preserving fingers and hands as well as the SEM sample.

Critical Point Drying

Critical point drying (CPD) uses liquid carbon dioxide as the solvent to remove any residual water and alcohol that remains in a sample. Specialized equipment is used for this technique, allowing unique changes in sample pressure and temperatures to create a condition where there is no difference between liquid and gas volumes. This allows the liquid to be removed from the sample using gas that does not cause any structural degradation to the sample.

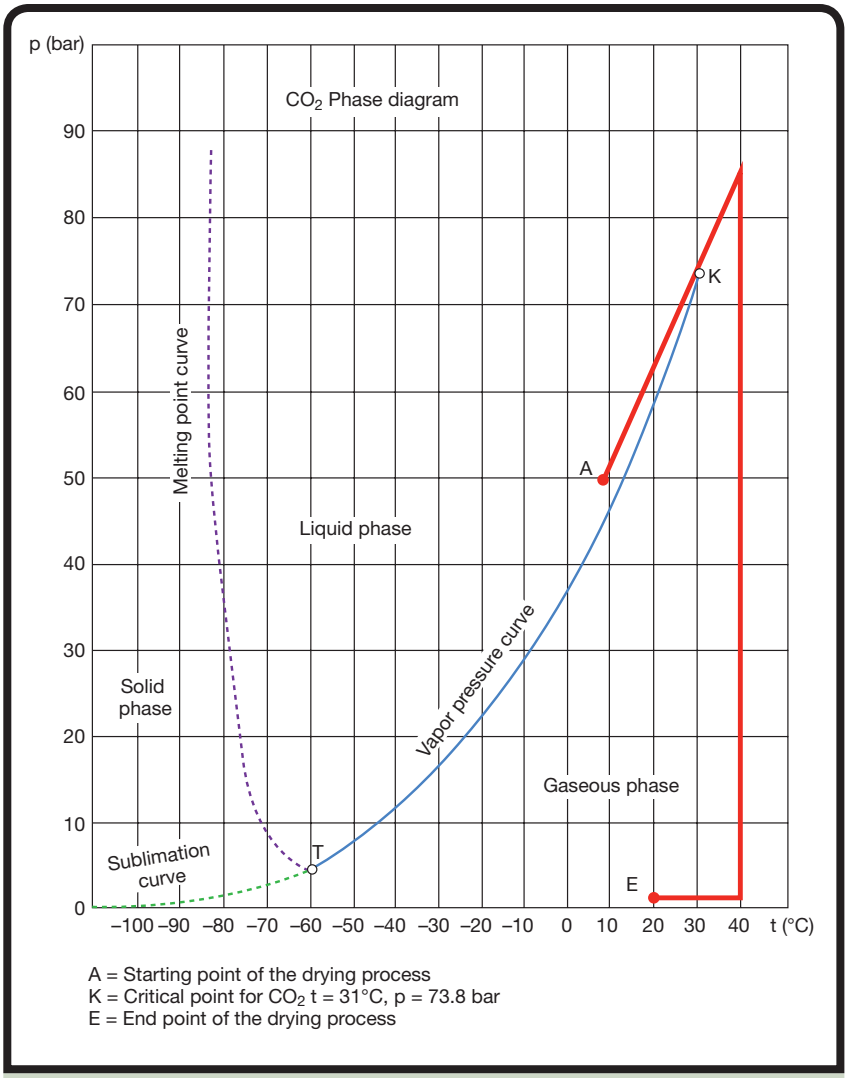


Figure 11.12 This phase diagram of carbon dioxide shows the special critical point required for dehydration. The critical point drying curve starts at location A, where the liquid carbon dioxide is allowed to condense on the samples at 8 degrees C. Temperature and pressure are increased to a point K where the carbon dioxide is beyond the critical point. The critical point for CO^2 is 31 degrees C at a pressure of 73.8 bar or 73.8 times atmospheric pressure. At this location, there is no surface tension to deform the sample. For this reason, the CO^2 is allowed to slowly vent to atmosphere pressure at point E. Using this information the specimen can be prepared to be extremely dry and not present any surface deformities as a consequence of liquid surface tension.

The critical point on the carbon dioxide phase diagram is shown in Figure 11.12. The series of steps in the diagram reveals how carbon dioxide changes from its liquid phase to its gas phase without ever boiling, a process called sublimation. This keeps the morphology and structure of the sample as near to the lifelike as possible.

Critical point drying begins by using ten containers, each filled with a different concentration of ethyl alcohol and water. Starting in a solution of 90 percent water and 10 percent ethanol alcohol, the specimen is stepped through a series of baths increasing by 10 percent until the same sample arrives at 100 percent ethyl alcohol. The sample is soaked in each solution for 10 to 30 minutes depending on the size of the sample. After soaking in the 100 percent ethanol alcohol for the required time, the specimen is moved to the critical point dryer instrument (CPD). The sample container used in the instrument is called "the Bomb" and usually has a glass window to allow inspection of the progress and the sample. Before the specimen enters the sample compartment, the specimen refrigerator is turned on to bring the specimen chamber to 8 degrees C, which allows the carbon dioxide to flow into the sample container as a liquid. The liquid carbon dioxide is allowed to flow in and out of the sample chamber at least three times. After the third exchange of liquid carbon dioxide, the refrigerator is turned off and the sample chamber is brought up to 40 degrees C. This raises the pressure to 85 bar or 1232 psi. When observing the sample in the chamber, the liquid carbon dioxide turns into a gas and oftentimes will form droplets of carbon dioxide that will wick in and out of the sample volume as if it was raining in the container. The sample container is held at the 40 degrees C temperature for 20 minutes. The carbon dioxide gas is allowed to escape slowly over the course of five minutes to an atmospheric pressure. The sample is now a solid shell and no

liquid should be evident. This procedure will work well with samples such as human blood cells or a mosquito. This sample preparation technique will yield excellent results. This technique might be modified slightly and should be compared in a side-by-side test before determining the best preparation technique used for a particular sample. The critical point drying process will also remove many soluble chemicals including chlorophyll found in leaf samples.

Recently, a technique has been published that gives similar results to critical point drying using the chemical hexamethyldisilazane (HMDS). The specimen is first placed in a 2.5 percent glutaraldehyde and water solution. The specimen is then moved to a container of 20 percent ethanol alcohol and distilled water. The specimen will then be submerged for 20 minutes in a series of baths of increasing ethanol and decreasing water strength until reaching a 100 percent ethanol solution. For the last step, the sample is placed in 100 percent HMDS and soaked for 30 minutes before being allowed to air dry in a fume hood.

An alternate method involves several steps of increasing HMDS solutions after dehydrating using the 100 percent ethanol. By increasing the concentration of HMDS in 25 percent concentration steps until 100 percent HMDS is obtained, better samples can sometimes be created. In some samples this gives comparable results to critical point drying when no critical point dryer is available. For new samples requiring preparation, it is useful to perform a literature review before experimenting with a preparation technique using an important sample. Some researchers have found the HMDS technique of sample preparation is preferred over the more commonly used CPD method. It is safer, cheaper and faster.

Sputter Coating

To create conductive samples that do not allow the build-up of electrons on the sample, the specimen needs to be conductive or be coated with a fine layer of a conductive material. Although several materials can be used for the coating process, gold is the preferred material because of its excellent electrical conductivity. Gold also does not oxidize. The gold used for sample coating is very thin and ranges between 50 and 500 nm. The gold that is used as the target in a sputter machine should last for a few years with normal use. In instances where sputter coating is desired, the sample will only need a very thin coating and this will give the specimen a dark appearance to the eye. A thicker coating will give the sample a more gold-like appearance. Samples that are observed under very high magnifications should use thinner coatings. Using a thick gold coating may mask extremely fine detail in a specimen.

The sputter coater uses a strong electric field to move the gold. A gold target is mounted a distance above the SEM samples that are to be gold-coated. A high negative potential of several thousand volts is applied to the gold target. Argon gas of 50 microns of pressure is allowed to bleed into the chamber and accelerates in the electric field when interacting with the gold. The collision of the argon atoms on the gold liberates individual gold atom clumps from the surface. These free gold atoms travel away from the gold's surface and toward the negatively charged and held SEM samples. The sputter rate of the target is linked to the gas in the sputter chamber. Argon is the gas of choice for this application.

To run the sputter coating system, the chamber is pumped down and argon is allowed to flow into the sample chamber. This is repeated twice until the argon atmosphere in the device is about 50 microns. The sputter coater is turned on and allowed to coat the sample for 30 seconds. This thin coating of gold will keep the sample from building up electrons (charging) when it is in the SEM chamber. It is best to use samples as soon as possible after coating. A sample left in the air will start to absorb water from the environment and change its size. This change in size will disrupt the conductive gold layer that has been recently formed as the sample's morphology expands or contracts with the addition of water. A sample left on a lab counter for a day or more may have to be sputter coated again. If samples will be observed over time using an SEM, it is suggested that the samples be kept under vacuum.

Conclusion

Scanning electron microscopes continue to explore the micro-world by combining breakthroughs in chemistry, physics, material science, and biology to make the world visible as never before. This is an exciting time in science and scanning electron microscopes are greatly contributing to understanding of the nano-world.

SUGGESTED READING

Goldstein, Joseph, Dale E. Newbury, David C. Joy, Charles E. Lyman, Patrick Echlin, Eric Lifshin, Linda Sawyer, and J.R. Michael, *Scanning Electron Microscopy and X-ray Microanalysis*, third edition. New York: Springer Science & Business Media, 2007; ISBN-13: 978-0306472923.

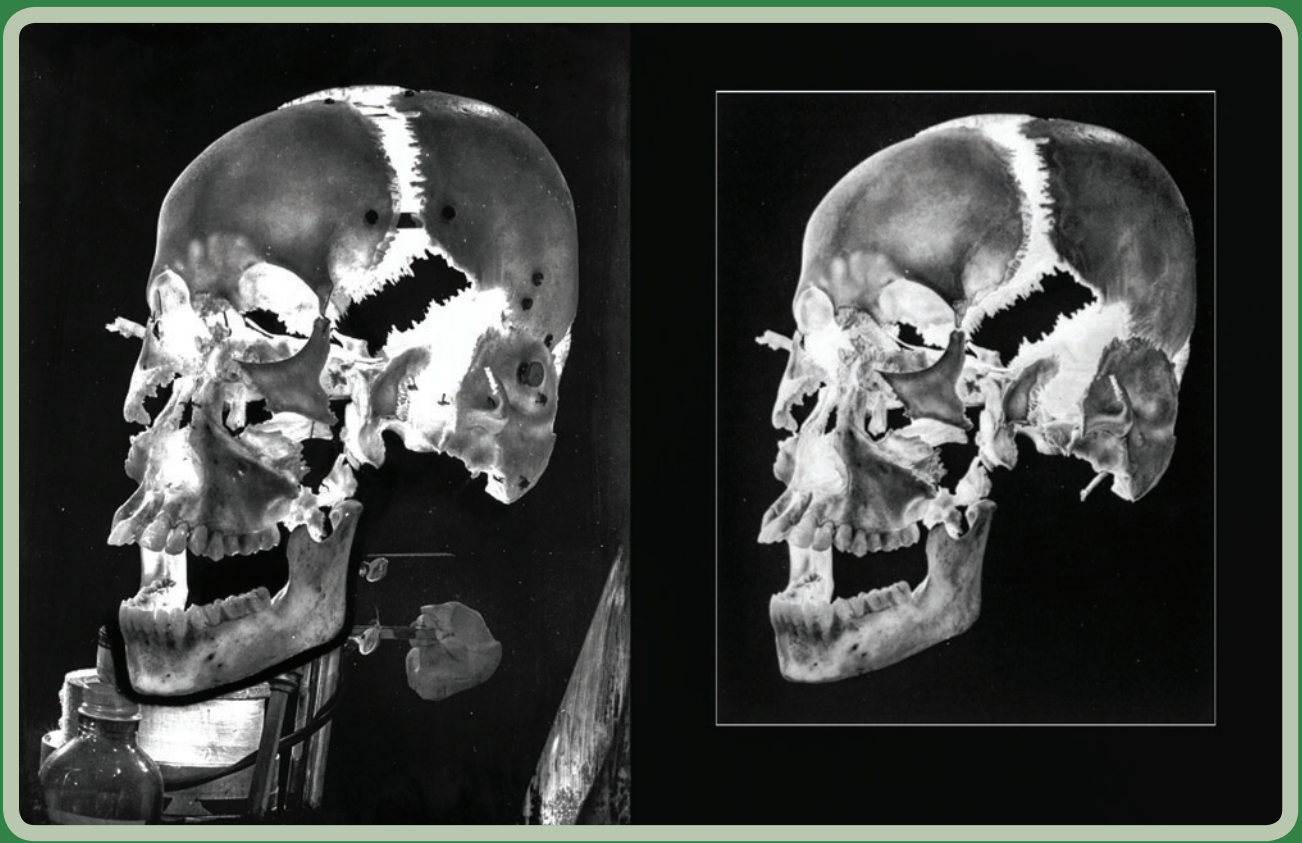
Postek, Michael T., Karen S. Howard, Arthur H. Johnson, and Kathlyn L. McMichael, *Scanning Electron Microscopy: A Student's Handbook*. Williston, VT: Ladd Research Industries, 1980; ASIN: B000H57L0K.

Chapter 12

Ethical Considerations in Scientific Photography

Why Ethics?

JAMES HAYDEN



There is a contemporary belief that modifying images in the digital era is more prevalent than was the case during the film era for science images. Image retouching and airbrushing were common practices for decades and were frequently used in high-end analog advertising applications. When practiced by the best professionals of the time, the results were astonishingly believable. These two images were created from scans of original prints. The photograph was made by Henry Lou Gibson (1906–1992). It is believed the photograph was created in the late 1940s or early 1950s. Gibson was a noted author and biomedical/technical photographer who worked at the Eastman Kodak Company. The image of the exploded skull was created to be used as an illustration for one of the countless Kodak books written by Gibson and published by Kodak. The photograph was titled *Beauchêne Skull*. The image on the left reveals what was removed by retouching when compared to the final photograph displayed on the right. Left image reproduced courtesy of the Howard Radzyner collection, image on the right reproduced courtesy of Eastman Kodak Company.

The Need for Protocols

Ethical protocols are not usually considered a prime concern in most photographic processes, but scientific imaging practices have different considerations when compared to traditional photography. At a minimum, scientific photographs are recordings that stand in proxy for the results of the experiments from which they were taken. At the extreme, results are extracted directly from the images themselves. As such, these types of photograph must stand up to the same rigorous standards and scrutiny as any other part of a scientific experiment. The images must collect and preserve data and be managed in a scientific workflow much like the chain of custody for evidence in a forensic environment. The images must be more than just properly focused, composed, and exposed: they are actually technical documents that must provide an objective rendering of a scientific truth. Just as importantly, these images must provide accurate and reproducible data. Failure to follow standardized protocols can result in an inaccurate representation of a subject, which can lead to incorrect interpretations of an experimental result. Unchecked, these misinterpretations can result in questioning of the published data. In addition, the temptation to artificially alter the original images with software to make them clearer can result in alteration of the objective record, investigations of scientific misconduct, retractions of published works, as well as professional and public censure. At worst, years of work can be lost, careers can be destroyed, and legal hurdles can become overwhelming.

Manipulation of the visual record is the primary cause of unethical behavior in scientific imaging. It stands to reason that, if the captured image is considered to be a direct representation of a scientific subject, then altering that image would also alter the interpretation of the data it represents. Unrestrained manipulation with image editing programs is generally believed to be the primary example of such behavior, but there is more to it than that. Manipulation begins with the decision of what to photograph, continues with the choice of lighting and camera settings, includes post-capture processing and culminates in the presentation of the data itself. Additionally, digital file storage and retrieval can have some bearing on the perceived veracity of the published results as well. If not stored as original files in the proper format, questions may be raised about the original work. Complete understanding of all of these processes is one of the keys to an objective and accurate recording of a scientific subject. Likewise, uncontrolled modification of these steps, either accidentally or on purpose, is the key to unethical imaging decisions and inaccurate scientific results. Ethical practices are, therefore, extremely important to proper scientific imaging.

Ethics, by definition, can be a rather subjective issue to describe. Merriam Webster defines it as "rules of behavior based on ideas about what is morally good and bad" or "a guiding philosophy." In either case, it essentially defines acceptable and unacceptable behavior based on pre-defined concepts. In the case of scientific imaging, ethics is used as a guide for approaching scientific image capture based on the application of the scientific method. By making an effort to understand all aspects of the imaging process and applying controlled conditions to the procedures involved, the photographer/scientist can maintain an unbiased approach to retrieving scientifically valid data from the images themselves.

The Image as Data

What makes an image "scientific"? When an image is used to represent data in the scientific record, it becomes a scientific document. Whether it is used as a "for the record" snapshot that only sees the inside of a lab notebook, as the raw data that provides a source of information for a graph in a paper, or as a final image in a publication figure, the scientific image represents something real. However, it must be kept in mind that the image is only a depiction of reality and not the reality itself. As such, there is always the danger of bias creeping into the recording and altering of the final interpretation. The goal of any scientific image is an accurate representation of the subject it captures and this can only occur when the image is treated as a series of raw, discreet data points.

Stripped down to its most basic units, any digital image can be represented as a table of specific numbers defined by individual pixels. Mapping out those values in a raster pattern based on their location in the recording chip identifies the information in an image (Figure 12.1). Each pixel has a defined location, intensity value and perhaps a hue associated with it. Changing any of those values changes the information in the pixel. This dilemma is the basic premise of looking at images as data.

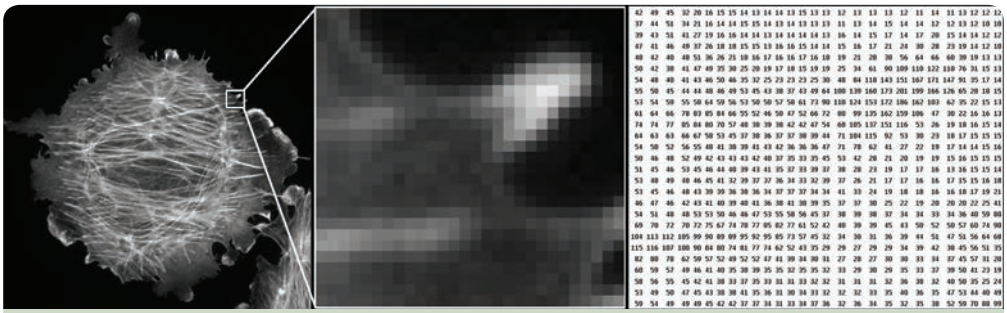


Figure 12.1 Images are data. Every pixel in a scientific image is represented by a specific numerical value.

There are a series of logical assumptions that are employed when analyzing the data in any scientific image: (1) the image is an accurate representation of the subject it depicts; (2) any data derived from the image is unbiased; and (3) the results can be replicated. The scientist/photographer can accurately control this perception by understanding the entire imaging process and paying attention to standardized imaging protocols. The data (in this case the image) can be standardized or altered through subject selection, choice of camera and camera settings, imaging techniques, file formatting as well as digital post-processing, so care must be taken to avoid unintended manipulations that can alter the interpretation.

In traditional analog photography, processes could also be standardized and side-by-side comparisons could be qualitatively visualized, but in the digital platform, visual comparisons have given way to a more quantitative approach. It is not enough to look at an image and say that it is *X* times brighter, or *N* times larger than another image; that number needs to be quantifiable and based on the intensity values of the individual pixels. To accurately

measure such changes, any part of the process that can be changed must be standardized. As with any set of experimental data, it also needs to be reproducible; results determined today must match results derived tomorrow. Some parts of the process are easy to understand and control, such as specific camera and lens selections, focus planes, camera settings such as shutter speed, ISO, aperture and white balance, location and intensity of lighting, etc. Some choices may become too subjective, such as exactly what part of a sample to image, and some issues may be difficult to measure and control, such as the effect of line current on the intensity of a mercury lamp in a fluorescence microscope. To minimize quenching, photograph first, stare later. Regardless of the difficulty, it is the responsibility of the scientist photographer to be as precise as possible and to accurately report all information regarding the acquisition, processing, and presentation of the scientific record.

Manipulation and Disclosure

If an image is considered data, then any modification of the content in the scientific image will be considered an alteration and, as such, any manipulation under the control of the scientist photographer must be taken into account. For the purposes of any review, image manipulation is the equivalent of data tampering and, depending on the severity, can be interpreted as anything from an unintentional mistake to fabrication of the scientific record. Manipulation of the data can happen at any point in the imaging process. Images when possible should be shot using the RAW format because the data in this file does not change; only the display of the RAW data commands is changed. It can later be converted to a TIF to clarify the image, but the RAW file itself remains unchanged and can be reverted to its native state by throwing away the .XMP file. A RAW file might be considered similar to a camera negative created during the film era. Specimen selection, hardware acquisition settings, software manipulation (digital manipulation), and data presentation all play a role. There is little agreement as to what level of manipulation is acceptable, so it is prudent to always err on the side of "none."

Falsification of the visual data doesn't discriminate between accidental or purposeful misconduct. Inaccurate results can be interpreted in both ways and it is up to the scientist photographer to make sure that the correct assessment is made. However, any image adjustments could potentially be deemed acceptable if all methods used to create the figure are explained in objective and reproducible detail. This can easily be accomplished by disclosing every step taken to acquire, extract, and present the imaging data. Scientific papers always include a materials and methods section to explain such usage. Captions are available when specific instances must be described. As the application of ethics implies intent, then full disclosure will show that no deception was intended. Lack of disclosure, however, leaves the results open to the interpretation that something is being hidden. Reviewers may disagree with the way an image was taken and dispute the findings it represents, but they cannot argue that it was intentionally deceptive if all steps were explained. To further support any claims, maintaining the original files in native form (RAW files) for inspection by reviewers will go a long way to prove the legitimacy of any questionable data.

Manipulation by Specimen Selection

The ultimate goal of any scientific image is to accurately represent the subject being photographed. This can be difficult to accomplish if the specimen is non-homogeneous and presents a variety of conflicting views. Different perspectives and magnifications can result in different interpretations of the experimental results, even if all other factors have been taken into account. Because of this issue, the first place a manipulation can take place is when the sample is selected. Therefore, ethical decisions should be considered about the sample's selection and the area to be imaged.

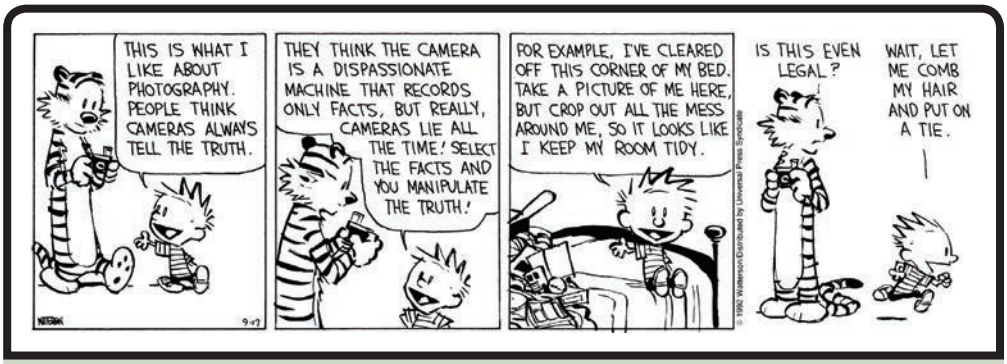


Figure 12.2 Acquired improperly, the information contained within a photographic recording can be misleading. If other images of Calvin's bed were taken from different points of view instead of only one narrowly selected point of view, the density of the accumulated debris could be more objectively analyzed and things would not look so tidy. CALVIN AND HOBBES © 1992 Watterson. Reprinted with permission of UNIVERSAL UCLICK. All rights reserved.

In scientific literature, the photographic record must be presented as an objective fact and then discussed for final interpretation. For this to happen, any reviewer must be able to confidently assess all of the data being presented and be persuaded by the arguments of the author. With a limited number of figures allowed in a paper, this means they must rely on any images chosen to be truly representative of the sample and sometimes this can be challenging and inadequate. This is why it has become more common to be expected to provide quantitative data. With an acceptable quantitative approach, the information in the image becomes numerical data, which can then be presented in graphical form, including such typical scientific annotations as standard deviations and error bars in a bar graph. It can be a stretch of the imagination for a photographer to think of their images as graphs, but this sort of presentation provides less ambiguity in data interpretation.

Most researchers are intimately aware of their experimental design and often begin the experiment with preconceived expectations about what the images will reveal. This is a necessary part of the scientific method where a theory must first be developed and then proven, but it often means the scientist photographer will impose their selection bias into the imaging of the specimen. Often, only areas that fit the expected result are chosen as representative of the experiment and the remaining areas are ignored. This restricted selection can skew the data and affect interpretation of the final results. For example, if

the expected result of a microscopy experiment is to show that all cells will stain positive for a condition but only a handful are found, then imaging those cells only and presenting them as representative in a paper would give a false impression that the sample contained a much higher percentage of positive cells than it did. This example demonstrates the need for being objective about what areas are chosen to photograph. Images of a single sample ($n = 1$) only provide information about one individual specimen or area and do not take into account variation in the subject. It is only by taking many images, averaging them, and then evaluating them with another set of conditions, that meaningful comparisons can be made. There are a variety of approaches that would provide a more unbiased view (Figure 12.3), including taking more images and averaging them (higher n), using a fixed pattern to choose an area to image regardless of what is there, using the same coordinates on comparable tissue slides, identifying and taking positive and negative areas, taking a lower magnification that shows the entire structure, or using stitching software to capture the entire specimen at higher magnification. The specifics of the sample will help to define the best approach and that method should then be applied exactly the same way for all specimens in the experiment.

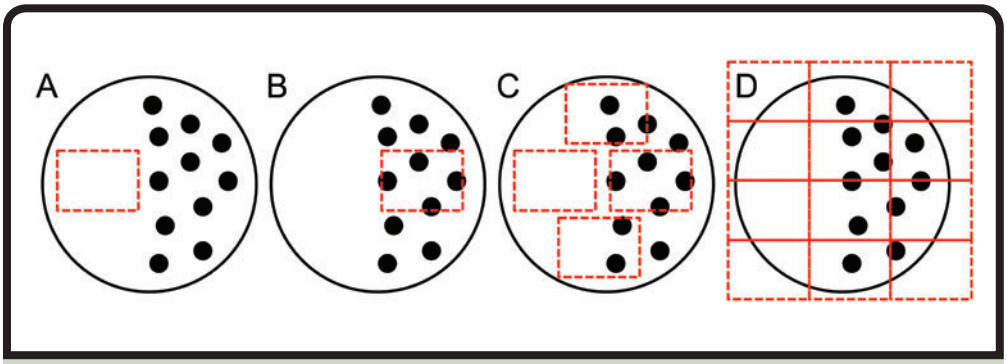


Figure 12.3 Making multiple images of various fields within a sample will avoid bias. Single images from a heterogeneous sample will show very different results. Dotted red lines represent individual images. Taking only one image (A or B) would demonstrate very different results. Taking multiple areas (C) and averaging the results will provide a more accurate count of spots per circle. For absolute precision, capturing the entire circle in a single, low magnification shot or as a series of stitched images (D) will provide the best results. The more images that are used, the more accurate the analysis.

Looking beyond the specific settings of the acquisition phase, any good imaging experiment must also include controls. Controls help determine whether a protocol actually worked. Deciding what makes a good control is part of the original experimental design, and it is not always the same for every experiment. For example, in a fluorescently labeled tissue preparation, it would be important to also create a negative control slide, prepared the same way as the sample but lacking the fluorescent label. In this fashion, scientist photographers can make sure that what might appear to be positive in the main sample is not actually some endogenous background staining caused by something like the tissue fixation. In another example, it would be just as important to have positive control slides, where known samples are prepared in the same way as the experimental slide. This would perhaps show

that nothing took up the stain, even though the control proves that the procedure was done correctly. Sometimes the answer to the scientific question is "there wasn't anything there." By ignoring controls, incorrect interpretation of the experimental results is quite possible.

After taking numerous images, some positive, some negative, some with lots of repetition, the question begs to be answered: what images should be ultimately chosen for presentation? There is only room to use a few. At this point, the process becomes subjective. In general, only images that represent the mean of the result should be used. This means that, after all the images are acquired and analyzed, the results of the analysis will dictate the choices. For example, if the quantitation shows that 30 percent of the cells in the sample are positive for a particular stain, then an image should be chosen that demonstrates 30 percent. It is important that the representative image should be close, but absolute accuracy is not essential, as the reviewers will most likely be more interested in the quantitative data. However, sometimes an experiment is designed in such a way that it is essential to show more of the original images. In cases such as this, most scientific journals invite the author to submit supplemental data that is posted online, but not in the hard copy version of the manuscript. Some journals even require that all original images used in a paper must be uploaded to the supplemental site. One great advantage to this opportunity is that it allows presentation of data that might otherwise not be available to the reader. Time-lapse movies, 3D reconstructions, and other digital media cannot be printed on paper but can easily be accessed and shared online.

Manipulation by Hardware Settings

A classic difficulty in scientific imaging is the understanding of exactly what happens when the button is pushed to record an image. Equipment manufacturers are constantly coming up with faster and better technological answers to address the needs of the scientist photographer. The general direction is to provide more automatic settings and remove operator options to aid in making exposure choices so that results can be standardized and better comparisons can be made. Unfortunately, all of this automation usually creates exactly the opposite problem. True image comparison requires that all acquisition parameters must be fixed and standardized. An imaging system that does not allow an operator to choose manual or override automatic settings will often provide non-comparable results. For example, many photographers will depend on a form of auto exposure to determine the best acquisition settings, but if multiple subjects are to be compared, the specimens themselves will affect the reading from the camera meter, resulting in non-comparable images (Figure 12.4).

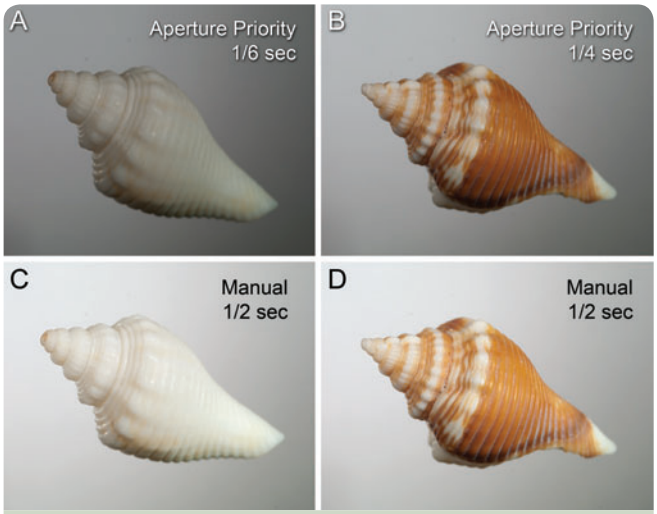


Figure 12.4 These images of two shells were taken independently using aperture priority and manual exposure modes. Each image was photographed at ƒ/16 and is presented here without adjustment. Auto exposure modes—when used to determine the settings—provide an average exposure to the scene. In this example, the meter was fooled by the amount of light tones in the background and the tones of different specimens. This resulted in variable shutter speeds (A and B), although neither of these combinations were optimal for the sample. Using a manual exposure mode (C and D) provided an accurate exposure as well as consistent and comparable results.

In general, it is usually best to select manual controls over all other variable settings in the camera and to make sure that no real-time adjustments are taking place while the images are being recorded.

Although it is not always the case, "auto" is a four-letter word. It is a bad word that indicates that the camera has been given authorization to autonomously select particular settings resulting in uncontrolled adjustments. When using auto settings, care must be taken to oversee any potential complications. Some auto features may seem good at first, but in the end analysis use of auto will depend on the circumstances for its selection. Autofocus seems harmless at first unless it selects the wrong plane to focus. Some Nikon and Canon cameras can control where autofocus settings are achieved, offering a bit more control. This can happen because the subject may have moved and the sensor is looking at a distant subject instead of the closer one. Auto-scaling and auto-histogram adjustments would both be useful to make dim samples more visible, unless intensities were being compared and a dim sample was artificially brightened even though it was a negative control that was supposed to look that way. As described above, auto exposure can provide inconsistent settings resulting in non-comparable results as well. The key to accurate and reproducible imaging is being aware of all of the variable parameters that can be changed and having total control of their settings. Table 12.1 illustrates the issues with some common variables in image acquisition. Some cameras may have many more.

Manipulation by Imaging Technique

The potential for image (data) manipulation does not end with merely adjusting the settings in the camera; it is just as important that those settings be chosen and used correctly. What good are standardized settings if they are overexposed or out of focus? What does it matter if the camera is set correctly but the subject is treated differently? Does it really matter if the lighting is different? The short answer to all of these questions is yes, it does matter, and it is the responsibility of the scientist photographer to know as much as possible about the subject being photographed, what is trying to be shown, and how best to capture that particular data. Once the camera choices have been established, they must be properly employed through standardized, reproducible technique.

How can accusations of manipulation be avoided and the best exposure defined for an image? With so many variables, how can it be made objective? The answer lies in the pixels. For any given image, every pixel has a specific numerical value, representing the intensity of the light on that pixel. If the pixel is at 0, then no light is falling on it and that translates to black. If the pixel value is at 255 (in an 8-bit image) then that pixel is considered saturated and translates as pure white. The problem with a saturated pixel, however, is that, once it has reached saturation, you do not know if more light (more data) may have continued to fall on that same pixel. Being fully saturated, the pixel cannot record any more information that may have fallen upon it. For this reason, the proper exposure would be one that does not have any saturated pixels. If the pixel had a value of 254, it would indicate that there was no more light to be captured, therefore no more signal to record. In the days of analog film photography, this was known as "exposing for the highlights" and still holds relevance in the digital era. The best image maintains tonal details throughout the range from dark to

Table 12.1 Examples of Adjustable Camera Settings and How They Can Affect Quantitative Documentation

Camera Setting	Settings and Complications
Bit depth	By default, most cameras capture at 8 bits (256 levels of grey). If the goal is to create accurate scientific quantitation, set the camera if possible to its highest setting: 12 bit (4,096 levels), 14 bit (16,384) or 16 bit (65,536). For digital SLR-type cameras, this means capturing in RAW (16 bit). Note that some scientific cameras capture at 14 bits and up-sample to 16 in Photoshop. Higher bit depth provides more precise tonal resolution.
Gain/ISO	Increasing the gain or ISO makes the camera more sensitive, allowing specimens to be acquired with lower light levels. However, the more the gain is increased, the more the noise increases and reduces the overall quality of the image. An ISO setting that is the lowest level appropriate for the sample should be selected and maintained. The camera should not be set to auto ISO, where the camera will automatically "gain up" in low light.
Shutter speed	Faster shutter speeds can reduce blurring caused by either the specimen or the imaging system. Normally, it is desirable to eliminate blur with a fast shutter speed. However, showing a specific amount of blur can be a good way to compare active specimens. The shutter/acquisition speed should be the same in each of the images that are going to be analyzed together. With different shutter speeds, any blur effects may be caused by the difference in shutter speed, not behavior of the specimen.
F-stop/aperture	Closing the f-stop in a digital SLR increases depth of field and can be useful, but not if comparable images have different depths of field. In a microscope, closing down the aperture diaphragm will result in increasing diffraction affecting information in the image. In each case, the aperture setting should be noted and should not be changed between images.
White balance	Known pre-sets, like tungsten or daylight, or custom readings should be chosen for each lighting situation. Auto-white balance is generally very useful in unknown lighting situations; however, it is affected by the colors of the subject and will produce inconsistent results.
Focal length/ focus plane/ magnification	When using a camera to make measurements of an object, the focal length and focus planes should be kept the same. Changing the focus changes the optics, the relative size of an object on the image plane, and the magnification. Changing the zoom may distort the image as well. When comparing macro specimens, the magnification ratio is fixed and focus is achieved by moving the entire camera forward and backward, ensuring that the same plane of focus is always maintained.
Spatial resolution	All images to be compared should be captured at the same spatial resolution so that all of the images are taken at the same size with the same number of pixels. Most cameras have choices ranging from the entire sensor to a much smaller option for web use.
Compression	Scientific images should first be captured in TIF format (uncompressed) or some other non-compressing format. With a DSLR, RAW should be used for scientific images. This makes sure that all of the original data for each individual pixel is saved. JPEG files may be smaller and easier to share, but they get that way by throwing out specific pixel information (data). Always save your original file and then work with copies.

light. In the case of comparing an entire data set of images, the proper exposure would be the one that reaches just below saturation on the brightest image in the group. If any images include saturated pixels, those images would be invalidated for quantitative analysis. It should also be noted that many images are used in a purely qualitative way in that they are a visual record only. However, if an image is to be used for quantitation, where numerical data is being derived, that analysis should only be performed with non-adjusted files.

