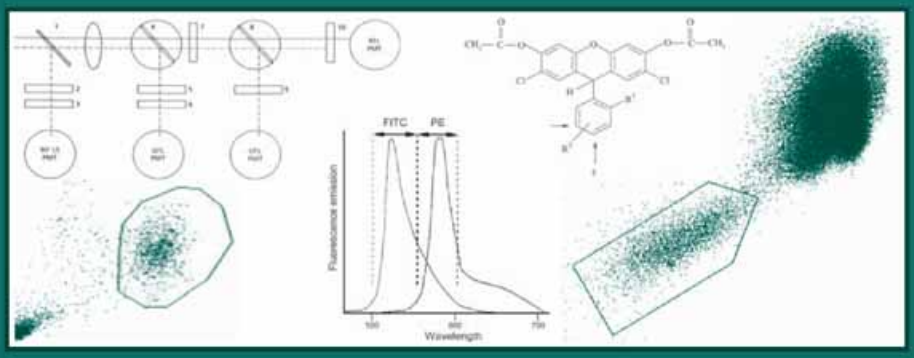
# Flow Cytometry

## *Principles and Applications*

*Edited by*

**Marion G. Macey**

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Edited by

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Cover design by Nancy K. Fallatt
Cover illustration:(*from left*) typical lens and mirror assembly for detection of 90° LS, FITC, PE, and ECD fluorescence (*above;* Fig. 8, Chap. 1, *see* full caption on p. 8); flow cytometric enumeration of cells that have migrated through wells containing HUVECs (*below;* Fig. 3, Chap. 10, *see* complete figure and caption on p. 230); fluorescence emission spectra for FITC and PE (Fig. 6, Chap. 1, *see* complete caption on p. 12); chemical structure of a probe for estimating intracellular oxidants (Fig. 10B, Chap. 3, *see* complete figure and caption on pp. 90–91); the light-scattering properties of platelets in diluted whole blood (Fig. 6, Chap. 10, *see* discussion on p. 244).

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# Preface

Improvements in instrument design and computing power and the increased availability of fluorescent agents have led to an increased usage of flow cytometry in both the research and clinical settings. *Flow Cytometry: Principles and Applications* provides a comprehensive introduction to data interpretation, quality control procedures, pitfalls and problems, in addition to detailed protocols from leading authorities with extensive practical experience in flow cytometry. *Flow Cytometry: Principles and Applications* also presents the principles and potential of flow cytometry to assess many functional aspects of cells.

This is an essential handbook and reference to both new and experienced flow cytometry users.

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# Contents

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# 1

# Principles of Flow Cytometry

**Marion G. Macey**

**Summary**

Flow cytometry is a powerful tool for interrogating the phenotype and characteristics of cells. It is based upon the light-scattering properties of the cells being analyzed and these include fluorescence emissions. This fluorescence may be associated with dyes or conjugated to mAbs specific for molecules either on the surface or in the intracellular components of the cell. Flow cytometry facilitates the identification of different cell types within a heterogeneous population. It was initially developed by immunologists wishing to separate out different cell populations for subsequent coculture experiments to determine the function of cells within the immune system. This was achieved by using fluorescence-activated cell sorting, or FACS, on the flow cytometer. The initial instruments were able to analyze one or two colors of fluorescence; today, instruments capable of analyzing 11 colors of fluorescence are available.

**Key Words:** Acquisition; amplification; fluorescence; histograms; light scatter.

## 1. History and Development of Flow Cytometry

Flow cytometry has developed over the last 60 yr from single-parameter instruments that detected only the size of cells to highly sophisticated machines capable of detecting 13 parameters simultaneously. A brief overview of the development of flow cytometry is given in **Table 1**.

## 2. Principles of Flow Cytometry

All forms of cytometry depend on the basic laws of physics, including those of fluidics, optics, and electronics ***(8)***. Flow cytometry is a system for sensing cells or particles as they move in a liquid stream through a laser (light amplification by stimulated emission of radiation)/light beam past a sensing area. The relative light-scattering and color-discriminated fluorescence of the microscopic particles is measured. Analysis and differentiation of the cells is based on size, granularity,

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**Table 1**
**A Brief History of Flow Cytometry**

| Year | Development |
|---|---|
| 1954 | An instrument in which an electronic measurement for cell counting and sizing was made on cells flowing in a conductive liquid with one cell at a time passing a measuring point was first described by Wallace Coulter ***(1)***. This became the basis of the first viable flow analyzer. |
| 1965 | Kamentsky et al. ***(2)*** described a two-parameter flow cytometer that measured absorption and back-scattered illumination of unstained cells, and this was used to determine cell nucleic acid content and size. This instrument represented the first multiparameter flow cytometer, and the first cell sorter was described that same year by Fulwyler ***(3)***. Use of an electrostatic deflection ink-jet recording technique (Sweet) ***(4)*** enabled the instrument to sort cells in volume at a rate of 1000 cells/s. |
| 1967 | Thompson ***(5)*** developed a system for the electrostatic charging of droplets which enhanced the development of cell sorters. Van Dilla et al. ***(6)*** exploited the real volume differences of cells to prepare suspensions of highly purified (>95%) human granulocytes and lymphocytes. |
| 1983 | First clinical flow cytometers were introduced. |
| 1990 | Advances in technology, including availability of mAbs and powerful but cheap computers, brought flow cytometry into routine use. Benchtop instruments developed with enclosed flow cells were developed. |
| 1995 | The ability to measure a minimum of five parameters on 25,000 cells in 1 s used routinely to enhance the diagnosis and management of various disease states and understanding of the pathogenesis of disease. |
| 1999 | Instruments equipped with lasers and capable of analyzing 11 fluorochromes developed by Bigos et al. ***(7)***. |
| 2003 | High-speed sorters using digital technology introduced. |

and whether the cell is carrying fluorescent molecules in the form of either antibodies or dyes. As the cell passes through the laser beam, light is scattered in all directions, and the light scattered in the forward direction at low angles (0.5–10°) from the axis is proportional to the square of the radius of a sphere ***(9)*** and so to the size of the cell or particle. Light may enter the cell and be reflected and refracted by the nucleus and other contents of the cell; thus, the 90° light (right-angled, side) scatter may be considered proportional to the granularity of the cell. The cells may be labeled with fluorochrome-linked antibodies or stained with fluorescent membrane, cytoplasmic, or nuclear dyes. Thus, differentiation of cell types, the presence of membrane receptors and antigens, membrane potential, pH, enzyme activity, and DNA content may be facilitated.

Flow cytometers are multiparameter, recording several measurements on each cell; therefore, it is possible to identify a homogeneous subpopulation within a heterogeneous population. This is one of the most useful features of flow cytometers and makes them preferable to other instruments such as spectrofluorimeters, in which measurements are based on analysis of the entire population.

Most commercial flow cytometers have the capacity to make five or more simultaneous measurements on every cell, but some specialized research instruments have considerably greater capacity, and with three lasers it is possible to analyze up to 11 parameters ***(7)***. A typical flow cytometer consists of three functional units: (1) one or more laser light sources and a sensing system that comprises the sample/flow chamber and optical assembly, (2) a hydraulic system that controls the passage of cells through the sensing system, and (3) a computer system that collects data and performs analytical routines on the electrical signals relayed from the sensing system (**Fig. 1**).

The flow chamber is instrumental in delivering the cells in suspension to the specific point that is intersected by the illuminating beam and the plane of focus of the optical assembly. Flow chambers may comprise flat-sided cuvets to minimize unwanted light reflections, and, where cell sorting is required, so-called stream or "jet in air" flow cells are used.

Cells suspended in isotonic fluid are transported through the sensing system. Most instruments use a lamina/sheath flow technique ***(10)*** to confine cells to the center of the flow stream; this also reduces blockage due to clumping. Cells enter the chamber under pressure through a small aperture that is surrounded by sheath fluid. The sheath fluid in the sample chamber creates a hydrodynamic focusing effect and draws the sample fluid into a stream. Accurate and precise positioning of the sample fluid within the sheath fluid is critical to efficient operation of the flow cytometer, and adjustment of the relative sheath and sample pressures ensures that cells pass one by one through the detection point. This alignment may be performed manually on some machines, but in most it is fixed.

Water-cooled laser sources with an output power in the range of 50 mW–5 W may be used for fluorescence and light-scatter measurements. Air-cooled lasers have a maximum output of 100 mW and are now more commonly used together with laser diodes in commercial instruments. Lasers have the advantage of producing an intense beam of monochromatic light which in some systems may be tuned to several different wavelengths. The most common lasers used in flow cytometry are argon lasers, which produce light between wavelengths of 351 and 528 nm. Other lasers used include UV lasers, which produce light between 325 and 363 nm; krypton lasers, which produce light between 350 and 799 nm; helium–neon lasers, which produce light at 543, 594, 611, and 633 nm; and helium–cadmium lasers, which produce light at 325 and 441 nm.

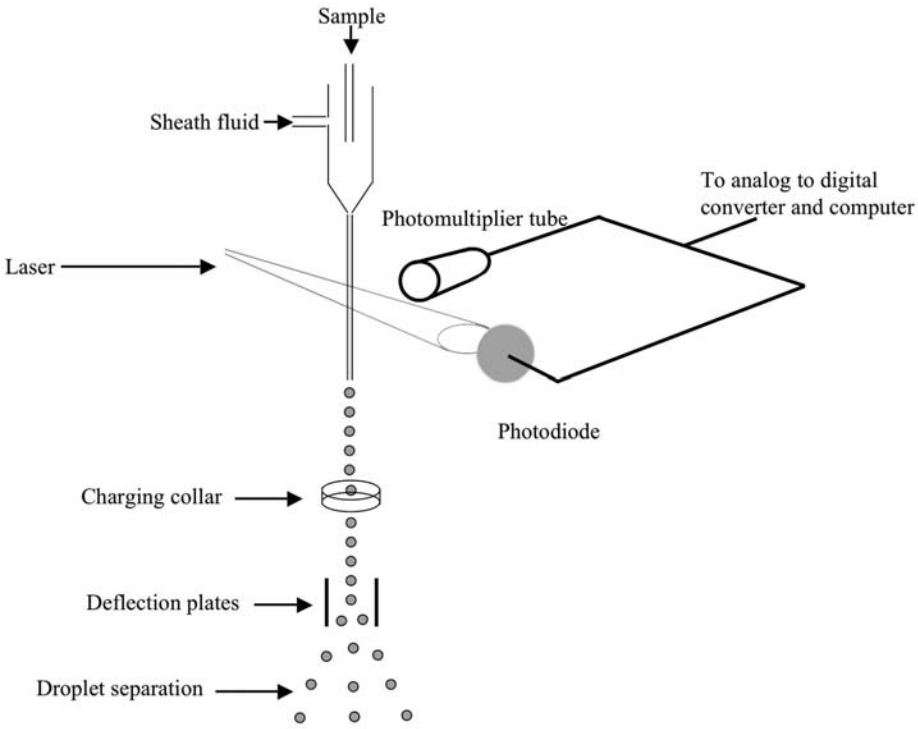


**Fig. 1.** Schematic representation of a flow cytometer, including the flow cell, sheath stream, laser beam, sensing system computer, deflection plates, and droplet collection.

## 3. Fluorescence Analysis

Fluorescence is excited as cells traverse the laser excitation beam, and this fluorescence is collected by optics at right angles to the incident beam. A barrier filter blocks laser excitation illumination, while dichroic mirrors and appropriate filters (*see* Section 4.1.) are used to select the required wavelengths of fluorescence for measurement. The photons of light falling upon the detectors are converted by photomultiplier tubes (PMTs) to an electrical impulse, and this signal is processed by an analog-to-digital (A-to-D) converter that changes the electrical pulse to a numerical signal. The quantity and intensity of the fluorescence are recorded by the computer system and displayed on a visual display unit as a frequency distribution that may be single-parameter (**Fig. 2**), dual-parameter (**Fig. 3**), or multiparameter. Single-parameter histograms usually convey information regarding the intensity of fluorescence and number of cells of a given fluorescence, so that weakly fluorescent cells are distinguished from those that are strongly fluorescent.

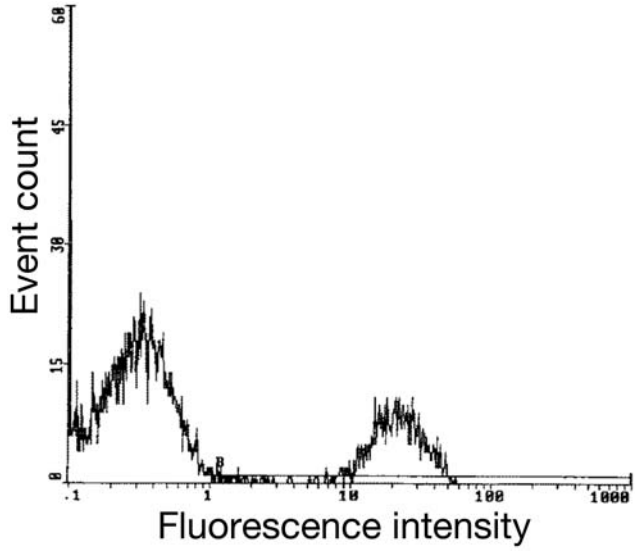


**Fig. 2.** Single-parameter histogram of fluorescence and cell count illustrating a typical distribution for weakly fluorescent and strongly fluorescent cells.

Dual-parameter histograms of forward angle scatter and 90° light scatter (90° LS) allow identification of the different cell types within the preparation, based on size and granularity. Right angle and side scatter are alternative names for 90° LS (**Fig. 3**).

## 4. Light-Scatter and Fluorescence Detection

### *4.1. Filters*

Light scattered by particles as they pass through a laser or light source must be efficiently detected, and fluorescent light of a given wavelength requires specific identification. The amount of light scattered is generally high in comparison with the amount of fluorescent light. Photodiodes are therefore used as forward angle light (FAL) sensors; they may be used with neutral density filters that proportionally reduce the amount of light received by the detector. A beam absorber (or diffuser or obscuration bar) is placed across the front of the detector to stop the laser beam itself and any diffracted light from entering the detector. The scattered light is focused by a collecting lens onto the photodiode(s) which converts the photons into voltage pulses proportional to the amount of light collected (integrated pulse). These pulses may be amplified by the operator. In some systems with multiple diodes, upper and lower light may be collected which may help separate populations of cells or particles.

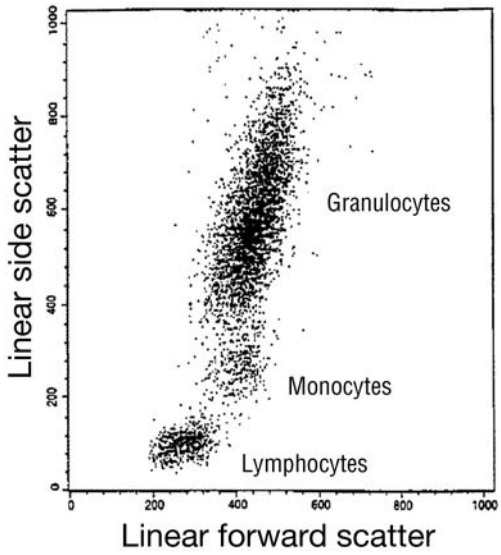


**Fig. 3.** Dual-parameter histogram of the forward-scatter and side-scatter analysis of leukocytes from peripheral blood. The characteristic distribution of lymphocytes, monocytes, and granulocytes is shown.

Fluorescence detectors are usually placed at right angles to the laser beam and sample stream. Stray light may be excluded by an obscuration bar in front of an aspheric (objective) lens which collects the light and refracts it into a parallel beam. To detect the components of the beam, filters and dichroic mirrors are used to remove unwanted wavelengths of light and direct light to the correct detector(s). **Table 2** describes some of the different types of lenses and filters, and **Table 3** lists suppliers.

**Figure 4** illustrates a possible lens configuration for detecting 90° LS, green (fluorescein), orange (phycoerythrin [PE]), and red (PE Texas Red [ECD]) fluorescence. Typically, the first filter used eliminates 488-nm laser light that still may have passed through. This light may then be diverted to a beam splitter or a dichroic mirror. This mirror reflects light in one band of wavelengths (usually long) while allowing another band (usually short) to pass through. It should be noted that there is no direct cutoff here between reflection and transmission. There is a middle band of wavelengths that will do both. For this reason, the color components are passed through other filters before entering the detector. These filters remove the unwanted wavelengths and allow the desired wavelengths to pass through to the detector. These filters are called band-pass filters and are designated by whether they transmit long wavelengths (long-pass) or shorter wavelengths (short-pass).

**Table 2**
**Types of Filters**

| Filter type | Comments |
|---|---|
| Absorbance | The transition from absorbance to transmission occurs over a set range of nanometers; the filters are therefore named at the 50% transmission point. Dye in glass band-pass filters have excellent blocking properties and very high (>50%) pass of light. They are inexpensive but fluoresce and so should not be used as primary blocking filters. They are always long-pass filters (i.e., they block short wavelengths and transmit long wavelengths). |
| Interference | These long-pass filters are manufactured by an etching process to give a raised and cut surface with ridges at set distances which cause interference in the wavelength of light transmitted. They are reflectance filters, so the shiny side is toward the laser. They do not fluoresce but have 90% efficiency at best, and they have poor transmittance. Also, the etching process allows light of incorrect wavelength to pass. They may be termed by the center wavelength, and band widths are usually given (e.g., 500/50). |
| Dichroic mirrors | These are a combination of a mirror and an interference filter that need to be placed at an angle of 458 to the beam. They reflect short wavelengths and let longer wavelengths pass. They are used with other filters. They are normally long-pass filters. |
| Beam splitters | These are metallic-coated quartz substrates and are designed to work at a 458 angle of incidence. Numbers indicate reflection/transmission values. |
| Band-pass | These filters allow light within certain wavelengths to pass. They are interference filters with two coatings and act as a long-pass and a short-pass filter. They transmit and reflect but may suffer from attenuation. |
| Neutral density | These attenuate all wavelengths and may be used for FALS and 90° LS. |

LS, light scatter.