In addition to affecting interpretation of pixel intensities, an overexposed image can also be used to alter the physical measurements of spatial values in an image. Pixel "blooming" results when signal from saturated pixels spreads to those pixels adjacent to it. The more overexposed, the more blooming and the more any feature appears to spread. In a properly exposed image, a feature of interest might be measured at, perhaps, 10 pixels wide, but overexposed it may measure 12 or 14 pixels because of the effects of blooming. That would result in a 20–40 percent variation just because of the chosen exposure. This problem is magnified with smaller features and higher magnifications, but the effect remains regardless of the size of the measurement and is another example of how important it is to use the imaging equipment correctly (Figure 14.5).

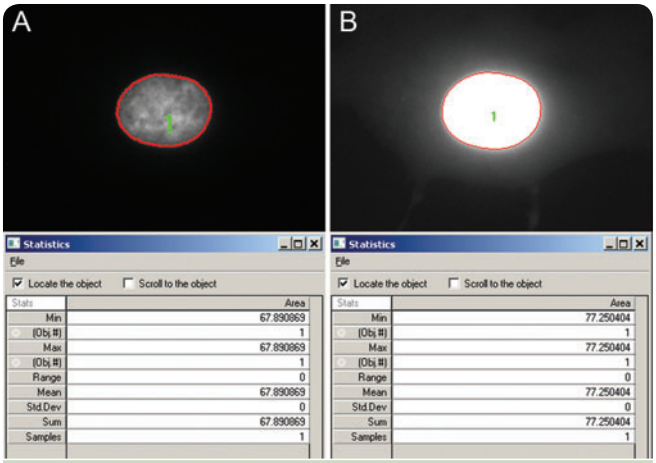


Figure 12.5 Two images of the same fluorescent nucleus were taken, (A) at the proper exposure and (B) for a very long exposure. The measurement of the nuclear area is 13.8 percent higher in the overexposed image due to the effects of blooming.

We've all seen it: the "before and after" advertisements of photos featuring weight loss, reduction of varicose veins, disappearing wrinkles, etc. Quite often, it is noticeable that the lighting is very different on the part of the subject which is most relevant. Possibly, the model in the first image is wearing skin-tight clothing with harsh lighting, but gauzy, flowing dresses with soft lighting in the next, or standing differently each time. In other situations, the "before" image may use a classic rake light with high contrast to enhance the apparent dimensionality of the subject, while the "after" image uses flat, low contrast lighting to minimize the appearance of the same thing. These are example of non-standardized protocols. Even if all camera settings are standardized and chosen properly, if the subject is not treated exactly the same way each time, then any results would be non-comparable as well. This is another example where the imaging process is used to alter the perceptions of the viewer and "distort" or at least modify what is actually shown.

Patient photography is a specialized application of scientific photography. Among other purposes, it is used to accurately record medical conditions for comparison to future time-points. With proper technique, measurements can be taken directly from a calibrated image to illustrate absolute size of a feature, like a mole on the skin, and see if it has enlarged, reduced, or otherwise changed when observed at another time. Without stringent application of photographic standards, however, it would be a simple matter to show a change in size simply by using a different magnification. Whether this is done accidentally or on purpose is irrelevant to the data—the data would be wrong. If treatment regimens depended on such results, an inaccurate set of photographs could affect patient treatments

and outcomes. Likewise, changing the lighting from a point source with texture lighting to a broad source with flat lighting to "show" a reduction in wrinkles is using the camera to imply a false assumption. The images are only comparable and scientifically valid if they are all taken the same way.

The subject itself can also have an effect on the implied veracity of a scientific image. An image of a patient taken from a slightly different angle or focal length could illustrate the difference between an apparently large nose or a smaller one. A patient wearing jewelry or clothing that hides a medical feature creates questionable distractions to the areas of interest. Distortions caused by taking the image at a different angle could also result in a different measurement and appearance. For these reasons, patient photography relies on standards that include fixed focal lengths, specific distances and magnifications, lighting angles, and specifically reproducible angles, such as straight on (0 degrees), as well as 45 and 90 degrees left and right.

Specimen response to the imaging procedure can also have a large effect on the results of an experiment. In fluorescence microscopy, for example, specimen bleaching is of primary concern. In general, the emitted fluorescent signal depends on the amount of excitation light falling on the subject. However, the longer the subject is exposed, the more the signal fades, so spending a longer time looking at and focusing on a specific area before actually capturing the image will result in different total intensities. In order to artificially show a difference between subject brightness levels, it would only be necessary to wait a little longer before taking the picture in one of the samples. In another example, some live cell imaging experiments are affected by phototoxicity, where the light used to make the exposures actually damages the cells so that they no longer respond to an experimental condition as expected. At the extreme, too much light will kill the cells and then it becomes difficult to know if the cell death was caused by the experimental conditions or by the imaging conditions.

These examples all illustrate that data manipulation can easily occur in an imaging experiment long before the shutter is ever tripped and the image has been captured. In the most benign situations, the cause is inexperience or accident, but an accomplished photographer can also make use of this knowledge in more nefarious ways to alter the perceived results of a scientific experiment. Usage of this more sinister approach translates into scientific misconduct. The difficulty for the unfortunate scientist photographer who is not aware of the effects of their imaging choices is that perceived misconduct does not discriminate between intended deception and accidental results. If the data is proven to be wrong, the assumption can just as easily be made that the result was an intended fabrication and so all of the work associated with the experiment would be placed into question.

Manipulation by Software

Most people think of Adobe Photoshop software when they think about image manipulation. Once an image has been captured, it can be a simple matter to make adjustments to the image using the software. This can be a very slippery slope to climb. Some adjustments like gamma correction, which may seem perfectly acceptable, can alter the data in an image profoundly. Other adjustments like copy/pasting and cloning are a bit more obvious

as unacceptable modifications for use on scientific documents, but even that doesn't stop people from using them. Regardless, as was evidenced in the hardware choices and standardized techniques, it is up to the scientist photographer to understand the limitations of the software and abide by the ethical constraints of scientific methods. Unfortunately, even this dogma is not universally applied. A thorough understanding of the consequences of poor image processing decisions is required.

When initially thinking about using editing software, protecting the original data must be the first consideration. When images were captured on film, the film itself was the repository of the information. It could be placed in storage and re-examined at a later date with expectations that the information never changed. The almost permanent medium of film was very useful in safeguarding the original information. With digital images however, the original file is much more tenuous and results from the first click of the capture button. Any changes to that specific file will change that original data. It is imperative, therefore, that all original files be immediately saved in a non-compressed format (because compression throws out information), before any adjustments are made, and only copies of the original file should be used to work with. Many digital cameras offer a RAW format that is excellent for this purpose. The RAW file will often posses a higher bit depth, and should be saved in a proprietary format that can't be adjusted without creating a duplicate file and needs to include all of the metadata from the camera. After capture using RAW, the original files regardless of camera should be saved as a TIF file (uncompressed) to retain all of the information. Understanding that to hit the "Save" button will immediately and irrevocably overwrite any file, it is also best to get into the habit of only using the "Save As" command instead. When employing "Save As," the software provides an additional step to choose location and file type before the save is actually made. This provides the safeguard before making a save that would eliminate the original data.

When critically considering image manipulation, the most objective description breaks the process down to the most basic unit, the pixel. Altering the numerical value of a pixel in a digital image changes the pixel and, objectively, then changes the data. Because an image can be broken down to numbers that are displayed in a spreadsheet, it would be the equivalent of going into that spreadsheet and changing some numbers after the fact. However, many scientific images are not broken down to that level of detail and many photographers work at a level that doesn't discriminate between individual pixels. As such, a more generic definition of software application is often used that takes into account a journal's reviewer board with a familiarity with photographic processes. In general, globally applied, linear adjustments to an image, such as those correcting for brightness and contrast, are acceptable, as long as those enhancements do not mask information in the image (Figure 12.6). Although there is no complete consensus on just what adjustments are acceptable in a scientific image, these traditional "darkroom adjustments", or adjustments that could traditionally be done

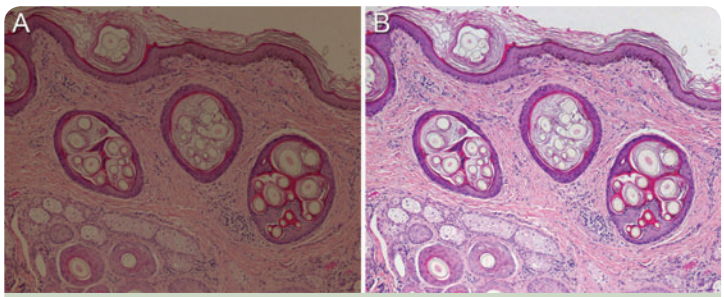


Figure 12.6 The original photomicrograph of canine skin (A) was underexposed and incorrectly white balanced. Adjustments to all pixels that affect brightness, contrast, and white balance (B) are generally acceptable changes when they do not mask details and as long as the changes affect all pixels equally.

in a darkroom with film, chemistry, and paper, are usually anticipated by reviewers. Digital imaging has also provided some new adjustments, like color balance correction that can be applied to an image without much concern. It must be kept in mind that once any correction has been made, that file should be immediately saved as a new, adjusted file and the original file should be saved in a folder as a native and unaltered file. There is additional content about this and image processing in Chapter 13.

While it can be difficult to define what level of manipulation is acceptable, it is often much easier to define what is unacceptable. When the manipulation is specifically used to alter or hide data in the image, it crosses the line and becomes unacceptable. By removing or changing pixel data individually and not globally, the altered image misrepresents the scientific record and is unacceptable. In general, manipulations that target a specific part of an image, but not all, are also considered unacceptable (Figure 12.7). The data within a scientific image resides in the relationship of each pixel to the pixels that surround it. Localization of the signal, relative intensity, and number of pixels involved in a feature are all examples of this relationship. By changing the relationship of these pixels to each other, the information they contain has been changed. This type of the alteration of pixels can be enabled by a variety of software by copying and pasting, cloning, erasing, painting, "healing," and some auto-adjustments such as red-eye removal. It can also be subtle, such as by using sharpening or noise reduction filters, which change the localized relationships of the pixel values that are used to enhance the appearance of the overall image. It will always be possible to come up with scenarios where the use of any or all of these tools might be deemed appropriate, but as their use is questionable at best, if they are used then full disclosure of the process is most important.

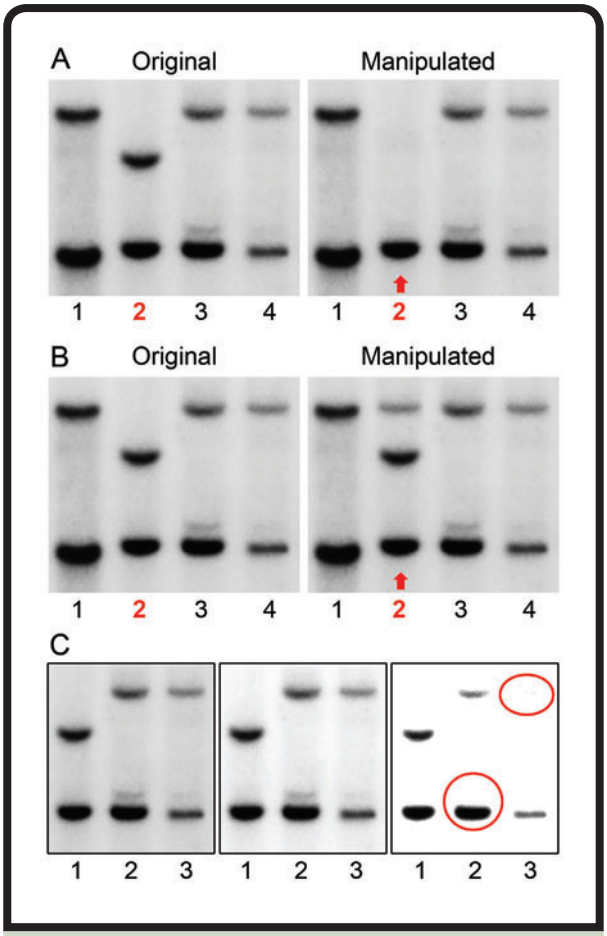


Figure 12.7 Several examples of fraudulent adjustments were made to a western blot. A single band was digitally removed from lane 2 in Example A, while a band was added to lane 2 in Example B. Contrast and brightness are normally acceptable adjustments until they go so far that the lighter bands in Example C are lost (circles).

Documented scientific experiments require detailed explanations of how they are performed and are needed to meet the need for credibility. The ultimate goal of any scientific image is that it can be replicated by following the steps that were used to create it the first time. To that end, there are many imaging practices that apparently meet the criteria for being unacceptable, but which are routinely used in imaging protocols. These controlled manipulations, without explanation, raise questions and should always be disclosed in the text, thereby making them acceptable instead. Examples include background subtraction, flattening, and noise reduction. These adjustments are termed non-linear adjustments because they affect different parts of the image in different ways (Figure 12.8). Background subtraction and flattening compensate for an uneven illumination in the sample and noise reduction removes transient pixel intensities in the

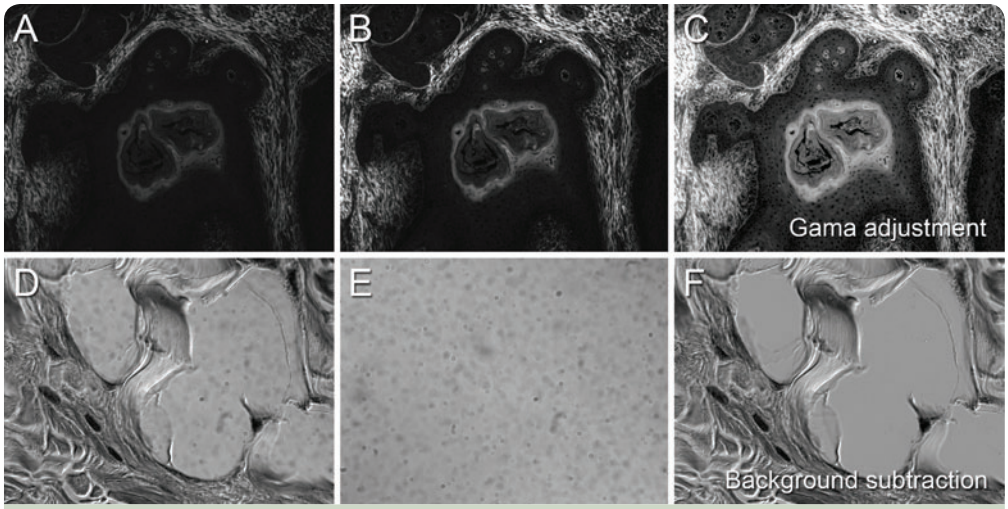


Figure 12.8 Non-linear adjustments affect each pixel in the image differently and adjustments are often disclosed in an image caption. (A) The original fluorescence micrograph of skin sample without image processing. (B) Brightness and contrast adjustments make some details more visible and (C) is a gamma adjustment shows the most detail. (D) This file shows the sample in Differential Interference Contrast (DIC) method featuring connective tissue. There were severe problems with condensation on the imaging sensor. (E) Background image of artifacts taken in clear area of the slide and (F) background subtraction to remove the artifacts from the original image.

image. Another common non-linear adjustment is gamma correction. From a practical view, adjusting gamma brings out details in part of an image, like the darkest areas, while leaving the other areas, like the highlights, untouched. Using gamma is an excellent way to compensate for images with a high dynamic range that are difficult to reproduce in print or even see on a computer screen, but because it is non-linear, it is one of the adjustments that must generally be disclosed in the image processing protocol to be acceptable.

Manipulation by Presentation

Regardless of how specific images are captured or processed, additional data manipulation can occur when those images are presented. Biased areas of selection, as noted previously in this chapter, is but one way this can happen. So is the comparing of files that were acquired or processed in different ways. A specific adjustment, like brightness, may be an acceptable manipulation, but if one image is adjusted a lot and another not at all, then those images are not comparable and any attempt to do so is a fabrication. Side-by-side files that were adjusted with the same settings are comparable, but only in a qualitative manner. True quantitation can only take place with original, non-adjusted files.

Figures are often created by compositing several images into one new image. Often, this is obvious, as the images may be non-continuous and separated by lines or space. Other times, however, a larger image is the result of multiple images stitched together and the individual images non-discernible. Chapter 14 explores this subject, sometimes called computational photography. It can be important for a reviewer to know this, and an image

that is created through compositing must be disclosed as a composite. With no reference as to how an image was created in the caption, there may be signs or evidence of changes that can't be explained, which then brings the entire figure into question. To be done properly, any composite image should state that it is, indeed, a composite, it should be made up of overlapping images, and all of those images should be taken with exactly the same settings and processed either all at once or exactly the same way for each individual section. If there is no overlap, it would be appropriate to separate the individual images with a line to show the individual parts of the figure.

Another consideration about the acceptability of an image manipulation would be image usage. An image that that will be used as part of a cover illustration does not have the same expectation for veracity as a figure embedded in the body of a paper would have. The cover is often used as a way to gain attention for the journal and so the splashier the better. For that reason more editorial-like images are often used as covers. Even so, it should be clearly stated in the caption if the image has been altered in any way, even to the point of referring to it merely as a "digital illustration" so that false impressions of the work are not created by such a showy presentation.

Forensic Examination: Uncovering Digital Image Fraud

There are many types of both willful and accidental manipulations that can occur to scientific images. Some are virtually impossible to discover, but the majority can actually be quite easy to identify. Simple examinations using Photoshop software can often uncover straightforward adjustments. For more difficult examples, there are macros available that can also be run in Photoshop to uncover such discrepancies. Simple pattern recognition algorithms are built into these macros, but the recognition by a sharp set of eyes can be quite effective as well. To discover problems within an image, the reviewer need only think like a cheater and look for the telltale signs. In the end, finding and identifying manipulations can only indicate that a particular inconsistency exists. Whether it was purposeful, accidental, marginal or severe requires more information. No one person or software can measure intent. By carefully observing image attributes, the de-authentication of data contained within can be implied, but determining whether there was intent to deceive would require the original file for comparison, as well as all other relevant information collected for the particular study.

It is perhaps ironic that Photoshop software, or other image editing software, can be the primary method for uncovering fraudulent image manipulations. However, it makes sense when you realize that a large majority of such adjustments are not done maliciously and that the scientist photographer was not actively trying to hide anything. Most often it is a simple matter of education. Many people do not realize the extent of the problem because they are not aware of what is happening to their images at the lowest levels and how that is affecting their data. The same tools used to create the manipulations can also be used to unmask them. The simplest such method is by examining the histogram.

The histogram is a very objective way of looking at the distribution of pixels in any image. One glance will reveal if there are saturated pixels (due to a spike at the 255 level) or

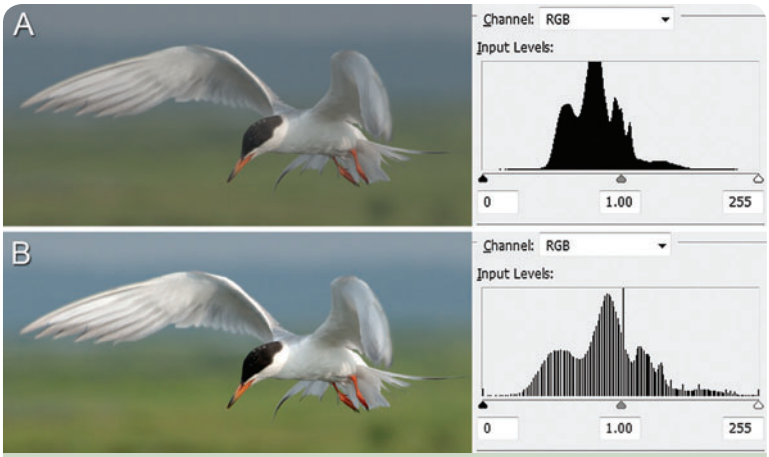


Figure 12.9 Histogram spikes and a non-continuous curve indicate that the tonal range of an image has been modified and the pixels redistributed. The histogram from an unadjusted image of a Forster's Tern (A) shows a continuous curve, while the adjusted file (B) shows the spikes and gaps. This is generally a sign of contrast enhancement.

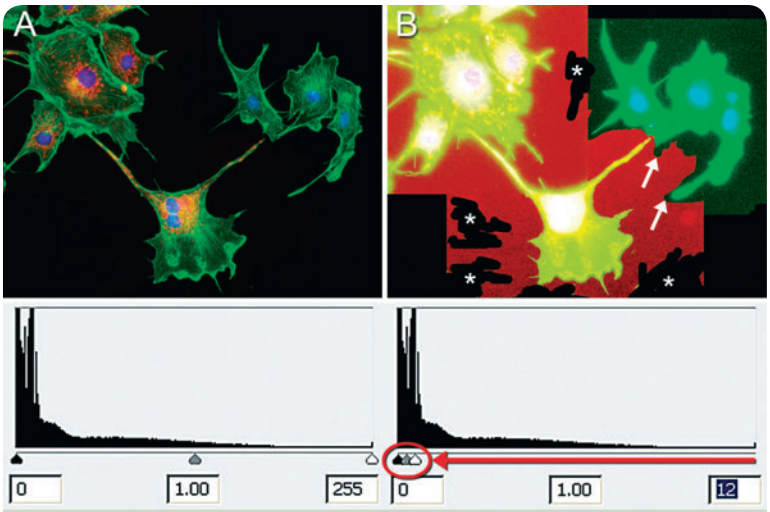


Figure 12.10 Stretching or compressing a histogram reveals that an image is *not* a single image but is actually a composite of three. Two of the individual images were captured with three channels, but the other one was only captured with two channels. There are also areas that were painted in (*) and other areas erased (arrows).

completely black pixels (due to a spike at the 0 level). These numbers assume one is looking at an 8-bit image, one that is most commonly presented. Interpretation of a problem curve can be very straightforward as well, but care must be taken. Perhaps the histogram is supposed to look the way it does because it reflects the actual image configuration. Maybe the image is simply overexposed, so a spike in saturated pixels is expected. Maybe the image is a fluorescence photomicrograph with a black background and it should have a large number of pixels near 0. More difficult is trying to determine if the aforementioned indications are due to external influences or post-processing modifications. If the histogram has gaps and is broken up, or has multiple spikes, it can be an indication that the original image was tampered with (Figure 12.9).

Adjusting the contrast or overall brightness or changing the color will demonstrate effects such as the ones shared in this chapter, but is this necessarily bad? As previously suggested, global changes are often deemed acceptable. In addition, compression artifacts can also manifest themselves in this way, so just how much the adjustment affects the data and whether the adjustments were noted in the text will have considerable bearing on the determination of any wrongdoing.

Using the histogram tool more deeply can also reveal otherwise hidden features and make these features stand out for examination. A "histogram stretch" can be performed, where the highlight level is moved from its standard position at 255 to the extreme left, to a level of approximately10 or 12. This adjustment is essentially an extreme adjustment of contrast, which enhances subtle variations in the image, especially in the darker areas (Figure 12.10). Stretching the black level to the extreme right, near a level of 245, will provide similar results with light-background images.

Many computer users assume (incorrectly) that their monitor is showing them all of the intensity levels in their image. Most monitors will be lucky to show sRGB let along Adobe RGB 1998. This holds true for video projectors, too. However, depending on the monitor's settings and the environment in which the screen is viewed, the tonal range may be compressed. Because of this shortened range, many of the levels in either the darkest or brightest regions will be cut off. When unacceptable manipulations such as erasing, painting, cloning, and copy/pasting are done to parts of the image at these far ends of the brightness graph, the results are not always seen directly on the monitor and the person making those changes may possibly assume that no one else will be able to see them either. Just because these levels cannot be easily observed does not mean that those levels do not exist and have been enhanced.

Photoshop software can be used in other ways to sleuth out incongruities in the images as well. If you were to copy a part of an image and paste it directly over the spot it was copied from, then all pixels would line up one-for-one. If that pasted section was then inverted and set to an opacity of 50 percent, it would completely negate the pixels that are located below this region and the result would be a solid gray block (this should be done at a display level of 100 percent). This is a useful way of comparing areas that are suspected to have been copied and pasted (Figure 12.11). Duplication is a common problem with images of western blots or other gels where there is the presence of a band pattern required to identify the data. Unscrupulous researchers will copy/paste individual bands or even whole lanes and create the gels in the computer that they couldn't create at the bench.

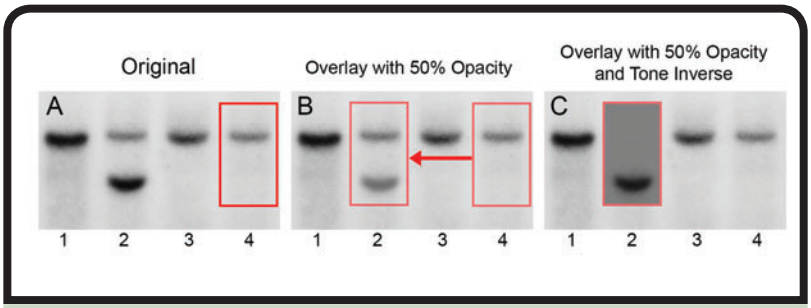


Figure 12.11 The top band in lane 4 of an original gel (A) is suspected of being copy/pasted into lane 2 in a similar location. The band and its immediate area are selected in Photoshop (red box), duplicated onto a new layer, which is adjusted to 50 percent opacity, and lined up over the questionable area in lane 2 (B). The new layer is then inverted (C). Notice that the top band, which lines up precisely with the original band, disappears, while the lower band remains visible, indicating the top band is an exact duplicate of lane 4.

Another way to examine a potentially compromised figure is by pattern recognition and simple visual comparison. A notorious example of flagrant manipulation occurred in 2005 when Dr. Hwang Woo-suk, a Korean stem cell researcher, published a groundbreaking paper in the journal *Science* in which he claimed to have created eleven human embryonic stem cells through a cloning process. The work came into question and started to fall apart when investigators noticed that two images in his published figures that were claimed to be unique stem cell colonies in fact appeared to be two halves of the same colony. When the two images were placed in just the right way, with just the right amount of overlap, the actual shape of the single colony was apparent. The images were actually inserted into two different figures which made the presentation easy to have overlooked. Only by the careful examination of every image in the paper and subsequent comparison of them to every other image in the paper did this deception rise to the surface. After this first falsehood was discovered, other discrepancies were soon noted and it was eventually revealed that most of the paper was a fabrication.

The comparison in the previous example was made possible in part because there were specific, small features of commonality between the two images in question. Patterns caused by tissue features, small bright or dark areas, artifacts from dust and other sources, or other morphological characteristics, all lend themselves to this sort of comparison. Although there are certainly commercially available programs that can look for such areas of consistency, it is often faster (and cheaper) to look through a set of images with the thought in mind that there may be problems. If you are looking for them in the first place, they will be easier to spot. In the early days of digital imaging, it was common practice to remove known artifacts through the use of cloning tools, or erasing. Today, these methods are unacceptable, not only because of the way they inherently change the pixel data, but also because they remove useful points of comparison, not to mention that each person's idea of what constitutes an artifact may differ. Ultimately, when any figure in a paper is brought into question, it raises questions about every other figure in the article and, by extension, the results of the paper itself are of concern as well.

Using the features in Photoshop software to analyze an image is a quick method to start the process of proving manipulation. Just as a scientific paper requires a degree of objectivity and reproducibility, so does the analysis of any potential problems in an image. Any method used to discover the discrepancies should be documented to the same degree of rigor that was required to create the images in the first place. This means noting the exact order of steps in the process and all of the numerical values set within those steps, as well as the results of the process. The Office of Research Integrity (ORI), a division of Health and Human Services, provides free "Forensic Image Tools" in the form of actions and droplets that are designed to be used in Photoshop; see https://ori.hhs.gov/forensic-tools. The tools found on this site standardize the actions used for discovery and are very helpful in documenting potential examples of inappropriate manipulations. They are provided with detailed instructions as well as test images to practice with and are often used by investigating committees looking into allegations of imaging misconduct.

Industry Oversight

Even though digital photography has been the primary method of acquiring photographic images since the late 1990s, the problems associated with image manipulation have been slow to be addressed. In what was a foreshadowing of things to come, arguably the first major example to illustrate the issue occurred February 1983, long before there was a computer on every desk. *National Geographic* magazine published an image of the pyramids at Giza that was digitally modified to better fit the image in the vertical format used on covers. A pyramid was moved for this reproduction. Strictly an editorial decision, this image nonetheless created a storm of controversy among Egyptologists who knew that there was no possible way for the view depicted on the cover to be actually seen from any point in the area. The debate that ensued forced *National Geographic* to issue an apology and create perhaps the first set of rules governing the acceptability (or not) of digital image manipulations within their publications. It is interesting to note that many of the same issues that relate to the use of images in scientific publications (objectivity, truthfulness, standardization, etc.) are also applicable to image reproduction in journalism. There are numerous examples of problems with manipulated images that come from photojournalism

as well. Examples from journalism tend to be very public and often find their way into the headlines.

In science, however, the main vehicle for finding, identifying, and responding to allegations of image manipulation tends to be the peer review process. This somewhat flawed system relies on the objectivity of scientists to police themselves. Papers written by researchers are reviewed by colleagues who may have a vested interest in the results being shown. Problems with images may be overlooked or ignored so as to validate the results and move their own research forward. Luckily, the investigators are not usually the publishers and so there is a second level of oversight in the publication process when the papers are chosen for inclusion in scientific journals. The journals rely on the reports from peer review, but they also have their own set of rules and policies that are generally listed in their "Instructions to Authors" statement.

As an example, the following is taken directly from the policies of the *Journal of Cell Biology* relative to their guidelines for handling digital images.

> No specific feature within an image may be enhanced, obscured, moved, removed, or introduced to the image. The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (i.e. using dividing lines) and in the text of the figure legend. If dividing lines are not included, they will be added by our production department, and may result in production delays. Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the whole image and as long as they do not obscure, eliminate, or misrepresent any information present in the original. Non-linear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend. All digital images in manuscripts accepted for publication will be scrutinized by the *Journal of Cell Biology* production department for any indication of improper manipulation. Questions raised by the production department will be referred to the Editors, who will request the original data from the authors for comparison to the prepared figures. If the original data cannot be produced, the acceptance of the manuscript may be revoked. Cases of deliberate misrepresentation of data will result in revocation of acceptance and will be reported to the corresponding author's home institution or funding agency.
>
> (*Journal of Cell Biology*, Instructions to Authors; http://www.jcb.org/misc/ifora.shtml)

The *Journal of Cell Biology* was one of the first well-known journals to initiate such standards, and most other large journals have since followed suit. However, there are no restrictions in the industry, where papers that have been rejected by some higher impact journals may still be able to be accepted by smaller journals willing to overlook potential problems. A seminal paper on the topic "What's in a Picture? The Temptation of Image Manipulation," published in 2004 in the *Journal of Cell Biology*, was written by Mike Rossner, former editor of the *Journal of Cell Biology* and Kenneth Yamada. Until that time, smaller papers in low-impact journals and editorial discussions on the subject in other journals had alerted readers and contributors to the potential dangers of image manipulation and attempted to create initial dialogue. The Rossner paper attempted to codify what was acceptable and what was not, providing specific examples based on editorial observations. The high impact of the journal

meant that the paper was widely disseminated and such standards for imaging ethics started to become more mainstream.

As the field and technology have matured, more research has been done on the science of analyzing scientific images for potential manipulation. Dr. Hany Farid, Professor and Chair of Computer Science at Dartmouth College, has led the way with an explosion of publications in a wide variety of media outlets. They run the gamut, from "A 3-D Stability Analysis of Lee Harvey Oswald in the Backyard Photo" in the *Journal of Digital Forensics, Security and Law*, 2015 to "A Perceptual Metric for Photo Retouching" in *Proceedings of the National Academy of Sciences*, 2011, which delves heavily into mathematical modeling. His website, Fourandsix, http://www.fourandsix.com/, explores the history and present-day use of manipulation and highlights an image authentication software he developed, called izitru, which will run and host an algorithm and certify whether an image has been manipulated.

Scientific journals have become increasingly dependent on their online presence to reach larger audiences. Digital journals have distinct advantages over traditional print magazines, in that they provide the opportunity to include supplemental data with every paper. This additional information could be video files, 3D data sets, or just more figures than could normally be accepted in the more limited space of a print run. The *Journal of Cell Biology* has led the way here as well, with the introduction of their JCB DataViewer, which requires that authors upload their original, non-adjusted images and allows the viewer to adjust them as they wish. This type of transparency in scientific publication is an excellent way to provide a more verifiable and unbiased approach to the presentation of published images.

Consequences

Honest and ethical practices in imaging and data presentation may at first seem to be a rather academic subject—nice to know but not necessary to follow. Nothing could be further from the truth. The vast majority of funding for scientific research in the United States comes through the National Institutes of Health (NIH), the National Science Foundation (NSF), and other government agencies, and they care very much how those funds are used. Following a series of cases of scientific misconduct that were investigated in the 1980s, the Department of Health and Human Services established a committee to oversee research integrity activities within the range of its organizations. The Office of Research Integrity (ORI) was created in 1989 to follow out these directives (http://ori.hhs.gov/). The ORI is responsible for "developing policies, procedures and regulations related to the detection, investigation, and prevention of research misconduct and the responsible conduct of research." The organization provides guidelines and training programs, investigative authority and recommendations for administrative actions to be taken by the Office of the Secretary of Health and Human Services in the cases where misconduct and, by extension, improper use of government funds, is established.

The Office of Research Integrity is quite specific about how misconduct is defined and all of the definitions, rules and regulations are published in the Federal Register. As stated in section 93.103 of these extensive documents, the definition of research misconduct is:

> Research misconduct means fabrication, falsification, or plagiarism in proposing, performing, or reviewing research, or in reporting research results.
>
> Fabrication is the making up of data or results and recording or reporting them.
>
> Falsification is manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.
>
> Plagiarism is the appropriation of another person's ideas, processes, results, or words without giving appropriate credit.
>
> Research misconduct does not include honest error or differences of opinion.

It is very important to note that the definitions do not discriminate about how the misconduct is performed, just that it is. Manipulating image data is considered just as damaging as any other form of misconduct. Indeed, in the last reported results on the subject, Dr. John Krueger, Scientist-Investigator for ORI, showed that "68 percent of the cases opened by ORI involved image data." This information was again confirmed in 2015 (personal correspondence).