**Table 3**
**Commercial Suppliers**

| Name | Internet address |
|---|---|
| Beckman Coulter | http://www.beckmancoulter.com |
| Becton Dickinson (BD) | http://www.bdbiosciences.com |
| DAKO Diagnostics (Cytomation) | http://www.dako.com |
| Partec | http://www.partec.de |

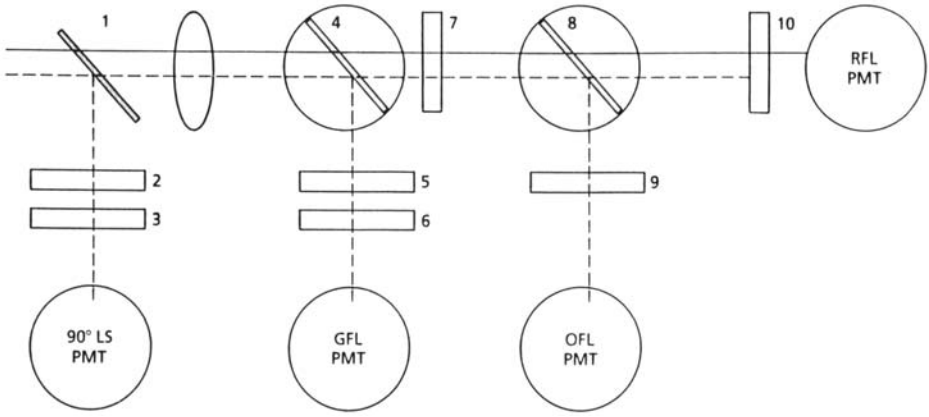


**Fig. 4.** Typical lens and mirror assembly for detection of 90° LS, FITC, PE, and ECD fluorescence. 1, beam splitter, normal glass; 2, laser line filter 396- to 496-nm band pass: 3, diffuser; 4, dichroic mirror 570-nm long pass; 5, laser cut filter 490-nm long pass; 6, green filter 515–530 nm band pass; 7, orange filter 600-nm long pass; 8, dichroic mirror 610-nm long pass; 9, orange filter 565- to 592-nm band pass; 10, red filter 620-nm long pass. GFL, green fluorescence; OFL, orange fluorescence; PMT, photomultiplier tube; RFL, red fluorescence.

The sensors used for side light scatter and fluorescence are PMTs. These tubes serve as detectors and also as amplifiers of the weak fluorescent signals. These tubes have their own high-voltage power supplies that provide the boost needed to amplify the signal internally within the PMT. The amount of high voltage, and therefore the amplification, is adjustable by the operator. A second amplification, also operator-controlled, may be made on the PMT signal external to the PMT. PMTs are used only under weak light conditions; they may be damaged by high-intensity light such as normal room light.

### *4.2. Filter Sets*

Filters are used in sets, usually in pairs of a band-pass filter with a dichroic mirror or beam splitter. Beam splitters are metallic-coated quartz substrates and are designed to work at a 458 angle of incidence. Filters have numbers that indicate reflection/transmission value for the center wavelength and bandwidth (nm). For example, a 500/50-nm band-pass filter would allow light between 450 and 550 nm to be transmitted.

## 5. Acquisition

Light-scatter signals may be a measure of a combination of parameters: (1) the size (projected surface area) of the particle, (2) the surface topography

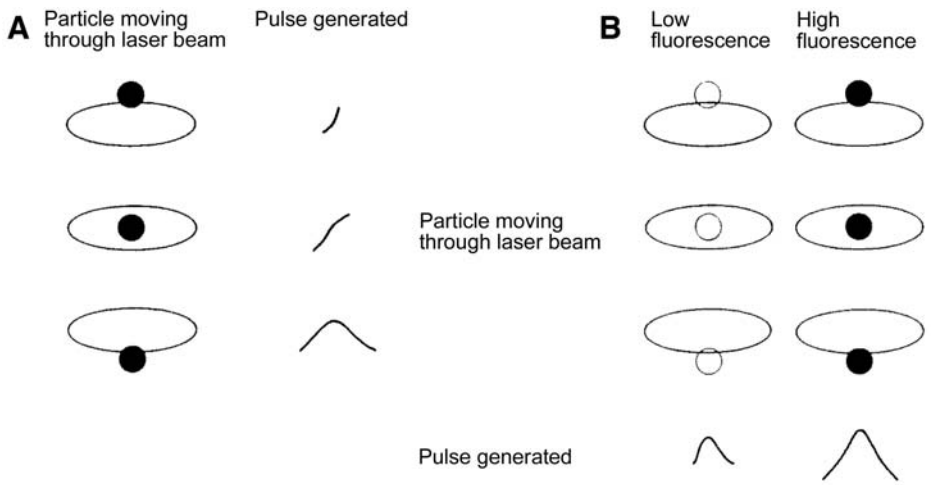


**Fig. 5. (A)** Illustration of the electronic pulse generated by a particle as it moves through a laser beam. **(B)** Illustration of the pulses generated by cells with high and low fluorescence intensities.

(rough or smooth), (3) the OD (OD will be influenced by the light absorbed and the refractive index will determine the light refracted through the particle), and (4) the internal structure of the particle (granular or uniform). Some of these components are present in all of the light scatter produced.

The purpose of analyzing the light-scatter or fluorescence signal is to determine the difference between particles in terms of voltage output from detectors. There are several methods of retrieving this information. A measure of the maximum voltage (or peak) level reached as the particle passes through the laser beam may be measured. The highest voltage level reached by the pulse (pulse height) may be a measure of the maximum fluorescence given off by a particle (**Fig. 5A**). Particles with different amounts of associated fluorescence have different pulse heights and therefore different peak pulses. A particle with fluorescent molecules spread uniformly over the surface will produce a wide peak pulse compared with a particle with fluorescence concentrated at one point. The latter will produce a narrower and sharper peak pulse (**Fig. 5B**). However, the peak of the pulse may be the same for both particles and so they become indistinguishable on the basis of this parameter. The area under the two pulses will, however, be different. The area under the pulse allows the generation of a second parameter, referred to as the integrated pulse. A third parameter may also be used if the ratio of the peak or integrated pulse is measured. This is termed time of flight.

## 6. Amplification

Some particles may also be better differentiated if the original peak or integrated pulse is amplified. Normal amplification accentuates the differences between pulses, but in some cases this may not be sufficient to differentiate between small changes in pulse height. The use of logarithmic amplification makes small pulses much larger while amplifying the larger pulses by a lesser amount. The result is that the differences in the smaller pulses are accentuated.

## 7. Histograms

Particles are analyzed individually but interpreted collectively. The collective picture is represented as a histogram. These may be single-, two-, or three-parameter. Single-parameter histograms are 2D graphs in which the parameter to be interpreted is represented on the horizontal (*x*-axis) and the number of events is represented on the vertical (*y*-axis). The parameter could be the peak pulse height, the integrated pulse height, or a ratio based on the first three parameters. Light-scatter or fluorescence pulses may also be used. As well as being displayed as data are accumulated, raw data acquired during "real-time analysis" can be written in a continuous stream onto disc. These listed data, or "list mode analysis," subsequently can be re-analyzed in more detail.

The production of a histogram relies on the measurement of pulses of a given value and their assignment to channels that represent different voltage levels. This type of analysis is referred to as "pulse processing." Each time a pulse falls into one of these channels, a counter increments the channel. The process of counting each pulse in the appropriate channel is known as A-to-D conversion. Most systems have 256 or 1024 channels for single-parameter histograms and may be generated based on fluorescence, FALS, or 90° LS. Some newer instruments (BD, Franklin Lakes, NJ) have 262,144 channels and this allows greater detection sensitivity. The pulses may be amplified, in a linear or logarithmic manner. In some experiments, the peak or integral pulses may vary widely in size. With linear amplification, small pulses will be bunched up into a few channels, making it difficult to distinguish differences between them. If the amplification is increased, this helps to distinguish between the small pulses but the larger pulses become pushed off the scale of the histogram. In such cases, the operator may elect to use pulses that have gone through a logarithmic amplification before plotting. The plot may include all pulses; however, the small pulses will be spread over more channels and the larger pulses over fewer channels. In this way, all pulses are brought onto the scale of the histogram.

## 8. Coefficients of Variation

Ideally, the same particle passing repeatedly through the laser beam should produce identical light-scatter or fluorescence pulses. Another particle might

produce a consistent but different set of pulses. Practically, there are always some variations within the instrument which causes some variation in the pulses even though the particles are the same. Any problems with the sample flow, laser intensity, laser alignment, beam focusing, and detection may result in variation of the pulses associated with a given particle. These variations lead to variation in histograms, and it is important to determine whether the variation is due to instrument or particle variation or both. The operator can assess the magnitude of the instrument variations by calculating a coefficient of variation (CV) on a good uniform test sample such as fluorescent beads available from Beckman Coulter (Fullerton, CA), DakoCytomation, (Glostrup, Denmark), and Polysciences (Warrington, PA). The basic equation for CV is:

$$CV = (SD/MEAN) \times 100$$

Where SD is the standard deviation and mean is the average value for the parameter measured for these particles. (For a Gaussian distribution, this would be the channel with the highest count.) Most instruments calculate the CV for the operator. If the operator knows what the CV is normally, any increase in CV will indicate that the instrument setting may be changing and result in broadening of histograms. However, if the beads have a good CV and a test sample has a broad histogram, the phenomenon is likely to be genuine. Fluorescent beads may be added to the biological sample to be tested provided that they do not interfere with the sample.

Once a histogram has been produced, the operator may now analyze it. The most common analysis is simply to determine what percentage a subpopulation is of the total population. This is possible if the populations are nicely separated, but in practice this may not be the case. However, sophisticated computer programs are available to analyze overlapping populations. Computers can also be used to compare one histogram with another and determine whether there are any significant differences.

All flow cytometry systems have the ability to analyze more than one signal on particles simultaneously and plot them as 3D histograms. Many combinations of signals might be used. The histogram is like a checkerboard of channels. Each channel, like the single-parameter histogram, has a counter but now two pulse heights for a particle must fall within a channel to increment that channel. A 3D histogram is therefore built up as the channels are incremented for a given sample. The two-parameter histogram can be converted to a single-parameter histogram by viewing from the side (either axis, *x*- or *y*-); this is called a projection. The two-parameter histogram may also be viewed from above, and in this format it is referred to as a scattergram or dot plot. It is also possible examine the distribution in all channels that have a certain operator-selectable minimum count in the channels; this is termed a slice, and an outline

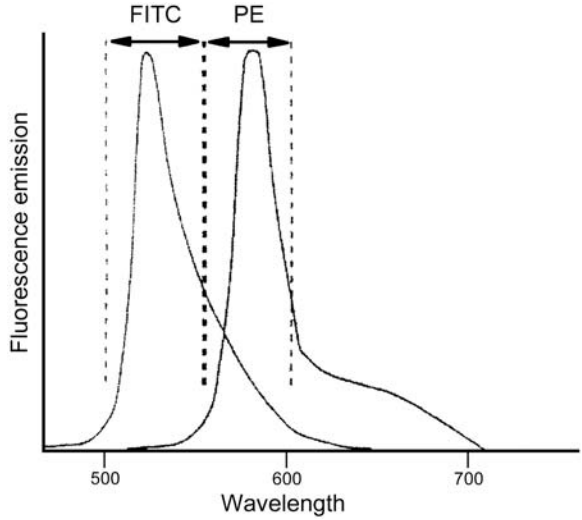


**Fig. 6.** Fluorescence emission spectra for FITC and PE showing the overlap in emission for the two fluorochromes.

of a series of slices from the bottom to the top of a two-parameter histogram is termed a contour plot.

## 9. Spectral Overlap and Compensation

Each fluorochrome has a wide emission spectrum. When multiple fluorochromes are used, parts of their emission spectra will probably be at the same wavelengths. This is referred to as "spectral overlap." **Figure 6** shows the emission spectra for fluorescein isothiocyanate (FITC) and PE. Superimposed on the spectra are the possible transmission characteristics of two band-pass filters. The FITC emission spectrum overlaps with that of PE, and some of its light will be transmitted by the PE filter and so enter the PMT for PE. This spectral overlap is corrected by subtracting a fraction of the FITC signal from the PE signal. Similarly, a fraction of the PE signal may be subtracted from the FITC signal. This is termed compensation.

**Figure 7** shows an example of the analysis of lymphocytes labeled with CD4 PE and CD8 FITC without and with compensation.

## 10. Safety Aspects of Lasers

Hazards from lasers can be summarized as follows:

1. Damage to the eye. The argon/krypton laser presents a possible hazard to the eye because "stray," diffuse, blue laser radiation can be focused by the eye onto the

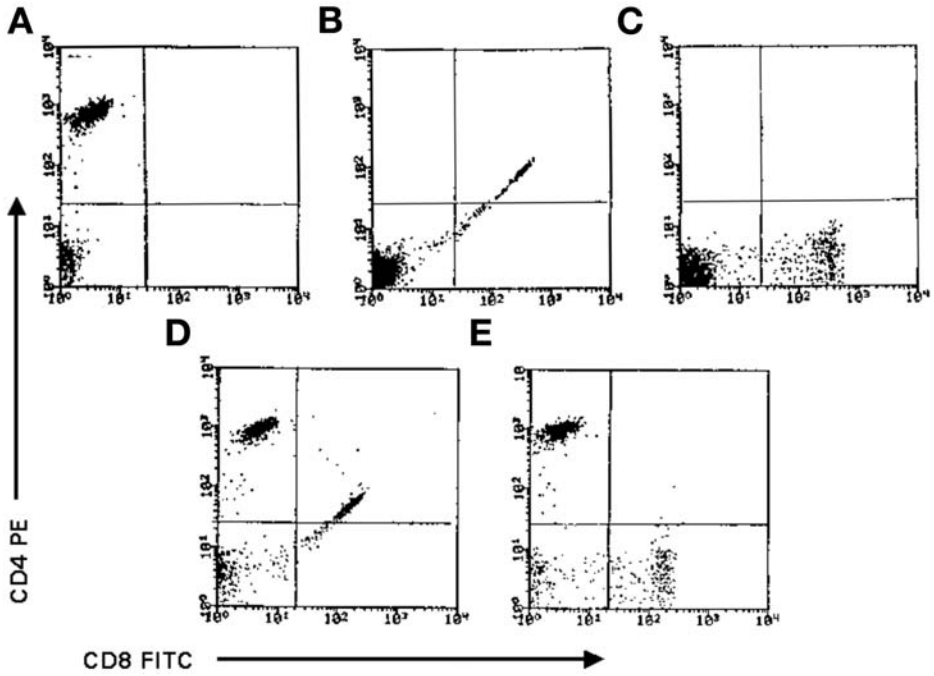


**Fig. 7.** Illustration of the analysis of **(A)** lymphocytes labeled with CD4 PE, **(B)** lymphocytes labeled with CD8FITC without compensation and **(C)** with compensation, and **(D)** lymphocytes dual-labeled with CD4PE and CD8 FITC without compensation and **(E)** with compensation.

retina. Damage can occur due to either thermal or photochemical effects, depending on exposure duration.

2. Skin burns. Interception of the beam by any part of the skin can cause damage due to thermal effects.
3. Material combustion. If irradiated, any unsuitable material in the vicinity of the beam can cause fire, emission of toxic gases, or explosion.

Under normal operation of commercially available flow cytometers, the laser requires that covers be installed and the laser output is interrupted if the cover is removed. Under these circumstances, the laser is classified as a class 1 product; there is therefore no potential hazard. The safety standard for British Radiation Hazard no. 21 in subchapter 1 applies ***(11)***. However, it is possible for the operator to defeat the interlock in the specimen irradiation area, thus allowing the beam to be seen, although direct (intrabeam) viewing is impossible (due to the equipment layout). A potential hazard exists from reflections. If the reflected beam is diffused in all directions, a hazard is not presented. If the beam remains focused after reflection, the maximum permissible exposure (MPE) (intrabeam

viewing) is 100/Jm$^2$. For a 60-s exposure, the MPE is 1.67/Wm$^2$; with a 2-W laser, a safe viewing distance for such exposure would be 0.2 m. Therefore, as a small source, an argon/krypton laser presents no hazard provided that the operator not view the beam reflection from any closer than 20 cm or for longer than 60 s. Should prolonged viewing of the beam be necessary, the use of safety spectacles is recommended.

## 11. Cell Sorting

An important function of flow cytometry is its ability to separate and collect a subpopulation of cells identified by multiparameter analysis. Classically, this sorting of cells is accomplished as the cells exit the sample chamber in a liquid jet. Savart ***(12)*** showed that when a small jet of fluid was vibrated at the correct frequency the stream could be broken into a series of uniform droplets. In the flow cytometer, the sheath stream is broken into a series of uniform droplets by vibrating the sample chamber with a piezoelectric crystal at a high frequency. Cells flowing through the flow cytometer are isolated in these tiny droplets. When the computer detects a cell that satisfies the parameters determined by the operator for sorting, an electrical charge is applied to the droplet ***(5)***. The polarity of the charge, positive or negative, is determined by the sorting criteria. As the charged droplet passes an electrostatic field, it is deflected to the right or left, carrying the sorted cell. Extremely pure populations of cells may be sorted at relatively rapid rates.

More recently, an alternative technique has become available for sorting cells. The Becton Dickinson machines employ a system in which the required cell is removed from the sheath stream by a small rotating catcher tube. Up to 300 cells per second may be sorted, but only one-way sorting is available at present. The technique is not dependent on droplet formation and takes place in an enclosed environment. Therefore, no aerosols are formed; this design eliminates the risk from biohazardous samples.

## 12. Commercial Flow Cytometers

There are four major manufacturers of flow cytometers: Beckman Coulter, Becton Dickinson, DakoCytomation, and Partec (Münster, Germany). Beckman Coulter, Becton Dickinson, and Cytomation originally introduced flow cytometers capable of sorting with water-cooled lasers. Beckman Coulter produced the EPICS (Electronically Programmable Individual Cell Sorter) series, Becton Dickinson marketed the FACS, and DakoCytomation promoted The MoFlo. DakoCytomation, Beckman Coulter, and Becton Dickinson have moved toward production of clinically orientated benchtop analysers, employing air-cooled lasers without the capacity to sort cells (i.e., Beckman Coulter EPICS XL, FC500, Becton Dickinson FACSCalibre, FACSCanto, and DakoCytomation

Cyan). The Partec flow cytometers are capable of determining absolute counts of cell subpopulations. Becton Dickinson recently launched the FACSAria high-speed cell sorter and the LSR II. Both of these instruments have the capacity to analyze up to 11 fluorescence signals simultaneously.

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# 2

# Cell Preparation

**Desmond A. McCarthy**

**Summary**

For any flow cytometry application, cells must be prepared so that they are single and suspended in an appropriate medium. There are a variety of procedures for preparing cells, and the method used will depend upon the cell type being investigated. Once prepared, the cells may then be labeled with a fluorochrome-labeled mAb or fluorescent dye. The labeling procedure may be influenced by a number of factors, including the specificity of the antibody and the density of the antigen being investigated on or in the cell. The concentration of the antibody used may need to be optimized, and appropriate negative and positive controls should be included in any analysis of cells.

**Key Words:** Antibody–antigen interaction; immunolabeling; permeabilization; preparation procedure.

## 1. Introduction

Flow cytometers can measure with high sensitivity the light that is scattered and emitted as fluorescence by single cells or particles that are present in a heterogeneous mixture. To do so, the cells must be uniformly suspended in a medium, which in most instances will be isotonic, approx pH 7.3, and (for maximum efficiency) at a concentration of $10^5$–$10^6$/mL, so that they flow through the analyzing light beam mostly one by one. Typically, only small sample volumes (0.2–1.0 mL) are needed for analysis, but the minimum volume will ultimately depend on the concentration of the cells of interest within the sample. If these comprise only a very low proportion of the total number of events, it can be an advantage to remove as many irrelevant cells as possible before analysis. Generally, the diameter of the cells to be analyzed must lie within the range of 1–30 μm because most commercial cytometers are not sensitive enough to detect smaller particles and their flow systems are blocked easily by larger ones. Small particles such as bacteria, sperm, DNA fragments, nuclei, and viruses can

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also be analyzed, but because their size and macromolecular content are much smaller (100- to 1000-fold) than those of mammalian cells, those components that are present in low concentrations may be undetectable or measurable with only low precision. If required, special cytometers with increased sensitivity to detect small particles or with wider flow streams to analyze larger cells are available. For these reasons, the technique is ideally suited for the analysis of blood, which contains platelets (approx 1 µm diameter) and cells (6- to 12-µm diameter) already in suspension. However, cells from lymphoid and solid tissues or in culture can be analyzed provided that they are first separated from each other or their substrate and that any aggregates are removed before analysis. What makes flow cytometry such a useful technique for clinical studies is that the sample stream velocity (typically, 5–50 m/s) allows 5000–50,000 cells to be measured each second and for most assays only 100 µL (or less) of blood is needed. For the results to be biologically meaningful, however, the sample must be processed and the flow cytometer operated correctly.

The first step in sample preparation, therefore, is to obtain, or produce, a cell suspension. Peripheral blood cells are already suspended in plasma and most of those collected from bone marrow can be resuspended easily by pipetting; however, the cell–cell and cell–matrix interactions of those in solid or lymphoid tissues need to be broken and the cells dispersed (*see* **Subheading 3.2.**). The second step is to maintain those cell properties, or functions, that are the subject of the investigation as closely as possible to their original *in vivo* state until they have been measured satisfactorily. For some components and certain cell types (e.g., many lineage-specific antigens on "resting" blood lymphocytes), this may be relatively simple and a number of different protocols will give essentially similar results. However, for others (e.g., "activation antigens" like CD62P and CD11b which can be upregulated rapidly on platelets and neutrophils, respectively), it is more problematic and different protocols may give quite different results, depending on the nature and magnitude of the preanalytical changes that can occur in vitro under different circumstances. Fortunately, for leukocyte immunophenotyping, the National Committee for Clinical Laboratory Standards (NCCLS) (in the United States), the National External Quality Assurance Scheme (in the United Kingdom), and the European Working Group for Clinical Cell Analysis recommend protocols and internal quality controls and also organize external quality control schemes. These measures have helped reduce interlaboratory variation and have enabled greater standardization ***(1)***. Similarly, guidelines for analysis of DNA content have been published by a North American group ***(2)*** and the European Society for Cellular Analytical Pathology ***(3)***. For many other assays (e.g., lymphocyte cytotoxicity, viability, apoptosis, neutrophil phagocytosis, and platelet activation), detailed protocols can be found in publications of the International Society for Analytical Cytology and Purdue University

Cytometry Laboratories ***(4,5)*** and in the volumes devoted to flow cytometry in treatises such as *Methods in Cell Biology* (Academic Press, New York, NY) and *Methods in Molecular Biology* (Humana Press, Totowa, NJ). However, when using/developing any protocol, one should always check preparations by light microscopy, include the appropriate negative (and if possible, positive) controls, and ensure that the method is repeatable and free from artifacts in the context in which it is being used. In this chapter, the basic elements of sample preparation and the principles underlying some of the more commonly encountered immunolabeling procedures for surface and internal antigens are discussed. Procedures for preparing samples for more specialized purposes (e.g., DNA analysis, functional assays, membrane integrity, and apoptosis) can be found in the relevant, later chapters.

## 2. Factors Influencing the Choice of Preparation Procedure: Live Versus Fixed Samples

It is obvious that when assessing some cell features or functions (e.g., viability by dye exclusion, phagocytic or cytotoxic ability, and responses to agonists in vitro) it is necessary to use live cells, suspend them in a physiological medium, and to examine them as soon as possible. However, there is no reason why live cells cannot also be used when assaying a number of other characteristics such as the expression of lineage-specific surface antigens or adhesion molecules, provided that they can be maintained in a quiescent state without significant change in structure and viability. Moreover, live cells have the advantage that they generally bind lower levels of antibodies in a nonspecific manner than do fixed, or dead, cells.

Phosphate-buffered saline (PBS) can often be used when cells need to be kept in vitro for only a brief period, but if cells are to be kept for longer, it is preferable to keep them in a buffered, isotonic, physiologically balanced salt solution that contains some protein. Balanced salt solutions such as Hanks' (HBSS) or Tyrode's can be buffered to pH 7.3 with 10–25 mmol/L HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]), which unlike the components of many conventional inorganic and organic buffer solutions, does not enter cells and will maintain the correct pH for several hours. Tissue culture media such as RPMI-1640 can also be used, but they should not contain phenol red, and unless it is possible to maintain a 5% $CO_2$ atmosphere in conjunction with the traditional carbonate–bicarbonate buffer system, they should be buffered with HEPES. Suitable proteins for many purposes are 0.5–5% bovine serum albumin (BSA) or 5–10% fetal bovine serum (FBS). However, for immunolabeling studies, 1% normal serum or 100 μg/mL of immunoglobulin (Ig) from the same species as the primary antibody can be used when it is necessary to reduce nonspecific antibody binding by blocking Fc receptors. But it must be kept in mind that some

surface antigens (e.g., CD14, CD16, CD32, CD62L, and tumor necrosis factor-α) can be released after cleavage by enzymes present in serum and/or by endogenous enzymes that can be activated by antibody or receptor binding ***(6)***. If cells (e.g., macrophages, neutrophils, and platelets) have a tendency to aggregate and/or to stick to plastic surfaces, they can often be kept in suspension by using media that are free of divalent cations ($Ca^{2+}$ and $Mg^{2+}$) and that, if necessary, also contain 5 m*M* EDTA. A lack of divalent cations and the presence of EDTA, however, are prejudicial to cell survival. Irrespective of whether live or fixed cells are to be analyzed, unless the suspension and staining media are to be kept sterile, it is good practice to make them up shortly before use (e.g., daily) and to keep them at 4°C while working, to minimize microbial growth. If BSA or FBS has been added to a simple medium (e.g., PBS or HBSS), it is worth checking it in the cytometer for the presence of protein aggregates and, if necessary, passing it through a 0.25-μm-diameter filter before use, a step that is particularly important when studying platelets and microparticles.

Although not appropriate for investigations of cell function, there can be some advantages to fixing cells for many other studies. If samples cannot be processed promptly or if molecules (e.g., CD11b on neutrophils ***[7]*** or CD62P on platelets ***[8]***, the expression of which is subject to artifactual change in vitro) are to be assayed, they can be fixed soon after collection and before immunolabeling. In fact, a transport medium (TransFix, Cytomark Ltd., Botolph Claydon, Buckingham, UK) suitable for the fixation of leukocytes and lysis of erythrocytes in peripheral blood samples ***(9)*** is available commercially. However, before employing this strategy routinely, it is important to validate it by comparison with samples that have been processed live and without delay; unfortunately, it can affect the ultrastructure of leukocytes, increase nonspecific antibody binding, and diminish antigenicity, leading to decreased antibody binding ***(10, 11)***. Also, in contrast to the findings of Hagberg and Lyberg ***(8)***, others ***(12,13)*** have found that, in platelets, formaldehyde fixation can cause ultrastructural changes and affect the expression of activation antigens (e.g., CD62P). More frequently, to preserve them until they can be analyzed conveniently, cells are fixed after they have been immunostained. Before deciding to fix a sample, it is worth becoming familiar with the mode of action of different fixatives because fixatives generally preserve ultrastructural integrity at the cost of impairing antigenicity, and it may be necessary to strike the correct balance between these two factors.

## 3. Factors Affecting Cell Preparation

### *3.1. Processing Blood and Bone Marrow Samples*

Peripheral blood is normally collected by venepuncture and must be mixed immediately with an anticoagulant – commonly heparin, sodium citrate, or

tripotassium ethlenediaminetetraacetic acid ($K_3$EDTA) – to prevent clotting. The choice of anticoagulant may be relatively unimportant for some purposes, but for others it may be crucial to obtaining reliable results because anticoagulants do more than just inhibit the coagulation pathway. $K_3$EDTA, which is routinely used for counting blood cells, is suitable for immunophenotyping leukocytes. It prevents platelet aggregation but does not preserve leukocyte cell morphology and light scatter characteristics as well as citrate and heparin do. However, because it is a strong chelator of divalent cations, it can diminish the antigenicity of $Ca^{2+}$-dependent epitopes such as CD11b and inhibit $Ca^{2+}$-dependent cell functions ***(14,15)***. Preservative-free heparin (10–50 U/mL) is generally preferred for functional studies of leukocytes, particularly when it is necessary to maintain physiological concentrations of $Ca^{2+}$ and $Mg^{2+}$, but it binds to platelets and enhances platelet activation and aggregation. For monitoring for platelet and neutrophil activation ex vivo, a combination of sodium citrate, theophylline, adenosine, and dipyridamole (CTAD) (available as Diatube-H® tubes; BD Biosciences, San Jose, CA) ***(16)*** and $K_3$EDTA (EDTA-CTAD) ***(17)*** will largely inhibit the spontaneous activation that occurs in vitro if samples are taken into citrate or heparin. Syringes and tubes made from polypropylene, rather than polystyrene, should be used when collecting and handling samples for those assays in which it is important to minimize platelet or myeloid cell activation and adherence. Bone marrow samples are usually aspirated into a medium containing heparin and contain a mixture of cell types, including hematopoietic cells, adipocytes, endothelial cells, and fibroblasts, together with some cells from the peripheral blood; however, it is not possible to distinguish their origin (i.e., marrow or blood).

The next decision to be made (after the choice of anticoagulant) is whether the cells in the sample are to be fixed soon after the sample has been collected, to prevent unwanted changes in vitro, or whether they are to be processed live (and if the latter, whether to enrich for, or to isolate, the cells of interest). If cells are analyzed live, they should be immunostained and analyzed as soon as possible after the samples have been collected or the cells isolated/enriched, because they will deteriorate when kept in vitro. Furthermore, the gating strategy can have a marked impact on immunophenotyping results that are obtained from samples that have been kept some days before analysis ***(18)***. If samples cannot be immunostained promptly, they must be kept at a suitable temperature until processed. Storage at 20–25°C is satisfactory for molecules (e.g., CD3) whose expression is stable ***(19,20)***, but storage at 4°C is preferable for molecules (e.g., CD11b and CD62L on myeloid cells) whose expression may change in vitro ***(21)***. Unless there are good reasons to use another temperature, immunostaining should be done at 4°C and, if necessary, the cells can

then be kept at that temperature for several hours before analysis or for considerably longer if they have been postfixed with formaldehyde after immunolabeling. In both blood and bone marrow samples, the high (approx 500:1) erythrocyte/leukocyte ratio slows down the analysis of leukocytes; consequently, to speed up the analysis, leukocytes are often isolated before staining or the erythrocytes are lysed after staining (and the leukocytes fixed). Unfortunately, the techniques used to isolate leukocytes can lead to selective cell losses of certain lymphocyte subsets ***(22)***, whereas the techniques used to lyse erythrocytes and fix leukocytes can lead to losses in antigenicity of certain epitopes/antigens ***(23–26)***. For these reasons, it is preferable to use live whole-blood procedures whenever possible because they are less prone to artifacts. **Figures 1** and **2** summarize the decisions to be made and their likely consequences.

### *3.1.1. Live Whole-Blood Procedures*

Leukocytes can be distinguished from mature erythrocytes and platelets in whole (unfractionated) blood because their nuclei can be stained with permeant DNA dyes such as LDS-751 and DRAQ5 and/or their plasma membranes can be stained with fluorochrome-conjugated CD45 (a pan-leukocyte antibody) ***(15,21,26–28)***. Nucleated erythrocytes, which are found in bone marrow samples and in the blood of newborn infants and which have light scattering properties similar to lymphocytes, can be distinguished from the latter because they stain positively with LDS-751, DRAQ5, and anti-glycophorin antibodies but not with CD45 antibodies. Only small amounts of sample and antibody are needed and the staining procedure is quick (10–15 min), but the analysis is slowed by the presence of erythrocytes. In practice, samples are simultaneously labeled with LDS-751 or DRAQ5 and other fluorescent probes. For instance, if LDS-751 (excitation/emission maxima, 543 nm/712 nm) is used, it can be excited by a 488-nm laser and monitored in one fluorescence channel, while the other fluorescence channels can be used to monitor fluorochrome-labeled antibodies or fluorescence probes that report physiological status (e.g., intracellular $Ca^{2+}$ concentrations) or $O_2^-$ generation ***(29,30)***. When analyzing the ex vivo expression of molecules that can be upregulated or downregulated rapidly on cells in vitro, inhibitors (e.g., 0.1% sodium azide) can be added to the staining medium. DRAQ5 (excitation/emission maxima, 646 nm/681 nm) can be suboptimally excited at 488 nm, has the advantage over LDS-751 that it will stain DNA quantitatively in situ, and can be used in conjunction with fluorescein- and phycoerythrin (PE)-labeled antibodies without the need for compensation ***(26)***.

#### 3.1.1.1. Live Whole-Blood Procedure for Direct Immunostaining in Conjunction with LDS-751 Staining of Leukocyte Nuclei

1. Incubate 10-µL samples of anticoagulated blood* with 2 µL (or the supplier's recommended volume) of fluorescein isothiocyanate (FITC)-conjugated and/or 2 µL of PE-conjugated specific or isotype control antibody at 4°C for 10 min.
2. Add 1 mL of HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3) containing 0.5% BSA and 0.1% (by volume) of a saturated methanolic solution of LDS-751. (A solution of LDS-751 in methanol can be kept almost indefinitely in the dark at 4°C.)
3. Allow the sample to stand for 2–3 min at 4°C so the LDS-751 can penetrate and stain the leukocytes.
4. Examine by flow cytometry and display the events in a plot of side light scatter (ordinate: can be either a logarithmic or a linear scale) versus LDS-751 fluorescence intensity (abscissa: logarithmic scale). Erythrocytes and platelets stain only weakly and should occur in the first two fluorescence intensity decades, whereas the leukocytes occur in the third and possibly fourth decade(s) (**Fig. 3A**). The former can be eliminated from further analysis by setting a threshold on LDS-751 fluorescence intensity and/or by setting a gate to encompass all leukocytes (e.g., R1 in **Fig. 3B**).
5. A gate can now be set around the cells of interest (e.g., R2, R3, or R4 in **Fig. 3B**) and the events displayed as a single-parameter histogram of number (ordinate: linear scale) versus fluorescence intensity (abscissa: logarithmic scale).
6. Use the fluorescence intensity of cells stained with the isotype control antibody (and/or that of any unstained "negative" internal control cells) to set the cursor so that no more than 1% of these events would be considered positive.

### *3.1.2. Leukocyte Isolation Techniques*

There are several ways in which leukocytes can be isolated from blood or bone marrow or the samples depleted of erythrocytes and/or platelets. However, when isolating lymphocytes or dendritic cell precursors, centrifuging can lead to selective losses of various subsets. Also, when isolating neutrophils, temperature changes, centrifuging ***(31,32)***, and using media that contain even trace amounts of bacterial lipopolysaccharide ***(33)*** can lead to activation. For this reason, it is advisable to use only endotoxin-free media containing >5% serum when isolating neutrophils and to check that expression of CD11b, or another indicator of activation, on the isolated cells is broadly comparable with that on the cells in unfractionated blood ***(34)***. Recktenwald and Radbruch ***(35)*** provide a fuller description of the methods available for cell separation and isolation, and the same authors ***(36)*** discuss the methods available for detecting and isolating rare cell populations. If isolated platelets are required, they can be prepared by differential centrifugation or by density gradient centrifugation ***(37)***.

*Pathological blood samples that have an abnormally high leukocyte count should first be diluted in medium with anticoagulant so that the leukocyte numbers fall within the normal range; alternatively, a proportionately larger volume of antibody should be used.