Investigating allegations of scientific misconduct generally falls to the institution within which the research occurred, with oversight and assistance from ORI. If the institution finds evidence of misconduct involving the use of government funds, the case is sent to ORI for final investigation and determination of administrative actions, if required. ORI has the authority to impose a range of punishments, based on the seriousness of the misconduct and the impact it has on the specific field of inquiry and the general public, as well the demonstration of a pattern of behaviors. These actions include the retraction and/or correction of published works, a ban on grant applications and funding opportunities, and a ban on serving on government advisory boards. Loss of funding is an obvious problem for research that relies on such funding. Without money, a laboratory might have to close and the investigator might lose their position. The ban from advisory boards is just as critical for a young researcher with a growing career, as study sections and boards provide the professional network and influence to get ahead. These actions can be imposed for any length of time, but are commonly one, three or five years. Some examples have been so severe that they warranted a lifetime ban and even jail time.

Conclusions

The advent of practical digital photography took place in the 1990s, and in a few short years scientific photography was converted from an analog process to a digital one. This created a whole new set of opportunities for quantitative imaging, real-time image capture, simplified multi-channel and multi-plane imaging, and many other techniques that were barely if even possible previously. Unfortunately, it also opened up a whole new way to cheat. Most students today have always captured images by pressing a button and getting immediate results. Most think nothing of tweaking the image to make it look better without ever realizing what is going on within the image's data set. It must always be kept in mind that whether performing a pixel-level manipulation to remove an artifact, or performing a global-level adjustment to correct for brightness and contrast, *any* adjustment changes the

specific values of the pixels, changes the data. There has been minimal education to explain the dangers associated with improper handling of the scientific record and this chapter has attempted to provide some practical guidelines.

In the end, the issue boils down to a few key elements: gaining an understanding of the subject, understanding the equipment, understanding protocols, and using standardized, reproducible techniques throughout the imaging process. Along the way, there are a few major considerations as well:

- Treat images as data.
- Capture images using standardized, objective, and reproducible ways.
- "Auto" is a four-letter word!
- Save original, unadjusted files in a non-compressing format in a safe place and work only with copies so that it is possible to go back and re-evaluate those originals.
- Only compare images that have been captured and processed in the same way.
- For quantitative evaluation, use only unadjusted files in a higher bit depth.
- Explain any manipulations in the text. A reviewer should be able to take your original files, follow your explanations and get the same results.

When all of these conditions are met, any images taken for the purposes of documentation, analysis, and presentation will be accurate, verifiable, and scientifically valid, which is the ultimate purpose for which they are intended.

SUGGESTED READING

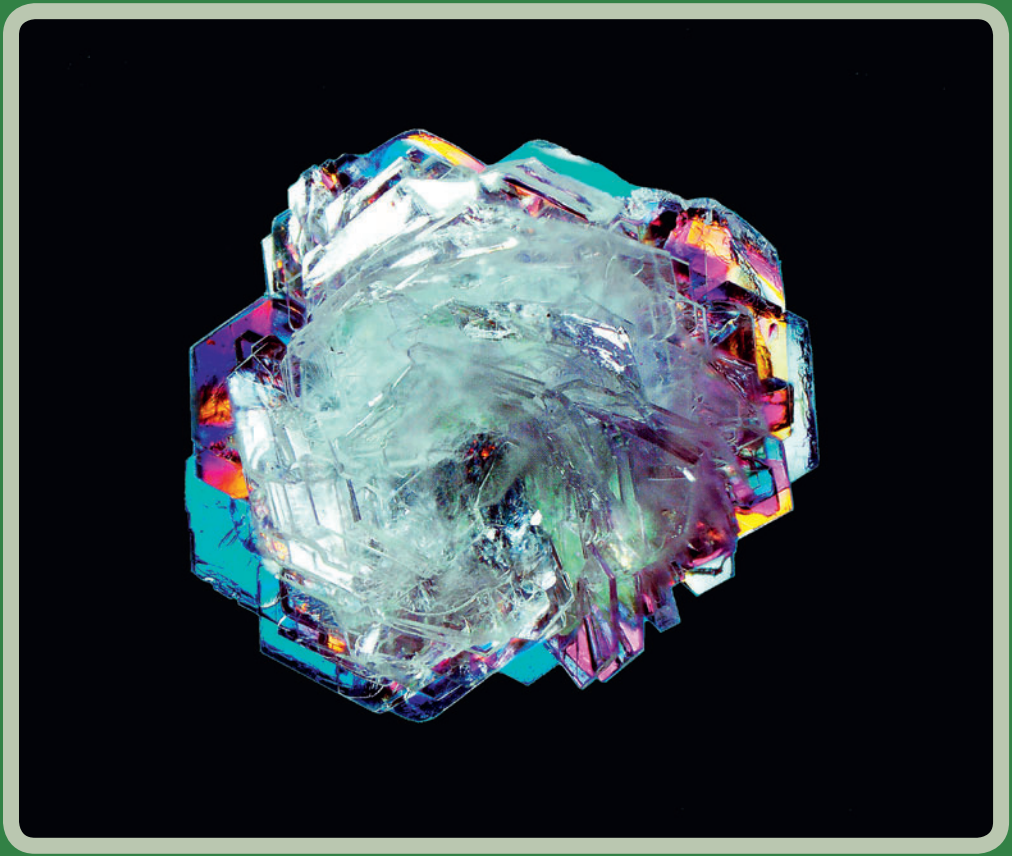
Pittala, S., E. Whiting, and H. Farid, "A 3-D Stability Analysis of Lee Harvey Oswald in the Backyard Photo," *Journal of Digital Forensics, Security and Law* (2015, in press).

Kee, E., and H. Farid, "A Perceptual Metric for Photo Retouching," *Proceedings of the National Academy of Sciences* (2011), 108(50): 19907–19912, 2011.

Chapter 13

Considerations and Methods for Image Processing in Science

STAFFAN LARSSON



This picture features a cystine crystal. These crystals are caused by cystinuria, an inherited (genetic) disorder that affects the transport of the amino acid cysteine in the body. This disorder leads to an excess of cystine in the urine (cystinuria) leading to the production of cystine crystals. Cystine crystals are hexagonal-shaped crystals that can only be seen using microscopic analysis of urine. This image was created using increased DOF methods. Fifteen image slices were captured using a 2x APO objective. Polarizing illumination was used to reveal birefringent properties of the crystal. The crystal was 1.5 mm. Image courtesy of Staffan Larsson.

Introduction

Science pictures are more than pictures, they are data. Science pictures contain visual information about an object or event that can be evaluated at a later date. To make a digital file's image data more visible, a file needs to be "processed" or clarified, important for viewing/analysis. Chapter 12 shares an excellent and comprehensive overview about the complex issues surrounding the processing of scientific images. Changing data comes with responsibilities that must be considered when evaluating how much a file should be "modified" before it misrepresents a subject's attributes. When images are being optimized or processed, data is being changed. Image processing can be destructive or non-destructive to native data. Image processing using non-destructive methods creates larger files by using the adjustment layers but maintains the captured data in the base, or background layer. Best—and scientifically accepted—practices are required when image processing files that are to be used in scientific publications.

It is expected by practitioners and established journals that some level of image processing is required for an image's reproduction in a printed article or featured on a website. Instrument cameras actually begin processing image data when the image capture attributes are selected. The process of improving visibility actually begins before ever making an exposure. Making things visible without distortion is a fundamental expectation in scientific imaging. Consider that right from the start of an imaging experiment, increasing of an object's visibility through optical means, lighting, or other scientifically accepted practices is required to make the "invisible, visible."

There is a distinction between image processing and image analysis. Image analysis is a process that extracts data/information about an image. For example, an image might be analyzed for the frequency of a certain cell type or other sample characteristic. Images are analyzed in everyday life, too. Bar codes are an example of an application of image analysis. This chapter will not include content about image analysis.

Terminology: Manipulation, Enhancement, Clarification

Digital image processing software allows scientist photographers to adjust data in ways that were never possible in the analogue world. The precision in which an image can be processed might be nearly impossible to detect by the vast majority of viewers. As a consequence, many journals require an image processing history to be submitted with an image for consideration to be included with an article. Unfortunately, while this protocol seems like extra work and a hassle, it serves to confirm the integrity of the image by describing the step-by-step image processing procedures that were used. Including some sort of image processing history can validate an image's truthfulness and, unfortunately, I do not see this requirement ever going away as we move forward into the future.

A fundamental decision about how much to process an image resides with the intent or objective of the creator of the image and its intended audience. A number of issues surrounding approaches and intent were covered in Chapter 1 and re-examined in Chapter

12. An image processing workflow (protocol) needs to be evaluated for procedures that will *not* lead to a negative perception about an image's credibility based on appearance. Another facet of image processing resides in the simple choice of words that are used to describe image processing in science. Image processing is often described as image manipulation and this is partly true; however, image clarification also describes image processing and so does enhancement. In the arts, creating an illusion, emotion, or fantasy can be the primary objective of an artist. Because the world of applied photography is large, possibly the term "manipulation" was suggested there many years ago and it stuck as an understandable way to describe image editing. Truthfully, there is no doubt that image content is being manipulated, but in science not for the purpose of misrepresentation.

"Image enhancement" is another term used to describe a process intended to change and sometimes improve the visual appearances of an image's data. In science, recorded data is processed to help reveal internal attributes, which makes structure and features more visible. As such, the process of improving visibility is more aptly described in science as image clarification. There might be an analogy when considering the wearing of glasses, which allows a person to see things more clearly. Looking at unprocessed files may be similar to seeing the world without glasses.

This conversation is not new. In 1997, the FBI (Federal Bureau of Investigation) formed the SWGIT group, designed to establish digital guidelines and protocols. SWGIT stood for Scientific Working Group—Imaging Technology and was formed in response to the very dynamic and constantly evolving digital—and new—technology. One of the points recommended by SWGIT was that term clarification should be used when describing image processing in any legal environment when discussing imaging or providing testimony. It is easy to understand that when testifying about an image in a judicial environment, the use of the word "manipulate" could evoke negative connotations from jurors or other officers of the court. The word "clarification" suggests a more noble outcome and implies less about deceit. Language, whether visual or written, has many subtleties that will flavor the perception of a maker's intent.

Software

Software is required to look at and process images. When moving images from a capture device to a computer, operating systems, software readers, or an image editing application are needed. These types of software products talk to files and display the pixels of an image in the manner in which it was created. Software can be basic or advanced. The more features a product offers, typically the higher the cost, although many shareware or open source products are particularly powerful. Image J and GIMP (General Image Manipulation Program) are examples of very useful and powerful open source software applications. Adobe Photoshop software is arguably the most common and well-known image processing software but there are others. Many instrument camera are shipped with software from companies such as Nikon or Zeiss, that can perform image editing in addition to operating the functions of the camera.

Image processing software accomplishes two very important tasks. It selects pixels or it adjusts the data in the selected pixels. Each of these tasks are segregated and fundamental

to using an image-editing program. Pixels can be selected using different tools both individually or as a group. Pixels can also be selected by choosing pixel brightness, location, or other characteristics that are similar across a few or many pixels.

Once pixels have been selected, adjustments or replacement of those pixels—sometimes called painting—can be accomplished. Depending on the software, various selection and/or paint features are available. The more powerful the software, the more features it will be capable of, including image analysis and video editing in some products in the Adobe Creative Cloud software suite, for example.

Image J

Image J is a powerful 64-bit image processing program. Launched in 1987 as NIH Image, scientists at the National Institutes of Health created this important tool for use in the earliest days of digital imaging. Now able to process 64-bit images, Image J is capable of performing many functions including image analysis. Image J is an open source image processing program that has been optimized for use with scientific multidimensional images. Image J is easily expanded and has numerous plug-ins and macros useful for performing a wide variety of image processing tasks. Limited only by space in this book, much more about Image J can be found online. There are countless useful websites dedicated to sharing methods useful to process images using Image J. This software is prevalent in science laboratories around the world and handles all the common file types such as TIF, JPEG and others that can be opened in other image editing programs.

GIMP

The General Image Manipulation Program (GIMP) is an open source bitmap and raster image editor launched in 1995 by Spencer Kimball and Peter Mattis. It allows for numerous pixel selection methods and pixel retouching including drawing, re-sizing, cropping, and many other important tasks. GIMP, which began as a college project, has become a highly respected and capable image editing software. Similar to Image J, it is easily expanded with plug-ins and macros. There is a relatively large GIMP following and a number of books have been published about using this software.

Some of GIMP's main features include how it describes color, how it creates selections and paths, its image editing, layers, masks and channels, as well as scripting and automation, to name a few. It is also free. Because GIMP is open source software, its features are frequently expanded. Because all of these image processing programs can run on various operating systems such as Windows, Unix, or Linux, it has led to such variant products as Cine Paint, GIMP Classis or GIMP Portable. Similar to Image J, a number of resources and user groups are available on line.

Adobe Photoshop

Photoshop is arguably the most well-known image processing software in the world.

It shares an excellent user interface and it is the de facto program for image processing in the photography, graphic arts, and publishing industries. Photoshop version 1.0 was launched in 1988 and has grown to now include basic video editing capabilities. As an imaging

editor software, Photoshop uses a raster mode, layers, and supports RGB, CMYK, lab color and spot color as a few of its countless features. There are many excellent books and web resources that discuss every nuance of Photoshop. Many terrific resources including Adobe TV and Lynda.com® are subscription and tutorial services as well.

The primary focus of this book has been on the making of scientific photographs; however, image processing is a fundamental step in the sequence of making things visible. The following pages will share methods and considerations using Photoshop software to process image files directed for use in science. Another Adobe product, Lightroom, can also be useful for image processing but will not be covered in this book.

Basic Color Theory

Since most digital cameras produce RGB image files except high-end monochromatic grayscale cameras, some color theory must form a part of any conversation about image processing. How a DSLR camera forms an RGB image was shared in Chapters 4 and 7. Primary colors are the foundation of a digital image and are defined as colors that can be added together to make other unique colors. Red, green, and blue are described as primary colors. When added together in equal amounts they produce white. Adding red and green together will make yellow; adding green and blue together will make cyan; and adding red and blue will make magenta. Primary colors are the basis of human vision. Human vision was covered in Chapter 2. RGB color is used in video projectors, all computer and tablet displays, TV displays, and in inkjet printing. The RGB color model is also used for web applications, in MS Office, and in imaging editing programs such as Photoshop software.

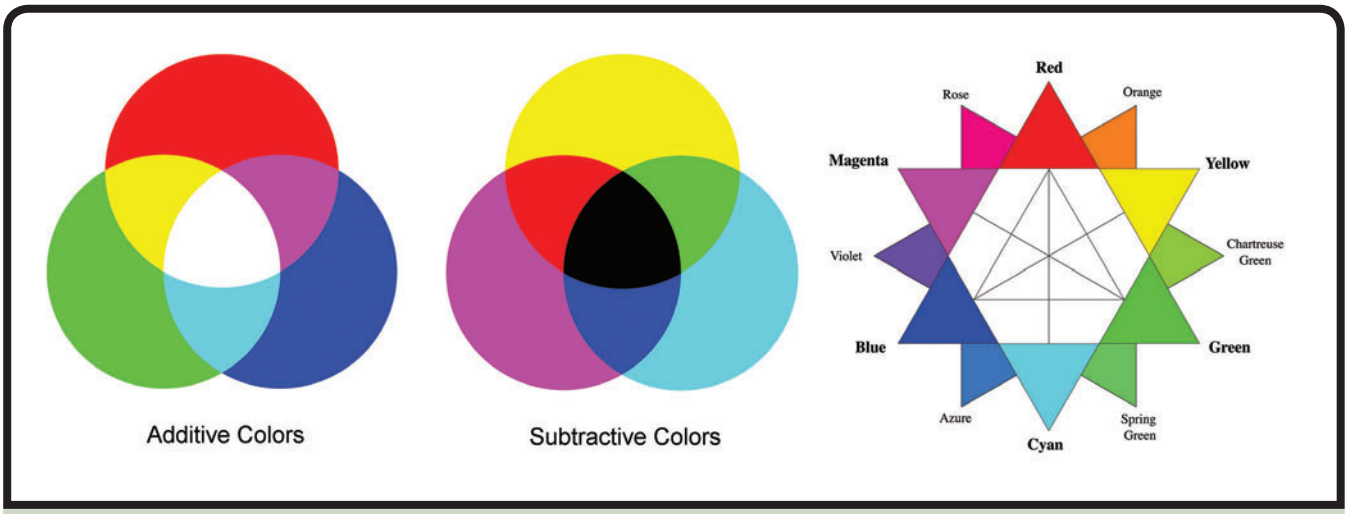


Figure 13.1 This figure shares the RGB primary colors sometimes called additives or primary colors (left), the subtractive or secondary colors (middle), and a color star (right). Additive colors when combined produce white, as seen on the left, and subtractive colors when combined form black, as evidenced in the center image. On the star, a color's compliment is situated 180 degrees across from it in the triangle. Primary or additive colors and secondary or subtractive colors are located on the star's points The RGB color model uses red, green, and blue. The subtractive or secondary colors include cyan, yellow, and magenta and when blended together create the absence of light, or black.

Subtractive colors conversely are described as secondary colors and include cyan, yellow, and magenta. When put together, these colors subtract light and lead to the creation of primary colors in varying degrees depending on the purity of the colors that are combined. Equal amounts of cyan, yellow, and magenta will make black, described as the absence of light. Magenta light and cyan light will create blue. Yellow and magenta will create red, and yellow and cyan lead to the creation of green. The C, M, Y, and K model is the basis of the offset printing process. Very few, if any, contemporary cameras produce CMYK files because of the smaller color gamut this produces. RGB images can easily be converted to CMYK images using an image editing software but the conversion can change the color gamut of an image. Sometimes there is a loss in color data during the conversion.

Complementary colors are colors that are opposites of one another. Combining complementary colors leads to the production of gray. Yellow is the complement to blue. Cyan is the complement of red, and magenta is the complement to green. When adjusting a color cast in a file, using the complementary color is one of the first steps to moderate the error in color reproduction. Changing a file's color bias can be accomplished by working in the highlights, the mid-tones, or in the shadows and can lead to a more neutral file color palate. Setting a camera's white balance is one step used in managing the RGB variances with the goal of creating accurate color.

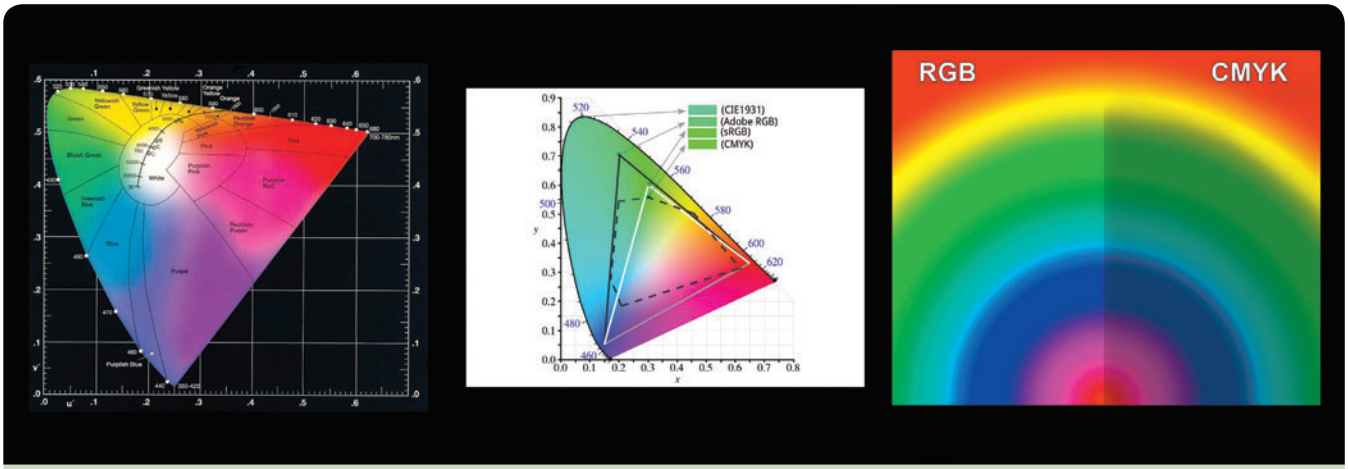


Figure 13.2 In this figure, the Comission Internationale de l'Eclairage or International Commission on Illumination (CIE) diagram is reproduced on the left. The commission was founded in 1913 to provide a group whose mission was to set standards for all things related to light and lighting. CIE formed a technical committee for Vision and Color that has been a leading force in colorimetry. The CIE color model was developed to be completely independent of any device or other means of emission or reproduction and is based as closely as possible on how humans perceive color. The key elements of the CIE model are the definitions of standard sources and the specifications for a standard observer. The middle image shares what color gamut is possible for specific file types including CMYK, sRGB, and Adobe 1998 when compared to the CIE diagram's potentials. In this drawing, Adobe 1998 can reproduce the most numbers of colors. The image on the right is a graphical representation of the loss of color data when an RGB image is converted to CMYK.

Fundamental Digital Color Models

Digital color data is described by imaging processing programs using a variety of modes or mathematical languages. The most common models are RGB, CMYK, grayscale, indexed color, and Lab color. There are others. Not all software programs can open and process all of these types of color languages. RGB color is the largest and most capable color language. It can record and display more than 16 million variances of color. The number 16 million is the multiplicative of three channels (RGB), each being 8 bit, and being capable of describing up to 256 tones per bit. CMYK is most commonly used for offset printing and creates a smaller color gamut than RGB. Grayscale images are 8-bit files; however, they have data present in only one channel, a monochromatic channel. Index color is very efficient and uses only 256 individual colors. Converting RGB to index files produces smaller files with the same pixel resolution. Indexed color is excellent model for web applications where color is not a critical component of the subject's features. Files can be saved as and converted back and forth across the various modes but may experience small or large amounts of data loss in the conversion process.

Channels

Channels display the color attributes of an image displaying different data from the red, green, and blue regions of the visible spectrum. Since pixels respond to brightness differences, the colors displayed on a monitor are mathematical models of the individual RGB values. When using the channel display window, each of the primary color components can be evaluated separately. The red component of an image can be seen by selecting the red channel, the green information can be seen by making the green channel active, and the blue channel would be handled in the same way. Older digital camera sensors were not as uniform in their sensitivities across the three spectrums as modern cameras. Older cameras recorded more data in the red channel than in the blue channel. Using channels can be useful for creating a black and white (B&W) image from an RGB file, pseudo-coloring an image, or for making stereo anaglyphs. When an RGB file is converted to CMYK, you will find cyan, magenta, yellow, and black channels instead of RGB. If an adjustment layer has been used, a mask will be added to the channel where the mask was used as well.

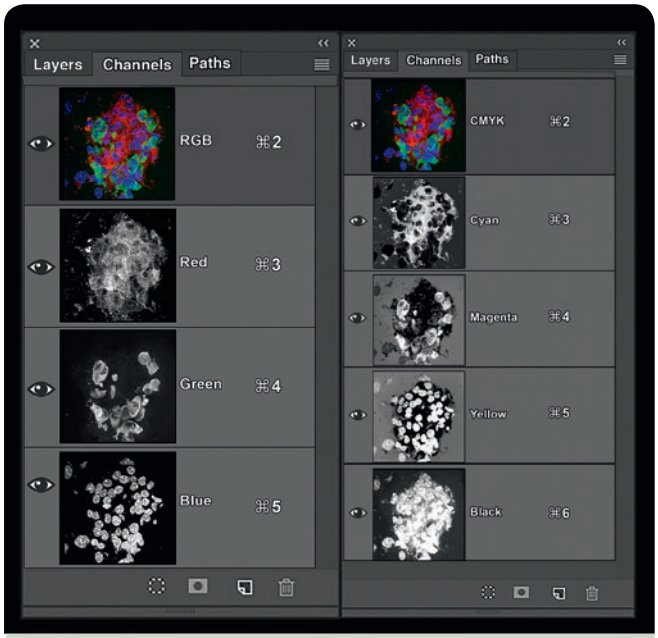


Figure 13.3 In this composite photograph, two versions of the channel display are shared. On the left is an RGB image broken down into its R, G, and B components. On the right, the CMYK version of the same file is broken down into its C, M, Y, and B components. Image courtesy of Staffan Larsson.

Layers

Layers in an image processing software might be considered to be like sheets of clear plastic that are stacked on top of one another. When image processing, working in layers is not destructive to the native file. The original recorded data remains unchanged in what is called the background layer. Each layer can be seen through the transparent areas of the layers located on top of, or down into, the layers below. Visibility of each layer is based on how the layers are merged or blended and then displayed. Layers can be positioned in a specific order, they can be moved, or they can be deleted without compromising the native data. It is possible to change a layer's opacity, which can make some of or all of the content transparent or visible through the layers. It is possible to use layers for compositing multiple images, adding text to an image, or adding vector graphic shapes. The manner in which layers provide transparency throughout the file is controlled by layer blending. Adjustment layers can also be combined using the Layer style for more precision and options for blending.

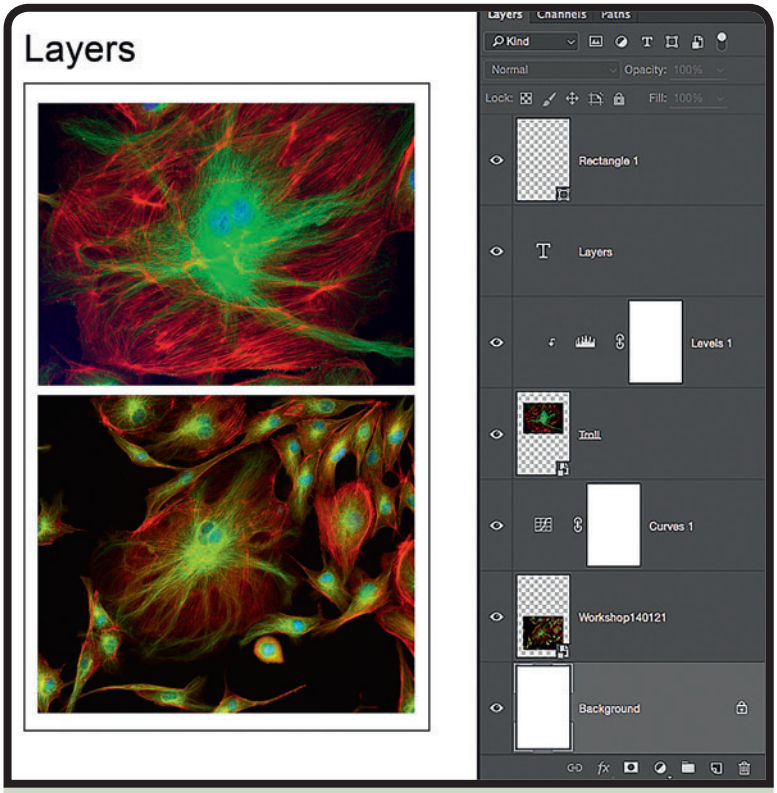


Figure 13.4 This composite image shares on the right side what individual layers were used to make the image featured on the left. In the composite file are two separate image files, one type layer, two image adjustment layers, and one frame layer. Once satisfied that the composite image is complete and no further work will be necessary, the layers can be flattened for use. Image courtesy of Staffan Larsson.

Chapter 14 covers computational photography. Computational photography is an approach that creates images composed of numerous and separately recorded image files. Widefield high resolution imaging, increased DOF imaging, and HDR are few of the topics that are included in the chapter. Additionally and of great importance in science is the use of batch processing used to create standard outcomes and efficiencies when working with large volumes of files that have similar attributes. Chapter 14 shares why, and how to batch process files, and how to create macros or actions that enable a scientist photographer to record repeatable sequences of steps, useful when automating a lot of similar tasks.

Fundamental/Global Image Editing Methods

Fundamental/global image editing methods are techniques that had direct counterparts in the traditional darkroom. These methods remain the most universal image processing methods that are accepted in science and used today. These methods include changing color, color balance and gray balance; dirt retouching; hue and saturation adjustments; adjusting

levels and curves for contrast and brightness management; RGB to grayscale conversions; RGB to CMYK conversions; and sharpening using unsharp masking and high band pass. There are also methods for retouching called cloning or pixel replacement, which might be considered similar to retouching a print. All of the above methods are considered to be traditional and acceptable image processing for science except for pixel replacement. Pixel replacement remains an area of debate and uncertainty about its appropriateness.

The remaining portion of this chapter will explain how to use imaging processing software to clarify scientific images. This book's focus from conception has been about image creation. There are many resources dedicated to Photoshop and image processing. When writing this chapter, I have chosen to include things that are specifically relevant to scientific publications and the clarification of scientific image data.

Chapters 5 and 15 suggest methods used to create files that require *less* image processing. These methods include establishing the optimal image capture settings, cleaning the subject such as a microscope slide and the imaging equipment, proper establishment of lighting and its corresponding color temperatures, using a good specimen, carefully controlling the exposure, and carefully establishing a white balance setting. This in turn minimizes the time spent on image processing.

The best practices used for image processing require the use of a calibrated monitor. This is fundamental to the control and precision in image processing outcomes. It will help to ensure that tones and colors are represented accurately on a monitor and that they match the expected outcomes.

Monitor Calibration

1. Set the color temperature to D50 or D55 ≈5000 K—5500 K and specify the color mode to ADOBE RGB 1998.
2. Set the gamma to 2.2.
3. Set the brightness to 120–140 cd/m^2.
4. Calibrate the monitor when and as needed. Chapter 15 shares methods and equipment required for this.
5. Balance the lighting in the image processing room.
6. Locate the monitor in a place without background light/flare and away from windows and not near brightly colored walls.

Selection and Tools Overview

To process digital images, all or some of the pixels can be adjusted. There are specific tools used to select all the pixels in a file, or a portion of a file down to one pixel if desired. These tools have various degrees of precision and ease of use. I have also learned that I can get very good results using only a few tools. This has helped me produce better work more quickly. It is not important to master every tool at once. I would suggest for the highly motivated scientist photographer to take a basic image processing workshop that covers basic image processing tools and methods. While the content in this type of course will not be specific to science, the general knowledge is transferrable to any environment.

One important step for image processing is setting up an effective workspace to display the necessary panels frequently needed and used most often during the editing process. To set up the workspace, go to the window where you will find the following panels.

The navigator panel provides an overview of where in the image you are working or can establish a position for working on an image when zooming in and out of an image. The info panel can provide the color values of pixels while you are making color corrections or the information provided from measurement tools such as the eyedropper or color sampler tool (up to four markers/samples at the same time). When you work with the adjustments panel or properties panel, the info panel displays two sets of color values for the pixels under the pointer. The value in the left column is the original color value and the value in the right column is that of the color after the adjustments have been made. The histogram tool displays the quality and the tonal range of the image. You can preview the effects of image processing when the image data is displayed in the histogram. The history panel displays the individual steps used to process the file during a work session and allows an operator the opportunity to go back in time and deselect a step or steps. Each time a new task is used on an image, it is added to the panel. It is also possible to create a document from the history as a screen shot or snapshot. Once the workspace set-up is correct, it is recommended to save that set-up of the workspace.

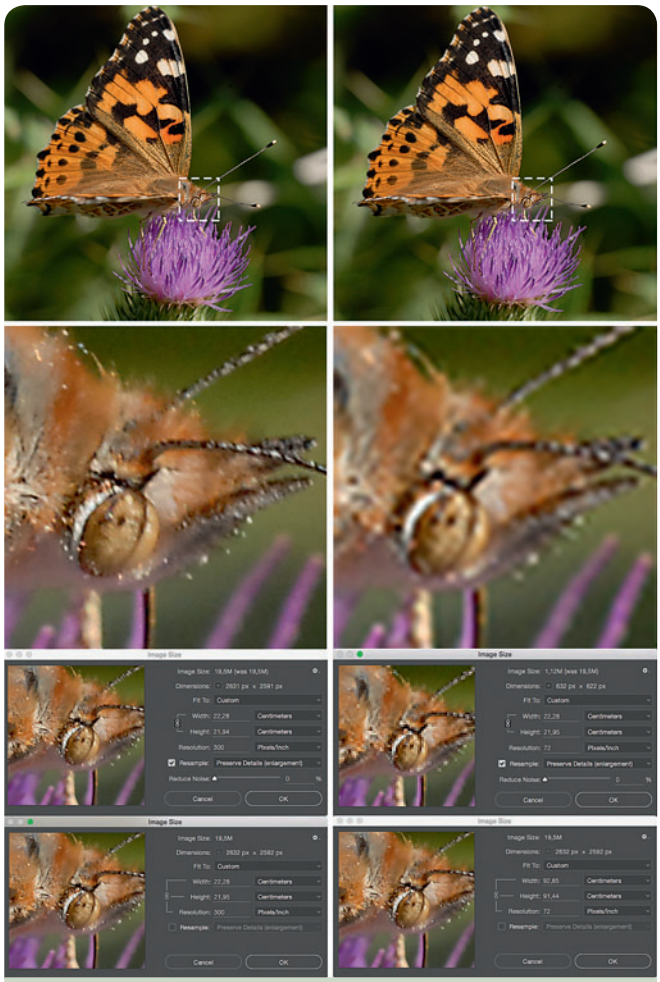


Figure 13.5 This illustration includes important information about an image's display resolution. Image attributes are shared in the dialogue boxes that are included below each image. Images on the left have a resolution of 300 dpi that was maintained when making changes through cropping. On the right, all images were 72 dpi. When cropped, the image falls apart because there are not enough pixels. When changing image size and resolution, it is important to evaluate image size, considering total number of pixels and output resolution requirements. Use the resample feature to achieve the right setting.

Image Size

One of the most fundamental considerations about a file is its pixel count or digital resolution. An image's pixel count can be easily determined by using the pull-down Image menu. Located there are a number of commands including image size and canvas. By launching the image size function, a dialogue box appears in the window sharing the file's size or number of pixels and other attributes. Image re-sizing can be accomplished in this window. Before modifying an image and its size, be certain what each image attribute in the control panel can accomplish. The image size window shares a file's size, the number of pixels in the file, both height and width, as well as the file's display resolution or DPI. Of utmost importance is the proper use of the resample function. A file will have a specific number of pixels that can be displayed in any number of resolutions. LCD or screen display requires a 72 dpi display resolution and printers will use a file resolution of between 200 and 300 dpi. Whenever resampling, pay close attention to the total file size. Sacrificing pixel resolution is not desirable unless mandated by image size specifications.

Image Editing Tools Overview

- Clone tool—creates a sampling of image data taken from one region in a file used to replace targeted pixels. This tool is frequently used for dirt removal.
- Crop tool—trims the outside part of a picture using a free or fixed aspect ratio.
- Dodge and burn tool—the dodge tool is used to lighten an area in an image. The burn tool is used to darken an area in an image. The size of the tool and the amount of lightening or darkening can be adjusted. Slow changes are best and should be targeted to the highlights, mid-tones or shadows.
- Eyedropper or color picker tool—measures pixel data in the active layer. It can go down to the pixel level or a grid of pixels such as 3 x 3.
- Healing brush—paints/blends pixels using a preselected sample or pattern to repair imperfections in an image. This is an excellent tool for blending background imperfections in photomicrographs.
- Lasso tool—lets the operator select pixels by drawing around a region to be selected. This selection can be freehand or magnetic.
- Magic wand—selects pixels with pre-determined similar brightness. May also be called the color wand tool, which selects pixels based on a pre-determined color or brightness characteristics.
- Magnify tool—changes the scale of the image display bigger or smaller. Allows for individual pixels to be observed.
- Marquee, circle, or rectangle tool—selects pixels in predefined shapes as a circle, rectangle, square, or line.
- Measure tool—measures distances in an image by counting the number of pixels in a specific distance. Calibrating the ruler tool can be helpful when calculating known distances.
- Move tool—moves the region of a file containing pre-selected pixels.
- Perspective tool—allows an image's shape to be changed. Might also be called skew or transform.