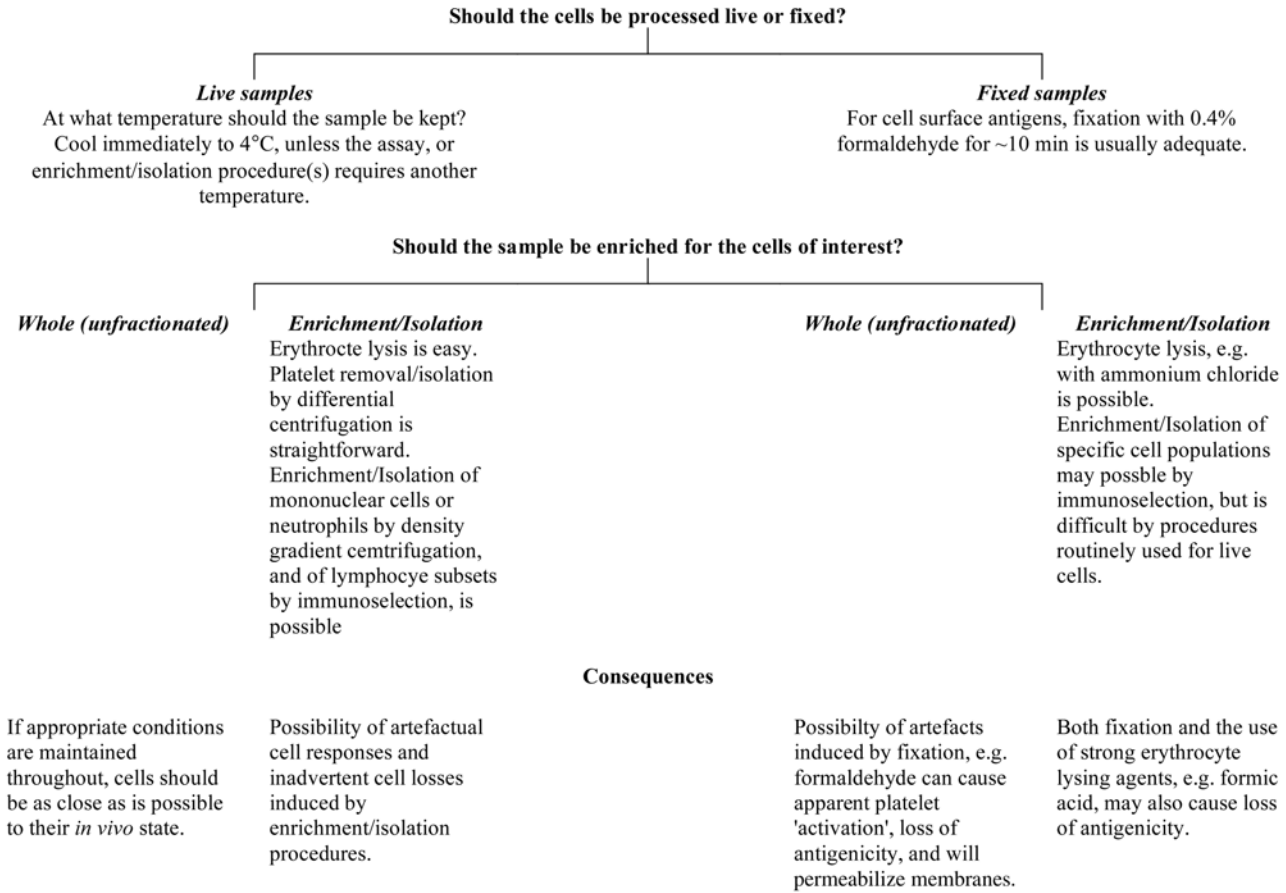


**Fig. 1.** The decisions that need to be made between sample collection and immunolabeling and their likely consequences.

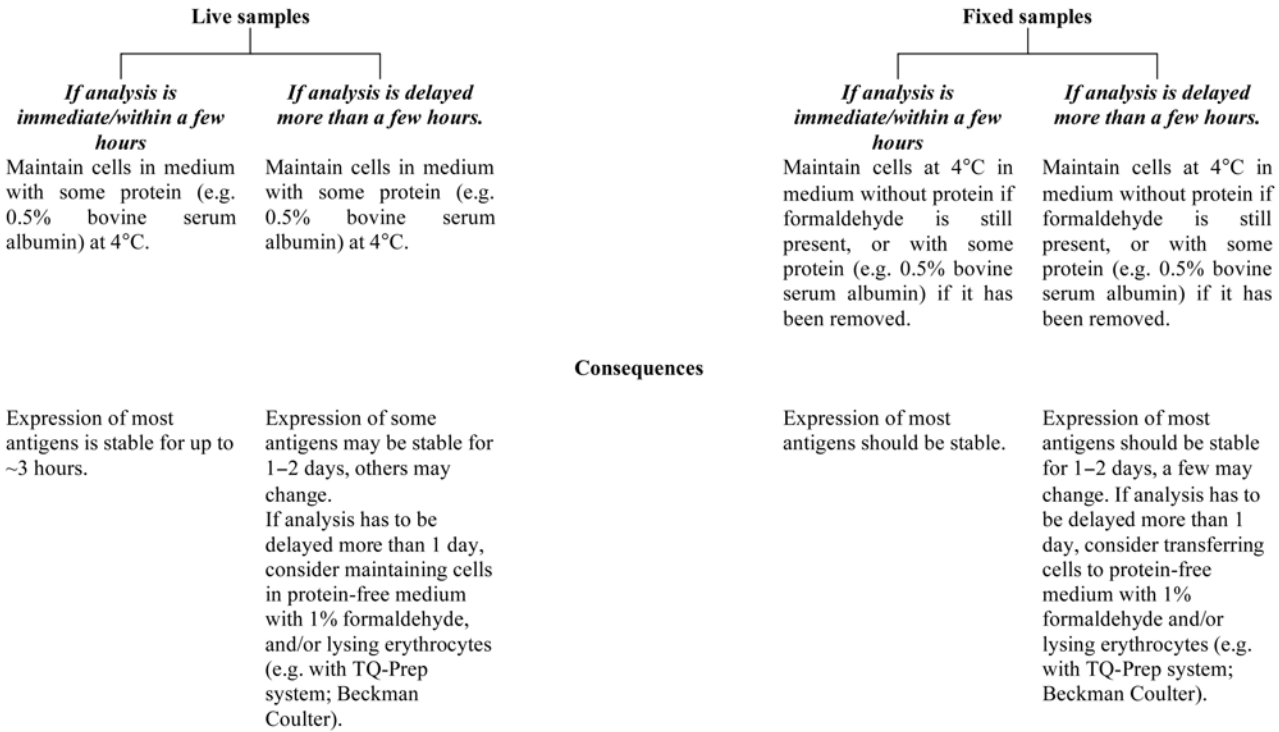


**Fig. 2.** The decisions that need to be made after processing and immunolabeling and their likely consequences.

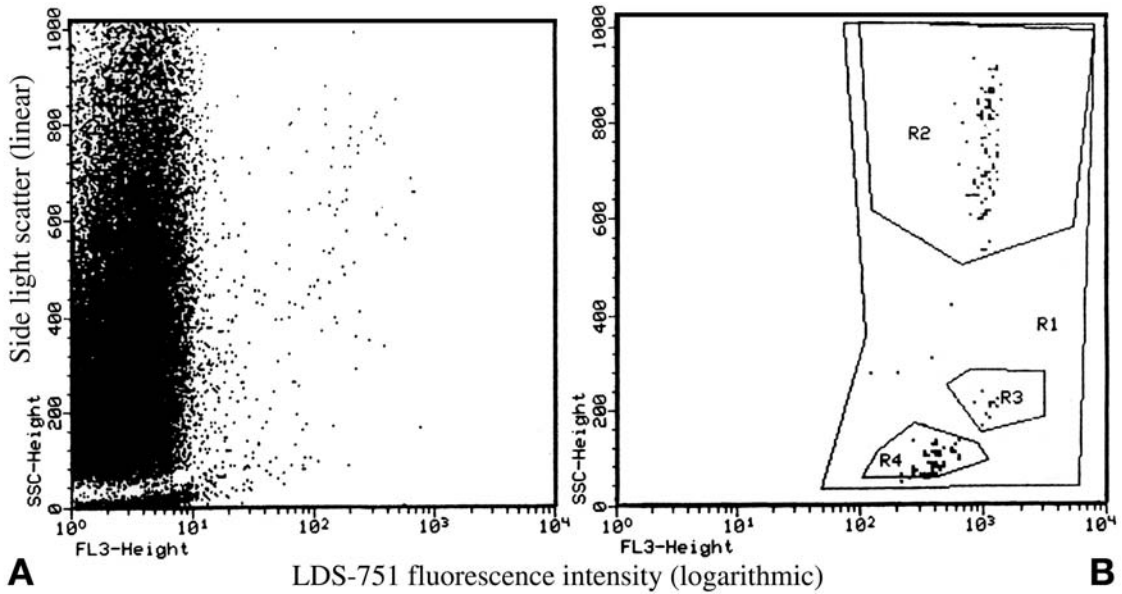


**Fig. 3.** The live whole-blood procedure using LDS-751 to stain leukocyte nuclei. **(A)** The ungated bit map demonstrates the very high numbers of weakly fluorescent events (predominantly red blood cells and platelets) present in whole-blood samples which can be excluded by setting a primary gate (labeled R1 in **[B]**). **(B)** Secondary gates within R1, designated R2, R3, and R4, were identified as containing neutrophils, monocytes, and lymphocytes, respectively, by their reactivity with lineage-specific antibodies. Reprinted with permission from Elsevier Science from **ref.** ***15***.

#### 3.1.2.1. Dextran Gravity Sedimentation

If anticoagulated blood is mixed with dextran or Plasmagel (a gelatine plasma substitute) and kept at 37°C, the erythrocytes aggregate to form rouleaux that sediment faster than the leukocytes, and after a while, the upper plasma layer containing mostly leukocytes and platelets can be recovered. The procedure involves few manipulations, and the few remaining erythrocytes can be removed by lysis if necessary, but it can lead to a small amount of neutrophil and monocyte activation ***(34,38)***. It is also possible to encourage rouleaux formation by using an anti-erythrocyte antibody (Red-Out; Robbins Scientific Corp., Sunngvale, CA) instead of dextran or Plasmagel.

#### 3.1.2.2. Preparation of Blood Leukocytes by Dextran Gravity Sedimentation

1. Add 2 mL of sterile nonpyrogenic dextran (Hespan; DuPont, Wilmington, DE) or Plasmagel to 8 mL of anticoagulated blood in a 10-mL plastic syringe fitted with a wide-bore (e.g., 19-gauge) needle that still has its plastic protective cover in place.
2. Leave the syringe for 45–60 min at 37°C in a vertical position for the erythrocytes to settle.
3. Using the protective cover, gently bend the needle through approx 110°. Remove the needle cover completely and, with the syringe in an upright position, gently press the plunger to deliver the leukocyte-enriched plasma into a centrifuge tube, leaving the erythrocyte layer behind in the syringe.
4. Wash the leukocytes twice by centrifuging at 400*g* for 5 min with 10 mL of HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3, containing 0.5% BSA. Resuspend in 1–2 mL of medium.

#### 3.1.2.3. Density Gradient Centrifugation

Leukocytes can be isolated from blood and bone marrow samples by density gradient centrifugation, but the procedure is not recommended for immunophenotyping lymphocytes, because it can result in variable recovery ***(39)***. Two types of density gradient medium are commonly used, Ficoll®-Hypaque® and Percoll®. Ficoll® is a dextran polymer that induces erythrocyte aggregation and is mixed with iodinated aromatic compounds (known commercially as Hypaque, Histopaque, or Isopaque) that increase the osmolarity and density of the solution ***(40,41)*** (**Table 1**). Anticoagulated blood is layered over the medium in a centrifuge tube. When the tube is centrifuged, cells contact the solution and the erythrocytes aggregate and shrink more rapidly than leukocytes and the resulting increase in density and decrease in frictional coefficient cause them to sediment faster than the leukocytes ***(42,43)***. Mononuclear cells can be isolated by protocols employing a single layer of medium (e.g., Ficoll-Hypaque, density 1.077 g/mL) because after centrifuging, they remain at the plasma-Ficoll-Hypaque interface whereas the erythrocytes and neutrophils pellet to the bottom of

**Table 1**
**Some Commercially Available Reagents for the Isolation of Blood Leukocytes**

| Product | Supplier | Comments |
|---|---|---|
| Ficoll–Paque™ PLUS | GE Healthcare (Chalfont, St. Giles, Buckingham, UK) | Ficoll®, sodium diatrizoate, and disodium calcium EDTA: density 1.077 g/mL, for lymphocyte isolation (maintains viability and B- and T-cell populations). |
| Ficoll-Paque™ PREMIUM | GE Healthcare | Ficoll®, sodium diatrizoate and disodium calcium EDTA: density 1.077 g/mL, for mononuclear cell isolation; manufactured to healthcare standards. |
| Histopaque® | Sigma-Aldrich (St. Louis, MD) | Polysucrose plus sodium diatrizoate, density 1.077 and 1.083 g/mL, for mononuclear cell or polymorph and mononuclear cell isolation. |
| Histodenz™ | Sigma-Aldrich | Ficoll® plus metrizoic acid (Nycodenz): density 1.077 g/mL for mononuclear cell isolation. |
| Lymphoprep™ | Axis-Shield (Dundee, Scotland, UK | Polysucrose plus sodium metrizoate: density 1.077 g/mL, for mononuclear cell isolation. |
| LymphoSep™ Lymphocyte separation medium | MP Biomedicals (Irvine, CA) | Ficoll® plus sodium metrizoate: density 1.077 g/mL, for mononuclear cell isolation. |
| Mono-Poly resolving medium | MP Biomedicals | Ficoll®–Hypaque: density 1.114 g/mL, for polymorph and mononuclear cell isolation. |
| OptiPrep | Axis-Shield | A 60% (w/v) solution of iodixanol in water: density 1.32 g/mL, mixed directly with blood for platelet and mononuclear cell isolation. |
| Percoll® | GE Healthcare | Can be used to separate a wide range of cells, subcellular particles, microorganisms, and viruses. |

Note: These media are intended for the isolation of human cells and it may be necessary to use media of slightly different composition for isolating mononuclear cells from rats, mice, and other mammals (e.g., Histopaque® 1.083).

the tube. With ACCUSPIN™ centrifuge tubes (Sigma-Aldrich, St. Louis, MO), which contain two chambers separated by a porous polyethylene "frit," it is easier to remove the mononuclear cell band without contamination by erythrocytes and neutrophils. For high-risk samples, tubes (VACUTAINER® CPT™; BD Bioscience) containing either sodium citrate or heparin; a separation gel; and a Ficoll®-Hypaque® density gradient liquid can be used. Blood is collected into the tube, which is then centrifuged as usual, and the gel forms a barrier between the mononuclear cell band and the erythrocytes and granulocytes beneath. It is also possible to isolate neutrophils by centrifuging blood over a layer(s) of medium in which the density and osmolarity has been adjusted, so that at the finish, the neutrophils and mononuclear cells form separate bands ***(42,44,45)***. Moreover, the yield and purity of neutrophil preparations can be increased if the height of the blood column is maximized and cells are centrifuged a second time over the density gradient medium ***(44,46)***. After the bands have been recovered, the cells should be washed thoroughly in normal medium to reverse the osmotic stress.

#### 3.1.2.4. Isolation of Blood Mononuclear Cells on Ficoll®–Hypaque

1. Pipet 3 mL of Histopaque 1.077 g/mL into a 10- or 15-mL polpropylene centrifuge tube and carefully overlay it with 3 mL of anticoagulated blood that has been diluted 1:1 with HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3.
2. Centrifuge at 400*g* for 30 min at room temperature (20–23°C) and allow the rotor to stop with the brake off.
3. The erythrocytes and neutrophils will sediment into/onto the Histopaque while the mononuclear cells form a band between the Histopaque and the plasma, and the platelets will remain largely in the plasma layer.
4. Remove the mononuclear cell band with a pipet, wash the cells by centrifuging twice with 10 mL of medium at 400*g* for 5 min, and resuspend in 1 mL of medium.

#### 3.1.2.5. Simultaneous Isolation of Neutrophils and Mononuclear Cells on Ficoll®–Hypaque (Fig. 4)

1. Pipet 3 mL of Histopaque 1.119 g/mL into a 10- or 15-mL polypropylene centrifuge tube and carefully overlay it with 3 mL of Histopaque 1.077 g/mL and then with 3 mL of anticoagulated blood diluted 1:1 HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3.
2. Centrifuge at 400*g* for 30 min at room temperature (20–23°C) and allow the rotor to stop with the brake off.
3. The erythrocytes will sediment into the lower Histopaque layer, the neutrophils will band in between the two Histopaque layers, the mononuclear cells will band at the interface between the plasma and the uppermost Histopaque layer, and the platelets will remain largely in the plasma layer.

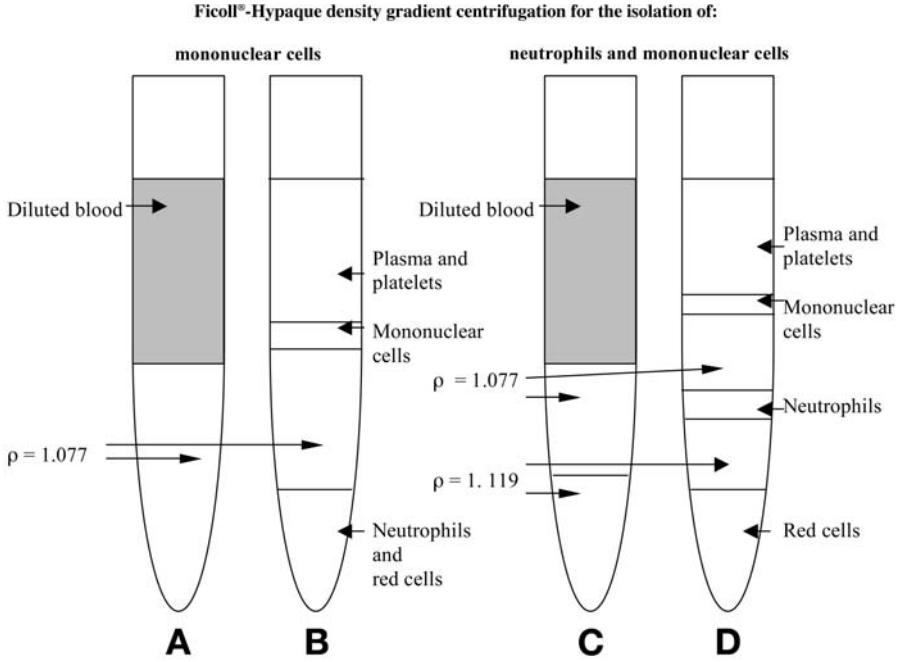


**Fig. 4.** Ficoll®–Hypaque density gradients for the isolation of blood leukocytes. (**A,B**) The isolation of mononuclear cells using a single-layer system and (**C,D**) the simultaneous isolation of neutrophils and mononuclear cells using a two-layer system. (**A**) and (**C**) represent the tube contents before centrifuging, and (**B**) and (**D**) represent the positions of the various components after centrifuging.

4. Remove the mononuclear and neutrophil bands separately with a pipet, wash the cells twice by centrifuging with 10 mL of medium at 400*g* for 5 min, and resuspend the cells from each band in 1 mL of medium.

Percoll® comprises polyvinylpyrrolidone-coated silica gel particles (mean diameter 21–22 nm) that form a density gradient when centrifuged in solution. It has the advantage that physiologically osmotic conditions can be maintained while gradients of appropriate density are formed for separating all of the different blood cell types ***(47)***. However, the density gradient centrifugation procedure using Percoll® is longer and more involved than that for Ficoll®–Hypaque centrifugation and for most purposes offers little additional advantage. It is most frequently used for isolating monocytes from mononuclear cell preparations that have been produced first by Ficoll®–Hypaque centrifugation (**Fig. 5**; **ref. *48***).

### 3.1.2.6. Immunoselection

If the cells of interest comprise only a small proportion of the total (e.g., $CD34^+$ stem cells or dendritic cell precursors in peripheral blood), they can be

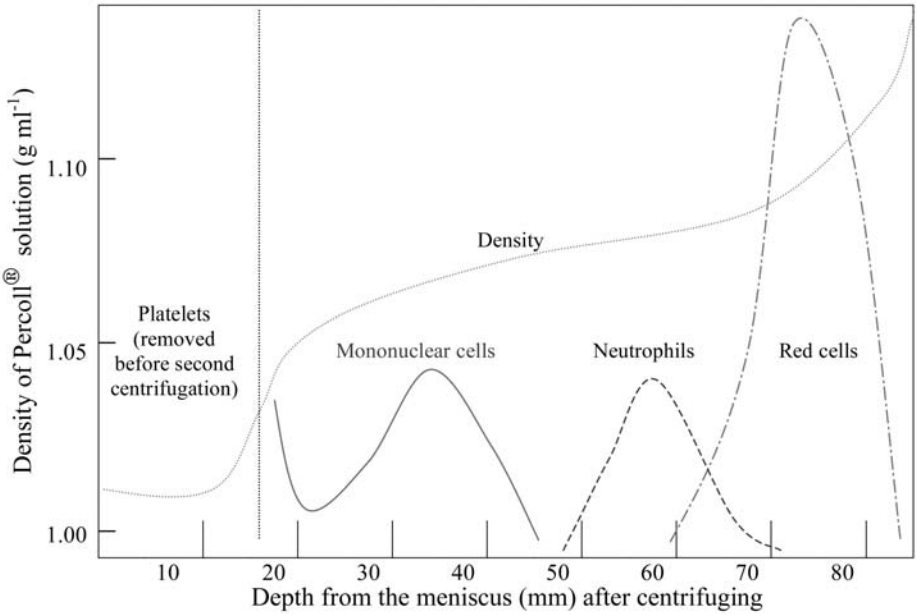


**Fig. 5.** Separation of human blood components by centrifuging on a Percoll® density gradient. Blood was overlaid on a preformed 70% (v/v) Percoll® density gradient in 0.15 mol/L NaCl and centrifuged at 400*g* for 5 min, and then the plasma layer containing platelets was removed and centrifugation was continued at 800*g* for 15 min, resulting in isopycnic banding of the blood cells. Redrawn from ***(47)***.

recovered by positive immunoselection, or irrelevant cells can be removed by negative selection using a cocktail of lineage-specific antibodies in a number of different ways (e.g., by immunoagglutination, with antibody-coated magnetic beads, or by immunoaffinity chromatography) (**Fig. 6**; **ref. *49***). When it is necessary to enrich samples in this way prior to cell-sorting (as it frequently is) or when preparations of 90–95% purity will suffice for experimental purposes, a commercially available automated immunomagnetic "pre-sorter" (autoMACS Pre-Sorter; Miltenyi Biotec Inc., Auburn, CA) can be used. Examples of some of the reagents and kits that are available commercially for this purpose are given in **Table 2**.

#### 3.1.2.7. Erythrocyte Lysis

Erythrocytes in whole blood can be lysed with water, isotonic ammonium chloride, and acid (e.g., formic acid) or by permeabilizing their membranes with saponin; however, 100% lysis is rarely achieved and a small number of erythrocyte "ghosts" usually contaminate the remaining leukocyte preparation.

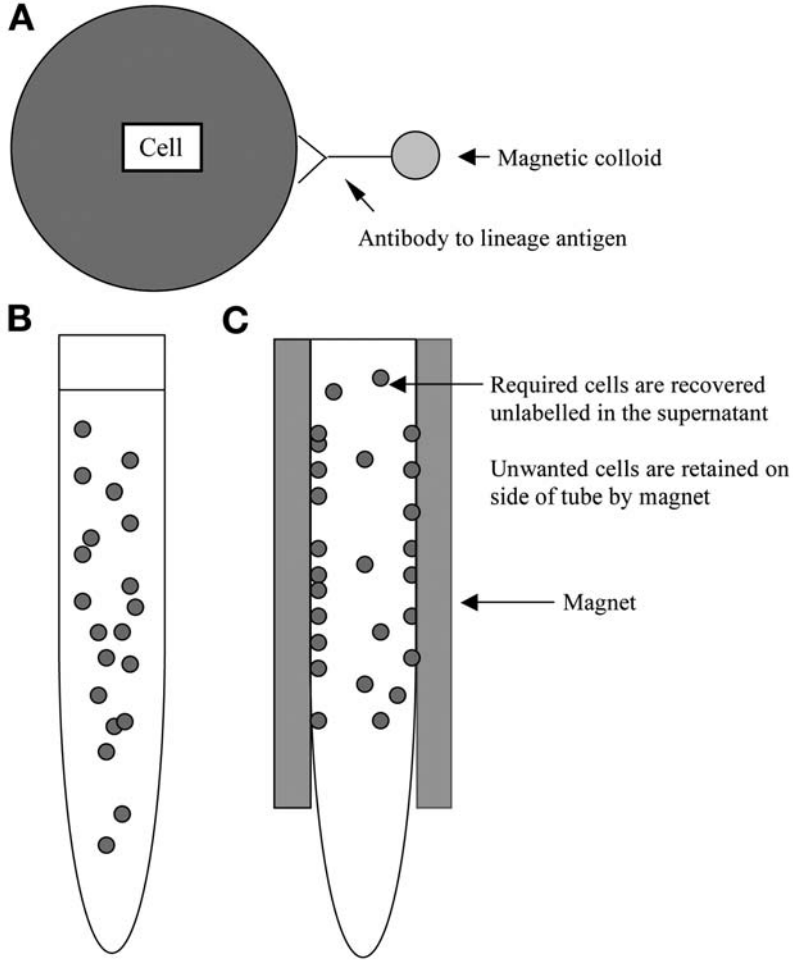


**Fig. 6.** Negative-selection principle using antibody-coated, superparamagnetic beads. The sample is labeled with an antibody specific for lineage antigen present on the unwanted cells which has been coupled to magnetic colloid beads **(A, B)**. Placing the tube containing the cell suspension in a strong magnet for a few minutes causes the unwanted cells to be held on the sides of the tube, allowing the unlabeled cells of interest to be decanted **(C)**.

The expression of many antigens is unaffected by these procedures, but some reagents can affect some leukocyte functions (e.g., oxidative burst and degranulation) and can affect the apparent expression of some platelet and neutrophil antigens ***(50)***. When considering whether to use these techniques, it is prudent to check their effects using live leukocytes that have been prepared by another procedure. Some commercially available erythrocyte lysing reagents are listed in **Table 3**.

**Table 2**
**Some Commercially Available Reagents for the Selection of Leukocyte Populations**

| Product | Supplier | Comments/intended use |
|---|---|---|
| BioMag® | Polysciences, Inc. (Washington, PA) | Magnetic particles (diameter, 1 μm), coated with secondary reagents (e.g., anti-fluorescein antibodies or streptavidin) for use in positive or negative selection procedure. |
| Cellect™ and Cellect™ plus | Cytovax Biotechnologies, Inc. (Edmonton, Alberta, Canada) | Columns that contain a glass bead matrix for the isolation of T cells or T-cell subpopulations by negative selection procedure. |
| Dynabeads® | Dynal Biotech (Invitrogen) | Superparamagnetic beads (diameter, 1, 2, or 4 μm) that can be coated with antibodies or secondary reagents that, using a magnet, can be retained in a tube. |
| MACS | Miltenyi Biotech (Bergisch Gladback, Germany) | Magnetic-activated cell sorting: using direct or indirect labeling and positive or negative immunoselection with superparamagnetic, antibody-coated microspheres (diameter, 50 nm). |
| MagSelect | R&D Systems, Inc. (Minneapolis, MN) | Streptavidin-coated superparamagnetic beads (diameter, 150 nm). |
| MICRA ACT-CES™ | Eichrom Technologies (Chicago, IL) | Advanced Cell Trap (ACT) tubes contain a porous silicone insert that allows cells to pass through. Use in negative-selection techniques for centrifugal separation of antibody-labeled cells that have been bound to nonporous beads coated with goat anti-mouse or streptavidin. |
| PrepaCyte™ | BioE Corporation (St. Paul, MN) | Antibody-mediated agglutination of other blood cells for the rapid one-step isolation of T cells and hematopoietic stem cells. |
| ProActive® | Bangs Laboratories, Inc. (Fishers, IN) | Streptavidin- and Protein A-coated superparamagnetic or nonmagnetic microspheres for use as secondary reagents. |
| RosetteSep | StemCell Technologies (Vancouver, BC, Canada) | Tetrameric antibody complexes that will couple unwanted cells to erythrocytes so that the rosettes can be removed by centrifugation on Ficoll®–Paque. |
| SeraMag™ | Serva GmbH (Heidelberg, Germany) | Streptavidin-coated superparamagnetic microspheres (diameter, 1 μm) for use as secondary reagents. |
| EasySep™, StemSep™ | StemCell Technologies | Tetrameric antibody complexes bound to colloidal magnetic dextran iron, which can be retained in a magnetic column in a one-step negative-selection procedure. |

**Table 3**
**Commercial Erythrocyte Lysis Reagents Without Fixative**

| Product | Supplier | Stated components/comments |
|---|---|---|
| EasyLyse™ | Dako (Glostrup, Denmark) | Ammonium chloride-based |
| Erythrocyte lysis solution | Genial Genetic Solutions Ltd (Rincorn, Cheshire, UK) | Not disclosed |
| Human erythrocyte lysing kit | R&D Systems, Inc. | Not disclosed |
| IQ Lyse™ | IQ Products | Not disclosed |
| Lysing buffer | Immunicon Corporation (Huntingdon Valley, PA) | Ammonium oxalate, pH 7.75, with additives |
| BD PharmLyse™ | BD Biosciences Pharmingen | Ammonium chloride-based |
| Quicklysis | Cytognos (Salamanca, Spain) | Not disclosed |
| Red blood cell lysing buffer | Sigma-Aldrich | Ammonium chloride, tris-HCl; intended for mouse erythrocytes |
| VitaLyse™ | BioE Corporation | Not disclosed, subsequent use of fixative optional |

#### 3.1.2.8. Distilled Water Lysis of Erythrocytes

1. Pipet 1 mL of anticoagulated blood into 9 mL of de-ionized water at room temperature.
2. After approx 15–30 s, add 10 mL of a double-strength medium (e.g., HBSS [without phenol red indicator] buffered with 10 mmol/L HEPES, pH 7.3, to restore the tonicity.
3. Centrifuge at 400*g* for 5 min at room temperature to collect the leukocytes. Resuspend the pellet in 1 mL of medium with 10% FBS.

#### 3.1.2.9. Ammonium Chloride Lysis of Erythrocytes

1. Gently mix 1 mL of anticoagulated blood with 15 mL of lysing solution* at room temperature (or at 37°C) for 3–5 min until the originally turbid suspension has become clear.
2. Collect the leukocytes by centrifuging at 400*g* for 5 min, resuspend the pellet, and wash the cells twice by centrifuging in 15 mL of HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3, containing 0.5% BSA (HHBSS-BSA) at 400*g* for 5 min.
3. Resuspend the leukocytes in 1 mL of HHBSS-BSA.

*The lysing solution is 154 mmol/L $NH_4Cl$, 10 mmol/L $NaHCO_3$, and 0.1 mmol/L EDTA, adjusted to pH 7.3 with a small amount of strong HCl or NaOH. It should be prepared daily because absorption of atmospheric $CO_2$ produces ammonium carbonate, which will not lyse erythrocytes.

#### 3.1.2.10. Saponin Lysis of Erythrocytes

1. Mix 1 mL of anticoagulated blood thoroughly with 2 mL of a 1% solution of saponin (Sigma-Aldrich) in a centrifuge tube on ice. Leave at 4°C.
2. After 5 s, add 10 mL of HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3), containing 0.5% BSA (HHBSS-BSA).
3. Cap the tube and invert it intermittently over the next 30–40 s until the suspension that was originally turbid has become clear.
4. Collect the leukocytes by centrifuging at 400*g* for 5 min at room temperature and resuspend them in 1 mL of HHBSS-BSA.

### *3.1.3. Lysed Whole-Blood Procedures*

For clinical investigations, it is often important to preserve the distribution of leukocyte that was present in blood in vivo as well as to determine their reactivity with an antibody or antibodies. For this purpose, leukocytes can be immunostained in unfractionated anticoagulated blood, the erythrocytes lysed, and the leukocytes then fixed. Once samples have been prepared in this way, they can be analyzed straight away or, if necessary, kept in the dark at 4°C and analyzed a few days later. One problem with keeping cells in formaldehyde is that their membranes become increasingly permeable, and if antibodies that were used for surface labeling are still present, they can bind specifically or nonspecifically to internal components. Reagent kits to facilitate processing samples are available from several suppliers; some examples are listed in **Table 4**. Some kits just require the reagents to be added sequentially to the labeled sample and are amenable to automation (e.g., the TQ-PREP™ system; Beckman Coulter, Fullerton, CA), whereas others require the cells to be washed and are more suited to manual processing unless a sample preparation system (e.g., the CellPrep system; Beckman Coulter) is available. Because the erythrocyte lysing reagents in these kits can have quite different effects on the forward light scatter and side light scatter profiles (**Fig. 7**) and on immunophenotyping results, some kits may be more suitable than others for certain applications ***(51)***.

### *3.1.4. Lysed Whole-Blood Procedure with Direct Immunostaining*

1. Incubate 100 µL samples of anticoagulated blood with 20 µL (or the supplier's recommended volume) of fluorochrome-conjugated–specific or isotype control antibody for 10 min at 4°C.
2. Lyse the erythrocytes and fix the leukocytes by processing the stained blood, using one of the reagent sets listed in **Table 4** in accordance with the supplier's recommendations.
3. Examine the cells by flow cytometry and display the events in a plot of side light scatter (ordinate: linear scale) versus forward light scatter (abscissa: linear scale).
4. Set a gate around the leukocytes of interest and display the fluorescence associated with these events as a single-parameter histogram of number (ordinate: linear scale)

**Table 4**
**Some Commercially Available Reagent Kits for Erythrocyte Lysis and Leukocyte Fixation**

| Product | Supplier | Stated components/comments | Time (min) |
|---|---|---|---|
| Cal-Lyse | Caltag Laboratories (Invitrogen) | Formaldehyde | 25 (no wash) or 30 (with washing) |
| ErythroLyse | Serotec Ltd. (Raleigh, NB) | Formaldehyde, diethylene glycol | 20 |
| FACS™ Lysing solution | BD Biosciences Pharmingen | Formaldehyde, diethylene glycol, buffer | 30 |
| Human erythrocyte lysing kit | R&D Systems, Inc. | Not disclosed | 20 |
| Immuno-Lyse® | Beckman Coulter (Fullerton, CA) | Not disclosed | Approx 10 |
| Immuno-Prep® | Beckman Coulter | Formic acid, buffer, formaldehyde | 1.5 |
| OptiLyse C | Beckman Coulter | Formaldehyde, diethylene glycol, buffer | 15 |
| Q-lyse™ | BioE Corporation | One-step, no wash procedure, lysing agent (not disclosed), and fixative (formaldehyde) | 30 |
| Uti-Lyse™ | Dako | Formaldehyde, buffer | 20 |

versus fluorescence intensity (abscissa: logarithmic scale) (**Fig. 7**). Use the fluorescence level of the isotype control antibody (and or that associated with any internal negative control cells) to set the cursor so that no more than 1% of these events would be considered positive.