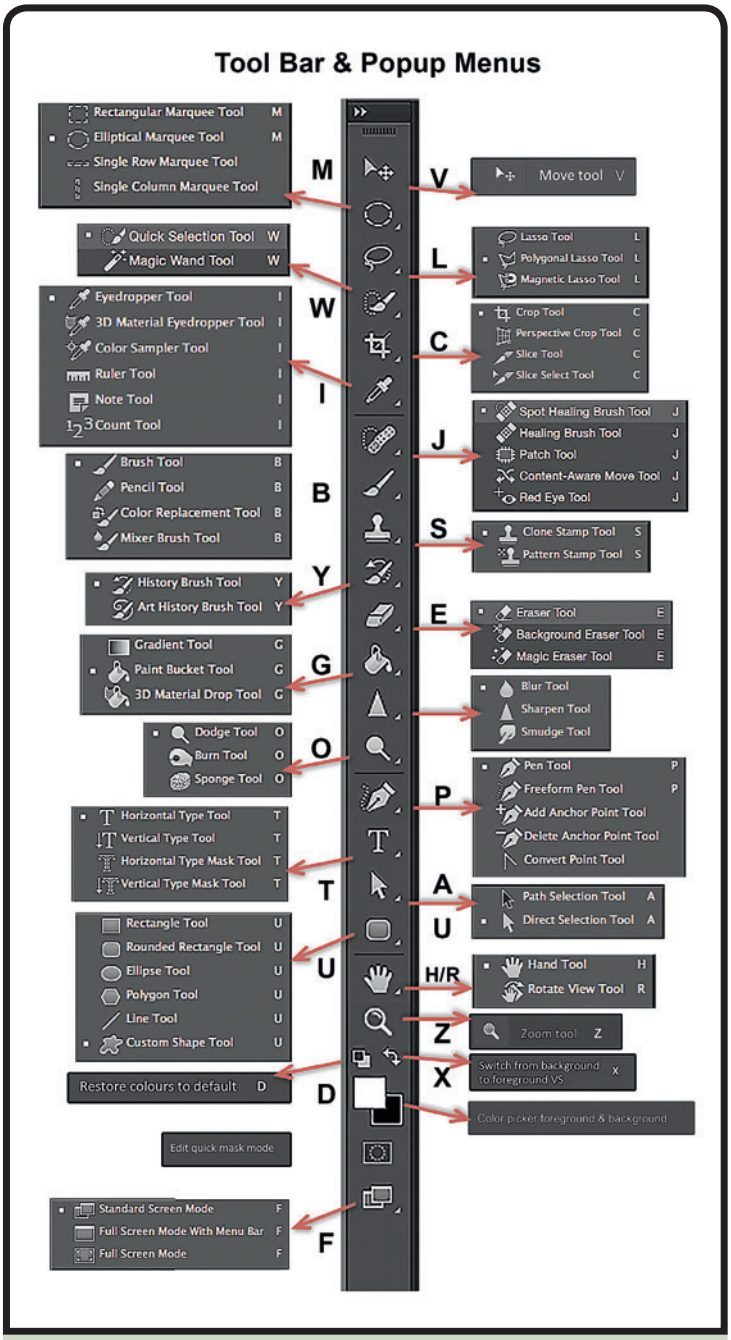


Figure 13.6 This figure shares the appearance of the Photoshop toolbar and the various tool options. Capital letters indicate keyboard short cuts that become activated when typing a specific letter on the keyboard. The H (hand) and Z (zoom) tools are particularly useful.

- Rotate tool—allows an image to be rotated clockwise or counter-clockwise in various degree increments. There are pre-set selections for 90, –90, and 180 degrees and custom.
- Select All—a tool for selecting all the pixels in the entire file.
- Text tool—allows text to be created in a new layer on top of the image or background layer. Fonts, colors, and size can all be modified. Adding type creates a new layer in a file and frequently will require the image to be flattened or collapsed before saving.

Image Processing

Making an image's data more visible is a two-step process. First, the entire file or parts of the file must be selected for processing, and once selected the pixels can then be modified. Pixels can be modified or changed in an image that produces two different outcomes. One outcome does not modify the pixels but creates a new image on top of the background or original file. This method uses adjustment layers and preserves the original data so it can always be re-evaluated as long as the layers are not collapsed. The downside to using adjustment layers is that files will become larger quite quickly. This strategy would be characterized as non-destructive image processing. Probably the most common method is to work directly on the native pixels themselves. Data is changed and going backwards is near impossible, but scientist photographers often subscribe to this method just because it is easier. This method creates files that often remain approximately the same in megabytes but pixels in the files are forever changed. If, for any reason, when working an image there is a need to revisit an older step of the image processing sequence, the scientist photographer cannot go back to the early steps. If that native file was not properly archived, the problem can be amplified. In either of these two scenarios, the edited file should always be "saved as," keeping all the individual steps/improvements in one file.

There are many image processing strategies that can be chosen to improve an image's contrast and appearance. The image processing can be easy and fast if the image has a good exposure from the beginning. Image processing can be more difficult and time-consuming if you start with files that have shortcomings.

Suggested Workflow Steps for Images that Require Minor Adjustments

Go to Image at the menu bar → Adjustments → Select: Levels/Curves/Color Balance.

Suggested Workflow Steps for Images that Require Major Adjustments

If an image requires more advanced adjustments, use the same steps shared above but use the adjustment layers instead. The adjustment layers will show up in the layer panel. It is an option to be able to go back to a layer and make additional corrections instead of starting all over again from the beginning. This can save a lot of time. The processed file should be saved as a TIF or PSD file that will maintain the layer information. Go to Layers at the menu bar → New Adjustment Layer → Select: Levels/Curves/ Color Balance, etc. You can also find adjustment layers at the bottom in the layer panel. Press ◑ and you get the same options as below.

Contrast and Color Balance Corrections

Method: Setting an Image's White Point using the Color Balance Function

An image will have a potential luminance ratio of 0 for a black or shadow and 256 for a white or highlight. It is impossible to print a pure black or pure white and so it is important to keep a touch of tone in the shadows and the highlights. A useful starting point is to set a shadow to a brightness value of 5 and the highlight brightness to a value of 240. The ultimate goal is to have the same brightness level and color values in each of the RGB channels for all files over time. By creating a targeted brightness value, consistency can be achieved file-to-file.

1. Use the color sampler tool on a neutral part on the image and the RGB values will show up in the info panel. Set a marker into this region. This tool can be found with the eyedropper tool.
2. Next go to the layer panel and create an adjustment layer for color balance.
3. A second panel is now added into the info panel. In this window, the changes for the color balance panel will be highlighted. Work on the highlights in the color balance panel.
4. Using the Complementary Controller slider, adjusting the small triangle in a direction that makes the sample appear more neutral. Keep an eye at the info panel and try to reach the value of 240 in a white region in the blue, green, and red channels.
5. You have now set the white point (gray balance).

Method: Setting a White Point using Levels

1. Begin by setting a marker with the color sampler (eyedropper). Locate the marker on a neutral part on the image and the selection will be visible in the info panel.
2. Go to the layer panel and choose Adjustment Layer for Levels.
3. A second window is now added to the info panel. Changes in the levels panel will be highlighted and visible there.

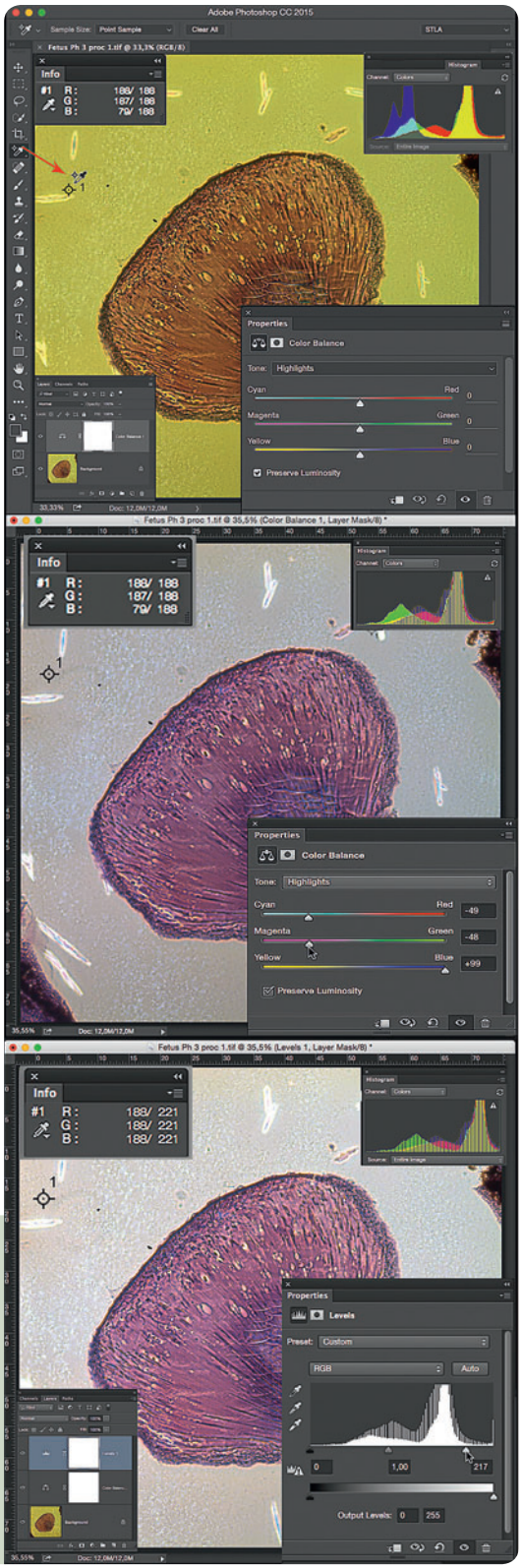
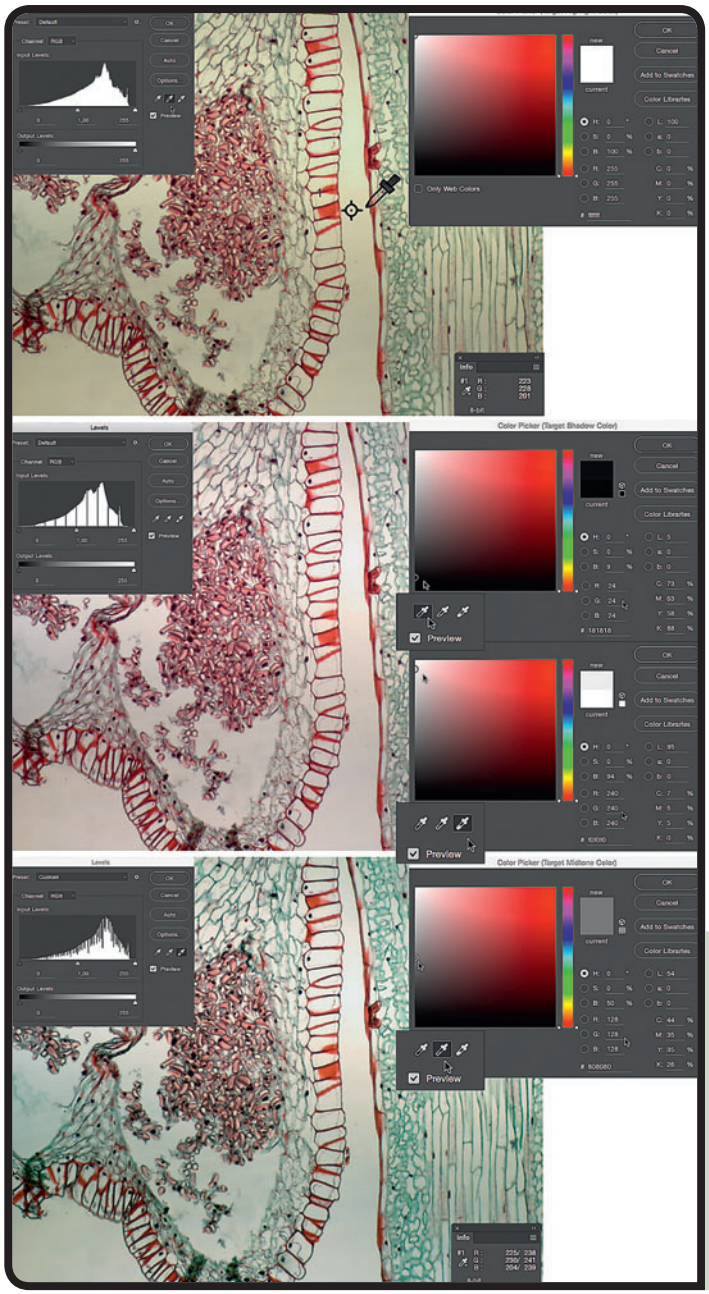


Figure 13.7 This series of pictures reveals how an image's color and contrast can be adjusted leading to more acceptable appearance. The photograph on the top reveals the histogram and the color directly out of the camera. A color maker has been added to the file and can be seen at the tip of the red arrow. In the dialogue box also on the top left, the RGB values are visible. The middle image shares how the RGB levels can be adjusted to produce a histogram that is more blended and without large color spikes. This data can also be seen in the RGB values in the dialogue box. The image on the bottom demonstrates how to optimize the image's contrast using a level adjustment level. This file's white point has been set to 221.

4. Move the small white triangle located on the bottom of the histogram on the right side to the left. This will reduce non-important information in the highlight region of the histogram.
5. Go to the RGB window. Press the left mouse button on the window and all the RGB channels will show up.
6. Choose the blue channel. Push the small white triangle at right side of the histogram (highlight) to the left. Stop where the slope starts at the histogram.
7. Continue to adjust the red. Go back to the RGB window again and change from blue to red channel.
8. You have now set the white point and some contrast (gray balance).



Method: Setting the White and Black Point with Levels Using a Simple Way

1. Begin by making a levels adjustment layer.
2. Select the white color sample (eyedropper) at the layer panel. Double click the eyedropper and it will open the color panel. Locate the small circle in the upper left corner. This location represents R 255—G 255—B 255 = total white.
3. Set a new standard default white point (gray point value) with the value of R 240, G 240, B 240. You can also set the luminescence L from default L 100 to a new default number of 95 and it will lead to the same result. Locate the small circle in the upper left corner and notice that it has moved. This new location in the white corner represents a new setting in the color space. Save by clicking OK.
   a. Go to the black eyedropper in the levels panel. Double click the eyedropper and it will open the color panel.
   b. Locate the small circle down in the left corner. This location represents R 0—G 0—B 0 = total black.

Figure 13.8 This figure shares how to use levels when setting a white and black point. In the top photomicrograph, the color marker has been located in a region that is primarily white. It is important to have the information window open when doing this. Once the levels tool has been activated, it is important to set a white, black, and gray point. Use the color picker set to 24 for the RGB in a black tone and the white point to 240. In the bottom photograph, a gray level was set to 124 and the image has taken on a new color balance.

c. Set a new standard default black point (gray point value) with the value of R 5, G 5, B 5.

4. You can also set the luminescence L from default L 0 to a new default L 5 and it will lead to the same result. Locate the small circle down in the left corner and notice that it has moved. This new place in the black corner represents the new value in the color space for black (gray point value). Save by clicking OK.
5. This will become the new default setting unless changed at another time. This setting is ready to be used as a fast and useful way to establish a white point setting on future files.
6. To use the new default setting adjusting white balance, go to the white eyedropper in the levels panel. Put the eyedropper on a neutral place at the specimen. Press the left button and take a sample. Now you have got a white point. Observe the new histogram.

Method: Changing Contrast by Setting White and Black Points using Levels

This is a method that can be used to reduce unnecessary information in the luminance ratio of the grayscale.

Setting a white point:

1. Open a file and initiate the levels control feature. To evaluate the highlight, hold down the alt key and grab the small white triangle at right side of the histogram (highlight) to the left.
2. Move the control while holding down the alt key. Stop when you see the first white spots in the black parts in the image. This is the start of highlight information in the image. You have now set the white point.

Setting a black point:

1. This is a way to reduce unnecessary information in the luminance ratio of the grayscale.
2. To evaluate the shadow, hold down the alt key and grab the small black triangle at left side of the histogram (shadows) to the left.
3. Move the control while holding down the alt key. Stop when you see the first black spots in the white parts in the image. This is the start of highlight information in the image. You have now set the black point.

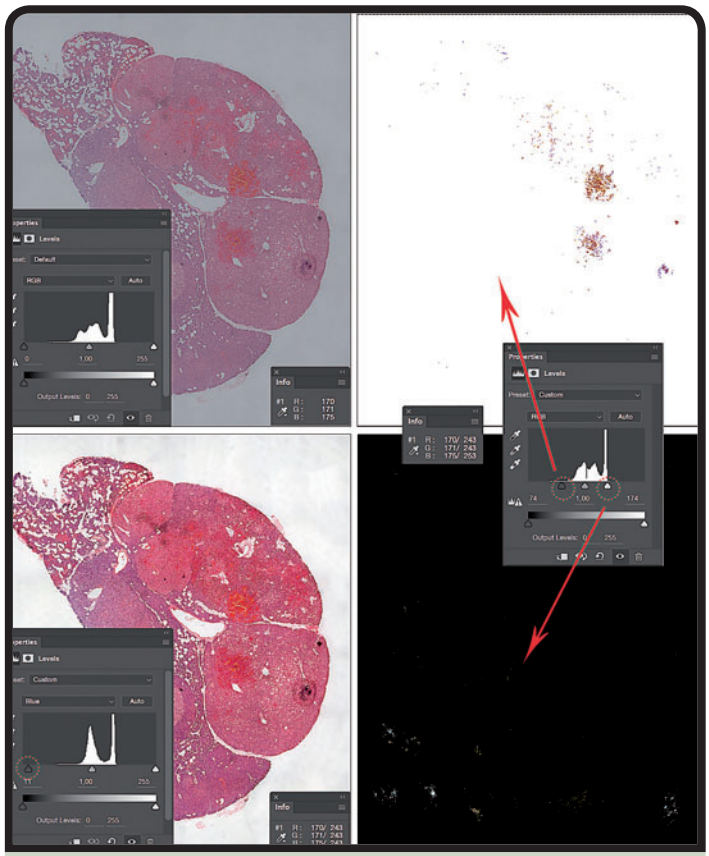


Figure 13.9 This composite file shows what image processing can be used on a file (upper left image) when setting the best black point and white point settings. The images on the right reveal how to adjust the black triangle and white triangle (point) on the image's histogram. The image on the bottom left reveals how these corrections would appear having a white point value of 243 in each channel.

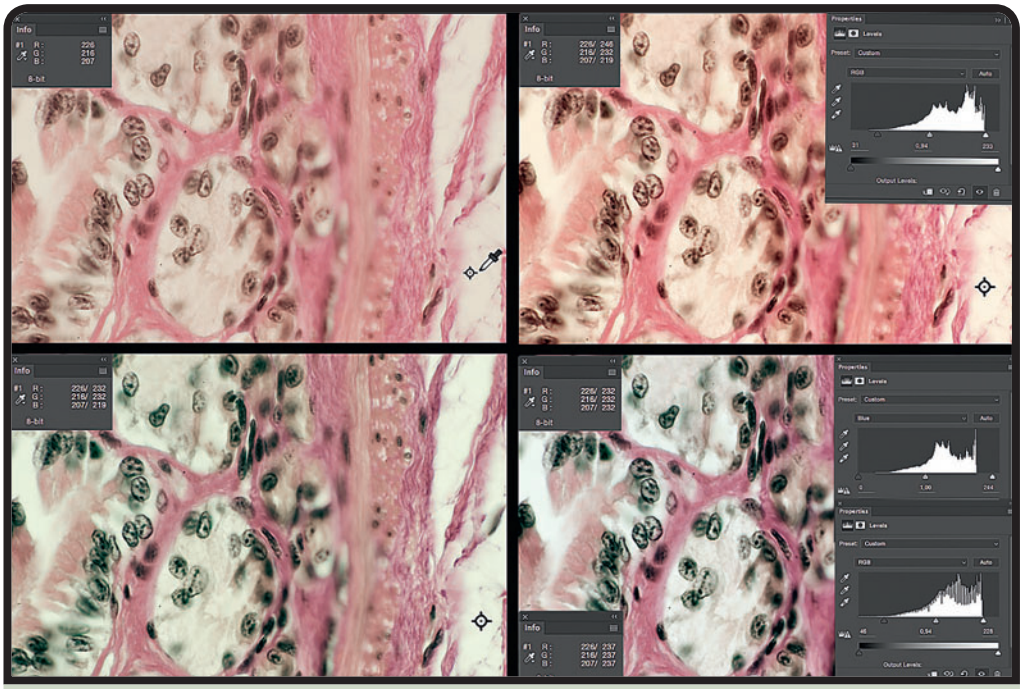


Figure 13.10 This composite figure shares the screens that would be visible when optimizing the file's brightness attributes leading to color correction. On the top left is how the file would appear as opened. Set a color marker/picker into a region of primarily white. On the top right, once the levels tool has been activated, the image's black point is set based on the histogram to 31 and the white point to 233. On the bottom left, selecting the red channel, the image's brightness has been set to 232. To complete the improvement (bottom right image), the blue channel is then set to 232 and small amount of contrast is added, adding brightness and a pixel value of 237.

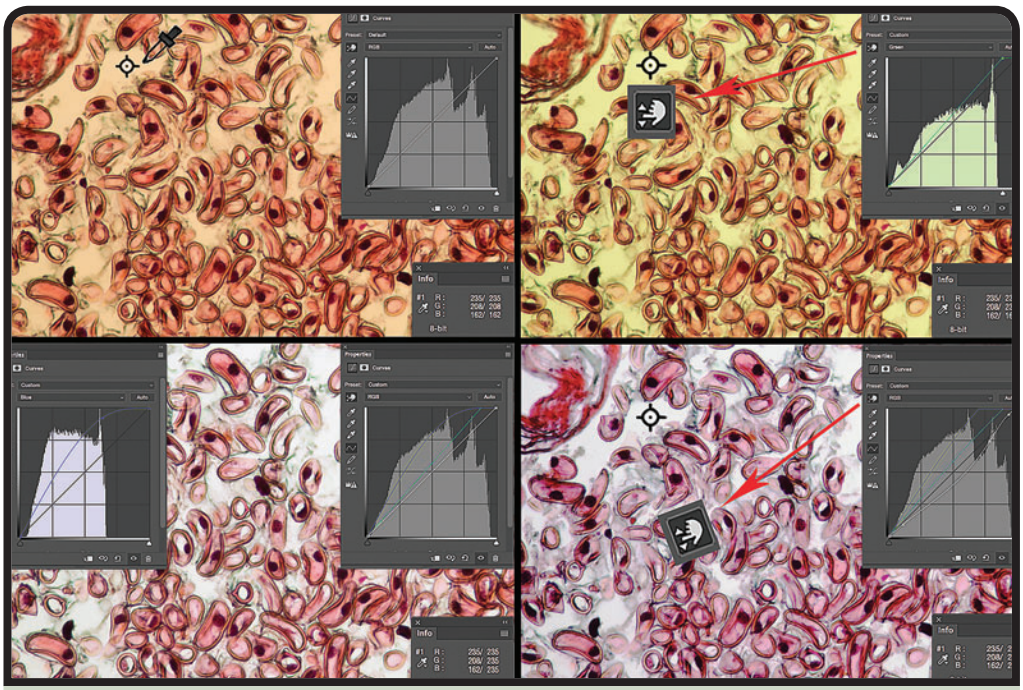


Figure 13.11 This figure demonstrates how to use curves for optimizing an image. In the photograph in the top left, the file is opened and the color marker/picker is added to a primarily white area. In the photo on the top right, using curves, the image is adjusted to create a value of 235 matching the brightness of the red channel when the file was initially opened. In the photo on the lower left, the blue channel is next set to 235 by adjusting the shape of the curve. The lower right image has been finally set to a 240 RGB value and a small refining of the black point setting. To make the work more visible, the directed adjustment tool was used (a small hand in the upper left former of the Curve menu). With this selection, it is possible to go into any part of the image, depress the mouse and the result is added as a marker on the image line, which changes the curve's shape.

Method: Changing Image Contrast and Correct Color using Curves and the Directed Adjustment Tool

1. Open the file and initiate access to the color marker tool located with the eyedropper. Set a color measurement marker in a highlight area with the color sampler tool.
2. Make a new curves adjustment layer.
3. Activate the hand in the left upper corner with a mouse click, the directed adjustment tool.
4. Move the mouse pointer into the area where in an image you want to change contrast. The pointer becomes an eyedropper (color sampling tool). Hold down the left mouse button and move the move the mouse up and down. The contrast changes will be visible and live real time. In the curves display window, small added markers for the adjustments and movements will be visible. Stop when you are satisfied with the result. Go to the next location you want to adjust and repeat the procedure
5. You can also use the same procedure in channels to adjust colors.

Converting RGB files to B&W (Grayscale)

Method: Convert Directly to Grayscale

1. File menu → Image → Mode → Grayscale. Once in this field, select Discard when prompted by the pop-up message menu. Say OK.
2. Next adjust image contrast. File menu → Image → Adjustment → Using Levels or Curves.
3. Levels histogram: move the small black triangle to the left (shadow region) and the small white triangle to the right (highlight).
4. Reduce the information that is not important in the image when displayed in the histogram.

Method: Split Channels—More Advanced

1. Activate channels panel.
2. Choose the channel with most B&W information.

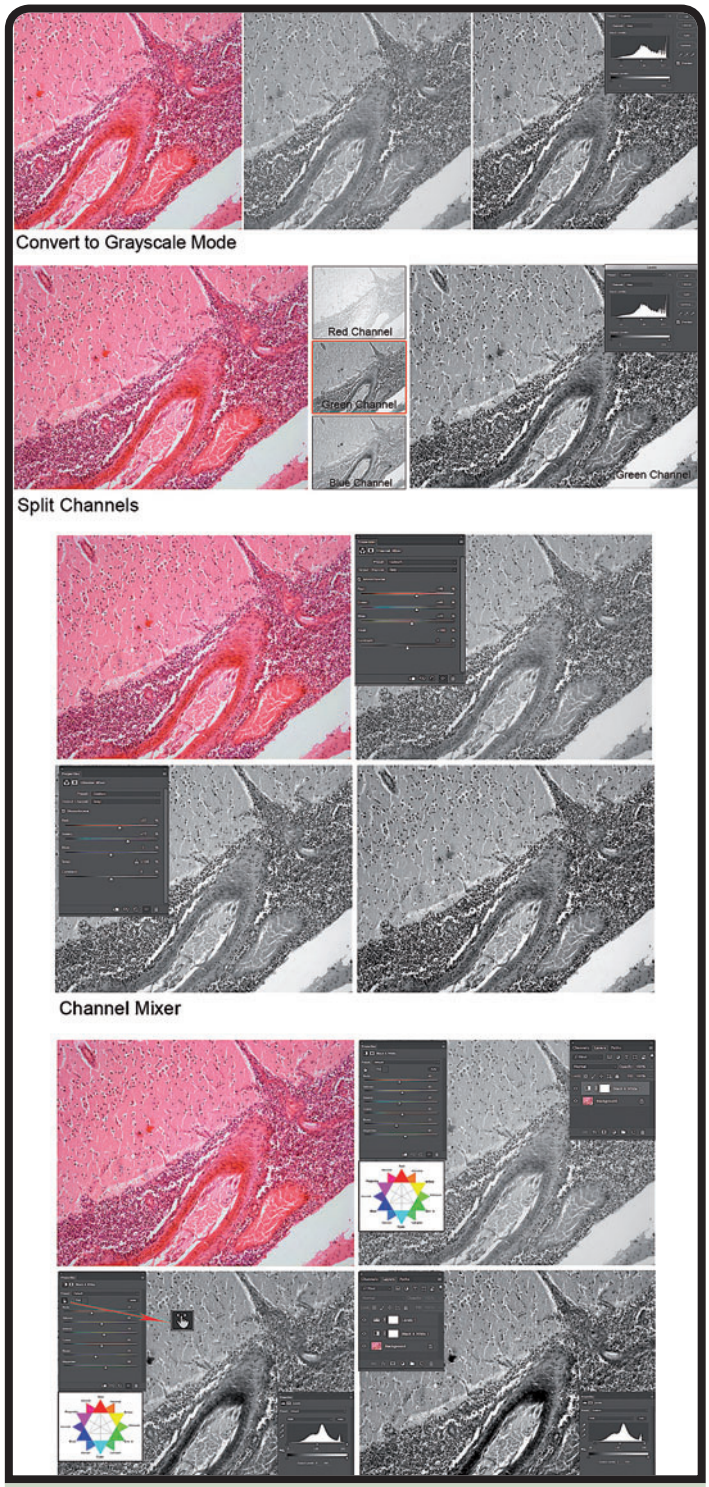


Figure 13.12 This figure demonstrates the outcomes of various methods that can be used to convert an RGB file to a grayscale image. A careful analysis of the results from top to bottom reveals how much more tonal information can be maintained during the conversion using each method. The fastest conversion can occur using the grayscale conversion mode and the best result was produced using the B&W adjustment layer.

3. Go to the right corner at the top, click the lined icon and open the menu. Choose Split Channels.
4. Now each RGB channel will become a separate B&W image. Compare and delete the weakest channels and maintain the channel with the best tonal data. Save using a new name.
5. Adjust contrast if needed. File menu → Image → Adjustment → Levels or curves
6. Go to Image → Mode → Grayscale and save.

Method: Channel Mixer—Most Precise

1. File menu → Image → Mode → Adjustments → Color mixer.
2. Activate monochrome option box at the bottom left of the channel mixer panel by checking that box.
3. The image switch will become grayscale but remains an RGB image. In the panel adjust the B&W information for each channel as needed. Adjust the small triangles until you reach the correct contrast/grayscale representation. Compare the RGB image and the B&W image and consider complementary colors when making the adjustments.
4. Convert finally to B&W. File menu → Image → Mode → Grayscale → choose Discard on the pop-up message menu.
5. Adjust the necessary final contrast adjustments. File menu → Image → Adjustment → Use Levels or Curves.
6. Save As.

Method: B&W Adjustment Layer—the Most Advanced

1. Layer menu → Adjustment Layers → Black & White.
2. Select the Black & White adjustment layer panel. In an adjustment layer it is possible to go backwards and change settings throughout the procedure.
3. The image will switch to grayscale but remains an RGB image. In the panel adjust the B&W information in RGB and CMYK colors as desired. This method is very precise. Push the small triangles until you reach the ideal contrast and tones. Compare the RGB image and the B&W image. Always consider complementary colors when performing the adjustments.
4. Convert finally to B&W. File menu → Image → Mode → Grayscale → choose Discard on the pop-up message menu.
5. If necessary adjust the final contrast. File menu → Image → Adjustment → Use Levels or Curves.
6. Save As.

Sharpening

It is possible to sharpen an image in several different ways based on output. In each method, different levels of sharpening and control can be achieved. It is suggested to sharpen an image using a separate layer on the top (command + alt + shift + e on Mac, ctrl + alt + shift + e on PC) so, if required, the sharpening can be changed if going to a different output device or to a different material, for example. If sharpening an image using a separate layer,

set the layer's blending mode to luminosity. This will avoid color shifts along edges. It might be useful to sharpen the image multiple times using small amounts. Over-sharpening an image leads to halos being created around the edges of structures and may appear similar to diffraction effects evident in photomicrographs that have aperture diaphragm effects.

Method: Unsharp Masking

The unsharp mask sharpens an image by increasing contrast along the edges in an image. It then increases the contrast of neighboring pixels by the amount that is chosen by the operator. Lighter pixels will get brighter and the darker pixels will get darker. In addition, it is important to specify the radius of the action. The greater the radius, the more pixels will be involved and the larger the edge effects. Less is more.

Using the unsharp mask filter:

1. Choose Filter → Sharpen → Unsharp Mask. Enable the Preview option. Click the image in the preview window and hold down the mouse to see how the image looks without sharpening.
2. Drag in the preview window to review different parts of an image, and click + or – to zoom in or out. Although there is a preview window in the unsharp mask dialog box, it's best to move the dialog box so you can preview the effects of the filter in the document window.
3. Drag the Radius slider or enter a value to control the number of pixels that will affect the sharpening. The greater the radius value, the wider the edge effect will be. The wider the edge, the more obvious the sharpening. The radius value varies according to the subject matter, the size of the final reproduction, and the selected output. For high-resolution images, a radius value between 1 and 2 is usually recommended. A lower value sharpens only the edge pixels, whereas a higher value sharpens a wider band of pixels. This effect will be less noticeable in print than on screen, because a 2-pixel radius represents a smaller area in a high-resolution printed image.
4. Drag the Amount slider or enter a value to determine how much to increase the contrast of pixels. For high resolution when printing images (300 dpi and large size files), an amount between

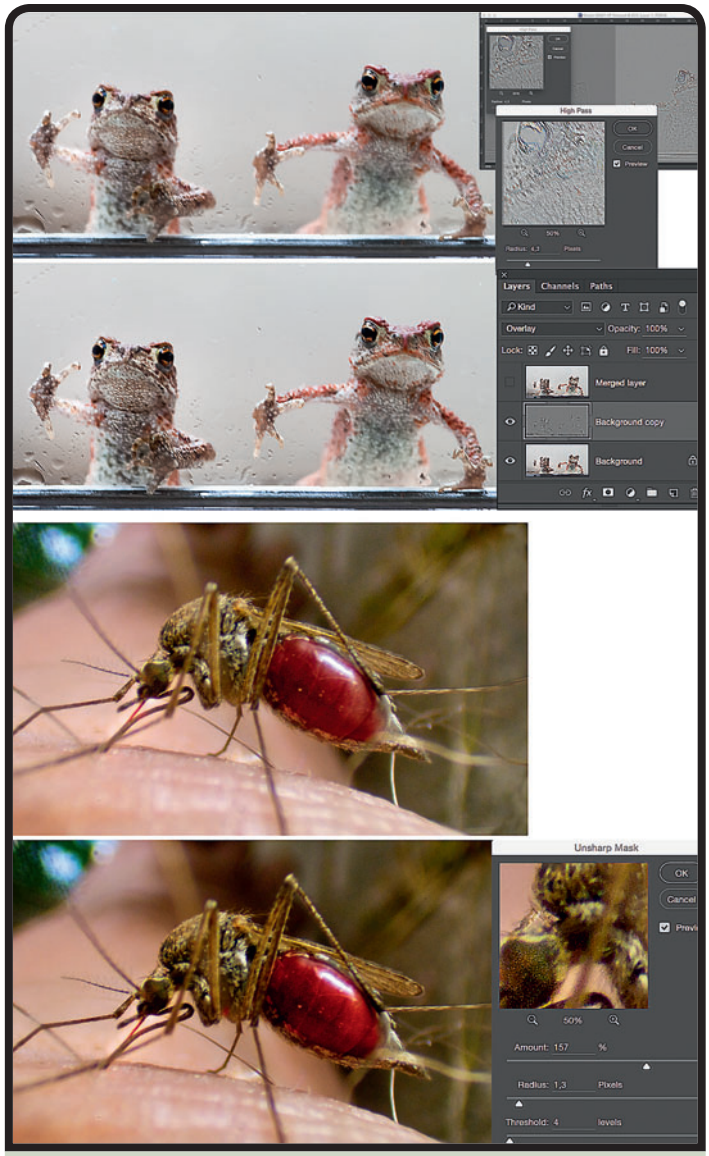


Figure 13.13 This figure shares two outcomes used for image sharpening. On the top is the high band pass method of sharpening and how the dialogue box will appear. Once the filter has been activated, it should be blended using the Overlay command. The lower image of the mosquito was sharpened using an unsharp mask filter.

150 and 200 percent is usually recommended. For low-resolution images (72 dpi and small size files) web, PPT, MS Word, etc., an amount between 50 and 100 percent is usually recommended.

5. Drag the Threshold slider or enter a value assessing how different sharpened pixels vary from surrounding areas before they become edge pixels and are sharpened by the filter. For instance, a threshold of 4 affects all pixels that have tonal values that differ by a value of 4 or more from a tonal scale of 0 to 255. So, if adjacent pixels have tonal values of 128 and 129, they will not be affected. This tool helps to control noise or posterization. For images with flesh tones, for example, use an edge mask or try experimenting with threshold values between 2 and 20. The default threshold value (0) sharpens all pixels in the image. To confirm the results of the sharpening, put the cursor in the sharpening window and the arrow will shift to a hand. Click the left mouse button up and down and you can evaluate the result before saving.

Method: The High Pass Filter

The high pass filter retains edge detail in the specified radius where sharp color transitions occur and it will suppress the information in the rest of the image. A radius of 0.1 pixel works on only edge pixels. The filter removes low-frequency detail from an image and has an effect opposite of the Gaussian blur filter. In many cases the high pass filter will give a better result on low contrast images when compared to the unsharp mask. It is helpful to apply the high pass filter on a continuous-tone image before using the Threshold command or converting an image to bitmap mode. The filter is very useful for extracting line art and large black-and-white areas from scanned images.

Using the high pass filter:

1. Open a file and create a copy of the image file in a new layer.
2. Choose Filter → Other → High Pass. In the layer panel, select the copy and notice the image will turn to gray. Make sure the preview option is selected.
3. Click the image in the preview window and hold down the mouse to see how the image looks without the sharpening.
4. You can move around in the preview window to review different parts of the image. It is possible to click + or – to zoom in or out. Although there is a preview window in the high pass dialog box, it's best to move the dialog box so you can preview the effects of the filter in the document window.
5. Drag the Radius slider or enter a value when just enough information of the image breaks through the gray tone. The radius value will vary according to the subject matter, the size of the final reproduction, and the output method. For high-resolution images, a radius value between 1 and 2 is usually recommended. Your experience will help guide how much is needed.
6. Next in the layer panel, click on the small window "normal" to left on the top. When it's open, try Overlay, Soft Light, and Hard Light. Each will blend the filter in different amounts and the image will turn from gray with structure to a visible and sharpened picture.
7. To complete the work use command + alt + shift + e on Mac, ctrl + alt + shift + e on PC to make a top layer of everything.

Noise Reduction using Photoshop Software

When photography transitioned to digital technology, a new image defect accompanied the new technology: the production of noise, the by-product of a sensor. Lack of light, low brightness of light (for example fluorescence), and a sensor's sensitivity to light are common sources of noise. To attempt to minimize noise, photographers can use a higher ISO setting or use camera gain or binning when recording an image. While these solutions can be helpful, they too can add noise to an image, especially in the regions where there are dark colors or regions with similar tones. Noise will be apparent as grainy specks of texture present across the colored RGB pixels. Noise can occur in chroma (color), which is evidenced as colored artifacts or luminance (grayscale) noise, which will make an image look grainy.

Before starting to image process and remove noise, zoom into an image at 100 percent and analyze the image to evaluate the presence and significance of the noise. When examining a JPEG file, there may also be JPEG compression artifacts in the image. JPEG compression artifacts will typically be visible as lines or squares of unusual blocks of pixels. Removing JPEG artifacts may or may not benefit from noise reduction methods.

Method: Eliminating Luminance Noise (Low Noise): Using the Despeckle Filter

In the Filter menu → Noise → Despeckle command.

In addition to the possible presence of RGB digital noise, luminance noise will appear as bright and dark patterns of specks. The despeckle filter evaluates edges in a file where significant color change occurs and it blurs the overall file except for those edges. Blurring a file removes noise and preserves detail. You can use this filter to remove banding or visual noise that results from scanning magazines or other printed materials. The Despeckle command has no dialog box and no options to choose from so it will not be particularly precise. It can be useful to apply the filter consecutively two or three times in a row.

Method: Smart Blur Filter

You can also try smart blur if there is more challenging noise. This works for only 8-bit color mode files. Filter → Blur → Smart Blur filter. In the dialogue box fractions for both the radius and threshold values to control the blurring will be visible. Try different settings in quality and mode to achieve the best result.

Method: Reducing Noise using the Reduce Noise Filter

Eliminating Luminance Noise and Chroma Noise

The reduce noise filter does a very good job of neutralizing the random red, green, and blue pixels while preserving detail in the image. Image parts remain sharp while eliminating RGB noise.

To open the reduce noise filter: Filter → Noise → Reduce Noise.

The reduce noise dialog box contains a large preview window on the left. The preview option box should be checked. To navigate inside the Preview area, you can zoom in and out, by clicking + and –, and navigate around the image by clicking the mouse button and moving the image around. The recommended starting point is 100 percent.

There are various tools within this tool:

- Strength and Preserve Details are used to remove luminance noise.
- The Reduce Color Noise slider reduces color noise.
- Sharpen Details—set to 0 percent and do not sharpen using this feature.
- Use Remove JPEG Artifact (blue) for reducing the appearance of JPEG compression artifacts.

Removing Color Noise

Reduce Color Noise is often the most obvious and easy place to start. Use the preview window and zoom in on an area that contains color noise. Check for red, green, and blue dots. Set Reduce Color Noise to 0 percent, then slowly move the slider towards the right until the color noise has blended into the image and the noise disappears. Do not move the adjustment too far. Keep an eye on the preview area when judging the result. Click in the preview area and check the result before and after. A value of around 60 percent is a good target to work towards. Release the mouse button to see the final effects of the reduce noise filter. The final value will depend entirely on the image amount of noise.

Reducing Luminance Noise

The next step is to reduce the luminance noise. Compared with color noise (different colored dots) luminance noise contains dots/speckles of varying brightness levels (black, white, and gray).

Removing luminance noise is a two-step process.

Start by setting the strength value to 0 percent. Use the same procedure that was used to reduce color noise. Do this until as much of the luminance noise as possible is removed. Click in the preview area and check the result before and after.

Next, begin moving the Preserve Details slider towards the right to bring back as much image detail as possible without reintroducing the noise.

The last step is to remove as much noise as possible with the Strength slider, then bring back detail with Preserve Details.

Advanced Options

If you think too much detail was lost using the basic options, try advanced options. Sometimes one channel contains more noise than the others. Frequently it is the blue channel. In Channels you determine where to concentrate the efforts based on the determination of which is the noisiest channel and leave the other cleaner image channels alone. Perform the same procedure in each RGB channel if needed. Start and set the Strength slider to 0. Select Advanced in the top right of the dialog box then click on the Per Channel tab. Work through channel by channel using the channel option and inspect each channel for noise.

The channel options Strength and Preserve Details work in the same way as earlier but they affect only the selected channel. Set the sliders to 0. Start moving the Strength slider towards the right to remove as much noise as possible in the channel. Click in the preview area and check the result before and after. When you've removed as much noise as possible, begin adjusting the Preserve Details slider towards the right to bring back some image detail without bringing back too much noise.

Apply different amounts of noise reduction to each channel to achieve the best result.

The next step in noise reduction is to switch back over to the main controls in the Overall tab. Fine-tune the Strength and Preserve Details sliders, trying to achieve even better results. When you're done with the individual channels, fine-tune things with the Overall sliders.

JPEG Artifacts

Some images will appear as though they have been divided into a pattern of pixels or squares. The visibility of artifacts depends on how much compression was applied when forming the initial file and how many times that file has been compressed and saved. The image size also influences the amount of JPEG artifacts. Large file sizes will have fewer JPEG artifacts and small sizes more. To reduce the appearance of the compression artifacts, select the Remove JPEG Artifact option.

Noise Reduction Using the Camera Raw Convertor

I recommend you remove/eliminate the noise using the Camera Raw convertor. I suggest this because you will have access to all of the image adjustment tools in the basic settings, such as white balance, contrast, saturation etc. The Detail tab includes noise reduction and sharpening. You also have tone curve and HSL (hue, saturation, and luminance) on a different tab. Note: these steps will work on all versions of Camera Raw.

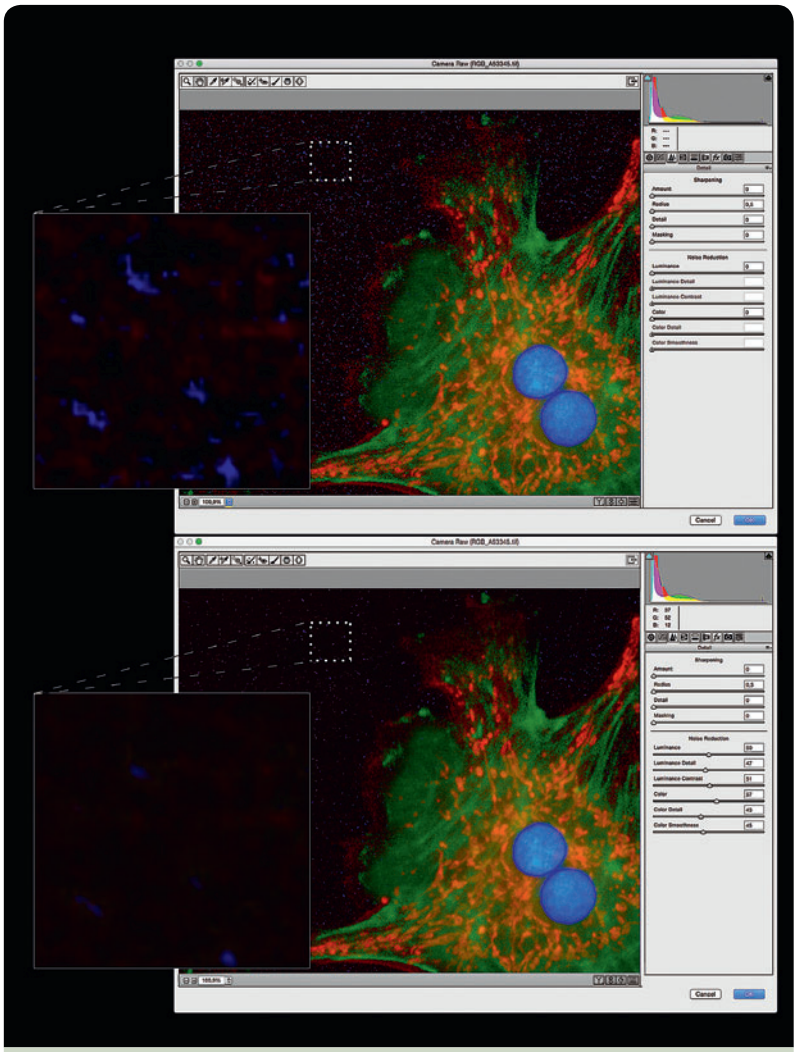


Figure 13.14 This illustration reveals the ability to minimize the presence of noise in a file using the Adobe Camera Raw file convertor. Notice the number of pixels containing noise in the top view and then how, when processed using the tools in the RAW file convertor tool, the noise can be suppressed.

Method: Using the Camera Raw Preprocessor Software Module

Open the file in Photoshop CC and choose Filter → Camera Raw Filter. In Photoshop CC, there is a filter that can be applied as a dynamic smart filter. To take advantage of this, you'll convert the image to a smart object in the following steps:

1. Make a copy of the background layer. Double-click the copy layer to convert it to a floating layer. Give the copy layer a new name and click OK.
2. Right-click in the image window and choose Convert to Smart Object. Choose Filter → Camera Raw Filter.
    a. Using CS6 or earlier, begin by opening the TIF or JPEG image in Camera Raw. Go to File → Browse in Bridge. When Bridge opens, right-click the image and choose Open in Camera Raw.
    b. As a side note, it is possible to open TIF or JPEG files directly in Photoshop Camera Raw by changing the settings in the software preferences. To do this, go to Photoshop preferences and launch File Handling → Camera Raw Preferences window and click. In the bottom of this window you find JPEG and TIF Handling. Choose Automatically Open Supported TIF. Do the same step with JPEG. Next time TIF or JPEG files will open up in Camera Raw.

The Camera Raw Window

- Basic tab: (location one) image controls for adjustments in exposure, temperature, tones, and color channel, etc. Produces image adjustments for optimizing what works best with a particular image.
- Detail tab: (two triangles icon) image controls for sharpening and noise reduction.
    - Sharpening selection: When a RAW file is opened in Camera Raw, you may notice some default settings are already applied to an image, for example sharpening. Sharpening is opposite to the effect of noise reduction. Sharpening enhances the edge contrast and texture detail of the file, which also enhances noise that might be present. To improve the noise reduction, decrease the sharpening and noise sliders to zero (0 percent) present in the Detail tab in Camera Raw. Sharpening should always be the last step of an imaging workflow.
    - Noise reduction selection: Image noise includes luminance (grayscale) noise, which makes an image look grainy, and chroma (color) noise, which is visible as colored artifacts in the image. Note: When performing noise reduction adjustments, first zoom into the preview image to 100 percent to see the noise reduction previewed. Noise reduction adjustment sliders present in the RAW filter converter detail window. These include:
        - Luminance: Reduces luminance noise.
        - Luminance Detail: Controls the luminance noise threshold. Higher values preserve more detail but can lead to noisier results. Lower values produce cleaner results but also remove some detail.
        - Luminance Contrast: Controls the luminance contrast. Higher values preserve contrast but can produce noisy blotches or mottling. Lower values produce a smooth result but can also exhibit less contrast.
        - Adjusting the Color and Color Detail sliders reduces chroma noise while preserving color detail.

- Color: Reduces color noise.
- Color Detail: Controls the color noise threshold. Higher values protect thin, detailed color edges but can result in color specking. Lower values remove color speckles but can result in color bleeding.

Using the Basic Panel

1. It is useful to adjust the image color, tone, and contrast adjustment at this time and determine what improves an image's visibility. Set the Blacks slider to 0, revealing hidden details in the shadows. Moving forward, the next adjustments become personal preferences using any other Camera Raw adjustments as required. It is practical to make minor edits before further significant edits are performed. This workflow will minimize the enhancing of noise artifacts.
2. Go to the detail panel and review the noise reduction options. Click the Detail tab and zoom in to 100 percent (using a PC, ctrl + alt + 0; using a Mac, command C + alt + 0) to see the enhancements.
   a. Luminance noise: First, reduce the luminance noise of an image. Luminance noise will be less distinct than color noise. Be careful not to over-eliminate noise, which will create blur on detail. Increase the luminance value until you see the noise drop out of the image. Evaluate the following settings: Luminance at 40, Luminance Detail at 50, leaving Luminance Contrast to 0 on an image.
   b. Reduce Luminance Detail to remove any "pockmarking" that is visible.
   c. Increase Luminance Contrast to regain any contrast that was lost when increasing luminance.
   d. Return to Luminance Detail and increase the adjustment until the pockmarking returns.
   e. Color noise: the last step is to reduce any color noise in an image. Color noise is generally more noticeable, so the Camera Raw default color noise reduction setting might be around 25 and not 0. A general rule is to match the Color Noise slider with the luminance setting.
   f. Before sharpening, switch back to the basic panel and locate Clarity and Vibrance. Increase the Clarity value to regain edge contrast. The Clarity slider adjusts mid-tone contrast. A good starting point is Clarity +20 and Vibrance +10. The Vibrance slider minimizes clipping when colors approach full saturation; by altering the saturation of lower-saturated colors without affecting the higher ones, more control is maintained.
3. Sharpening should always be the last step in the sequence of image processing steps.
   a. Increase Sharpening to its max value.
   b. Take Detail down to 0. Set Amount to 40, increase Radius to approximately 1.5. Never over-sharpen an image. It can overdo things and add noise back into the file. When satisfied, click the OK button and complete additional image processing work in Photoshop.

Combining Separate Fluorescence Images

Sometimes it is difficult to create a single exposure that can optimize the recorded information for each stain when the sample has been triple stained. Combining individual fluorescence images that feature red, green, blue exposures can be accomplished using camera software or using Photoshop software.

To begin, open all three of the individual RGB files.

1. For the red fluorescence image, make the red channel active and save the file using Save As and a new name.
2. For the blue fluorescence image, make the blue channel active, select and copy the blue channel and past this file into the newly named file created in Step 1 into the blue channel.
3. For the green fluorescence image, make the green channel active, select and copy the green channel.
4. Make the green channel active in the new file and paste the green channel into the active green channel in the new file created in Step 1.
5. It is possible to adjust the contrast with levels/curves and colors/hue/saturations if needed.
6. Once satisfied, Save As.

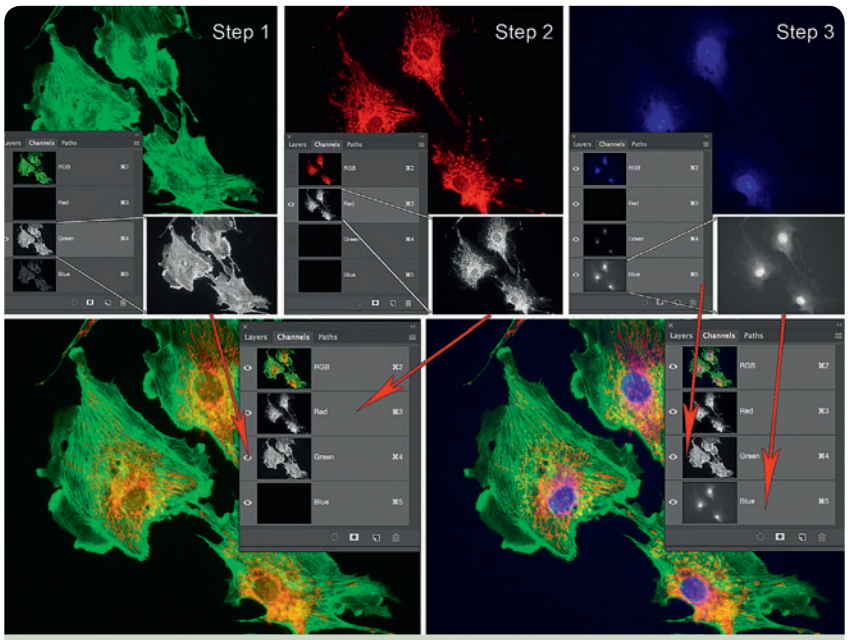


Figure 13.15 This composite photograph shares the steps used to combine individual RGB images of three different fluorescence photomicrographs. Step 1 is to highlight the green channel in the first file. Step 2, select the red channel from the photomicrograph made from the red component of the image. Copy that channel and paste it into the first image file in the red channel. Step 3, next select the blue channel in the blue image, copy and paste that image into the blue channel in the first file (the green image in this example). The bottom right image reveals the three channels, taken from each separate file and pasted into the first green image file.

RGB Files from Monochromatic Cameras

It is possible to also produce a high quality color RGB composite image when using a monochromatic camera when photographing fluorescence specimens. Once the files have been recorded for each fluorophore as grayscale images, you can basically use the same method shared above to get an RGB image.

1. Go to Image and Mode and convert each of the grayscale files to RGB. Start with, say, the red fluorescence image and save, using Save As and a new name.
2. Continue as Step 2 and 3 in the previous list to copy and paste each green and blue grayscale image into the correct channel in the new file. This will create a merged RGB color image.
3. Adjust the contrast with levels/curves and colors/hue/saturations as needed.

Pseudo-coloring B&W Images

1. Determine the size of the B&W file to be colorized and create a new image document using the RGB color mode at the same resolution and size as the original B&W image. Give the file a name. Save As.
2. In the Toolbar menu, select set the foreground color. It is the two squares near to the bottom of the toolbar. Determine the color for pseudo-coloring using the color picker tool. Locate the paint bucket tool, select that tool, and hover over the new document and click. The background should be filled with that color.
3. Make the B&W image file active by clicking on it and select all. Next copy the B&W file using the pull-down menu or use the command C keys. Paste the B&W into the newly colored document. This action will create a new layer.
4. The two layers must have their opacities changed for the color to be seen. Near the top of the layers action window is a small action panel that has the normal displayed. This panel controls the blending of layers. Use Multiply Blend the Layers. When satisfied go to the Layer pull-down menu and Flatten Layer.
5. Adjust the contrast and other image attributes as needed. Complete the image processing by sharpening.

Pseudo-coloring B&W Images Using the Gradient Map Adjustment Layer

1. In the Layer menu, select the New Fill layer and then the Gradient Map adjustment layer.
2. Add a Gradient Map adjustment layer and click the gradient icon in the Gradient Map layer; Properties pops up.
3. Next, click the default gradient window at the top and the gradient editor opens up. At the top, you will find a number of pre-sets. Under the small gear at the top right, you will find more. In the editor you can move the small colored squares where you can adjust the setting.
4. Double-clicking the square opens the color picker and you can choose any color. To add a new color square, click any place just below the editor. To remove a square, press the mouse button on the square and drag it down or press the delete button down in the right corner.

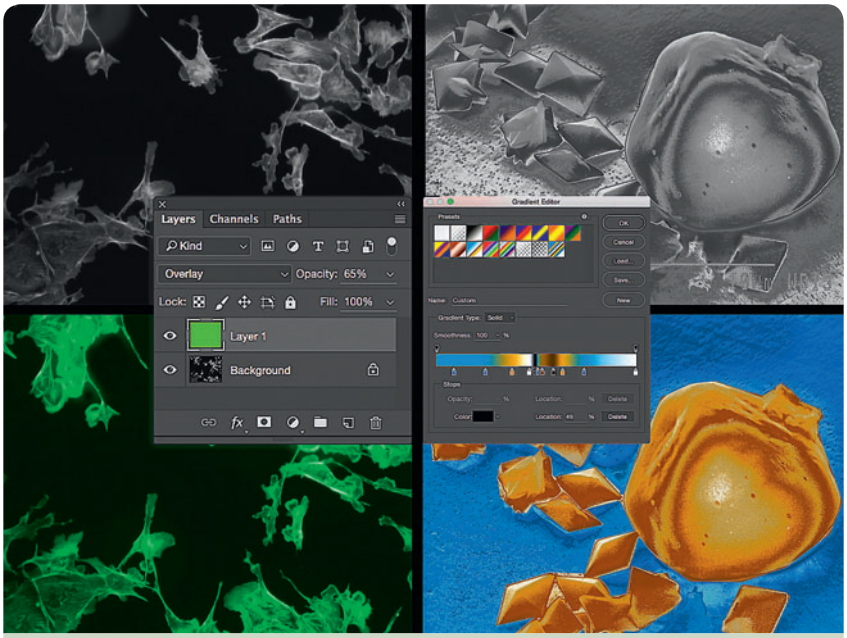


Figure 13.16 This figure shares how grayscale images might appear when pseudo-colored, sometimes called false colorizing. On the left a B&W image is converted to a green image that is more representative of the appearance of this stain. The two layers were blended using Overlay. On the right is a more complex colorization using a gradient map adjustment layer.

5. For a more sophisticated result you can add another gradient layer with other settings. If you activate the adjustment mask to the right in the layer you can paint opaque black or white to open or cover parts, and new opportunities open up.

Creating a Composite Image for Publication

Including multiple images in a journal article remains one of the more expensive elements of publishing. Once an article has been accepted by a journal, the acceptance is typically based on allowing only a certain number of images to be included with the article. Additional images can be included but often an author must pay to include the extras. One way to resolve the dilemma is to create a composite image that includes multiple images. Making an educational point using a single image that has multiple frames is an excellent way to provide readers with a more complete experience with the material for less money.

Composites are relatively easy to assemble in Photoshop or any image editing program. Image selection is a crucial step in the process. It is sometimes useful to include a variety of views that provide an overview image and then several close-up photos that reference details, for example. Labels, arrows, and frames can also be helpful to complete the composite.

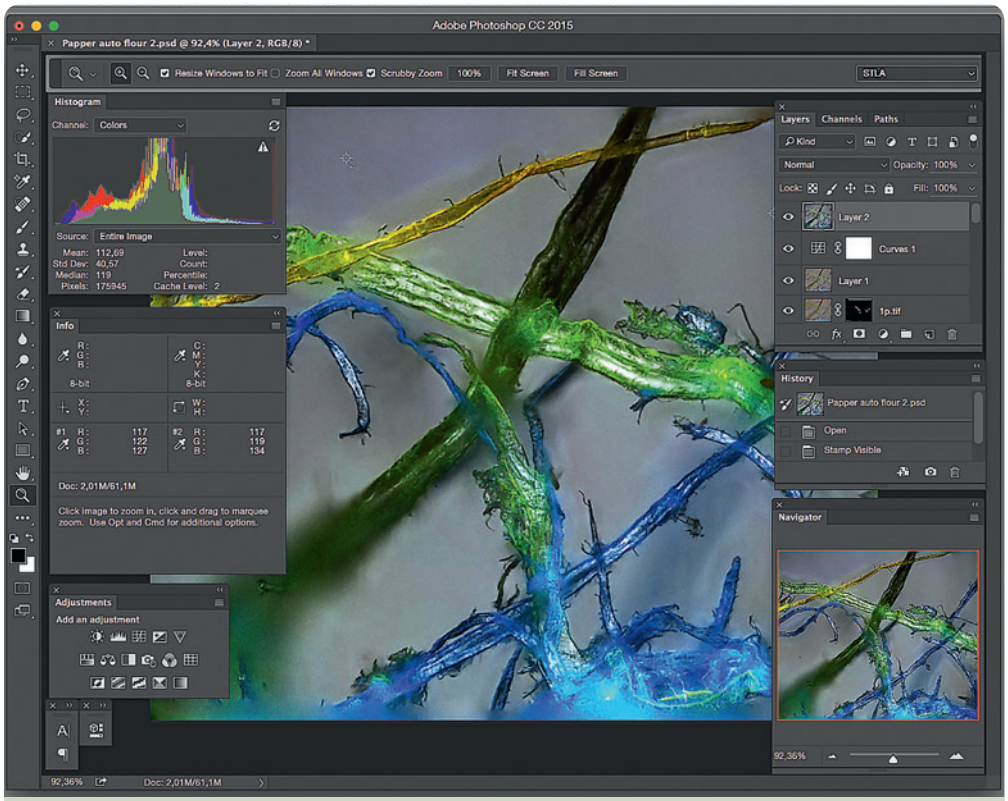


Figure 13.17 This figure shares the author's preferred Photoshop workspace set-up. Each window is useful to monitoring image processing activities. Info, Histograms, Adjustment Layers, Layers and Channels, History, and Navigator have all been opened and placed in regions that do not obscure image visibility. Your settings can be saved in the menu in the upper right corner of the working space. The photomicrograph features auto-fluorescent paper fibers found in office copy paper. Image courtesy of Staffan Larsson.

You can create a composite using Adobe Photoshop in several ways and the following steps are used as an example.

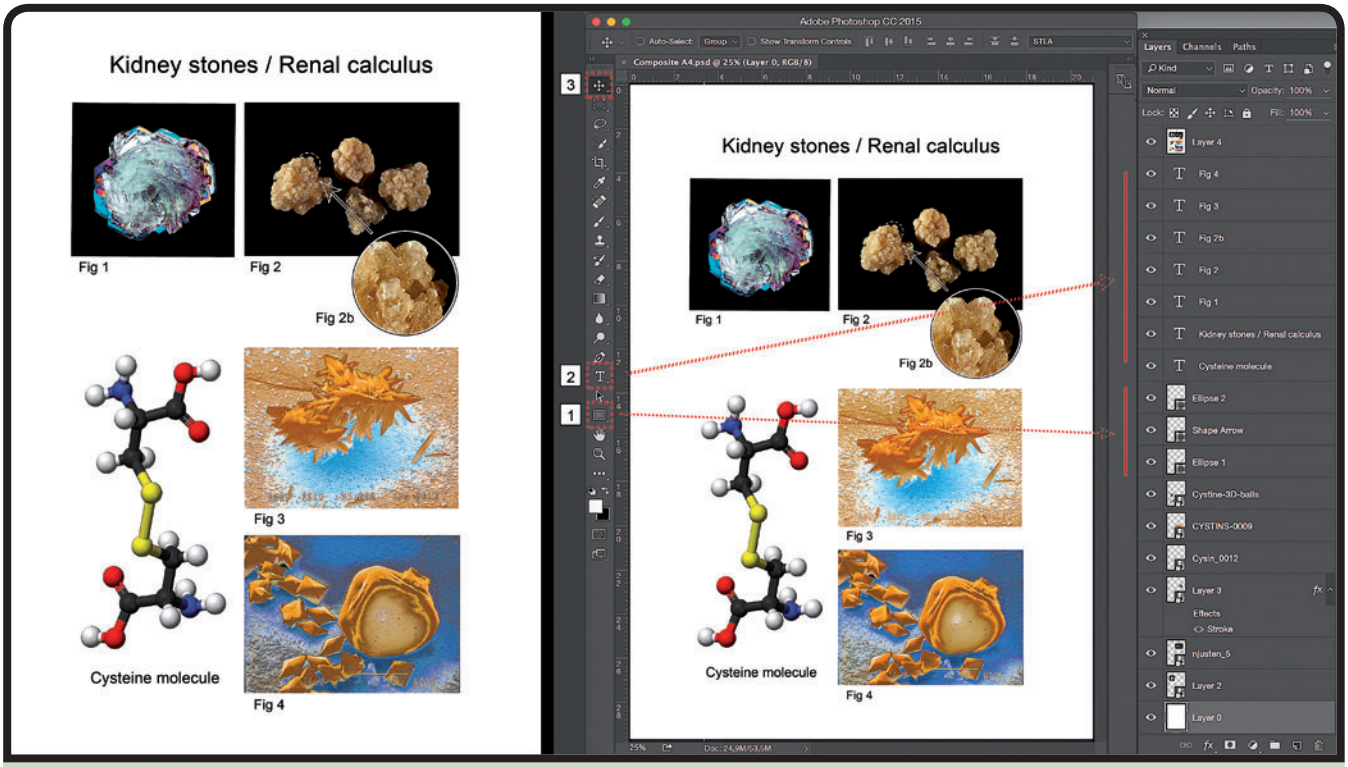


Figure 13.18 This composite reveals the numerous steps and components required to make the graphical image on the left. Highlighted with red lines are the various tools and the layers that indicate where they were applied. Image courtesy of Staffan Larsson.

Method: Making a Composite

1. Before starting to make the composite, it can be helpful to add show extras to the workspace and use the toolbar that displays grid, rulers and guides in the display Photoshop window. To do this go to View → Rulers and if you want Grid, go to Show and Activate Grid. You can turn it on or off using Click Extras, located under the View menu. (You will find the setting of the grid in Preferences/Guides, Grid & Slices.) You can save it as a pre-set as well, choosing OK. To work in Guides, use the moving tool, go to and touch upper or left rulers, press the left mouse button and drag a line or add more into the working space. To turn on or off, go to View Show.
2. Make the file or background that will become the composite's workspace. To do this choose File → New to create a new blank image. Specify image size based on the desired print size. Be sure to make it a bit larger than the size you want to produce. For this example we will design an 8 x 11.5 inch file. It is recommended to Show the Grid when making a composite for alignment.
   a. To start, go to File menu → New.

   b. In the dialogue box, set the new file parameters.
      i. Set the width and height to 8.5 inches x 11 inches.
      ii. Set the resolution to 300 pixels/inch.
      iii. Set the color mode to 8 bit.
      iv. Set the background to white.
      v. Set the color profile to Adobe RGB 1998.
2. Next, prepare the images you want to insert into the composite. Size, resolution, white balance, and contrast should be adjusted and confirmed to have the correct file attributes shared above, with the exception of image size. It is important to use unsharpened images. If the desired images for the composite differ in resolution or pixels per inch, they will be displayed in different sizes in the composite image. There are several ways to resize an image and a method you may be familiar with is shared below.
   a. It is important to have all the images preselected or organized in a folder or some other manner where they are readily available. Open each of the files prior to starting to build the composite and determine the file attributes that were shared above. Depending on the number of images that will be included in the composite, assess each image's size. It is imperative to have a plan, and frequently it is better to use fewer and bigger images rather than more and smaller images to make a point. If for example, four images will be composited, it is easy to see why each file should be 4.25 inches wide and 5.5 inches tall if they are to be the same size. It is also possible to scale images directly into the new document. Another point to consider is whether to include white borders between the images or not. If the files have been resized, they can either be saved as or the files can be kept open for use in the next step. If the files will be sized in the new document, the method to resize will be shared below.
3. Add each new image to the composite image as a separate layer. This process will allow you to position and adjust each image individually throughout the process. When all of the images have been assembled into the final image, you can resize the composite file, or move the layers (the added images). If the layer palette does not appear as a window with software, it can be made visible using Window → Layers.
4. You can add the images to the composite in several ways. It can be practical to limit the number of images used in a composite. It is important to keep in mind that the more photos that are included, the more difficult it will be to see the image detail because the images will become smaller. To add the images:
   a. Choose File → Open, and open the images to be added to the composite. The composite background image and all of the images should be open at the same time.
   b. The first image that will be added to the composite needs to be copied and moved into the new image. To do this, go to Select Menu → Select All; in the Edit menu → Copy; and in the Edit menu → Paste into the new file. The copied file will create a layer in the composite file.
   c. Another way to move a file into a new file is by dragging that individual file into the composite file workspace in the following way. Select the move tool from the toolbar. Click and hold the cursor anywhere in the image that is to be brought

into the composite and, holding the mouse down, drag the first image into the composite image window. Once it is located over that space, release the mouse button. The image should become displayed in the composite image window as a new layer. Bring the remaining images into the composite at this time.

d. It is very practical to label the layers to help with future edits of the file if needed. To label a layer, double-click the word Layer 1 in the layer palette, and rename each layer as needed to track your layers.

e. Add all images, one at a time. Go to one of the open photos files and, using the marquee tool, select all (Select → Select All), copy (Edit → Copy). Click into the new document and Edit → Paste to paste. This will bring each photo into the new document in its own layer.

5. If the image resolutions of the photos that were imported to the composite need to be adjusted, it is possible to resize them in the composite in the following way. First select the image layer that will be resized. Go to the Select menu and then choose Select All. Once the file has been selected, go the Edit menu and select Free Transform. Once made active, use the left corner node of the selected file and, while pressing shift, adjust the scale of the image until it is sized correctly for the new document composite. When you have finished, double-click the image. It remains possible to resize the images prior to importing them into the composite as well. If you convert the image layer to a smart object, an option on the Located Layer menu, it will maintain the integrity of the image quality as you size up and down.
6. Close the original image windows so that only the composite image file remains open. Your layer palette now contains a list including Background Layer and the new layers that may have been renamed. Select a layer as needed and then reposition the layer using the move tool.

It is also possible to rotate images in the following fashion:

1. In the layer palette, click once on the layer that you want to adjust, to target the layer. Choose Edit → Free Transform (Photoshop) or Image → Transform → Free Transform. Be certain that the correct layer is targeted before you transform a layer in the layer palette.
2. Once the image is selected, under the Edit menu select Transform. Note the anchor points on all four corners and sides (eight in total) around the edges of the bounding box of the layer. You resize the layer by dragging the anchor points and then click inside the bounding box and drag the layer to be repositioned. If a layer displays larger than the collage image, drag the layer in any direction until you can see a corner of the image. Use the anchor point on the visible corner to transform the image.
3. Rotate the layer by positioning your cursor just outside the bounding box, clicking, and dragging. You can rotate the layer when your cursor changes to a curved double-headed arrow. In the option bar at the top of the screen, click the check box to accept or commit the transformation. Or, click the circle with the slash through it to cancel the transformation. The layers can be reordered if needed. To change the order of the layers or the way the layers overlap, select a layer and drag the layer in the layer palette above or below the other layers as needed.