### *3.2. Preparation of Cell Suspensions from Solid Tissues and Cell Cultures*

The usual aim when preparing cell suspensions from tissues or cultures is to obtain a good recovery of all of the cell types present while preserving the plasma membrane and intracellular components. It is relatively easy to produce cell suspensions from monolayer cultures using the same procedures that are used for resuspending cells when they are being passaged. It is more difficult, however, to produce them from other tissues, because the tissue must first be disrupted by mechanical means and/or by enzymic digestion. Gentle mechanical disaggregation (e.g., by grinding between the frosted ends of two microscope slides or by forcing through stainless steel mesh) is sufficient to release lymphocytes from lymphoid tissue such as lymph nodes, spleen, and thymus. However, it damages a proportion of the larger blast cells and leaves a proportion of several

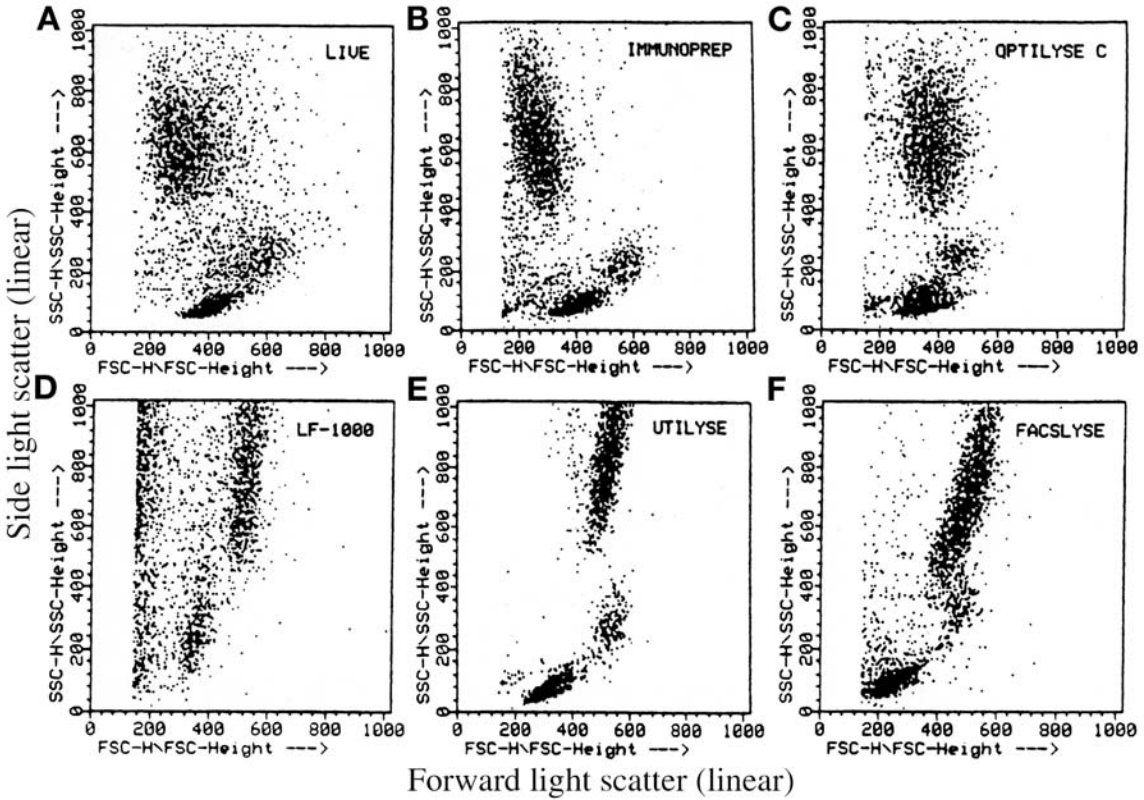


**Fig. 7.** Forward light scatter and side light scatter profiles from the peripheral blood leukocytes of a healthy control subject. **(A)** Leukocytes analyzed by the live whole-blood procedure. The histogram is produced by gating on a region similar to that denoted R1 in **Figure 1B**. Distributions obtained when cells were prepared using the **(B)** ImmunoPrep® (Beckman Coulter), **(C)** OptiLyse C (Beckman Coulter), **(D)** LF-1000 Lyse and Flow (Harlan Sera-Lab), **(E)** Utilyse™ (Dako), and **(F)** FACS™ Lysing Solution (BD Biosciences) reagents. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc., from **ref. *23***.

cell types, such as dendritic cells, in the debris; consequently, such cells are best obtained by a combination of gentle mechanical and enzymic digestion. Greater mechanical force (e.g., mincing with a scalpel blade and/or grinding in a Dounce homogenizer (Blasting Glass Co., Rochester, NY), or forcing through a fine-bore syringe needle) is usually needed together with digestion by collagenase, neutral protease, or pepsin to release cells from other solid tissues. Unfortunately, if proteases are used, there is always the possibility that they may cleave and affect the antigenicity of some of the cell surface glycoproteins, and strong mechanical disruption usually results in cell suspensions with lower viability than does enzymic digestion. Samples that contain a high number of dead cells tend also to contain DNA in solution, which if not removed by treatment with DNAase (approx 10 U/mL in a $Mg^{2+}$-containing buffer), will bind to the cells and lead to clumping. Irrespective of how a cell suspension has been obtained, it should be checked microscopically to confirm that the cells are representative of the original tissue. The suspension must also be filtered through nylon mesh or single cells must be recovered by density gradient centrifugation (e.g., on Ficoll®–Hypaque) to avoid blocking the flow cell during cytometry. If solid tissue samples are to be analyzed frequently, it may be worth purchasing a commercially available (e.g., Medimachine System; BD Biosciences, San Jose, CA) mechanical tissue disaggregation system that is designed specifically for preparing samples for flow cytometry.

### *3.2.1. Preparing a Suspension of Small Lymphocytes from Lymphoid Tissue (e.g., Lymph Nodes, Spleen, or Thymus)*

1. Tease the tissue apart in 10 mL of RPMI-1640 medium at 4°C using blunt instruments and then squash between the frosted ends of two microscope slides or with the rubber end of a plastic syringe plunger to release the cells.
2. Strain the suspension through nylon mesh (e.g., Falcon 2350 (Becton Dickinson) or CellMicroSieve [Bio Design, Carmel, NY]) to remove clumps and debris.
3. Collect the cells by centrifuging at 400*g* for 5 min and discard the supernatant fluid.
4. Resuspend cells in a known volume of medium. If spleen has been used, the erythrocytes can be lysed at this time. Determine the cell (leukocyte) concentration by using a hemocytometer.
5. Wash the cells by centrifuging the suspension again, discard the supernatant fluid, and resuspend the cells in fresh medium at a concentration of $1 \times 10^7$/mL.

### *3.2.2. Preparing a Cell Suspension from Solid Tissues*

1. Collect tissue into 10 mL of RPMI-1640 medium at 4°C and keep on ice until used no more than 6 h later.
2. Place approx 100 mg in the base of a Petri dish, cover with 5 mL of RPMI medium at 4°C, cut into small (1-$mm^3$) pieces with a McIlwain tissue chopper (Mickle

Laboratory Enginnering, Gomshall, Survey, UK) or a scalpel blade (e.g., no. 20), and scrape the cells from the tissue surfaces with the scalpel blade.
3. Disperse the tissue fragments in the medium by pipetting up and down using a plastic pipet (e.g., Pastette) and collect the cells and tissue fragments by centrifuging.
4. Resuspend the pellet in 1 mL of RPMI-1640 containing collagenase type II (0.5 mg/mL), trypsin or dispase (2.5 mg/mL), and DNAase I (20 g/mL) ***(52)***. Incubate at 37°C with intermittent agitation for 1 h. Add 2 mL of FBS to stop further proteolysis.
5. Let the larger fragments settle. Filter the supernatant through a nylon mesh with a pore size of 35–100 µm diameter (e.g., BioDesign "CellMicroSieve").
6. Wash the cells twice by centrifuging at 400*g* for 5–10 min in approx 15 mL of medium containing 10% FBS and resuspend in 1 mL of medium*.

### *3.2.3. Preparing a Cell Suspension From Anchorage-Dependent Cells*

1. Remove the culture medium and wash the monolayer with PBS without $Ca^{2+}$ and $Mg^{2+}$.
2. Add a small volume of 0.1% trypsin and 0.2% EDTA in PBS and resuspend the cells gently as for passaging.
3. Wash the cells by centrifuging at 400*g* for 5–10 min in approx 15 mL of PBS containing 10% FBS to stop further protease action.
4. Resuspend the pellet in 1 mL of medium.

## 4. Fixation: Commonly Used Fixatives and Their Effects

Two main types of fixative are used in flow cytometry: alcohols and acetone (which are precipitating or coagulative fixatives) and formaldehyde (which is a crosslinking fixative). Unfortunately, glutaraldehyde, a dialdehyde that is used so successfully in electron microscopy, is unsuitable for flow cytometric applications because it causes a high background autofluorescence.

### *4.1. Alcohols and Acetone*

Alcohols and acetone precipitate proteins but not nucleic acids or carbohydrates, which will leach out of the cells and at the same time solubilize membrane lipids so that the cells and organelles are permeabilized and cell ultrastructure is poorly preserved. The precipitation of intracellular proteins results in an increase in light scattering, which can help to discriminate cell types by this criterion. Methanol is a better fixative than ethanol for most purposes and can be used satisfactorily for blood, bone marrow, and cell suspensions made from tissues. A disadvantage of these fixatives is that they cause cells to aggregate and to stick to the sides of plastic (polystyrene rather than polypropylene) tubes; moreover, certain plastics may dissolve in these solvents.

*The amount of tissue and the enzyme cocktail used may need to be varied to optimize the yield of cells from different tissues.

### 4.2. Formaldehyde

Formaldehyde ($CH_2O$) is a water-soluble gas at room temperature which reacts rapidly with most biological macromolecules to yield covalent adducts (hemiacetals); however, the free amino groups on protein amino acids and nucleic acid bases are probably the most important targets ***(53)***. Where hemiacetal derivatives have been formed close together, slow condensation reactions create methylene bridges between them that crosslink the macromolecules. Formaldehyde is also a good fixative for lipids but not carbohydrates; however, because of its reactions with proteins and nucleic acids, it often adversely affects antigenicity and interferes with DNA staining and also enhances the autofluorescence of cells. Solutions containing formaldehyde for use in flow cytometry can be prepared from the grade that is supplied for electron microscopy and that is free of additives. Alternatively, they can be made by dissolving paraformaldehyde (a polymerized form of formaldehyde which is solid at room temperature) in water or aqueous solutions at 60°C for several hours. In aqueous solutions, formaldehyde gradually forms polymers, so solutions should be prepared fresh daily or (if necessary) kept at room temperature and used within a few days. Cells to be fixed with formaldehyde preferably should be washed free of protein and fixed at 37°C or room temperature rather than at 4°C. If the purpose of fixation is just to inhibit metabolic or energy-dependent changes, cells can be fixed with <1% formaldehyde for 10 min and washed by centrifugating in PBS or a balanced salt solution. Brief fixation with low concentrations of formaldehyde (e.g., 0.2% for 4 min at 37°C) causes cells to become permeable to small molecules but not to antibodies ***(15)***. Longer fixation with higher concentrations of formaldehyde causes blebbing of the plasma membrane, which also becomes permeable to antibodies. When immunolabeling cells that have been fixed with formaldehyde, any free formaldehyde must be removed by washing and free aldehyde groups quenched (e.g., by reaction with glycine) before adding the antibody, or high levels of nonspecific binding are likely to occur. After cells have been immunostained with a fluorochrome-conjugated antibody, they can be preserved for subsequent analysis if they are washed free of extraneous protein and stored in 1% formaldehyde in PBS. In time, crosslinks will form between adjacent macromolecules, preventing loss of the antibody and, hence, of the covalently bound fluorescent label. Fixative solutions containing formaldehyde are often available commercially as separate reagents within erythrocyte lysis and leukocyte fixation kits (**Table 4**) or may be obtained separately (e.g., StabilCyte™; BioE, St Paul, MN).

## 5. Permeabilization and the Detection of Intracellular Components

It is sometimes necessary to detect or quantify intracellular components, including DNA sequences, enzymes (e.g., nuclear terminal deoxynucleotidyl

transferase), glycoproteins destined for display on the cell surface or secretion (e.g., cytokines), regulatory and transcription factors (e.g., cyclins and p53), and structural components (e.g., filamentous actin). If internal components are to be detected by immunolabeling, the plasma (and perhaps organelle) membranes must be permeabilized prior to or during staining so that antibodies are allowed to enter the cell. At the same time, the antigen must be retained in the cell and both its antigenicity and the light scattering characteristics of the cell must be preserved. Generally, cells are fixed with formaldehyde, which will stabilize the membrane and increase its permeability, and are further permeabilized with another agent(s). It is necessary to quench excess formaldehyde (e.g., with glycine) and to minimize nonspecific binding to internal antigens by incorporating BSA or reconstituted nonfat dried milk powder in the medium. A coagulative fixative such as methanol may also be used if the cells are to be stained for nucleic acids.

Commonly used permeabilizing agents include saponin and the nonionic detergents. Saponins have large hydrophobic regions that insert into cholesterol-containing membranes to form complexes with 12- to 15-nm-diameter holes that enable macromolecules to enter the cytoplasm. The plasma membrane is left otherwise relatively intact, but because pore formation is reversible and saponin-treated cells are only permeable to antibodies that have previously bound saponin, saponin must be included in the antibody-containing solutions when labeling. Saponin is incapable of permeabilizing those membranes that lack cholesterol (e.g., the inner nuclear membrane and mitochondrial membranes). The nonionic detergents such as Nonidet (NP40), Tween® 20, and Triton® X-100 are weak protein denaturants that insert into plasma and organelle membranes and solubilize both the lipids and the transmembrane proteins. At low concentrations (0.01–0.1%), they permeabilize cells but at higher concentrations can cause lysis and loss of the cytoplasmic contents. Unfortunately, no single permeabilization procedure is universally applicable and high levels of nonspecific intracellular staining frequently occur. The best technique to use for a particular antigen, including the optimum fixative/detergent concentration, exposure time, and temperature, may have to be determined by comparative experiments ***(54,55)***. There are a number of excellent detailed accounts of strategies and methods for detecting intracellular components which should be consulted before attempting to design and evaluate a procedure ***(56–59)***. A list of some commercially available reagent kits for permeabilizing leukocytes is given in **Table 5**.

### *5.1. Detection of Leukocyte Cell Surface and Intracellular Antigens After Fixation and Permeabilization*

1. Incubate samples containing approx $2 \times 10^6$ leukocytes in 100 µL of HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3, containing

**Table 5**
**Commercial Permeabilization Reagents**

| Product | Supplier | Comments | Time (min)[a] |
|---|---|---|---|
| BD Perm/Wash™ | BD Biosciences Pharmingen | Saponin-containing buffer for immunolabeling. | Approx 60 |
| Cytodetect kit | IQ Products (Groningen, The Netherlands) | Intended for the detection of intracellular. cytokines: includes monensin for blocking export via the Golgi), fixative, permeabilizing reagent. | Approx 600 (including time for cell stimulation) |
| Cytofix™/Cytoperm™ | BD Biosciences Pharmingen | Formaldehyde-containing fixation buffer and permeabilizing agent for use in the detection of intracellular antigens. | 60 |
| Cytofix/Cytoperm™ Plus | BD Biosciences Pharmingen | Fixation-permeabilization kit including BD GolgiPlug™. GolgiStop™ for blocking export via the Golgi. | Approx 600 (including time for cell stimulation) |
| FACS™ Lysing Solution | BD Biosciences Pharmingen | Although not intended for the detection of intracellular antigens it can be used for this purpose. | 60 |
| FACS™ Permeabilizing Solution | BD Biosciences Pharmingen | Contains formaldehyde and diethylene glycol. | 50 |
| Fix & Perm® | Caltag Laboratories (Invitrogen) | Components not disclosed but described as FIXation and PERMeabilization media; a modification incorporating methanol is recommended for cell cycle antigens when using FITC but not PE antibodies. | 40 |

| | | | |
|---|---|---|---|
| IntraPrep™ Permeabilization Reagent | Beckman Coulter | Formaldehyde and saponin. | 45 |
| IntraStain | Dako | Reagent A (fixation) formaldehyde, Reagent B (permeabilization) not disclosed. | 40 |
| IntraStainCell | Bender MedSystems | Four reagents including monensin for blocking export via the Golgi, fixative, permeabilizing reagent and resuspension buffer. | 70–85 (not including time for cell stimulation) |
| IQ Starfiqs™ | IQ Products | Reagent F (fixation) formaldehyde. Reagent P (permeabilization) saponin. | 45 |
| Leucoperm™ | Serotec Ltd. (Ruleigh, NC) | Not disclosed. | 60 |
| PermaCyte™ | BioE Corporation | Not disclosed. | 95 |
| Permeafix | Ortho Diagnostic Systems (Raritan, NJ) | Not disclosed. | 105 |

[a]Time for permeabilization and intracellular staining, to which must be added the time taken for surface antigen staining if required.

5% FBS (HHBSS-FBS) and 0.1% sodium azide with 20 µL (or the supplier's recommended volume) of a fluorochrome-labeled cell surface–specific or isotype control antibody for 10 min at 4°C.
2. Wash the cells by centrifuging at 400*g* for 5 min with 4 mL of HHBSS-FBS. Resuspend the pellet in 200 µL of HHBSS containing 4% formaldehyde. Fix for 20 min at room temperature.
3. Wash the cells with 4 mL of HHBSS-FBS.
4. Resuspend in 100 µL of HHBSS-FBS and 0.1% saponin. Add the appropriate volume of the second fluorochrome-labeled intracellular antigen–specific or isotype control antibody. Incubate for 30–60 min at 4°C.
5. Wash the cells by suspending in HHBSS-FBS and 0.1% saponin and centrifuging at 400*g* for 5 min, but allow adequate time for any unbound second antibody to diffuse out.
6. Resuspend the pellet in HHBSS-FBS. Analyze by flow cytometry.

## 6. Immunolabeling

### *6.1. Antibodies*

Antibodies or Igs comprise light (κ or λ) and heavy (α, δ, ε, γ, or µ) chains, which define the class or isotype (IgA, IgD, IgE, IgG, or IgM) and subclass (e.g., $IgG_1$ to $IgG_4$) of the antibody. The structure of an antibody can be exemplified by IgG, which has two identical light chains and two identical heavy chains with disulphide bonds linking the heavy and light chains and the two heavy chains (**Fig. 8**). Each IgG molecule thus has two identical antigen-combining sites that are created by the N-terminal (variable) domains of each pair of heavy and light chains. These antibody-combining sites are preserved in the monomeric F(ab′) or dimeric $F(ab')_2$ fragments that can be produced by digesting IgG with papain or pepsin, respectively. The remainder of the molecule (the Fc portion), which comprises the second and third constant domains of the heavy chains, is responsible for binding to Fc receptors on cells (**Fig. 8**). Immunologic specificity arises because antibodies recognize specifically (by means of their antibody-combining sites, or paratopes) only relatively small structural features of an antigen, which are known as epitopes.