It is time to finalize the layout. When you have added all images that you want into the collage, it's the right time to make the fine adjustments to the placement of the individual components. It can be helpful to have borders between images to provide space between the images. The amount of white space is governed by personal preferences. To move files, select the image to be moved then, using the arrow keys, shift the file in the direction that is wanted. Using the arrow keys can allow the spaces to be carefully managed across all of the photos. You can align vertical and horizontal positions using the guides. When the move tool is selected, the align control panel appears at the top. To select the images you want to align, hold down the cmd key (Mac) or ctrl (PC), select the layers in the layer palette and reposition the images for lining up to the left RAW and then next the images to the right RAW. Do the same procedure for the upper RAW and lower RAW.

The Type Tool

Before using the type tool there are a few features that can be helpful to start with. The Text menu will display most of the tools and can be seen clicking the text tool in the tools palette. In the workspace window, text options will be evident in the header.

1. Horizontal and vertical text switcher.
2. Font size: There are number of pre-sets of font sizes but you can also type any number size in here.
3. Anti-aliasing options.
4. Alignment of the text.
5. Text adjustments pop-out. More extended options.

There are also extended characters and a paragraphs panel. Go to Window → Characters and Paragraphs.

Go to the tool panel and choose T as in "Type (Text)" tool. There are two options, a horizontal type tool and a vertical type tool. If you start using the horizontal type tool, you can always revert to vertical text. Go to the Context menu at the top of the software window and click the switcher to the left, or go to Type → Orientation.

This will be visible as a text layer. There are two ways of adding text to the file. The first and most common way is to use the point text tool. Click on the text tool in the tools palette, click back on your image and start typing. The second way is to use Paragraph Text.

The Point Text Tool

Start typing letters and your text will have an underline and a cursor will show where you are inserting the next letter. When you keep on typing, the text will continue in one long single line. To get text on a new line, press Enter.

The Paragraph Text Tool

Select the text tool, drag it out and make a rectangle. Once the box is formed begin typing the text. The text will be constrained by the size of the box. Once the typing is completed, you can grab the text box handles (the little boxes on the middles and corners) and define

the size of the area in which you want the text to appear. This can be important for larger areas of text. The paragraph text tool will provide more flexibility because of the ability to change the size of the text box.

Once the text has been created, the move tool can be used to position the block of text where desired. A paragraph text block is easy to center on a file. To center the text, grab one of the handles and drag it to the right edge of your page. Drag the left box to the left edge of your page. Go up into the Context menu at the top. Click the Center Text icon. The text will now be centered horizontally on your document.

Editing Text and Transforming Letters

It is possible to change the spacing of the individual letters or to change their height or width. It is also possible to change a block of uppercase text to lowercase, or vice versa. To make these changes, activate the text layer. Double-click the T icon and the text box is open for editing: changing type, size, colors, subscripts, switching between all caps and small caps, style and much more.

Rotating the Text Block

Sometimes it might be desired to rotate a text block. To rotate text, use the move tool. Select the text layer and click. Grab a handle on one of the corners and rotate. You can also use Edit → Free Transform and also Scale.

Basic Shapes

Creating circles, rectangle, borders, backing boards, arrows, and frames can provide more impact to a composite panel. Photoshop offers basic shape tools for working with your images. An easy way is to create rectangles and circles. First create a new empty layer. Go to the marquee tool. Select a rectangle. Drag it to a suitable dimension. Notice the marching ants surrounding the marquee. You can adjust the form later using Free Transform.

To start, go to Edit → Stroke. In the stroke window you have choices to fill in.

Width: Fill in the stroke width in pixels. It might be useful to make a few tests.

Color: Click in the small square and the color picker opens up. Drag the small handle and refine the color scale and choice. Stop at the color you want. Go to the shade window and move the small circle with your mouse. Stop when you can see a useful color in the small right window. Press OK.

Location: Click outside of the rectangle and click OK.

An unfilled rectangle with a green border will be created. If you want a wider stroke, go back and add a larger pixel size. If you want to fill it with another color, keep the marching ants and go to Edit → Fill. In the fill window, go to Contents and click. You can choose between default black, gray, and white. At the upper part you will find Color. Do the same color choice as in Stroke. To scale, rotate, or transform the shape, use the move tool. Select the layer and click. Grab a handle on one of the corners and rotate. You can do the same action with Edit → Free Transform.

Custom Shape Tool Functions

Photoshop features some basic shape tools that can be used when working on photographs or artwork. Rectangles, rectangles with rounded corners, circles and ovals, multi-sided polygons, straight lines and arrows, and a number of other custom shapes are available to use by clicking and dragging controls.

1. To use a shape tool, select the appropriate shape tool or a pen tool from the toolbox. If the pen tool is selected for use with a special predesigned shape, be certain that the specific shape is selected from the menu in the Options toolbar. Select the desired shape from the Options toolbar, and then click and drag to create the shape in the document. It is easy to create the size and appearance of the shape, too. If you know where you want the center of the shape to be located, position the pointer at that location and draw the shape by clicking and holding the mouse while moving outward from the center and pressing the alt (Windows) or option (Mac OS) key. The path of the drag using a diagonal direction towards a corner or edge of the frame will affect the final shape. Once satisfied with the size and shape, stop dragging the edge of the shape. It will take a few practices to get the feel of the tool.
2. You can maintain the proportion of a shape by pressing the shift key (both Mac and Windows) while you drag the cursor when making the shape. This action will maintain the width-to-height ratio. With the shift key held, the rectangle tool creates squares; the ellipse tool creates circles; the polygon tool creates proportional polygons; the line tool creates horizontal or vertical lines (or diagonal lines).
3. In the Options toolbar or in the properties window, double-click the shape layer or go to Window → Properties where you will find the options. To choose the color of the shape, click the color swatches for Fill and Stroke in the Options toolbar, and then choose a color from the color picker. Once the color has been selected, the stroke width option and width and height for more exact sizes of the shape can be located to the right of the color swatches.
4. When using custom shapes, pressing the shift key will ensure that the shape will maintain a specific height-to-width ratio. More about the custom shape tool can be found in Step 15.
5. The option (Mac) or the alt (Windows) key will create an object that is centered on the point where a mouse is clicked. Without using the option/alt key, the object will be created in whichever direction you drag.
6. Pressing the shift and option/alt keys together helps you create a proportionally constrained object, centered on the point at which you click.
7. To constrain a rectangle or rounded rectangle to a square, to constrain an ellipse to a circle, or to constrain the line angle to a multiple of 45 degrees, hold down shift when using the tool.
8. If a shape tool is selected and you click rather than drag the tool, you will open a small dialog box that allows you to enter the exact dimensions of the new shape. Click the OK button once the shape has been created to the lower right of (or centered on) the point where you clicked. The dialog box is visible in the figure.
9. When you are dragging a shape, keep the mouse button down and press the spacebar. You can then drag to reposition the shape while you are creating it. Continue to keep the mouse held down, release the spacebar, and finish dragging the object.

10. In the Options toolbar various properties become visible when switching shape tools. The options bar can allow characteristics of the various shapes to be managed. For example, with the rounded rectangle tool active, you can choose the radius of the rounded corners. The polygon tool offers a simple field in which you choose the number of sides for the shape. When you're using the line tool, choose the thickness (weight) of the line in the options bar. It is possible to add arrows to lines by clicking the button to the left of the weight field in the options bar.
11. It is possible to change the layer content. With a shape layer selected in the layer panel, select any shape tool and change the shape's attributes in the options bar and properties. You can change or remove both the fill or stroke. If you need to adjust the line, for example, use the direct selecting tool (inverted arrow) and click on the line and the option bar will open. The shape options will now be visible in the options bar.
12. You can create your own designed shapes with the pen tool making work paths. Choosing one of the three options in the menu on the left in the options bar, you can elect to create shapes or work paths (temporary paths used to make selections or masks), or add pixels in the selected shape to your currently active layer. This is done by editing the vector path. You can use the direct selection tool to change the course of the path, customizing the appearance of the shape.
13. It is easy to find the shape layers in the layer panel. The default name of the layer is the name of the shape (Circle 1, Rectangle 2, etc.). You can change the layer name, if useful, by double-clicking it in the layer panel. In the layer panel you can see the shape layer thumbnail includes the shape badge in the lower-right corner. When a shape layer is selected in the layer panel, that shape's path is visible in the paths panel.
14. To scale, rotate or transform the shape, use the direct selection tool or move tool, or select the layer and click. Grab a handle on one of the corners and rotate. You can accomplish the same action by using Edit → Free Transform.
15. The custom shape tool has additional features that are different from the basic shape tools. The custom shape is located as the last choice in the Shape sub-menu. With the custom tool selected, go to the right of the options bar to open sample shapes in custom shape picker. The custom shape picker offers a number of ready-to-use shapes. When you click the gear icon in the upper-right corner, you can choose from a variety of other sets of shapes listed at the bottom of the Custom Shape Picker menu. The custom shape option bar is similar to the basic shapes. You can find and click on the Fill field and the Stroke field to open the options, and customize the shape. When you select custom shapes from the menu (or use the Load Shapes command to add a set of shapes not in the menu), you are asked whether you want to add the new shapes to the current content of the custom shape picker (Append), replace the current shapes (OK), or not add the shapes after all (Cancel). If you select All from the Custom Shape Picker menu and click OK, all shapes will be loaded.

Preparing Files for Publication

There are a number of things that can be done when preparing files for printing, which can improve outcomes and meet expectations. Soft proofing or making a virtual image proof is one way to predict how an image will appear in specific conditions when displayed on a

computer screen. This simulation provides a visual display that emulates how the image will look when printed on a specific printing press or when using a specific inkjet printer. "Hard proofs" are generally physical prints made from a calibrated inkjet printer and are not the same as soft proofs, which feature electronic display only.

Sometimes when an article is published, authors receive a surprise when, upon opening the journal for the first time, they see the colors of their images on the printed images appearing differently than expected. Typically, the images do not display the same color saturation or contrast when compared to the images that were prepared on the computer and observed on the monitor. The difference in this outcome often is the result of a monitor's color space being RGB, which has a much wider color space (gamut) when compared to a CMYK color space.

Method: Converting RGB to CMYK

When the RGB colors are converted into CMYK colors, many highly colored images will lose saturation and contrast. The expression "what you see is what you get" is not always truthful. Because images can be important evidence used to support a research outcome, using soft proofing can assist scientist photographers in the optimizing of a file prior to submission for reproduction.

It is impossible to print a complete black (0) or white (255) in the grayscale because of limitations of printing techniques. For this reason, when preparing files, initially it is useful to begin file evaluation by checking the image's info panel when adjusting contrast in curves or levels. To produce a printable image, it is imperative to keep each of the RGB channels between 5 in the black and 240 in the white. Once established, these numbers can be translated into typical ink limits (maximum separation in black) for journals and magazines. A coated paper should be set to a range of 300–320 percent and newsprint, or uncoated paper to a range of 240–260 percent. More has been shared about this recommendation throughout the chapter.

Method: Evaluating an Image's Black Point

Black is an important element in an image and ensuring it is properly displayed/reproduced is important. To set a black point, first set a measuring point using the color sample tool located in blackest part of an image or in the shadows. Go to the info panel and review the sum of the CMYK result. If it's more than 300 percent try to bring down the total (C X% + M X% + Y X% + K X% = X%) using the levels or curves.

There are numerous standards that have been created to assist in achieving good printing outcomes. One of these standards is the FOGRA39, which is a characterization data set developed by the German graphic technology research organization FOGRA in 2006. This standard was registered in the ICC Characterization Data Registry. It represents the colors used in a typical print made by the commercial offset litho press or onto gloss or matte coated papers that have a maximum ink coverage for the separation of 300 percent.

A color press uses a combination of four printing plates that represents each color featuring cyan, magenta, yellow, and black (CMYK). The color reproduction is built up by the CMYK pattern of screen dots that are laid down using different sizes. The spaces with very small

clusters of mixed CMYK dots will display the white highlight, and when the color dots are larger they represent variations of colors depending on the CMYK mix. As the dot size grows, it continues into the blacks or shadows of an image.

A good quality desktop monitor will assist scientist photographers in preparing for better output files. Calibration is required. A monitor's default is generally sRGB, which is smaller than Adobe RGB 1998 but can be adequate for basic image output for video display. For print, it is important to set a monitor's color display to Adobe RGB 1998. Today, it is possible to have monitors with a large color space such as Adobe RGB 1998 but frequently these monitors will be expensive and are mostly used by dedicated high-end professional users.

Monitor Settings

An ordinary LCD monitor typically will have a default color temperature of 6500 K and only a few will have the capability to make adjustments. If a display with a 6500 K color temperature is used for image processing, the results will lead to incorrect colors in the publication. A more standard color temperature for color reproduction is 5000–5500 K. Calibrating the display will help to ensure that colors are represented more accurately when anticipating the reproduction gamut. Be sure when buying a new monitor that, at the least, brightness, color temperature, gamma and color gamut can be adjusted.

The basic monitor set-up was shared earlier in the chapter.

Profiles

Another trick can be, when launching Photoshop for the first time, to set the standard color space in Preferences. This will remain important for future work and will allow you to have control of the color space for input and output settings from the beginning. To do this, go to the Edit menu and select Color Settings. You can choose from a variety of pre-sets or you can configure your own and save it. Depending on whether you work in the USA or Europe, there may be slightly different settings. The most common standard setting used with RGB both in the USA and Europe is Adobe RGB 1998. When using CMYK, in the USA, US Web Coated (SWOP) V2 is the common default, and in Europe, Coated FOGRA39. They are also called ICC profiles. There are many specific profiles available.

As an example, select Europe Prepress 3 in the settings at the top to the left. This sets your RGB working color space to Adobe RGB 1998 and the CMYK working color space to Coated FOGRA39. In older versions of Photoshop, it is called Coated FOGRA27 but can be replaced with Coated FOGRA39 through manual selection. You can find this profile easily on the Internet if it is not installed on the system. sRGB, with its smaller color space, is recommended to use for web publications and in PowerPoint and MS Word applications. When you are preparing to use an inkjet printer, Generic ICC Profiles will have been automatically installed when the appropriate printer software was installed in the computer. Select the profile that conforms to the paper of choice.

If you use Adobe Bridge and Managing your Color Settings it will also synchronize and match the settings in all Adobe CC/CS applications. Find the setting in Bridge: Edit → Color Settings, select and choose your color space. Continue the final settings in the Photoshop Color Settings. A recommendation is to select Preserve Embedded Profiles in Color

Management Policies as, which allows you to open a file with an embedded ICC profile. If you are given an image having a sRGB color space (rather than Adobe RGB), leave it as sRGB until it is converted to CMYK. If you need to increase the saturation of color, converting the image to Adobe RGB 1998 will be required.

When editing images in Photoshop, stay in RGB mode as long as possible! It is always best to stay in RGB mode when color-correcting an image. When your image has been color-corrected, save a master copy in the RGB mode. If needed you can later modify the file once the maximum color information has been processed. The last step in the workflow should be the conversion to a CMYK profile.

Proofing

It has been stated that an image can change a lot in its color when converted from the primary RGB image to the printed CMYK image. To avoid having to make color adjustments after the image has been converted to CMYK, be sure to proof and make sure that your proof settings are correct. This can minimize this outcome.

To make a soft proof file, go to View → Proof Setup → Working CMYK or select another profile if you have a preference. Working on CMYK files will be similar to your Photoshop settings. There are a lot more choices of profiles available and designed for print. There are ICC profiles that are associated with specific ink jet printers when used with different papers. In the option Custom (Customize Proof Condition) you can customize and design your own settings. Find your ICC profile in the window "Device to Simulate." In Rendering Intent chose Relative Colorimetric (for images). If the file will be printed on simpler paper quality or includes illustrations use Perceptual and select Black Point Compensation. There can be two useful options for use when selecting proof settings that are found under Custom. The Simulate Paper Color and Simulate Black Ink options will reduce the contrast range of the file and can assist in creating a proof view that will be most similar to ink on paper. If desired, you can save the settings for future applications. Saved settings will appear in the Custom Proof Condition drop-down menu at the top. To revisit this, go to View → Proof Setup and you will see the custom saved name at the bottom of the list and the image will be displayed using these proof colors.

Proofing Colors

To use proofing colors, go to select View → Proof Colors. Making this selection will simulate the result of converting an RGB file to a CMYK profile. The feature is active when there is a check mark visible next to "Proof Colors" in the menu. When you are finished with the evaluation, it is best to turn it off, as when it is turned off the visible contrast range of the monitor will be extended. This is helpful when working on image detail. Using this tool will allow Photoshop to simulate on the screen how an image will look when printed! The proofed image may reveal a wide range from a small change to a significant difference, with some differences to be expected.

Compare the Image Before and After

To compare a result before and after proofing, duplicate the window showing the two images in a side-by-side comparison. To do this, click Window → Arrange → New Window displaying the desired file. Photoshop can display a duplicate window of the same file but this is not a duplicate file, just a duplicate window. Arrange the windows so you can see them easily together. One image will displayed normally and the other image will reveal the soft proofing since the proof colors are active. You may see that the images are similar but there may also be some important differences. Some of the bright, saturated colors will often have shifted and the blue regions will have lost saturation and can become gray and magenta.

Check the Gamut Warning

Sometimes colors are difficult to display or print. This may be caused because the gamut is too large and this can be predicted using the gamut warning. The definition of gamut is a range of colors and tones that can be captured and are available for a particular device such as a monitor or printer. A monitor which displays RGB signals will typically have a greater color gamut than a printer, which uses CMYK inks and is limited by an ICC profile. When the color in an image is "out of gamut," it cannot be properly converted for display or output to the selected device. To evaluate gamut warning, select View → Gamut Warning: Using this function, you will be able see colors that cannot be reproduced by the chosen device and will be displayed as gray. Remember, the device being simulated is configured under View → Proof Setup. Photoshop will reference the selected ICC profile in this set-up when determining the gamut of the output device.

Gray areas will be created in the gamut warning and will indicate all the colors that the printer will not be able to print. Typically these will be colors that are saturated (rich/strong). Un-checking the Gamut Warning feature removes the gray warning tint and the normal presentation of colors appears again. If Photoshop is displaying with proof colors (View → Proof Colors), you can see exactly how they will print.

Adjusting Out-of-Gamut Colors

There is a feature in Photoshop that forewarns operators of issues that may arise when an image is outside of the print gamut and that can be used to help with trouble-shooting these

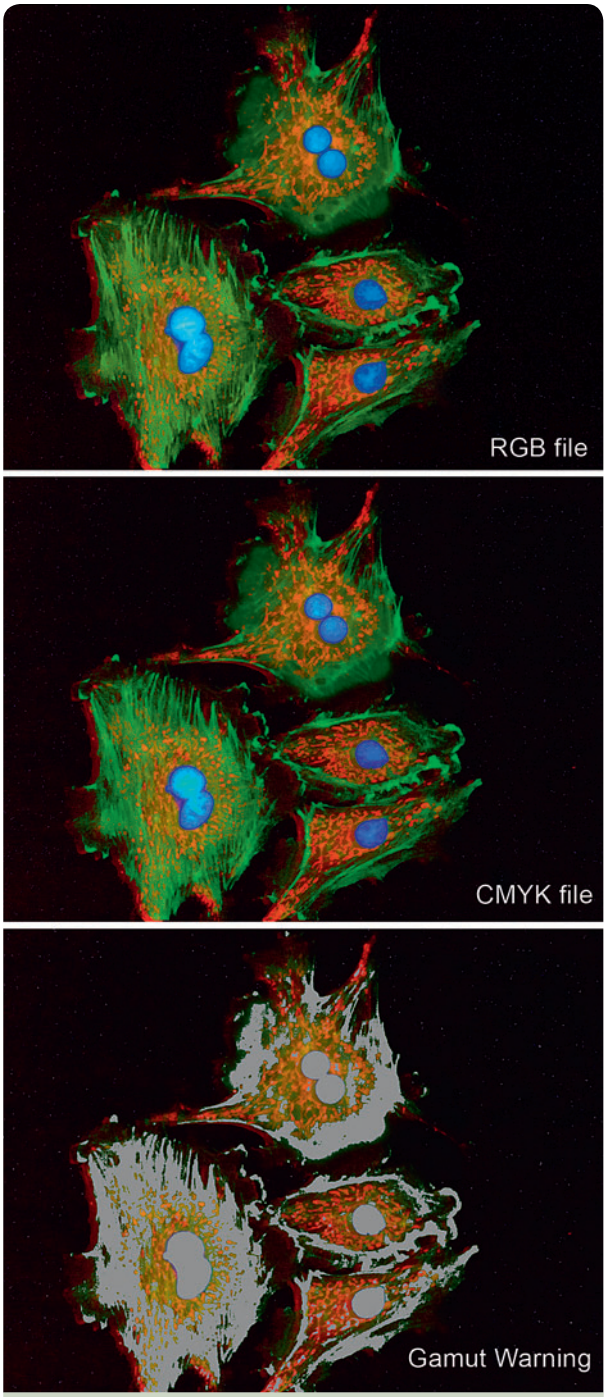


Figure 13.19 This illustration shares how RGB and CMYK files will appear when prepared for soft proof viewing. The gamut warning shared in the bottom file reveals the areas of the files that will not reproduce with detail in print.

problems. The subject in the image might have some colors that are simply too saturated for reproduction in print. It is the role of the ICC profile to manage these colors, and so you may choose to leave things as they are and go ahead with printing anyway. You may decide to make some manual tweaks to the file using the appropriate tools if you feel you can improve the image. By turning the Gamut Warning on and off, you can watch the gray areas disappear or reappear while making adjustments. Adjustment layers for hue and saturation, curves and levels can be flexible and should be first choices for use. Selective Colors, Channel Mixer, and the addition of an alpha channel mask can be used to cover sensitive regions for enhancing or decreasing color and could also be used as an alternative route. Small gamut warnings can be ignored if some areas that have turned gray don't have a strong presence in the image.

Once a print has been made, compare the print and the image displayed on the monitor. As your eyes adjust to changes in lighting conditions, hold the print next to the monitor and evaluate for a good match. It can be important to evaluate the print under the right white-light conditions. Humans will perceive light in the 5000–5500 K color temperature range as white. A light bulb with 5000 K CRI of 90 is recommended as a daylight-equivalent print-viewing light source.

SUGGESTED READING

Adobe Creative Team, *Adobe Photoshop CS6 Classroom in a Book*. New York: Adobe Press, part of the Pearson Custom Library, 2012; ISBN-10: 0-321-82733-3.

Goelker, Klaus, *GIMP 2.6 for Photographers. Image Editing with Open Source Software*. Santa Barbara, CA: Rocky Nook Press, 2011; ISBN-13: 978-1-933952-49-9.

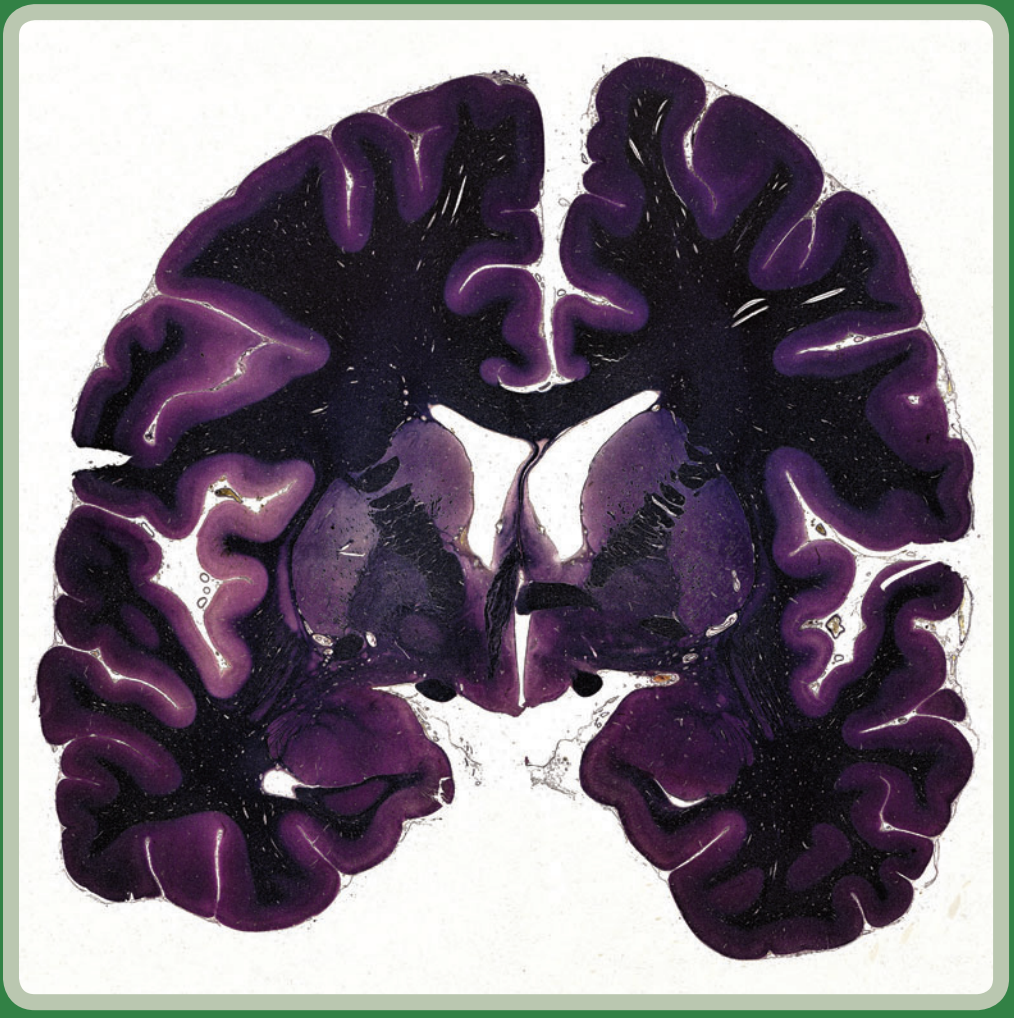
Lecarme, Olivier, and Karine Delvare, *The Book of GIMP: A Complete Guide to Nearly Everything*, first edition. San Francisco: No Starch Press, 2013; ISBN-10: 1593273835.

Mateos Perez, José María, and Javier Pascau, *Image Processing with Image J*. Birmingham: Packt Publishing, 2013; ISBN-10: 1783283955.

Russ, John C., *The Image Processing Handbook*, sixth edition. New York: CRC Press, 2011; ISBN-13: 978-1-4398-4063-4.

Sedgewick, Jerry, *Scientific Imaging with Photoshop: Methods, Measurement, and Output*. New York: New Riders Press, 2010; ASIN B003FJ64HG.

Chapter 14 Applications of Computational Photography for Scientist Photographers



This photograph features a human cerebrum in coronal view. An average human brain is 140 mm or 5.5 inches in width. Image courtesy of Michael R. Peres.

Making a new picture that is created using other pictures is not a completely new idea, but because of how good the results can be when using contemporary digital practices and software, people have become spoiled in what is expected. In the film era, the tiling of smaller prints to make a larger print was not unheard of either. Great skills were needed to expose and develop the film in the same way creating negatives that were matched in density and contrast. Once the developed negatives were ready for printing, each individual frame had to be printed to the same darkness and contrast. This tone matching was quite a production, and finally came the making the new large print composed of the many individual smaller prints. It was slow, expensive, and required a skilled operator who assembled the individual pieces into the final photograph.

Making a derivative or computational picture using today's powerful imaging tools can provide both pleasure and pain and presents scientist photographers with new ways to make unique and highly informative images. This chapter will share the best practices used for increased depth of field (DOF) imaging; widefield high resolution imaging; high dynamic range (HDR) photography; and time-based imaging. Photographs that are the results of a derivative process might be called computational photographs. While the term "computational photography" is not universally used to describe these processes in particular, it suggests that the final image contains a lot of image processing that was used to create the derivative image.

Batch Processing

Because there are sometimes many image frames needed to create a computational picture, it can be practical to batch process the individual files prior to integrating them into the new file rather than processing each of them individually. When the images are processed the same way, their characteristics will also be similar. Automating the same "task" can be a huge time saver for scientist photographers who are always seeking ways to multitask. Automation in this instance would be called batch processing. Downsizing a file is a common image processing function that lends itself nicely to batch processing. There are others but they should be chosen carefully and might require a test to determine effectiveness when used on all the files.

Making an Action

Once it has been decided that batch processing a folder of image files is a desirable idea, image processing steps must be selected. Batch processing files requires the making of an action using Photoshop software, which is a series of commands that can be programmed by Photoshop or other image processing programs and repeated as needed for future applications. To program an action in Photoshop, open the action panel, which can be found in the Window menu. It can be also be found as an Automate function in the pull-down menu under File. Once launched, to make a new action there is a button option located in the dialog window that will open. Once named, push the Record an Action button. It is recommended to have any file open prior to starting the recording. If the Open File button is recorded in the action, it will need to be deleted prior to running the action.

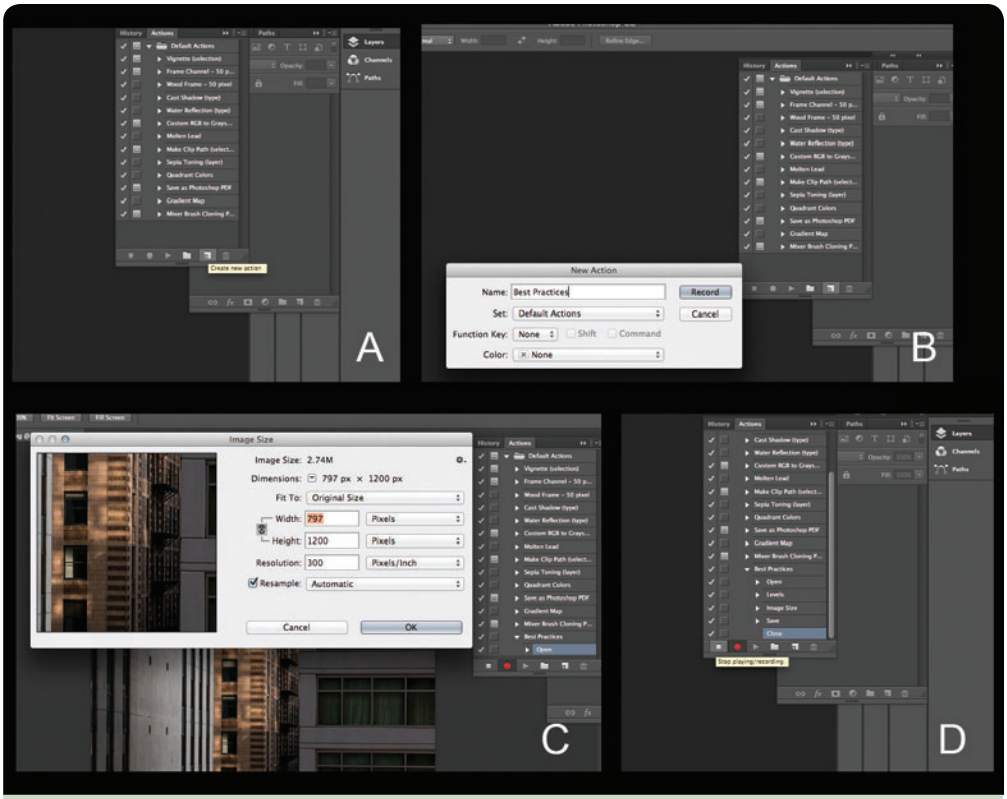


Figure 14.1 Creating an action used for batch processing requires using many small steps that can be monitored in the action dialog box. The following composite shares what each window includes. Step 1: Create an action. Step 2: Name the action. Step 3: Record the sequence. In this example, Step 4: Image resize. Step 5: Stop recording.

Once started, as the image attributes are programmed to change, each of the various adjustments is recorded by the action. It is recommended to record using the Save As feature to preserve native files. It is also recommended to make a new folder where the newly saved files will be located. Once the image processing steps have concluded, stop recording the action. The action should be named. When finalizing an action it remains important to include a close file step, as well.

To use the action, go to the Automate Software function located in the File menu. Once in Automate, select Batch.

- Choose the action that has been recorded for the image processing.
- Select the source folder where the images to be batch processed are located.
- Select the output folder where the saved as files will be redirected.
- Start running the action by pushing the OK button and Photoshop software will batch process all the files located in the source folder with the steps programmed into the action.

Adobe Lightroom software also allows the batch processing of photos. First, import the files into Lightroom. Once all the files have been imported, one file can be adjusted as needed using the Develop mode of the software. Following satisfactory completion of the image adjustments, select all the files and run Sync on the selected files.

Increased Depth of Field Methods

In close-up photography and other magnified imaging techniques, a limited amount of DOF is produced by the lens. As the magnification of an image is increased, the image's DOF will go down proportionately. Some images may have only a few microns of DOF. There are optical methods useful for increasing an image's DOF, but these often lead to a loss of critical edge sharpness because of the influences of diffraction; see Figure 3.19. Closing the lens's aperture—or the aperture diaphragm of a microscope—can increase the image's DOF but the images created when using small apertures will have less optical resolution; see Figure 6.8. A useful approach for creating images with more DOF is using focus stacking or increased depth of field imaging software. Sometimes this method is referred to as Z-stacking. As its name implies, it produces an image with greater DOF, that blends individual image slices by first placing them on top of each other or in layers using an image processing software. Once the files have been placed into individual layers or transparent images, the software will seek out contrast differences between frames—the sharpest focus in adjacent pixels (edges)—and isolate these regions, making a new derivative image from the high contrast regions of the individual image slices. A tremendous amount of computing power is needed to remove the out-of-focus areas and then re-blend the in-focus areas into a new and completely focused image.

This method—like anything undertaken in science—requires careful attention to every detail, and even then it may not work as expected. There are several software products that I have used for increased DOF imaging. It is also possible to use Image J and GIMP software. Research-grade photomicroscopes also come with software that can produce increased DOF images to a very high degree of accuracy using automated features. Increased DOF images can be programmed into the instrument's camera software and the automation in the changes of the working distance, and then subsequent image processing creates excellent outcomes.

Helicon Focus®, Zerene Stacker®, and Adobe Photoshop software are commercially available increased DOF software products and will be further discussed in this chapter. Each of these products works well for different types of samples and magnifications. I have found it necessary sometimes to try each of them on difficult samples because each will work better with different types of images and sample attributes. They are not overly complicated to use and knowledge about how to use one can easily be transferred from one to another.

Making Image Focus Slices

It is important to create a camera and lighting approach where nothing changes when the exposures for each individual frame are being made. It is important for all of the exposures to be identical in all image attributes. To minimize any deviation in exposure and other image attributes, it is best to use the camera's manual exposure mode and set the f-stop, white balance, shutter speed, and corresponding ISO to the same settings. To that end, it is important not to adjust the lighting or brightness of the light(s) during the photography either. As found light is sometimes changeable, photographing with ambient light can be difficult because of exposure variances. It is also important to focus the lens in the manual

mode in each of the individual exposures. An aperture setting 1.5 to 2 stops from wide open is ideal. Often this might be an aperture setting of approximately $f/5.6$. Never use the aperture completely closed down for this work. A lens will have an optimal aperture setting that creates the maximum of lines per millimeter or optical resolution. A lens's optimal diaphragm setting can be determined from its specifications data sheet.

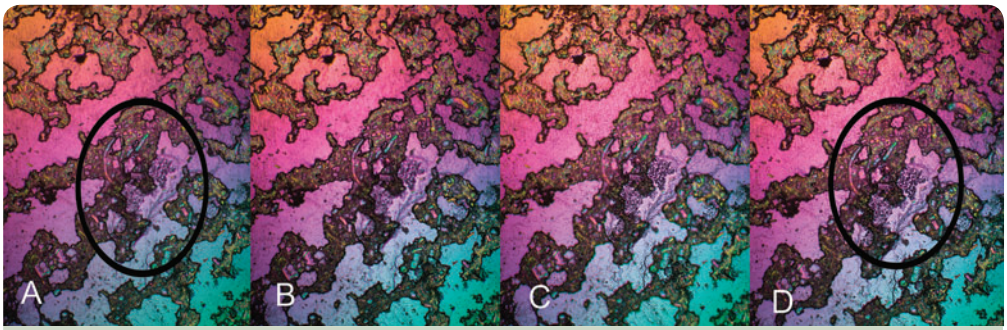


Figure 14.2 This composite photograph includes an increased DOF image (D) and features a hepatitis medicine examined using polarized light. The colors are evidence of birefringence from a material that is composed of multiple refractive indices (anisotropic). If the material were a single refractive index, it would be called isotropic. The focus in each image (A, B, and C) was placed in a different plane by changing the working distance by 1 µm. The circle indicates a region where significant change in image focus was evident. Image D is the composited file.