Either polyclonal antisera or mAbs can be used for immunolabeling. Polyclonal antisera contain a mixture of antibodies, which will react with different epitopes on the antigen and are subject to a degree of natural variation. In contrast, mAbs, which are produced by a single B-cell clone, are chemically identical and react with just a single epitope on the antigen. Those mAbs that react with one particular human leukocyte surface antigen are assigned to the same cluster of differentiation (CD), which is designated by a number (e.g., CD3). Even though mAbs might belong to the same CD, unless they are from the same clone, they may recognize different epitopes on the antigen, and their isotype, binding affinity, and propensity to bind nonspecifically to cells may also differ. It is generally preferable to use mAbs (if they are available) rather than polyclonal antisera

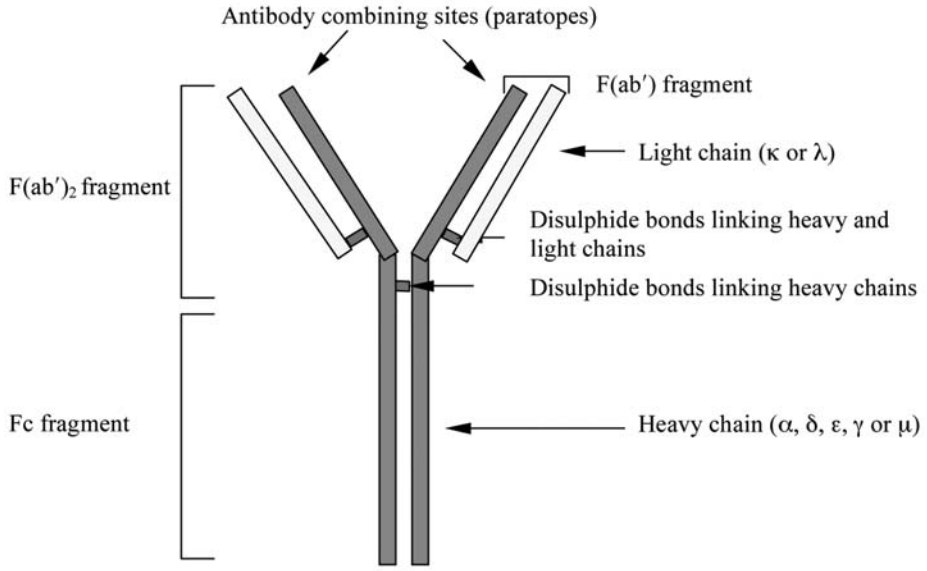


**Fig. 8.** Schematic diagram of an antibody molecule, showing regions corresponding to $F(ab')_2$ and Fc fragments that would be produced by proteolytic cleavage and the interchain disulphide bonds that help to stabilize the structure.

because mAbs are more consistent reagents and are less likely to give nonspecific background staining by crossreacting with irrelevant antigens. Unlabeled antibodies dissolved in aqueous solvents retain their reactivity for decades if kept frozen at –20°C and for several years if kept with a preservative at 4°C, but they should not be repeatedly frozen and thawed. Fluorochrome-labeled antibodies are considerably less stable; they are best kept in the dark at 4°C and used before their expiration date if they have been obtained commercially. It is good practice to monitor fluorochrome-conjugated antibodies for the presence of Ig aggregates (e.g., by flow cytometry) and, if necessary, to remove any aggregates by centrifuging before use.

### *6.2. Antibody–Antigen Interactions*

Antibody–antigen interactions are noncovalent, are potentially reversible, and take a finite time to reach equilibrium. For a typical mAb of moderate affinity (e.g., $10^8$–$10^9$ L/mol) with kinetic association ($k_1$) and dissociation ($k_2$) rate constants of $2 \times 10^6$/s and $4 \times 10^{-3}$/s, respectively, Karlsson and Roos ***(60)*** have calculated that the binding would be 98.3% complete in 8.3 min. A value that agrees with empirical (unpublished) observations that near maximum staining of leukocytes is obtained within approx 5 min when using commercially available

fluorochrome-conjugated mAbs in accordance with the supplier's recommendations. Thus, a staining time of approx 10 min should be suitable for most cell surface antigens when using antibodies at the recommended dilution, although longer incubations (30–60 min) will be needed when staining intracellular antigens. It is usually unnecessary to wash the cells after direct immunostaining, because unbound antibody in solution will not contribute significantly to the fluorescence signal coming from the cells ***(61)***. However, if cells are washed after immunostaining, it is prudent to analyze them promptly because some previously bound antibody will dissociate as a new equilibrium is established after washing.

### *6.3. Antibody Titration*

To maximize the ratio of specific-to-nonspecific (signal-to-noise) antibody binding, antibodies for use in flow cytometry are titrated to determine the concentration that will saturate all of the available antigen sites yet give the least nonspecific staining. Commercial suppliers of antibodies to human leukocyte antigens usually do this in advance and, in their product data sheets, provide recommendations regarding the concentration to use. In practice, the recommended concentrations usually provide for an antibody excess that is twofold to fivefold over the level to achieve saturation. However, if antibodies have been obtained from other sources or no recommendation is available, it may be necessary to titrate them to ensure that an appropriate amount is used in later studies. The simplest procedure for titrating a fluorochrome-labeled antibody is to produce a doubling dilution series of the antibody and to mix a set volume (e.g., 20 µL) of each antibody concentration with a set number of cells (e.g., $5 \times 10^5$ cells) in a set volume (e.g., 100 µL) of medium. The samples are analyzed by flow cytometry and the mean or median fluorescence intensity (MFI) is determined for the positively stained and unstained cells at each antibody concentration. The MFI values for both populations are then plotted graphically against antibody concentration by means of logarithmic scales. With increasing antibody concentration, the MFI of the positively stained cells increases markedly until it reaches a plateau, but around this antibody concentration, the MFI of the unstained population will also be seen to increase. The optimum antibody concentration is one that gives the maximum difference in MFI between the two cell populations. It will vary according to the protocol being used, but in most instances it will be one or two concentration steps higher than that which just gives the plateau value for the positively stained cells (**Fig. 9**). When judging what is an appropriate amount of antibody to use, it must be remembered that the effective concentration of some antibodies can be decreased by binding to a soluble form of the antigen in the sample. For instance, some cell surface antigens can also be shed (e.g., CD8, CD25, and CD62L) or secreted (e.g., IgM on B cells) and it may be necessary to wash the

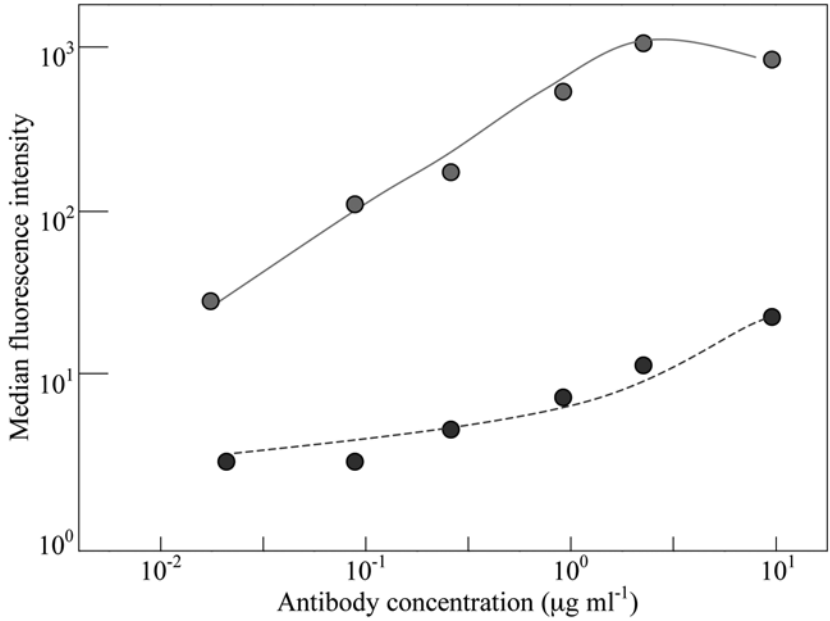


**Fig. 9.** Titration of a fluorochrome-labeled antibody. Relative fluorescence intensity (logarithmic scale) of the positively stained (solid line) and unstained (dotted line) cell populations is plotted against antibody concentration (logarithmic scale). The optimum antibody concentrations for staining is that which gives the highest signal-to-noise ratio (approx 1–5 μg/mL in this example).

cells before immunolabeling. It may also be necessary to include 0.1% sodium azide in the medium during and after immunolabeling to inhibit the internalization of antigens.

### *6.4. Sensitivity of Detection and the Measurement of Cell Surface Antigens*

Currently, the sensitivity of most commercial cytometers theoretically allows them to detect approx $10^2$–$10^3$ molecules (or approx $10^{-18}$ g) of a fluorochrome on a fluorochrome-labeled cell. However, the sensitivity with which antigens can be detected by immunofluorescence is somewhat lower than this because it is affected by the intensity of the fluorescence emitted by the fluorochrome-labeled antibody (*see* **Chapter 3**) and by the extent of nonspecific binding, which will increase with antibody (and hence fluorochrome) concentration. A number of cell components will bind antibodies nonspecifically and with low affinity by a variety of mechanisms. Cell surface Fc receptors will bind different classes of antibody, and especially aggregates formed during manufacture or

storage with high affinity. Although it is possible to remove antibody aggregates by brief ultracentrifugation (e.g., 10 min in a benchtop microfuge), this is not recommended for IgM antibodies or PE-conjugated antibodies, because their large size means that significant amounts of unaggregated antibody would also be lost from solution. Nonspecific and Fc-mediated antibody binding can be greatly diminished by pretreating cells with unlabeled normal (control) Ig or serum from the same species as the antibodies being used for immunolabeling. For example, approx $10^6$ cells can be incubated with 10 µg of purified normal IgG for 10 min at 4°C or resuspended in 2–5% normal serum before adding the specific antibody. In experimental studies in which Ig or serum cannot be used, 0.5–5% BSA can be used instead to reduce nonspecific binding but it will not, of course, block Fc receptors. Dead cells bind high levels of antibody nonspecifically, but if an appropriate fluorescence channel is available, they can be excluded from the analysis on the basis of their permeability to propidium iodide (5 ng/mL) or 7-aminoactinomycin D.

It would be extremely useful if immunolabeling in conjunction with flow cytometry could report the absolute number of a particular molecule (antigen) on the surface of a cell. Unfortunately, this is not possible at present; first, there are too many unknown factors in the binding of antibodies to cell surface antigens ***(62,63)***, and second, there are a number of problems in quantifying the fluorescence intensity from cells. The nature of antibody binding under experimental circumstances is unknown because the majority of mAbs used in cytometry are IgGs and can bind, therefore, in a monovalent or bivalent manner; if the latter happens, they will do so with up to 100-fold greater affinity than their corresponding monovalent F(ab′) fragments. It might be assumed that when fully saturating concentrations of antibody are used there will be only monovalent binding, but the true ratio of antigen to antibody is not usually known. Moreover, an antibody like fluorochrome-labeled IgG cannot easily be used to count cell surface molecules; for most practical purposes, the antibody concentrations required to achieve fully saturated monovalent binding are experimentally unattainable (approx 100 µg/mL) and would result in unacceptably high "background" signals. The use of F(ab′) fragments can overcome the problem of not knowing whether binding is in a monovalent or bivalent manner, but their lower affinity is accompanied by a faster dissociation rate, which can lead to appreciable loss during washing and secondary antibody staining. Further complications are whether the antigen can exist as multimers as well as monomers and whether it can diffuse in the membrane to facilitate bivalent interactions with the antibody ***(64–67)***. The problems associated with quantifying immunofluorescence in an instrument-independent manner that would allow interlaboratory comparisons are not as great, and a number of attempts have been made to solve them ***(68)***. Quantitative estimates of immunofluorescence intensity are commonly made in the following

two ways. In the first method, the cytometer is calibrated before use with beads containing different known amounts of fluorochrome. The logarithmic values for mean or median channel numbers for immunofluorescence are then converted to an equivalent number of molecules of soluble fluorochrome (MESFs) by interpolation on a calibration curve constructed from the relative fluorescence of calibration beads and data concerning their fluorochrome content provided by the supplier. In the second method, cell immunofluorescence is monitored as before and converted to units of antibody-binding capacity (ABC) by interpolation with a calibration curve of the fluorescence emitted from microbead populations (e.g., Quantum Simply Cellular®; Flow Cytometry Standards Corporation, now part of Bangs Laboratories, Inc., Fishers, IN) that have bound different but defined numbers of antibody molecules under similar conditions. A number of factors, including staining (direct versus indirect) technique, affect the results of ABC assays, so it has been recommended that direct staining with FITC-conjugated antibodies be used as the "benchmark" for this procedure ***(69)***. Clearly, if the number of molecules of equivalent soluble fluorochrome per antibody molecule (akin but not identical to the fluorochrome/protein ratio) is known, it is possible to convert MESF values to ABC units.

### *6.5. Direct and Indirect Immunostaining*

Cells can be labeled either directly or indirectly with antibodies linked to a fluorescent reporter molecule. In direct immunolabeling, samples are incubated with a specific antibody that has been coupled covalently to a fluorochrome; unbound antibody does not usually need to be removed for flow cytometry but can be removed by washing if preferred. It is necessary, however, to include a negative control that is a nonspecific, or irrelevant, antibody (e.g., to keyhole limpet hemocyanin) of the same isotype as the specific antibody being used and that has been labeled with the same fluorochrome. In practice, samples are often labeled simultaneously using a premixed "cocktail" of two or more antibodies that have been conjugated to different fluorochromes (multicolor analysis), and both single-color and multicolor isotype controls should be included for setting the compensation for spectral overlap between fluorescence channels. Because there is the potential for interactions between reagents when samples are labeled simultaneously with different antibodies, it is also advisable to check that similar results are obtained and cells are labeled sequentially with the same reagents. Premixed, two-, three-, and four-color cocktails (e.g., BD Biosciences Simultest™, TriTEST™, and MultiTEST™ reagents, respectively) containing antibodies to many commonly analyzed combinations of lymphocyte antigens (e.g., CD3 and CD4; or CD3, CD4, and CD45; or CD3, CD4, CD8, and CD45 and their corresponding isotype control antibodies) are now available commercially. Protocols for direct immunolabeling can be found above (*see* **Subheadings 3.1.1.1.** and **3.1.4.**). Indirect

immunolabeling is a more protracted technique because the sample must first be incubated with an unlabeled specific primary antibody, washed, and then incubated with a fluorochrome-labeled secondary antibody specific to the Igs of the species from which the primary antibody was obtained. The negative control sample is labeled with an unconjugated isotype-matched nonspecific primary antibody revealed with the same fluorochrome-conjugated secondary antibody. In the indirect immunolabeling procedure, it is also possible to use a biotinylated primary antibody so that it can be revealed by a fluorochrome (e.g., PE) conjugated to avidin or streptavidin (*see* **Chapter 3**). Indeed, it is claimed that as few as 50 antigenic sites per cell can be detected by a triple-staining variant of this system, the "super avidin-biotin system," in which an anti-streptavidin antibody is used to bridge two layers of PE-streptavidin ***(70)***.

However, because biotin may be present in serum, it may be necessary when using this procedure to wash cells free of serum and to maintain them in a medium containing BSA instead of serum, to avoid adventitious binding to endogenous biotin. If problems resulting from high nonspecific binding are encountered, they can be decreased by blocking nonspecific antibody binding sites with Ig or serum from an animal species other than that from which the primary antibody was obtained; by blocking Fc receptors with normal serum from the host species of the secondary antibody or by using a fluorochrome-conjugated $F(ab')_2$ fragment of an affinity-purified secondary antibody; It is possible to label samples using mouse mAbs and a combination of the indirect and direct procedures; however, if this is done, the indirect procedure must be completed first and any unoccupied anti-mouse Ig-binding sites must be blocked with unlabeled normal mouse IgG before direct immunolabeling is attempted (**Fig. 10**).

In summary, direct immunolabeling is a simpler, quicker, and less error-prone technique than indirect immunolabeling because it requires only that the sample and antibody be mixed in the correct proportions. Its disadvantage is that each specific primary antibody needs to be available in a fluorescent-labeled form, which is considerably more expensive than using an unlabeled antibody (or antibodies). Although indirect immunolabeling requires more manipulations and takes longer, it results in amplification (typically approx fivefold) of the fluorescence signal because several fluorochrome-conjugated secondary antibody molecules can bind to a single primary antibody molecule. Secondary antibodies labeled with a variety of fluorochromes are readily available commercially and, because they are produced in large amounts, are relatively inexpensive.

### 6.5.1. Indirect Immunostaining of Live Whole Blood

1. Incubate 100 µL of anticoagulated blood with 20 µL of unlabeled specific or isotype control antibody* for 10 min at 4°C.