Once the correct exposure has been determined and an effective aperture selected, the making of the image slices for the increased DOF image can begin. It is possible to photograph moving the sample up or down. There is no advantage to either direction. The more individual slices, the better the results. Producing too few image slices can be a problem because the software may struggle to form data when insufficient pixel data is available. It is also important to try and move the sample or stage the same distance for each image slice—to the extent that is possible when doing this by eye/hand and without automation. A focusing rail is an excellent accessory for this task. Many focusing rails are calibrated and include printed distance increments. When making each exposure of the individual frames, nothing can change in the set-up except the working distance. If any structures in or on the sample move, ghosting or other image defects will be created in the new derivative file. When making the individual image slices, it is important to create overlap of the in-focus areas. It is important to capture all of the exposures needed when shooting because it will be impossible to re-create a replacement image in the same precise manner—if needed—for integration into the stack of the previously captured images. Image acquisition should include regions above and below the subject. It is recommended to capture image files using a RAW file format when using DSLR cameras.

Stackshot® is an electronically controlled focusing close-up rail that is a terrific tool used for increased DOF imaging. It is sold by Cognisys®. It is designed to help produce tiny and precise working distance changes automatically once programmed. The focusing rail is capable of moving the lens/camera system a 2 um distance and can cover up to a 10 cm distance. A USB port on a computer is required for operation of this device.

Global Image Processing of Files Used in Computational Images

Once the set of image slices has been successfully formed, recorded, and moved to a named folder, image optimization is suggested prior to building the Z-stack. It is fundamental to the success of the final image that all the slices can be batch processed together making the same adjustments to each file when helpful. This is mostly useful for image size and changing file types. Depending on what image processing software is being used, it is possible to select all files and adjust them all together, leading to matched image tones and color balance. It is also common to slightly pre-sharpen at this stage. It is suggested to also adjust the image's exposure or other image attributes if helpful. Once all of the image characteristics of each frame are matched satisfactorily, the files should be saved as TIF files or, less desirable, JPEG files. Helicon Focus and Adobe Photoshop can manage RAW files, but without testing, do not assume that all increased DOF software will be able to process RAW files.

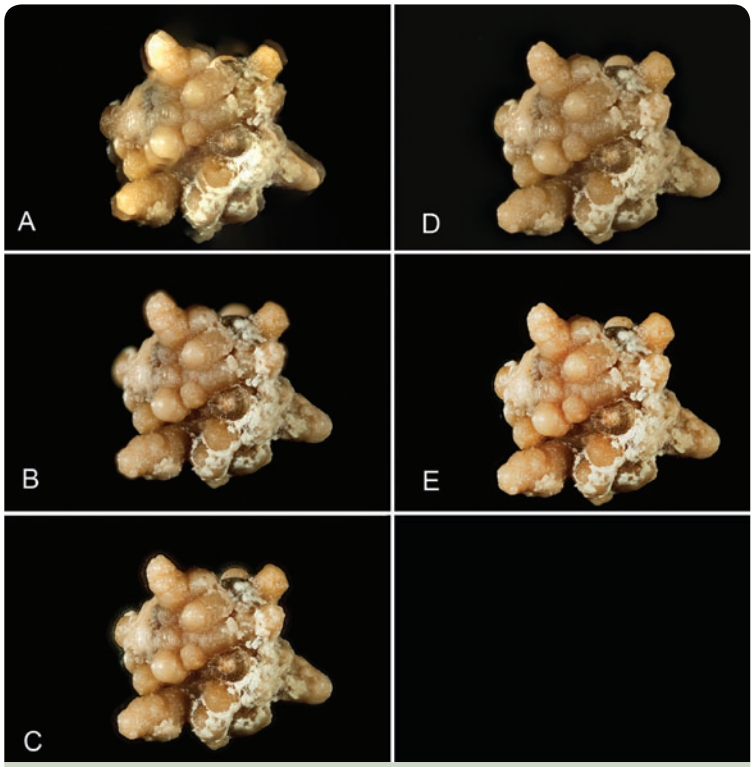


Figure 14.3 This composite photograph shows the results produced by Photoshop, Helicon Focus, and Zerene Stacker. Image A reveals artifacts using Photoshop. Image B used Helicon Focus in the Depth Map mode and also contains artifacts. Image C used Helicon Focus in the Average mode and shows improvements. Image D was created using Zerene Stacker in the PMAX mode, and for Image E Zerene Stacker was used in the Depth Map mode. The subject is a kidney stone. Image D created the most successful result. Image slices courtesy of Staffan Larsson.

One other consideration for building an increased DOF file may be the individual image's capture resolution for subsequent importing and processing. Many contemporary DSLR cameras are capable of recording more than 7000 pixels in the long dimension of the sensor. Depending on the software and number of slices, the amount of cumulative image data may exceed the software's and computer's RAM capabilities to process. Unfortunately the largest number of pixels that can be processed by the computer will be determined using trial and error. Once a file is too large, it may cause a computer to freeze. How many pixels are required for a particular output is always going to be a prevailing question that may remain ambiguous for practitioners of scientific photography. An HD monitor will use 1900 pixels, while a VGA monitor will display just over 1000 pixels. Output will drive input. Downsizing large files can be very helpful for screen display applications.

Z-Stack File Processing Using Photoshop Software

The individual files that will be used for the Z-stack need to be brought into Photoshop software as layers. You can select all of the images using Adobe Bridge and then, under the Tool

menu choice, select the Photoshop Open In Layers option. Once the files have been loaded into Photoshop layers, the images must be aligned and/or blended. Selecting all the files can be accomplished by manually clicking each layer while holding the shift key on the first and last file or, under the toolbar, selecting all layers. Once selected, using Photoshop and under the Edit menu, the Auto Align feature can be executed. Once aligned, the Auto-Blend feature should be chosen by selecting the Stack Images option. In that window, check the Seamless Tones and Color dialog box. The software will then work through each file, isolating the pixels that have the highest contrast, which will be used to build a new file from the individual files.

In a relatively short time, the stacked image with its blended in-focus areas is available for review. The final image is created from the individual layers and each file still can be reviewed within an individual layer by clicking on the eyeball in the software window if desired. If the result is satisfactory, the file's layers can be flattened. Save as a copy. You may have to do some touch-up in Photoshop but for the most part you should see a image with noticeably greater depth of field. Increased DOF images may appear more sharp to a viewer.

Z-Stack File Processing Using Helicon Focus Software

Using Helicon Focus software can be easier and faster than Photoshop. There is one command to add the image files, the Add Image command, or the files can be added by dragging them into the program's load window. Once the files have been added, the files can be rendered using the Density Map or Average modes. The stacked file will appear in a window while it is being constructed. Some subjects will benefit from using the Density Map method while others will do better using the Average selection. A visual inspection of the stacked file will reveal which mode is best for the type of subject of the photograph. Do not sharpen or perform other image processing using this image stacking software. Image adjustments to the stack file will be better managed using image processing including sharpening. Once satisfied with the stacked file, save as a TIF file and flatten the file if needed.

Z-Stack File Processing Using Zerene Stacker Software

Using Zerene Stacker is easier and faster than Photoshop as well. You can add image files using the Add Image command or you can add them to the software by dragging the files into the program's load window. Once the files have been added, the files should be aligned before they are rendered using DMap, PMAX, or both modes. Similar to the recommendation when using Helicon Focus, do not sharpen the files or perform other image processing using this image stacking software. Image adjustments to the stacked file will be better managed using image processing that including sharpening. This software can save its output images as 16-bit TIF files.

Widefield High Resolution or Image Mapping

A picture—when based on intended use—may need more digital or more optical resolution or both. When photographing using low power objectives, the optical resolution of the images will be limited by the objective's finite and inherently low resolution. This reality

may lead to results that are woefully unacceptable for publishing. It is relatively easy and fast to make a single photograph of an entire sample using a low power lens but the image may not delineate structures within the sample, and after image processing only the gross structures will be clearly visible in the image.

The interest to create pictures that are more resolved and interactive is not new but modern tools continue to evolve, making it easier to make widefield high resolution files. Many years ago, Kodak—when in the digital photography business—focused on a type of strategy they called immersive imaging. The core concept of the initiative was to allow users to view images in interactive ways. Producing high resolution digital files today, sometimes more commonly called widefield high resolution images, is much less complicated. The images produced from widefield techniques are scalable and allow viewers to zoom and move around the image as they choose. This allows users to see much more information within the image and allows the images to be published on the Internet in a similar fashion, as Google maps where the images are tiled.

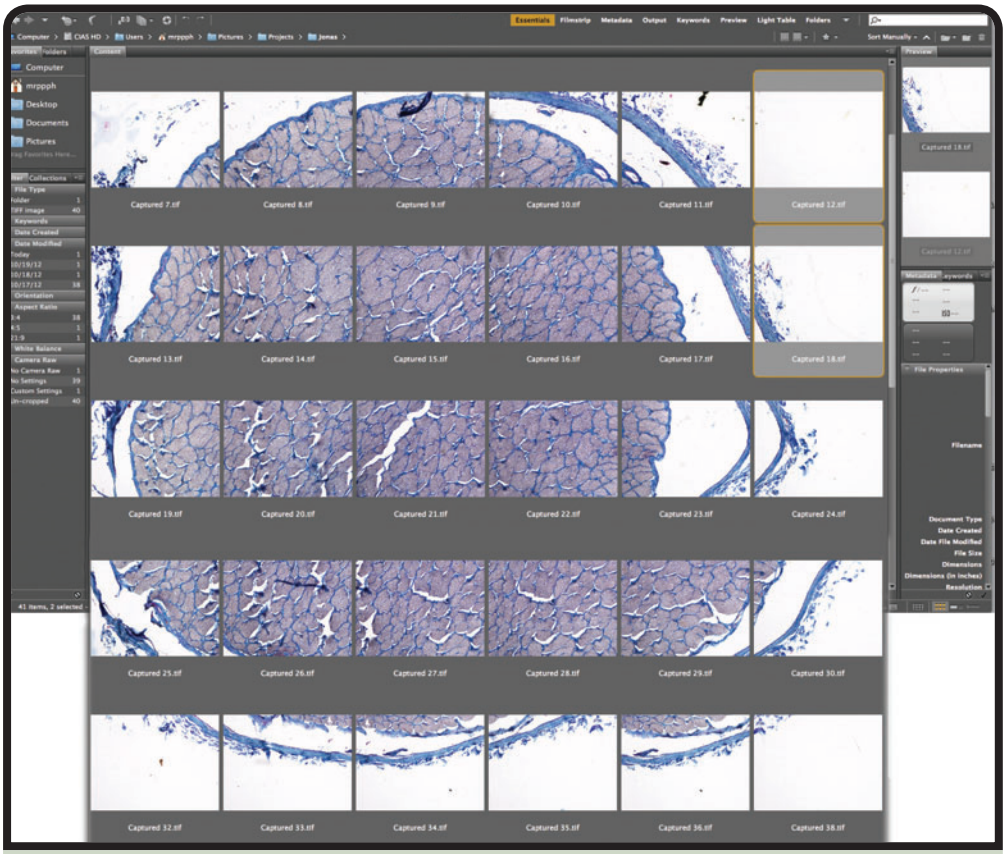


Figure 14.4 This figure shares the screen appearance of thirty files ready to be imported to Photoshop's Photomerge function. When shooting files to be used as a map, it is important to overlap each of the individual frames. Staying organized during the image recording process is tedious and requires attention to location on the sample. Some photomicroscopes have an automated stage allowing for precise movements to be programmed.

Methods

Producing the individual files required to make a widefield high resolution image is not particularly difficult. Creating the individual files needed for the process—sometimes called a map—needs to be carefully managed. Each field should contain some overlap to the adjacent regions included in each frame. In any process that is derivative, the end result will be influenced by the sum and quality of the parts.

The numerical aperture (NA) of an objective or the aperture of a macro lens will play a significant role in the optical resolution of the image a lens forms. The higher the magnification of the objective, the higher the objective's numerical aperture must be. The higher the magnification of the image, the narrower the field of view that will be included in the image. This becomes a core problem in this type of work. Images made from objectives with a widefield view will have low optical resolution and images that are more highly magnified will be well resolved but will not include the entire sample.

To create both outcomes, many individual images photographed using a higher NA lens can be made and then combined to form an improved image. It is important for all of the exposures for each frame to be identical in all the image characteristics. It is vital to minimize any deviation in exposure and other image attributes when making the frames, and this could include using the camera's manual exposure mode, white balance, and corresponding ISO when determining the best exposure settings. It is important not to adjust the lighting, aperture diaphragm settings, or brightness of the light(s) during the photography either. Functionally, only the field of view of the sample and the focus of the lens using the manual mode in each of the individual exposures should be different. I like to overlap images approximately 15–20 percent. It might be practical to consider the sample as a grid and work back and forth to the extent that is possible; see Figure 14.4. When using an instrument camera's software, all aspects of the process of image slice formation, including focus, can be programmed and managed by the software and microscope.

Many high-end research-grade photomicroscopes are equipped with automated stages that can be programmed to make a map. These systems are very precise and typically produce highly successful images. The challenge for any system will be the number of files that are created. Using low NA objectives will create fewer image tiles but the widefield image will be less resolved. Using a very high NA and magnification will mean making many more individual tile images that will require a significant amount of

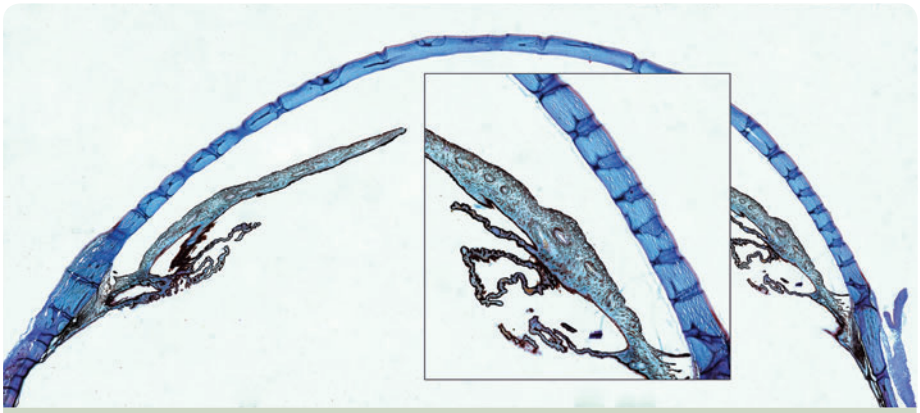


Figure 14.5 This widefield photomicrograph features a cornea and iris in cross-section. This image is the product of nearly sixty individual frames. It was highly resolved and downsized for reproduction. The smaller inset window has been overlaid on the image to emulate how zooming might look. Image courtesy of Jonas Brane.

computing to create the derivative image. Separate from that, the number of image files selected also depends on the camera's digital resolution. Files from big sensors will tax even the most robust computers. It is not uncommon to make an image file that is larger than 1 GB.

Once the correct exposure has been locked in and an effective setting of the aperture diaphragm established, the making of the image tiles can begin. It is possible to photograph left to right and then shift down or up and repeat the process. There is no advantage in any one way of photographing the frames in the grid. The only absolute is that the more the individual frames overlap each other, the better the blended result will be. Creating too few image tiles can tax the software to stitch the files. Having too few individual frames may create false data when insufficient pixel data is available to overlay. It is also very important to move the sample or stage the same distance for each tile to the extent that is possible when doing this by eye/hand and without automation.

When making the individual frame's exposure, nothing can change in the set-up except the working distance. If any structures in or on the sample move, ghosting or other image defects will be created in the new derivative file. Images should include the regions outside of the subject. The ideal image capture quality setting should be the RAW file format when using DSLR cameras or TIF when using an imaging photomicroscope's instrument camera.

Global Image Processing

Once the set of images across the sample have been successfully formed, recorded, and moved to a named folder, image optimization is required prior to building the high resolution widefield image. It is fundamental to the success of the final image that all the frames be processed with the same adjustments. More about these consideration can be read in the increased DOF section of this chapter. The ultimate goal is—to the extent the similarities can be created—that all image file attributes are the same.

Creating the Image Map

Creating an image map is easily accomplished using Adobe Lightroom or Photoshop software. Once Photoshop has been launched, the file can be built using the Photomerge function. This function can be launched using the Automate menu bar located in the File menu. Once loaded, the procedure is pretty straightforward. The software will bring up a dialog box that allows the images to be blended using auto, perspective, cylindrical, spherical, or collage, or allows the user the ability to reposition mode. Check the Blend Images Together feature. Once the images are added, simply click OK and the software will produce the computational photograph. I prefer to use the Reposition mode of the Photomerge function.

It is also possible to use Panorama Maker®, PTGui®, Image J, or Fiji—an image processing package that bundles Java, Java3D, and plug-ins to make a widefield image. The main focus of Fiji is to assist research in life sciences. It is also possible to stitch images using the Stitch Panorama or using GIMP software. There are other products that can be visited online, shareware, or proprietary products.

High Dynamic Range Images

Many times objects or scenes will have a luminance ratio that is beyond the sensor's ability to record details in both the darkest and brightest regions. A camera's sensor will have a finite exposure range. Often this range might have an approximate 120:1 brightness ratio or approximately a 6+ f-stop range. Once the brightness exceeds this range, the sensor cannot record useful exposures in both the brightest and darkest regions. The middle gray tones are not typically affected by the dynamic range. You can read more about a sensor's dynamic range in Chapter 4.

High dynamic range photography is a method used to create a derivative image built using two or more files that each have different exposures. The human eye—being dynamic—can see into dark shadows and bright highlights almost simultaneously. Because a sensor creates a static capture, this is not possible in photography. HDR images record sample information from both the darkest and brightest regions in the sample in the result. Fluorescence imaging is a type of application that can challenge a sensor to capture the entire brightness range in one exposure.

Making Photographic Exposures

The various exposures—the ones above and below those recommended and needed for an HDR file—can be produced either using a manual or an automatic exposure mode. Many cameras will have an HDR exposure setting and will create multiple exposures very quickly and efficiently without effort. The camera can be programmed to make what is called a bracketed series of exposures. This exposure range is typically –2, –1, 0, +1, and +2 f-stops. In an ideal situation, nothing about the subject can change during the image recording phase of the process. Since the images are being blended, it is important to change exposures using time only. Using the lens aperture will create images with different amounts of depth of field. For this reason, if using a camera's automatic exposure mode, use A priority.

Blending the Images

Once the images have been created, creating the HDR file can be accomplished using a number of image processing software packages including Photoshop, Bridge, Image J, GIMP, PTGui, and HDR Studio. These programs are simple to use. Many of the same concerns resident in stitching files or increased DOF application should be addressed when pursuing global image processing prior to launching the program. Care should be always be taken when image processing.

Blending of images with various exposures is accomplished by what is called tone mapping. Generally, tone mapping applies an algorithm to a computational image based on how images were recorded and stored. The derivative image will be influenced by bit depth. In HDR image processing, the image data is derivative from a digital file rather than a scene. In this operation, all the variances resident in similarly located pixels are averaged to create a new file lowering contrast across the subject's dynamic range. Tone mapping is the process by which the software accomplishes this blending files of differing exposures.

Creating the HDR file is easily accomplished using Adobe Photoshop software. Once Photoshop has been launched, the file can be built using the Merge to HDR Pro function. This function can be found in the Automate menu located in the File pull-down menu. Once the files are loaded, the procedure is pretty straightforward. The software will bring up a dialog box that allows the images to be loaded simply by clicking OK. The software will produce the computational photograph. Use the Align Images features as well.

Other software products will have a different workflow but in the end will accomplish much of the same tasks.

While they are useful for making images that contain information in the highlight and shadow regions of the subject, there may be some compromises in HDR images as well. Exposure and image brightness is often not a singular element of an image file. Image contrast and color data is also associated with exposure. Because of these linked image attributes, HDR files will often exhibit a unique color reproduction and shadow characteristics. They exhibit a bit of a glow and the color palate might be described as pastel. HDR images that are overworked can have a characteristic look. Many times, this appearance is negative and suggests the image is not displayed in its correct tonality. It looks like a computer image, and because of this, HDR might not be ideal for scientific applications. Testing should be done to assess your tolerance and the appearance of the images that are created. Fluorescence photomicrographs may appear false and may simply not look correct.

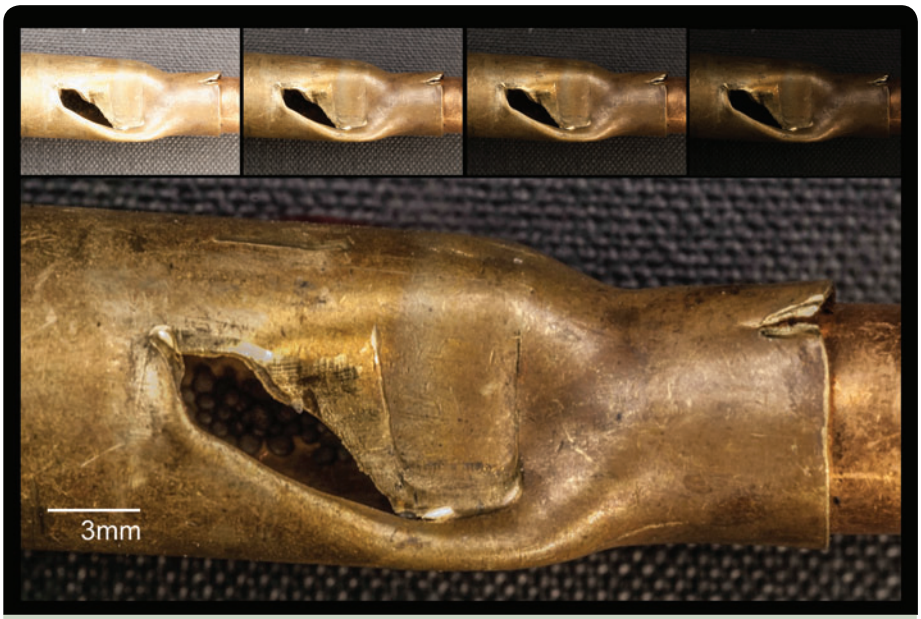


Figure 14.6 This HDR photograph features a 7.62 mm cartridge fired from an M143D six-barreled Gatling gun, often called a minigun. The illustration reveals damage to the shell as a result of a feeding malfunction. The HDR image was beneficial for recording detail in both the highlights from the bent metal and the interior of the cavity, where there still remains unburned powder. This would not be possible using a conventional single-shot photograph. The four exposures were made using ƒ/25 for 1/4, 1/8, 1/20, and 1/50 sec. times. Image courtesy of Adam Hartley.

Time-based Imaging

Time-lapse photography can be a useful tool when photographing an event that occurs over a longer period of time. An example of an event that can benefit from compressing time would be the opening of a flower or cells dividing that can be observed using a light microscope. Many times but not always, time-based images are displayed using videos with frame rates that allow the event to be watched using various amounts of time delay or acceleration.

Prior to making the first exposure, several fundamental questions need to be answered when considering the making of a time-lapse sequence. How long does the event last from start to finish? Is the process/event linear or logarithmic in its behavior(s), does it have an acceleration or deceleration component, or does it occur at the same rate throughout? And lastly, how long will the video's playtime be? The answers to these questions will influence decisions about the time increment between exposures and how many frames are needed to produce a screen time of the desired length.

If individual single frames of the event will be used for print applications, moving forward is relatively straightforward. Select the best frames and built a composite; see Figure 1.12. If a time-lapse video is going to be used for the distribution to show the images, the term "frame rate" will be used when considering the time-lapse movie. Traditionally a video playback setting would be 24 or 30 frames per second (fps); allowing viewers to have persistence of image and perceiving a change in location of an element is practical. A 10-second video will require 240 frames if shown using 24 fps. If the event takes two days from start to finish, the event would last forty-eight hours or 2880 minutes. Photographing an event that lasts 2880 minutes that will be displayed as a 10-second video clip (240 individual frames) requires one picture to be taken every 12 minutes if the event has a constant rate of change. Time is shortened or lengthened in time-based imaging and events can be speeded up or slowed down by changing the frame rate of display or by changing the number of the individual images captured. Both elements of the process will influence the effectiveness of the work. Time-lapse applications allow the compression of time and high-speed events allow the expansion of time.

To make movies that are smooth and less choppy, more frames are needed that possess shorter times between frames. Jumpy video sequences are created using fewer frames that have longer time increments between frames. The final determination of time increment between photos is subjective.

Photographic Considerations

Similar to increased DOF imaging or widefield high resolution applications, nothing should change to the camera recording settings or to the artificial lighting (if used). If exposures change during capture, the images when projected will exhibit a "flicker" or brightness variance throughout. This can be very distracting when watching the time-lapse video. When time-lapse work is done using ambient or found light, this flicker or variance of the lighting may be part of the event. When recording the changes in cells that are dividing, for example, or the growing of crystals, the only change that should occur should be to the subject and not the background or exposure. If the lighting and its brightness remains constant during the event, it is recommended to use a camera's manual mode to minimize any variances that may be produced by the automatic light measurement features of the camera. If the lighting does change during the event, then using the automatic camera exposure mode will be more practical, allowing for better management of the variances of times when responding to the brightness changes.

To that end, image capture settings should be selected through considering how much post-processing will be required. Less is more. Since the vast majority of time-lapse work is shown in video format, the files will—in all probability—be shot as JPEG. Making hundreds

or thousands of TIF or RAW files that will subsequently require image processing and then have to be imported to video editing software cannot be discounted in building a best-practice strategy for this application in a lab environment. Image processing takes time, and unless a scientist photographer aspires to spend a considerable amount of time image processing, efficiencies should always be sought out. There are compromises when using JPEG in data compression or artifacts.

Instrument cameras, smartphones and DSLR cameras are all capable of creating time-lapse recordings directly. These next few paragraphs will focus on considerations for using instrument and DSLR cameras. Unfortunately, every camera and software program will have differences in how they are set up and how they operate for this type of work. It will be impossible to offer specifics for all products. I will use some examples in trying to address the most global considerations and settings. These suggestions will need to be adapted as necessary to your equipment and applications. Using a DSLR camera can be a very good choice for time-lapse photography applications because of its features, the high quality output, and excellent controls. Instrument cameras offer advantages because of the constant access to stable power. Effective time-lapse work mandates that the camera or optics cannot change position during imaging. Even the smallest changes can be disruptive to the sequence. A tripod or vertical camera stand is required.

Intervalometer

Once the camera position has been determined and the various exposure attributes set, it is important to practice once before attempting to complete important work. Making a time-lapse movie is the product of many individual exposures. Producing this type of media without automation can be accomplished but it will be fraught with challenges. It is more practical to program an electronic controller to make the exposures using an internal timing application called an intervalometer. This device allows an operator to program a camera to take pictures using specific time increments between exposures as needed. Intervalometers are valuable tools and allow precise and autonomous operation of the camera over a long period of time. An intervalometer can be embedded in camera software or operate as a remote trigger.

Many high-end Canon cameras can use a Canon TC80N3 trigger and newer Nikon cameras have a time-lapse mode built into the camera features. Older Nikon cameras will have an interval-timer shooting mode. Regardless of the camera, make a test exposure with it to ensure that all functions work correctly.

Photographing a Time-lapse Sequence

To set the time-lapse shooting function in the computer software or in the camera's menu:

1. Set when to start the shooting sequence.
2. Set time increment between exposures.
3. Set how long the session experiment will last.
4. Consider the size and capacity of the memory card. Photographing for prolonged exposures over time will create significant amounts of data. It would be a catastrophe if the event could not be exposed from start to finish as a consequence of lack of storage on a media card.

5. Disable all power-off settings, such as: turn off LCD preview, turn off autofocus, and turn off the image stabilization on the lens.
6. Reformat the card before exposing a time-lapse sequence. Losing a few critical frames can render the project unusable. Fast cards are also suggested.
7. Select the quality of the file format. There are advantages to RAW vs JPEG. RAW allows the files to be very effectively managed but will create larger files and require more time for processing. JPEG files have great advantages because of speed in handling but data will be compressed and artifacts can be added.
8. Do not use auto white balance settings. Custom white balance settings will provide the cleanest files.
9. Set the camera's exposure attributes including ISO, aperture, and shutter speed. It is vital to expose for the whites using a test exposure frame. Shadows can be adjusted for exposure in image processing but white details can never be added back to a file. Select the aperture based on the DOF requirements.

Once considerations for all the above aspects of the process have been finalized, the individual frames can be exposed. Some cameras will produce a series of exposures that may require a small amount of image optimizing before they are inserted into a time-lapse assembly software such as Apple Quicktime Pro® software. Some cameras are capable of creating the time-lapse movie within its features when attributes are selected from the video modes within the camera itself.

Image Editing Time-lapse Photos

After the shooting has been completed, there could be hundreds or more image files. The number of frames is a function of the settings that were selected. In some folders, there can be advantages to improving the image attributes before merging them into time-lapse video. This might include brightness, cropping, white balance, etc. Editing each photo one at a time would be time-prohibitive, but there are ways to batch process the individual files using Lightroom or Photoshop software. To create a time-lapse movie directly in Lightroom, a pre-set module for time-lapse will need to be added to the software. These pre-sets can be found and downloaded. Once a module has been added to the pre-sets folder, the choices are quite intuitive. There will be a frame rate choice and other export mode choices will also be located in the module. It is a relatively easy activity to work through.

There are software products that can assemble a time-lapse movie when using older cameras. Apple Quicktime Pro 7 software is a very simple and effective choice. It operates on both Windows and Mac operating systems. To produce a time-lapse using Quicktime, go to File and Open Image Sequence. Locate the folder containing the time-lapse files, select the first photo file and hit the Open button. Select the 24 fps output and hit OK. The software will compile the individual files into the video at this time. Select File Fit to Screen to preview video. Once satisfied, it is time to export and name the file. Save the file to an appropriate location selecting the desired export settings.

There are various video codecs that can be chosen. A codec is a process that compresses videos based on size for playback rates both from a hard drive or when the media will be viewed online. Many professionals choose the Apple ProRes codec and save file.

SUGGESTED READING

Bloch, Christian, *The HDRI Handbook 2.0: High Dynamic Range Imaging for Photographers and CG Artists*. Santa Barbara, CA: Rocky Nook Press, 2013; ISBN-10: 1937538168.

Chylinski, Ryan A., *Time-lapse Photography: A Complete Introduction to Shooting, Processing and Rendering Time-lapse Movies with a DSLR Camera* (Volume 1). No location: Cedar Wings Creative Press, 2012; ISBN-10: 098537571X.

Davis, Harold, *Creating HDR Photos: The Complete Guide to High Dynamic Range Photography*. New York: Amphoto Books, 2012; ISBN-10: 0823085864.

Chapter 15 Best Practices

This picture features a simulation of insect vision. Photography was used in this experiment as a tool to emulate what insect vision might look like. For this, 4,500 black drinking straws were bundled together selectively allowing light reflected from the subject to be directed onto a 14 inch x 14 inch ground glass screen. The resolution of the system was designed to be equivalent to that of a fly. The image formed on the ground glass was then photographed using a medium-format digital camera. The portrait featured Professor W.R. Muntz, a Monash University biologist who studied cephalopod vision, specifically in *Nautilus* spp. Image courtesy of Susanne K. Williams and Adrian Dyer, 2001. RMIT University, Bio-inspired digital systems LAB, Melbourne, Australia.

Introduction

This book was written to demystify the tools and processes required to make scientific images. Chapters were dedicated to tools, methods, and strategies. These ideas have been thematically repeated throughout the pages of this book. Sample selection, proper use of selected radiated energy and its related behaviors, knowledge, skills, and a personal motivation can all collectively—or individually—play a role in the successful creation and distribution of scientific images. Sometimes it simply takes good luck, too.

More Thoughts about Best Practices and Workflow

The forming of an image, the recording of an image, the processing of an image, and the viewing of an image are all steps in the image chain. In each step, degradation of an image's quality can occur. There are, however, other things a scientist photographer can adopt when developing a holistic approach using proper tools and methods consistently each and every time a new image is made. This approach is called best practices and is a strategy that is well accepted across many industries. It requires processes to be optimized and managed from the beginning to the end, eliminating, to the extent possible, variables that will degrade results. How samples are handled, what lens or lenses are selected and used, what aperture is selected, what file type is chosen for saving files in camera, and so on, all become part of this working method used to form and record the best image possible.

It might not be typical for a scientist to think like a "photographer" if they have come to photographic practices without formal training. Today it is common for professional staff and researchers to take on imaging responsibilities as part of their work, and once core knowledge about image formation using standardized approaches becomes habit, other improvements can be adopted, moving forward into new challenges as experience is gained. No element of the process is too minor to pay attention to. Best practices optimizes what is possible while taking into consideration the accepted practices of the scientific community at large. There are a number of best practices that may not be considered specific to a method or lab and can contribute to improved outcomes.

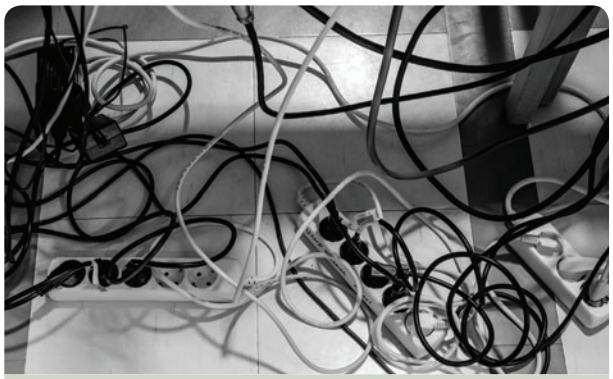


Figure 15.1 Clutter and lack of outlets seems to be a recurring theme found in modern imaging facilities. Care should be taken to keep a space and related outlets organized.

The Laboratory and Environmental Conditions

Imaging starts out in a place. A room. Unfortunately, many contemporary imaging laboratories are situated in less than ideal rooms, sometimes located in small spaces with a lot of equipment. Older rooms are frequently re-purposed for imaging and not designed for this type of work. Frequently there are not enough wall outlets for all the various devices that require electricity. Computers, fiber optic lights, microscopes, and the other electrical devices all take up space on tables, generate heat, create vibrations, and can produce electrical feedback. Often overlooked—and much needed—is simply

an uncluttered worktable for sample preparation with close proximity to the imaging workstation. Staying organized and having access to various tools and accessories while performing an experiment can be very important to an outcome. Needing to change an experiment to modify the activity can be catastrophic to imaging outcomes and continuity.

Laboratories can be dynamic places to work. People are coming and going and things get moved around. Sometimes, because there are multiple users of the same piece of equipment, settings might be changed or folders moved. Worse yet, a folder or file gets deleted. Unless one operator has total control of all the pieces of imaging equipment in the lab, best practices include confirming the settings on every piece of equipment used for an imaging experiment before starting new work. Check the camera settings, the lens settings, the lamp or other light source settings, and any other controls that are adjustable. The height of a gas-lift chair used at the photomicroscope should also be a part of the "start-up" protocol. Best practices will include managing the room lighting and ensuring the window illumination is effectively moderated as needed. Since the human viewer plays a vital role in imaging outcomes, creating an environment and approach that helps an operator see "better" can contribute significantly to achieving better results. While each of these things seems relatively innocuous by itself, each can contribute to the creation of better photographic work.

Tables can also be an important foundation for best practices and successful outcomes. Some tables are not good for imaging activities. Their legs or their stability may simply be woefully inadequate and contribute vibrations to the system and image. A strong and sturdy imaging table is vital to success. Heavy tables are much more desirable than light and portable tables. Tables with wheels can also be problematic. Where a table is located can also negatively influence results of highly sensitive systems. It might be helpful to use a piece of Formica kitchen counter or a similar dense, heavy, or inert countertop type material to increase mass and absorb, but not eliminate, table shake. Always strive to create structure and stability.

Cleanliness is Imperative

Certainly of no less importance is having clean surfaces in the work area. One of the most commonly photographed subjects in science unfortunately is dirt and particulate matter. Dirt will be a chronic problem and one that needs frequent attention. A weekly, or certainly monthly, washing down of all tabletop surfaces using lint-free cloths can be helpful in managing the presence of dirt that might find its way into samples or the imaging equipment, camera, or other locations. Plan to wash floors monthly as part of best practices. Dirt can be brought into a lab on shoes or on clothing as fibers, and sometimes a ventilation system—if a forced air system is used—will bring in dust and debris. Whether dirt is big problem or a minor problem, being aware of its potential presence will lead to better results. It has been my experience that dirt will ultimately find its way into images one way or another. I sometimes use pieces of white copy paper as surfaces to prepare and work on samples even when I believe tables have clean surfaces.

Carpets should never be located in imaging rooms. Carpets are made of fibers which over time break down, and the fibers will become airborne and can land on samples. Creating any new sources of particulate matter should be avoided and this would include fabrics

used on chairs. In my lab, we have placed added additional air filters on the inflowing HVAC vents.

Optimizing the Camera's Settings

It was previously shared that best practices for starting a new imaging session require that all possible capture settings are confirmed prior to doing anything. This includes checking the ISO or sensitivity setting, white balance setting, the selection of the file type, color space choice, the status of sharpening filters, or other camera image enhancement settings including noise reduction, etc. Changes can occur to capture settings by choice or accident. This happens frequently when cameras have multiple users. Also practical is to clean the sensor on a DSLR camera and reformat the media card(s) frequently. These are small steps that can absolutely contribute to the formation of images that are less prone to corruption and will require less post-processing.

There are significant advantages to operating a camera using manual settings whenever possible for scientific work. While automatic features offer some advantages and efficiencies, automatic can create variability in file attributes that may require additional post-processing needed to equalize image nuances across the images. Auto white balance setting is one of those choices that can produce image file variability. Using the manual mode may be a bit more time-consuming at first but will create more precision and uniformity across images during capture.

Always record a file using the camera's highest bit depth and using the camera's largest color space. Many devices default to sRGB. This color space is the smallest possible choice and produces the most compressed color. Colors are squeezed together when recorded using sRGB and the color gamut is less than ideal. For DSLR cameras, Adobe RGB 1998 is a better choice than sRGB and allows more colors to be discriminated. Instrument cameras will use different color mode choices. Selecting a color space may not be possible on all models of cameras.

There are many locations in the image chain where color space is addressable including monitors, printers, and other capture devices such as scanners. Other color spaces might be found in various devices. A few of them would include RG Chromaticity, Wide Gamut, and ProPhoto. Color space is a complex subject and scientific imaging activities require the use of the largest color space possible using any capture device.

Cleaning a Lens

Lenses and other optical elements might require surface cleaning—using an appropriate brush or lens tissue—before taking any pictures. It is rather remarkable how fast airborne particles can find their way onto the front surface of a lens. If fingerprints or other oily marks are located on the surface of the lens, use an approved lens cleaner or isopropyl alcohol applied on a cotton-tipped wooden stick. Never rub a lens using a lot of pressure but rather use a circular motion using a light touch. Pretend you are polishing the lens surface softly rather than rubbing it using pressure. Glass and other optical elements

are vulnerable to scratching and care should be taken when cleaning and removing oily marks. Any cleaning process should not create new and more permanent artifacts such as scratches. There are special lens cleaning tissues or swabs that can be very effective when encountering stubborn spots.

Monitors and Video Displays

Digital images cannot be seen unless they are displayed. They remain invisible as digital data, which is a significant difference from film images. The digital image, once captured, is stored and requires the use of software and a display to make the file visible. Display devices exist on the back of cameras, on smartphones, or on monitors from laptops, tablets, and other flat-screen devices. It was not so long ago that cathode ray tubes (CRT) were used as monitors. They were heavy, sometimes noisy, and always created heat. They were also large and took up a sizeable footprint on desks. More importantly, their displays drifted frequently. The drift could be in brightness, or color, or both. Since the digital image can only be seen when displayed—and if the monitor is not properly managed in its brightness and color—characteristics of the digital image's display attributes can be skewed by the monitor's lack of accuracy. Some additional content sharing how to set up a monitor's display characteristics is included in Chapter 13.

Liquid Crystal Display (LCD) technology has moved CRT technology to closets. One advantage of LCD displays is that they experience less image variability and they use less power. The drift of brightness and color does still occur to LCD monitors but more slowly and in smaller increments. CRT monitors seemingly could change overnight. Because images are processed using the display characteristics of the monitor, brightness, color and gamma, it is necessary to calibrate a display screen using appropriate tools. In a perfect world, image processing would be accomplished using pixel brightness information and other image metrics shared as numbers. The reality, though, is that the majority of image processing is done using the displayed image on a monitor as a guide, and it is assumed that the monitor is correct. While significantly better than CRT technology, LCD screens are vulnerable to burn-in or image persistence so don't trust a monitor display completely until it is calibrated.

Since the image is displayed on a monitor and decisions about image processing are based largely on the display, errors can occur. It can be frustrating when image processing is performed on one device and evaluated on another; often it will look different. In Chapter 2, much was shared about human vision and perception. Because of the variables of the human visual system and environmental influences on seeing, monitor calibration using a digital calibration tool can be useful and precise. Calibrating a monitor—or at the least being aware of the importance of calibration and what is required to calibrate—can be very helpful in the production of more effective results displayed across various platforms. The tool most useful for monitor calibration is called a colorimeter. Since first introduced, they have come down in price to less than $200 including shipping in the US. It is also possible to prepare a basic profile for a laptop computer's screen using the computer's software. This can be a useful place to start the process of screen calibration but it will not be as accurate as a colorimeter. More about this is shared in Chapter 13.

The importance of monitor calibration cannot be overstated and there are a number of books dedicated to the topic. The following is a short list of products used for monitor calibration: Colormunki Display or Smile or i1Display Pro sold by X-Rite, leaders in this industry or Datacolor, another important company in the calibration market that sells the Spyder 4Pro, 5Express, and 5Elite systems. Eye One was an early innovator in the field and sells a very good product.

In summary, color calibration has never been easier or cheaper. In the earliest times of digital imaging, it seemed like a PhD in color science was required to understand all of the nuances needed for managing numerous variables. However, over time, innovation has led to products and software that are more intuitive and it is possible to calibrate a monitor in less than five minutes. For anyone who has worked carefully optimizing a file on one monitor and then displayed the file in another environment using another software such as Microsoft Powerpoint, the disparity of an image's appearance can be shocking.

Color Management

Since digital images are recorded by one device, displayed on another device, and possibly printed on a third device, variances to a file's color appearance can occur. In each location, a lot can happen to the file's color, which affects the appearance of the image. The way an image's color and color space is managed on each device and across the various devices can be a challenge. The process used by the various devices and their conversion of the digital color language used to describe an image is called color management. The appearance of color occurs from the use of algorithms and mathematical models. Because these languages are coordinated, color profile standards have been created to work managing colors across all the platforms where images will be used. To help users who work with images on various computers that use different operating systems, color profiles have been created and managed by the International Color Consortium or ICC. Assigning an image profile will allow its image attributes to remain the same regardless of the device.

There are books and college courses that explore color management. It is a very complex subject. Calibrating monitors is an important first step in managing the color of a file but assigning a color profile to a file can be effective in minimizing departures from an acceptable result. Color profiles can be assigned at capture or during image processing. Color spaces were briefly mentioned earlier in the chapter, and when selecting a larger color space the color changes/losses can be closely monitored. When color compression occurs it is sometimes called the clipping (loss) and colors are minimized.

When considering color gamut, color management facilitates the translation of the colors across platforms working to preserve them. This translation is managed by a process called gamut mapping and includes the use of rendering intents. Since some colors are difficult to describe (mathematically) there are ICC specifications to allow all devices and users to manage these losses or gains in the same way. Color language uses four types of rendering when the color gamut is outside the capabilities of a device. These include absolute colorimetric, relative colormetric, perceptual, and saturation intents. Each description provides a user with advantages and environments where choosing one over the others makes sense. Rendering is a protocol that helps to describe an image's appearance. Where

images are displayed/used will play a role in the determination of which profile to select, sometimes using a personal preference. There are a number of good web resources that can suggest more on the subject. LYNDA.com is a terrific resource for learning about this subject and many other applications.

A very simple and useful tool for improving color reproduction and subsequent color management is to include a calibrated color target in an image when possible. XRite sells a very small target called the ProPassport, which can be easily added to some scenes. Relative to some scientific subjects, a color checker might be too large to include—but if not this product, anything standard can be helpful. A simple white and black patch can be used based on the magnification and imaging equipment. Using some type of target such as this or others is necessary to set proper color reproduction for scientific images, whether color is required for interpretation or not.

Figure 15.2 Including a color checker such as the Macbeth ProPassport can be a useful tool in managing accurate color reproduction.

Software, Upgrades, and Optimizing the Computer

There will be times when new software products will be released with a high frequency and it will seem as if acquiring these new products or other related software is required to create "better" work. Almost everyone I talk to about upgrades and new products has experienced the angst about how to upgrade, when to upgrade, and why to upgrade. It can be likened to being on a treadmill. This may or may not be true for you. Digital imaging is now at an interesting period in its evolution. It is now a very mature, powerful technology. New features, lower costs, and more simple operations are all driving innovation, growth, and adoption. In the beginning of this technology, cameras, software, and devices were explosive in new development and frequent changes that were expensive were needed to acquire the basic tools. Rapid changes and lack of standards made it complicated to use cameras and the related software. The hardware had its own set of challenges to communicate across the devices. Since that time, camera sensors have gone from 640 pixels to 7000 pixels in a DSLR in a few short years and prices went from tens of thousands to a thousand dollars. We are not in a period of explosive change any more. Big changes in devices are not so frequent and the cost benefit to upgrade might be worth the investment, or not. There are a number of considerations.

Having a reliable workflow that uses the right tools and software is practical. It will not be possible to stay current on everything, all of the time. Software seems to need updating each year and scientist photographers will be challenged to resist the pressure to upgrade each year. Hardware considerations, software compatibility, and a few other factors all play a role in assessing the benefits of an upgrade and when it makes sense to upgrade. If a system is working and just a little slower than a brand new product, that may not be reason alone to upgrade.

Many universities upgrade their software packages annually because of site licenses. This can make sense, while other organizations do not have a unified approach to managing software with an institutional point of view. Individual personal users have different challenges that are often rooted in the cost of buying upgrades. It seems that sometimes the differences in an upgrade might be slight with changes to features not relevant to the type of work being produced. There is no one answer about these considerations for everyone.

Operating systems on a CPU, like all software, can be upgraded as needed. An upgrade to an operating system may have consequences to hardware and other software, affecting everything including the learning curve needed to learn it. Drivers, plug-ins, transfer protocols, and a host of other compatibilities come into play when evaluating upgrades. Each year countless dollars' worth of products and services become obsolete because devices can no longer talk to each other or drivers for scanners, for example, may not work with new operating systems. Going backwards once an upgrade has been made can lead to other unexpected challenges. It is vital to back up important data before proceeding with software changes. This can all be maddening.

Cameras, computers, software, media, monitors, and many peripherals will all have life expectancies separate from operating software considerations as well. A three- or four-year life expectancy seems typical for much digital equipment. This is in part because everything is related. When new sensors produce more pixels, more pixels lead to more data, which makes bigger files. This in turn requires more computing and processing power. Over time some CPUs and RAMs may become inadequate to function without bumping up the RAM. The expression "Don't fix what is not broken" may have some value when considering when to upgrade and what to upgrade. The sheer cost of converting all of the tools and software every few years can be prohibitive.

Image Workflow, Folders, and Naming Files

Staying organized remains an absolute requirement for digital photographers. Creating workflows that use the same protocols such as practical file-naming conventions and a folder management system will pay measurable dividends over time. Looking for files without using flexible organizing file management strategies can create challenges when trying to locate a file or folders many months, or years, after creation. Many imaging experiments are conducted over time and creating a practical and easy-to-use approach is invaluable.

It seems obvious but, for the record, each experiment or event should have its own folder. In this folder, if needed, should be the creation of the other new folders. My foldering/filing system uses a main folder for each session. It might be named Snowflakes_January_2_2014, for example. As soon as I make this folder, I then make three new subfolders within that folder. One of the folders is used to hold the native files, another folder is used for the image-processed TIF or PSD files, and the third folder is used for resized JPEG files used for social media applications. As soon as the photography session is completed, I copy the camera native files to the RAW folder where they will live forever. I *never* rename them. Once a RAW file is opened and the image processed, it is saved as a TIF or JPEG using the command Save As into another folder named "processed" or something useful. Overwriting a native file may have terrible consequences if, at some later date, additional or different image

processing strategies become required. You can never go backwards. RAW file formats are excellent file types for science applications so long as the original file is preserved. It can always be reset to its native capture characteristics by discarding the sidecar or XMP file, which reverts its data to the unprocessed capture.

It is also crucial to keep the native camera-generated file number in the naming convention used for the individual newly processed image files. For example, winter_2013_14_jan_2_DSC3190.tif is how I might name processed files. By keeping the camera RAW DSC number in the file name, locating the native file—should the folders ever become lost—is much easier. It is relatively easy to search for a file with a very specific name such as a number. It makes me smile to think back to when I first started making digital pictures in the mid 1990s. Twenty years ago, there were no norms for naming. Sometimes I would call my file "best" or, in the case of family pictures, Jon_1 (my son's first name), for example. After a year, I had countless Jon_1 files—it seemed such a perfect plan when launched, but quickly was not.

There are a number of programs that can help a photographer stay organized, such as Adobe Lightroom, Adobe Bridge or other image database applications. There are too many to list. One valuable consideration for selection of any product is its ability to talk to the larger IT environment— back-up file locations—in labs or servers and whether it can be accessed remotely or shared with others. Unfortunately there is no one answer or strategy for all situations. Numerous variables and personal preferences will play a role in developing a best practices solution for the environment where images are archived and for the operator who will oversee its management.

Having an organizational point of view can be practical when developing a plan going forward. Introducing departures to previously accepted practices leads to challenges and time *not* well spent reconciling variances. Digital images are not visible except when using software. A carefully constructed plan that takes into account the entire environment and investigates where the protocol has vulnerabilities, such as naming conventions or file types should an operating system change, should be planned for. Always consider that the file types need to be backwards compatible with new software and operating systems.

Adding metadata should always be expected and can be invaluable. All of this takes time and can be boring work for some. There is tedium to these tasks but they are required for science and will be time well spent. The more data that can be added to the metadata, the more mileage it will provide. I have found often, when publishing, the information added as metadata has been invaluable.

Archiving, Data Redundancy, and Backing Up

When storing physical documents such as film or photographic prints, they could be touched, retrieved, or preserved using visible processes. A material's laboratory pH could be measured, temperatures could be maintained, humidity monitored, and the brightness of the environmental light could be managed. When these factors were properly handled, images could last for what was believed to be forever. Archivists developed strategic methods for filing, storing, retrieving, and handling documents gained from decades of experiences to guide them. Degradation was visible and systems could be physically checked. Physical

degradation from water, humidity, the environment, or from fire were the primary contributors to image loss. Although some emulsions were attractive to certain species of fungi, most survived just fine. Most importantly, photographers knew without too much effort where an image—whether a print or film—was located.

Digital technology and digital file storage is a very different beast and requires an operator to consider how to create and manage appropriate methods. Because the data is electronic, it is vulnerable to different problems and not so many physical concerns. I am not an engineer but rather a user. And so I do not really enjoy tech talk but staying aware of trends, products, and monitoring a product's functionality remains an important element to success. The most important element of this topic is the need to preserve digital assets. Digital data is stored on hard drives, which are electronic devices. Hard drive technology—like all technology—is still an evolving technology. Hard disks or drives contain one or more spinning "platters" that are used to store and retrieve digital data as needed. The image data is "written" to the drive using a specific code on a particular region of the disk that is determined by the operating system. Spinning disk technology is slowly being replaced by a newer, more expensive, and more stable hard drive technology that uses solid-state drives. Solid-state drives are less prone to failures and are faster but also more expensive.

Images can be stored on internal hard drives or external drives, sometimes called portable hard drives. Both types of drives have similar concerns. Interestingly, there are specialists who work as a digital conservationist or image archivist. Medical and scientific stock photography companies, university and research organizations, and many other institutions may have literally billions of images to manage, store, preserve, and access. Data has both financial and emotional importance to an organization or scientist photographer. Preservation remains vitally important.

Archiving software and technologies are changing continually and the ability to maintain access to digital data is something to pay attention to. Older products and devices might be frequently replaced with newer, faster and cheaper devices. Scholars refer to this as technological obsolescence because, while the devices physically operate, they do not operate effectively. Once a new product gains a place in the market, it may not always be backwards compatible with older devices or file types. As such, there may not be backwards compatibility for reading many older file types or all folders. Specifically, files made with proprietary formats are problematic. Obsolescence can happen to storage media as well. Simply consider the life of the 3½ inch or 5¼ inch floppy drive or other diskettes and related drives that are now virtually non-existent.

When considering hard drives, certain themes recur, such as capacity, size, or how they communicate with the CPU. Both internal and external drives continue to grow in capacity. When storage media were first marketed, the average cost of one megabyte in 1999 was approximately $1. Today a one terabyte hard drive may cost as little as $100. Storage and not size of files should be the only concern in this era. Bigger drives are simply going to be needed when making bigger images. Bigger image files will be the norm for the foreseeable future and there are currently DSLR cameras that create 70 MB TIF files right out of the camera. There are not universally accepted strategies for the handling of electronic media; however, at one time or another, people talk about their methods used for digital conservation.

Planning for Data Loss and Failures

Hard disks, and all computer hardware for that matter, are vulnerable to electronic fatigue and ultimately failure. It is not a question of if a device will fail but rather when. Planning for failure and managing data preservation is an incredibly important task. Data should exist in multiple places and be redundant. Redundancy and archiving data takes time and commitment, and may seem like time not well spent—until there is a problem. Because data is invisible, the notion of archiving it is not important until you need to use it. I liken it to having insurance. For the record, jump or thumb drives and memory sticks should *never* be considered for use as long-term storage devices. These devices, while incredibly useful, are vulnerable to problems and are not permanent.

Specific challenges and solutions are unique to a person and environment. An important strategy for storage is resident in whether multiple users will need access on location or remotely. Answer to questions such as these will influence decisions about purchase. A valuable type of hard drive for minimizing data loss is a RAID drive. RAID is short for redundant array of independent disks. In this system, data is written to separate partitions on two drives when the data is copied to the RAID. In this manner, should Drive A fail, Drive B will have the same data. Each drive is large and matched. I have seen drives as large as 6 TB, and I imagine larger drives on are the horizon. When necessary, I manually back up data from my computer's drive once a month or as needed. Being very concerned with data loss, I also maintain another set of hard drives kept in a different location. I use the same foldering and naming convention on all drives. This all takes time. I have lost data only once and it was as though I had lost a relative. I also learned that the data rarely can be recovered. There are companies that provide recovery services but the cost and lack of certainty about prospects for success looms as a real obstacle. Lost data cannot be replaced. And so experiments, images, articles, and the work of a lifetime requires a certain responsibility to preserve and manage going forward for its storage. Various products exist for backing up drives directly to a computer's drive. In the Mac environment, Time Machine is an excellent choice for backing up during the time when the external Time Machine drive is physically attached to the CPU. There are similar products for the Windows environment. This aspect of working the digital environment should never be ignored and requires frequent attention.

Removable and writeable media can also degrade. The material that removable media is made from will not last forever. It is not impervious to use, damage, or corruption. In some cases, the degradation will occur more quickly than might be imagined. A complete but not modern list of removable media would include magnetic tapes, floppy disks, optical disks, or some less common other products. An example of a DVD or other removable media being unusable is evidenced in a DVD that was scratched or delaminated. A scratch results in the disk being unreadable and the data lost. This happens more often than you would imagine and the loss of media holding countless digitized images has a variety of costs. Hard drives are much better for storing data long term than DVDs. Many modern laptop computers no longer come with CD drives, an interesting trend to be aware of. CDs and DVDs were the gold standard for storage in the fairly recent past. Nothing stays the same forever with this technology.

As an endnote to this topic about digital data preservation, one of the most important strategies required for digital conservation separate from the specific hardware and tools is the continual migration of data. It will always be critical to migrate data from older technologies to new technologies before the ability to do so no longer exists. Never wait until the bitter end of a technology's life to consider moving it. It can be helpful to use a specific time of year to migrate older data and important files forward. This might include consolidating older hard drives or other related media. I know organizations that keep older machines working for the sole purpose of transferring and reading files on media that is no longer modern.

Digital Housekeeping

As evidenced by the suggestions in this chapter, being successful requires time and attention to details that might not be measureable or important at the moment. Paying attention to them will lead to better outcomes over time. When talking about these concepts I sometimes compare an imaging session to cooking. There is a preparation time, there is the cooking time, and then the dreaded clean-up activities.

Making images can make digital "messes" within a computer. Digital bits and bytes, files and folders from the process can end up all over a drive and in many folders. There can also be invisible "residual digital artifacts" that are left in RAM, in the CPU, or in the caches in the software that was used. I frequently use my desktop and other locations in my CPU while working on projects and it becomes a catchall for files and folders over time. While writing this book, it was not uncommon for me to have countless files opened and saved to the desktop before moving them to my more permanent filing system. Sometimes my desktop held so many folders and files, I simply could not easily find anything. This unfortunately happens to most of us at one time or another. I do know photographers who keep all folders and images in the operating system's picture folder, no matter what. In this fashion, every image file can be copied to other drives with no concern for location or leaving things behind. I also know other scientist photographers who make shortcuts from their desktop to their image folders, facilitating a simple workflow.

Any digital clutter over time is not good for a machine's performance. It is good to tidy things up as often as necessary. Many folders and files will seem to just show up and busy science photographers can end up with a mess on the desktop or in the main directory. Having an imaging session can be dynamic and it is easy to fail to clean up after a session because, if you are like me, you move on to the next crisis. Over time and by not routinely paying attention to keeping things together and organized, the digital environment can become similar to the aftermath of a digital hurricane. It is not an overly large chore to keep things tidy when there is only a small volume of data to manage, but it takes considerably more time and interest to do so when there is more. This can be especially true when there are a great number of folders and files haphazardly organized. Developing good work habits will pay dividends in efficiencies over time, and it helps to ensure all files are in the proper folders and the folders are located in the proper directories. It has already been shared that it is useful to use an effective naming convention. Things can be renamed later, but thinking through what is practical can be very helpful from the

beginning. You might find it useful to create a foldering system that will be used for months or years. There is no right or wrong method, simply one that achieves the goals of the organization/photographer. Image directories should be considered a sort of personal Dewey Decimal System.

Keeping Things Tuned Up

Apple and Windows operating systems are not the same in how they accomplish certain tasks but both create files and random artifacts during use. These outcomes in turn affect performance. It is nearly impossible to prevent files from creating this outcome no matter how careful an operator is. Every time a computer is used, this happens to one degree or another. The residual pieces of files will, over time, slow down the processor and clock speed of a computer. There is software to remove this digital clutter.

With regular attention and a concerted effort, it is possible to have a system perform at its peak levels for most of a computer's life. There are core things to do, which include, as already mentioned, keeping files and folders where you want them, and removing the digital data artifacts and corrupted time codes and hierarchies when possible using the appropriate software tools.

Something helpful can be to keep directories optimized and fixed when corruptions occur. Macintosh computers use a program called Disk Utility, which looks for problems across directories. It fixes dates and times so that all applications and files are correct and do not cause conflicts within the computer's operating system and software. When the operating directories are corrected, the computer will perform operations more efficiently. Windows operating systems use a product called Disk Cleanup. This software helps users free up space on a hard disk by finding files that can be safely deleted. Disk Cleanup performs the following: removes temporary Internet files; removes downloaded program files; empties the Recycle Bin; removes Windows temporary files; removes optional Windows components no longer in use; and removes installed programs no longer in use. While each of these activities individually is minor, together they can degrade a computer's overall performance.

It is important to run anti-malware or anti-virus programs frequently as well. There are many companies that sell these products, including McAfee Total Protection for Windows. These products can block the installation of viruses, malware, ransomware, spyware, and unwanted programs. Once a computer has been infected with any of the above "bugs", it may run like a turtle if at all. Also disable any start-up programs that are not required. Virus Barrier is an excellent product for the Mac environment.

Smartphone Photography

Using smartphones for photographing in science has become an everyday occurrence. The ability to broadcast real-time video using applications such as Facetime or other video teleconferencing applications including Skype has both economical and time benefits for scientists and is a practice that is well entrenched. Workers in medicine were early adopters of this technology and in particular the fields of dermatology, otolaryngology,

and some applications in ophthalmic photography. In 2013, the British Institute of Medical Illustrators (imi.org.uk) published a set of recommended suggestions for use of smartphones for clinical photography.

Smartphone sensors and image capture capabilities continue to improve but because of the compactness of the camera/phone design, digital space is very limited. For this reason, smartphone sensors, while creating 12–16 megapixel files, are relatively small. Because sensors are small, they cannot respond to low light in the same way as larger sensors, which collect more light because of larger surface areas in a pixel. This makes smartphones vulnerable to challenges when operating in low light. Low light imaging leads to images that are high in noise.

The lenses used in smartphones will have a fixed focal length and fixed aperture as well. Most smartphone cameras have an approximately 35 mm focal length lens when calculated for the size of the sensor. The image that the camera forms and displays on the camera's LCD screen can be digitally scaled to crop into the image area more if desired. Increasing the image size in this manner can make operator shake more visible. Since the focal length is fixed and camera's lens aperture is also fixed, locating the subject in the relative middle of the frame and not getting too close or too far away will play a role in the subsequent photograph's quality.

Figure 15.3 This plate was coated with the bacteria *Serratia marcescens* on EMB agar in the shape of a crab. The photograph—while revealing of the growth pattern—also exhibits a few other subjects. The photographer and ceiling are both reflected in the surface of the plate with the bacteria. It is possible to see the thumb and forehead of the photographer next to the reflection of the phone. It can be relatively easy to solve this problem by photographing through a black piece of cardboard that has a small hole located in front of the lens. The size of the cardboard should be large enough to block the entire reflective surface of the agar plate by casting a shadow of itself there. Photograph courtesy of Laura Solomon.

Exposures are calculated automatically by smartphone software. Image brightness or darkness is affected by how much light falls onto the sensor. Since these cameras use electronic global or rolling shutters, the preview image is the same exposure as the captured image will be. The camera creates the exposure as a function of time and the amount of light that is falling onto the subject. The camera operator can touch the displayed preview image to change the brightness but this happens globally across the entire sensor. Touching a dark tone will brighten the exposure at the expense of already bright objects. If the operator touches a bright part of the displayed image, the exposure will be darker. The camera's light measuring system—like all meters—tries to create an 18 percent gray exposure unless operated in the HDR mode. The basic exposure-making capability has a finite acceptance range. HDR methods have improved significantly in the last two years across Apple, Samsung, Lenovo-Motorola, and LG Electronics.

White balance also does not operate perfectly when challenged. Since a camera's operation is automatic, the camera alone will assign the light source's color temperature when it tries to white balance an image. When operated in bright light and a single type of light, smartphone cameras produce relatively neutral and useable results. When operated in challenging and mixed light environments, the results can be variable.

Smartphone image file attribute constancy will be a challenge when used in science. With some attention to camera placement, working distance management and use of preferred light sources, reasonably adequate results can be obtained. It is possible to color correct picture files using the smartphone camera. The corrections are typically global and do not offer the precision of more traditional products. I frequently use Dropbox to move files from phone to CPU and vice versa.

Accessories

There are many companies that sell accessories for smartphones including macro and telephoto lenses. Momentlens.co makes some very excellent products and other practical accessories can be purchased at photojojo.com/store/.

Smartphone Camera Protocols

If a smartphone is determined useful, there are a few things that a scientist photographer can do to achieve better results.

1. Smartphone cameras are vulnerable to dirt and debris being on the lens since they spend much unprotected time in pockets and purses. Often, when not in use, the lens is not covered. Lenses should be dusted off prior to using. It is very common to see dust on a lens and dust will introduce flare and other image degradation
2. Use composition and other techniques shared in Chapter 6. Place a subject on a neutral background and remove any distracting elements in the composition if possible. Try and make the camera parallel to the subject's surface. Alignment of the camera to the subject will affect the image's shape.
3. Found light can be ideal but this is not always possible. Window light or overhead lighting can work effectively. Mixed lights all will have different spectral compositions, such as daylight, tungsten and fluorescent, and should be avoided. Using a single source of light will make the most neutral images.
4. Never use the camera's flash unless there is no alternative. It is not very powerful.

Social Media

No contemporary book would be complete without at least some reference to social media and its positive and negative opportunities for scientist photographers. Social media is very real and becoming more engrained in contemporary society and in science as well. Facebook, Instagram, Twitter, and other start-up companies have ginormous user groups. Without performing exhaustive research on the subject, it seems safe to say that almost every important organization in the world has a Facebook presence and is exploring how to maximize its reach, dialoguing with followers engaged with the organization/person. Similarly Instagram users are always thinking about new ways to create dialogues with their followers.

Why people use social media is an on-going study by scholars. We are living right now with how social media operates and the strategies used to work using social media are unfolding right in front of us. Whether personally interested in this outlet or not, having a presence

on social media can have professional benefits for science and scientist photographers as well as their organizations.

Creating a few achievable goals or at the least determining why you (professionally) are going to be on social media is a first step. This consideration is separate from maintaining a personal account. Sometimes not being on social media speaks more about a person or an organization than being on social media. Having a presence on social media is considered by many to be the norm and requires some time and effort to have basic success. People are engaged with the people they follow and having conversations with followers is a component of the engagement. Once committed to having an account, it will in time grow followers. Like everything, being on social media takes time.

I am frequently reminded that my pictures of the invisible world are unique. I appreciate that and sometimes take that for granted. People love to be surprised and see new things. How many new photographs of a cake, a cup of coffee, or a cheeseburger can engage people outside of a user's inner circle of friends and family? Photographs of never-before-seen objects, views from space, or cells dividing under a microscope can cause intrigue, inspire or simply amaze people. Sharing your work on social media, if curated, can be a powerful new voice for science and science images. NASA astronaut Scott Kelly has grown his Instagram following to nearly 500 K. That is extraordinary, keeps science in front of those people, and maybe in some small way is contributing to increasing an interest in STEM education and young people pursuing science careers.

Social media has been very helpful to my career and has become a powerful voice for my photography. While not in control of who follows me, I have found it helpful to use social media in a planned way. This has led to increased exposure of my work and new publishing opportunities. In fact, several photographs in this book were provided at my invitation after I saw them in my Instagram feed as another benefit to new opportunities. I must confess that I was cynical at first about the value of social media but now I have become a firm believer in its benefits.

A few strategies have been helpful to me in creating positive outcomes when using social media:

1. Distinguish yourself in how you make your posts. For two years I have used the handle #tinythings and I work hard not to dilute my posts with anything "not small."
2. Curate your posts. There are billions of pictures of food and of people on vacation, but many fewer posts of scientific things. There are not billions of photographs of the types you may make. Make them special and share them.
3. Use appropriate hash tags on all posts. Hash tags are the new normal. They have found their way into TV ads and other venues. For example, #science, #scienceart, #artscience, #microscopy, #birefringence, #crystals, and #snowflakes are a few of the hash tags that I use. They have been helpful and have given my work more visibility across many different audiences. This is in turn creates followers, exposure, and new opportunities. I am continually seeking new hash tags.
4. Publish at the same time of day. I have found people enjoy seeing work at the same time of day, each day. Mornings, lunchtime or evenings can mesh with people's schedules, when they steal a few moments to look at pictures.

This future is one full of change, challenge, and new opportunities. I have found it important to maintain some level of engagement and housekeeping to social media activities once started. Out of sight, out of mind. At this moment, while you are reading this chapter, researchers, archivists, and picture editors are looking for new content. An opportunity might be lost when a visitor stumbles onto a dated or no longer relevant page containing information about you. Keep your website and LinkedIn information current. If you create and to want have a presence on social media for professional reasons, monitoring content and updates is part of your best practice.

Conclusion

This chapter has tried to summarize all the various non-photographic components required to create the best results. Sometimes the devil is in the details. Making effective scientific images requires paying attention to details and the desire to do so. Creating successful outcomes takes time and effort. Like many, it is easy to lose track of time when making photographs. Time will simply evaporate right before your eyes. It is not overly difficult to develop best practices with experience but the interest to do so must drive the further development and adoption. Being told to do something will never produce the expected results. The desire to improve must be a personal ambition and emanate from your personal pride in doing so.

SUGGESTED READING

Austerberry, David, *Digital Asset Management*. London: Focal Press, 2007; ASIN: B00EKZ1VP4.

Hagman, Anna and Riedberg, Sander, *Guidelines for Smartphone Use in Telemedical Photography*. Thesis project: Medical Engineering, Karolinska Institute.

Hinkel, Brad, *Color Management in Digital Photography; Ten Easy Steps to True Colors in Photoshop*. Santa Barbara, CA: Rocky Nook Press, 2006; ISBN-13: 978-1933952024.

A CHEAT SHEET FOR BEST PRACTICES

- Bring an attitude that will allow success no matter what the challenges.
- Be patient. Successful imaging outcomes take time.
- Prepare for the imaging experiment by assembling all the necessary supplies and equipment before starting. Anticipate what will happen and what will be needed to finish.
- Evaluate the sample and select the best choice possible from the inventory. Always consider the ethical aspects of sample selection.
- Develop an appropriate sample treatment plan.
- Select the correct background for the sample and intended use of the image.
- Select the ideal light source and organize lighting modifiers if needed.
- Once established, never change the lamp brightness during a photography session.
- Select the best camera for the job.
- Review all the capture settings on the camera prior to starting.
- Select RAW file capture if possible.
- Select the largest color space possible, such as Adobe RGB 1998.
- Select the highest bit depth.
- Select the proper lens for the imaging requirements of the size and scope of the sample.
- Establish the best aperture setting for the sample and imaging objective. Closing an aperture to a smaller opening will create more DOF but images will not be as sharp.
- Always clean a lens.
- When using a light microscope, establish Köhler illumination.
- Measure the available light and select the best shutter speed/aperture combination for the sample and imaging requirements. There are a number of combinations of time and brightness that can be used to create reciprocal exposures.
- Evaluate the surface of the lens and determine if cleaning surface debris is required.
- Manage the environmental conditions such as the presence of ambient light.
- Never focus the camera system using the autofocus capability.
- Carefully place focus in the best location and align the subject to the sensor.
- Establish the proper white balance setting. Never use auto-white.
- Trigger the camera using methods that do not create or minimize vibration.
- Calibrate a monitor prior to image processing.
- Create an appropriate image processing workflow that creates acceptable outcomes.
- Always archive native files.
- Always save image processed files using Save As.
- Stay organized.
- Practice digital conservation.
- Stay current.

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