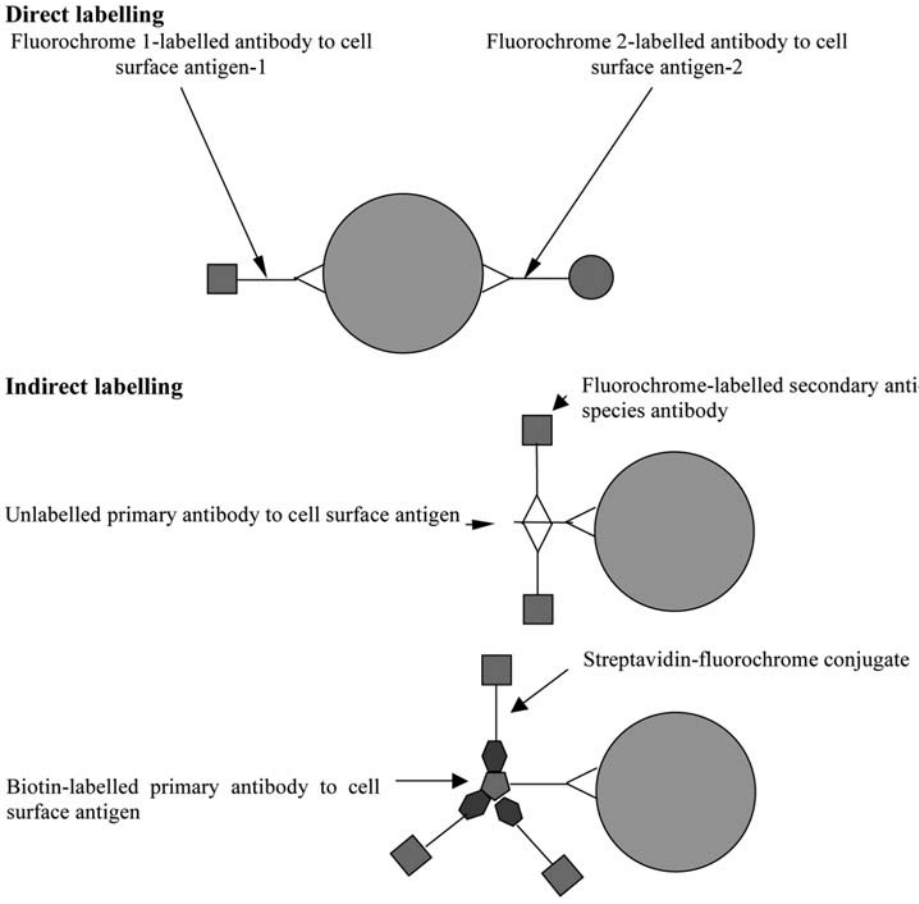


**Fig. 10.** Direct and indirect immunostaining. **(Upper diagram)** In the direct labeling procedure, two different antibodies each conjugated to a different fluorochrome (1 and 2) have been used to simultaneously recognize two different antigens (1 and 2). **(Middle diagram)** In an example of the indirect labeling procedure, an unlabeled (primary) antibody (e.g., a mouse mAb) that has bound to a cell surface antigen has been subsequently revealed by a fluorochrome-conjugated (secondary) antibody specific for the Igs of the species from which the primary antibody was obtained (e.g., a FITC-conjugated goat anti-mouse Ig antibody). **(Lower diagram)** Also in the indirect labeling procedure, a biotin-labeled (primary) antibody (e.g., a mouse mAb) that has bound to a cell surface antigen has been subsequently revealed by a streptavidin-fluorochrome conjugate.

2. Wash the cells twice by centrifuging with 4 mL of HBSS (without phenol red indicator) buffered with 10 mmol/L HEPES, pH 7.3, containing 1.0% BSA (HHBSS-BSA) at 400*g* for 5 min at 4*°C.

3. Resuspend the pellet in 100 µL of HHBSS-BSA, add 30 µg per $10^6$ cells of normal unlabeled Ig (blocking antibody) from the same species as the secondary antibody that is to be used, and incubate for 10 min at 4°C.
4. Add 20 µL of fluorochrome-conjugated secondary (anti-species) antibody and incubate for 10 min at 4°C. (The optimum concentrations of antibody must be determined by prior titration unless specified by the supplier.)
5. Wash the cells twice by centrifuging with 4 mL of HHBSS-BSA at 400*g* for 5 min at 4°C and resuspend the cells in 1 mL of HHBSS-BSA containing 0.1% by volume of a saturated solution of LDS-751 in methanol. (A saturated solution of LDS-751 in methanol can be kept almost indefinitely in the dark at 4°C.)
6. Alternatively, the erythrocytes can be lysed and the leukocytes fixed using one of the reagent sets listed in **Table 4** in accordance with the supplier's recommendations.
7. Analyze by flow cytometry using the gating strategy described for the live whole-blood procedure (**Subheading 3.1.1.**) or, if the erythrocytes have been lysed, by gating on the relevant leukocytes in a display of forward and side light scatter.

### 6.6. Determining Absolute Cell Counts

It is sometimes necessary to determine the concentration of a particular cell type in a sample (e.g., the concentration of $CD4^+$ lymphocytes when monitoring the progress of HIV infection) and this can be done in two main ways. First, all of the cells of that type can be counted in a volume of sample that has been set or that has been computed from the flow rate and analysis time (e.g., using the volumetric Ortho CytoronAbsolute). Second, the sample can be mixed with a known concentration (as particles per milliliter) of fluorescent microbeads, cells and beads counted, and the cell concentration computed from knowledge of the bead concentration. Suitable microbeads are available as suspensions (e.g., Flow-Count Beckman Coulter) so that a set volume can be mixed directly with a set volume of the sample ***(71)*** or lyophilized in tubes (e.g., TruCOUNT™; BD Biosciences) so that they are resuspended when a set volume of sample is added to the tube ***(72)***. The "single-platform" bead-based methods require that the sample and beads be thoroughly mixed and the ratio of beads to cells to remain unchanged during data acquisition. Assumptions can now be subjected to internal quality control using the Perfect Count system (Quest Biomedical, Solihull, West Midlands, UK), which comprises two bead populations with different densities to allow inadequate mixing to be detected ***(73)***.

### 6.7. Safety

When preparing clinical samples for flow cytometry, both infectious and chemical hazards will be encountered. The risks associated with each operation must be assessed in advance and appropriate precautions taken to minimize them. The Advisory Committee on Dangerous Pathogens and the Health Services Advisory Committee (in the United Kingdom) and the NCCLS (in the

United States) publish guidelines on these aspects of laboratory safety. To minimize the risk of infection, samples must be collected, handled, and disposed of correctly. The level of containment and personal protection required depends on the nature of the sample and the risk that it poses. Briefly, as a minimum, a Dowsett and Heggie-style laboratory coat and disposable gloves must be worn, together with safety spectacles when there is the possibility of eye contamination through splashing. Samples must be kept in sealed containers (e.g., capped tubes). Pipettors fitted with tip ejectors should be used for transferring liquids. If samples are to be centrifuged, they must be contained in suitably robust capped/sealed tubes placed within sealed centrifuge buckets that will prevent the release of an aerosol if the tube should break. If laboratories process a high number of samples by standard protocols, it may be worth using laboratory automation systems (e.g., the Beckman Coulter CellPrep™ and TQ-Prep™ systems) for as many of the pre-analytical procedures as possible, for reasons both of efficiency and safety. All biohazardous waste, including that from the flow cytometer/sorter, must be disposed of in a safe manner in accordance with local practice; items that are to be re-used should be decontaminated with a suitable disinfectant (e.g., Virkon; Antec International, a subsidiarg of DuPont Animal Health Solutions, Sudburg, Saffolk, UK) and/or by autoclaving. Some of the safety concerns for shared flow cytometry core facilities and the special precautions to be taken when flow-sorting potentially biohazardous samples are discussed by Schmid et al. ***(74,75)***. In the United Kingdom, legislation requires the supplier to provide details of the particular hazards posed by different chemicals and the precautions to be taken when handling them and for their disposal. A more detailed discussion of the safety aspects of sample processing can be found in the books by Owens and Loken ***(76)*** or Valenstein and Collinge ***(77)*** and in the Purdue Cytometry CD-ROM ***(3,4)***.

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# 3

# Fluorochromes and Fluorescence

**Desmond A. McCarthy**

**Summary**

To facilitate flow cytometry, there is a need for cells to be labeled with fluorochromes. However, because of the spectral properties of fluorochromes, the fluorescence associated with one fluorochrome may be detected by more than one detector, producing spectral overlap which requires compensation. However, the number of useful fluorochromes has increased steadily over the years, and more recently there have been significant advances in techniques for compensating for spectral overlap between several fluorescent signals, so it is likely that the number of fluorochromes used in analysis will increase further. The basic principles of how light and matter interact, the nature of fluorescence, and the properties of various fluorochromes that are used for different purposes are described in this chapter. Some examples of the many parameters that can be measured using fluorescence and the fluorescent probes that can be used are also given.

**Key Words:** Fluorescein; phycobiliproteins; quantum dots; tandem dyes.

## 1. Introduction

The absorption of light of one color (or wavelength) by a substance and the subsequent emission of light of a different color, usually of a longer wavelength, is termed fluorescence. Very few cellular components are intrinsically fluorescent, and those that are (e.g., pyridine- and flavin-containing nucleotides) provide only limited information, so cells are usually stained with fluorescent probes (called fluorochromes, fluorophores, or fluors) that are able to reveal the presence of components that would not otherwise be visible ***(1)***. For example, antigens, within or on the surface of cells, can be identified using fluorochrome-labeled antibodies, whereas DNA or intracellular $Ca^{2+}$ concentrations can be quantified using fluorochromes that change their fluorescence properties when bound to particular components. The earliest fluorochromes to

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be used were compounds of relatively low molecular weight such as fluorescein, but since then, many other dyes with different spectral properties, higher quantum yields, and greater stabilities have been developed. Other fluorochromes in current usage are high–molecular weight cyanobacterial or algal proteins (e.g., phycoerythrin [PE]) and the "tandem dyes" that are formed by coupling them covalently to another (usually low–molecular weight) fluorescent dye to produce a substance with novel fluorescence characteristics. It is the combination of fluorescence detection with flow cytometry that creates such a powerful technique, because it enables several different parameters to be measured simultaneously on many thousands of cells per minute. At present, in diagnostic immunohematology laboratories, samples are commonly stained with two, three, or more fluorochromes (two-color staining, three-color staining, etc.). They are then analyzed usually in commercial flow cytometers, equipped with one, two, or three laser light sources, that can detect forward and side light scatter signals and between three and eight fluorescence signals. Over the years, the trend in instrument development has been to increase the number of fluorescence signals that can be detected; the recently introduced FACS Aria (BD Biosciences, San Jose, CA) can acquire data in up to 16 channels (12 different fluorescence signals/colors). The main limitation on the number of different fluorochromes (and hence cell properties and/or functions) that can be measured simultaneously, however, is the shortage of fluorochromes with distinct emission spectra and the problems in distinguishing the origin of their fluorescence from those with overlapping emission spectra. Over the last two decades, however, the number of useful fluorochromes has increased steadily, and more recently there have been significant advances in techniques for compensating for spectral overlap between several fluorescent signals, so it is likely that the number of fluorochromes used in routine immunohematological analysis will increase further. In this chapter, the basic principles of how light and matter interact, the nature of fluorescence, and the properties of various fluorochromes that are used for different purposes are discussed. More detailed information on the structure, properties, and uses of fluorochromes used in flow cytometry can be found in the comprehensive publications by Haugland ***(2)*** and Shapiro ***(3)***. Some examples of the many parameters that can be measured using fluorescence and the fluorescent probes that can be used are given below (**Table 1**).

## 2. Interactions Between Light and Matter

Light can be regarded as a form of electromagnetic radiation that travels in waves and that can be defined by electric and magnetic vectors that are at right angles both to each other and to the direction of propagation. The relationship between the frequency and wavelength (the distance between wave crests) of the radiation is then expressed by a simple equation:

$\lambda \times \nu = c$ (in which $\lambda$ is the wavelength in meters, $\nu$ is the frequency in cycles/s, and $c$ is the velocity in meters per second, approx $3 \times 10^8$ m/s, the "speed of light").

However, only light that has a frequency between approx $0.4 \times 10^{15}$ Hz and $0.75 \times 10^{15}$ Hz (a Hz, or hertz, is one cycle per second) or, alternatively, that has a wavelength between approx 400 nm (near UV) and 700 nm (red) is visible to the human eye. Although for many purposes it is convenient to consider light as a continuous long wave, it really comprises discrete short "wave trains," called "photons," that can also be considered as particles that have electromagnetic energy, but no mass. The electric and magnetic vectors of the wave arise from the force field that surrounds a pair of charges, the oscillating electric dipole, which can be considered as a positive and a negative charge oscillating toward and away from each other so that their separation distance is a sine wave function. The oscillating dipole, therefore, generates a moving wavelike force field that radiates out in all directions with the electric vector in the same plane as the axis of the dipole to produce a single wave train. The process, from most sources, lasts for only approx $10^{-9}$ s, and as the force field travels at the speed of light, it would travel approx $3 \times 10^8 \times 10^{-9}$ m, or 0.3 m, in that time (**Fig. 1**).

The concept of the oscillating dipole is useful when considering the movement of electrons in atoms and molecules and their interaction with light, because it corresponds to the relative movements of the electrons (negatively charged) and nuclei (positively charged) in matter. To cause a dipole to oscillate, work must be done, but a corresponding (or smaller) amount of energy can then be radiated as light, which if absorbed by other dipoles, can also start them oscillating, so that, in turn, they can also radiate. It is the exchange of energy between oscillating dipoles and the radiation field that governs the interactions between light and matter. The emission of light from matter (e.g., a metal) heated to high temperature is termed incandescence, but light emitted without recourse to heating is known as luminescence, a term that encompasses chemiluminescence (or bioluminescence if it occurs in vivo) and two forms of photoluminescence, which are fluorescence and phosphorescence. Chemiluminescence results when the enthalpy (heat) of a chemical reaction raises an electron in an atom or molecule to a vibrationally excited state, and light is emitted when the electron decays to its ground state. In contrast, photoluminescence results when the energy acquired from the absorption of light (typically, the UV, visible, or near-infrared) causes an electron to be raised to a higher energy state in an unoccupied orbital and (as in chemiluminescence) its return to the ground state is accompanied by light emission. A feature of the exchange of energy between light and matter is that it can occur only in discrete quantities (or packets) (**Fig. 2**) known as quanta or photons, the energy of which is related to the frequency of the radiation by the Planck-Einstein equation:

**Table 1**
**Examples of the Cellular Parameters That Can Be Measured by Flow Cytometry**

| Parameter | Fluorochromes and/or techniques used[a] |
|---|---|
| **Antigens** | |
| Presence on cell surface or internally | Many fluorochromes (e.g., fluorescein, phycoerythrin, or Qdots®) can be conjugated to specific antibodies |
| Proximity of different antigens | Fluorescence resonance energy transfer between different fluorochrome-labeled antibodies |
| Apoptosis/necrosis/viability | *See* DNA content, Membrane asymmetry, integrity/permeability, lipid packing, potential |
| Cell division (generation number) | Lipophilic dyes, or CFDA-SE, which binds covalently to labeled cells |
| **DNA** | |
| Content | DAPI, DRAQ5, Hoechst dyes, propidium iodide |
| Base ratio | Dyes that bind preferentially to A-T or G-C pairs (e.g., chromomycin A and Hoechst 33258) |
| Synthesis | Incorporation of BrUdR detected with fluorescent anti-BrUdR antibodies |
| Breakdown (e.g., during apoptosis) | Incorporation of labeled nucleotides at strand breaks (TUNEL) |
| Nucleotide sequence | Fluorescent-labeled oligonucleotides |
| Enzyme activity | Fluorogenic substrate analogs |
| Glutatione/sulphydryl groups | Fluorescent bimanes |
| Intracellular ion concentrations | |
| pH | "Ratio" dyes (e.g., BCECF, SNARF-1) |
| $Ca^{2+}$ | Fluo-3 and "ratio" dyes (e.g., INDO-1) |

| | |
|---|---|
| **Membrane** | |
| Asymmetry | Fluorochrome-labeled annexin V |
| Integrity/permeability | Fluorescein diacetate, and nonpermeant DNA dyes (e.g., ethidium bromide, propidium iodide) |
| Lipid packing | Merocyanine 540 |
| Potential | Rhodamine 123, JC1 |
| Net charge | Fluorescent polyanions |
| Receptors | Fluorochrome (e.g., fluorescein)-conjugated ligands |
| Oxidative metabolism | Dichlorofluorescein |
| Phagocytosis/endocytosis | Fluorescent bacteria, fluorescent beads |
| Pinocytosis | Fluorescent low–molecular weight dextran |
| Reporter gene expression | Green fluorescent protein |
| RNA content | Acridine orange, pyroninY, thiazole orange |

[a]BCECF, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein; BrUdR, bromodeoxyuridine; CFDA-SE, 5(6)-carboxyfluorescein diacetate succidimyl ester; DAPI, 4′, 6-diamidino-2-phenylindole; SNARF-1, carboxy-seminaphthorhodafluor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

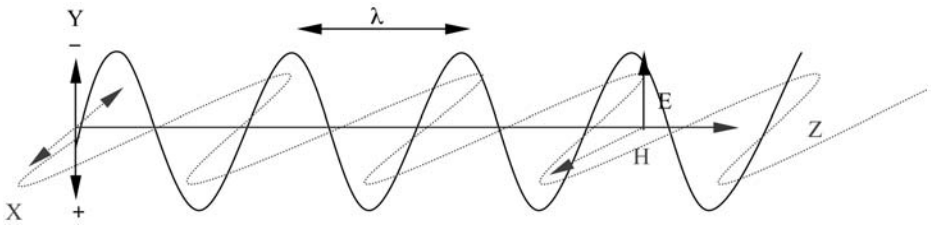


**Fig. 1.** The generation of electromagnetic radiation from an oscillating dipole. The dipole (shown by the vertical line with arrows and charge symbols at the left) generates an electromagnetic wave with a magnetic vector (*H*) in the plane *YZ* that is at right angles to, and in phase with, the electric vector (*E*) in the plane *XZ*. The wavelength (λ) is maintained as the wave moves in the direction *Z*, but its amplitude decreases as the distance from its origin increases.

$E = h \times v$, in which $E$ is the energy (in Joules) in one quantum of radiation that has a frequency of $v$ (cycles/s), and $h$ is Planck's constant, $6.626 \times 10^{-34}$ J-s (i.e., Joule-seconds); for light in a vacuum, $E = h \times (c/\lambda)$ (because $v = c/\lambda$, from above).

Blue-green light with a wavelength of 488 nm has a frequency of $3 \times 10^8$ m/s divided by $488 \times 10^{-9}$ m, or $6.148 \times 10^{14}$/s. Consequently, the energy (in Joules) of a single photon/quantum of blue-green light with a wavelength of 488 nm is $6.626 \times 10^{-34}$ J-s $\times$ $6.148 \times 10^{14}$/s, or $4.07 \times 10^{-19}$ J. A 488-nm laser operating at 15 mW will, therefore, produce approx $3.68 \times 10^{16}$ photons or quanta per second (because 1 W is equivalent to 1 J/s). Also, as a cell is illuminated for between $5 \times 10^{-6}$ to $5 \times 10^{-5}$ s when it passes through the analyzing beam of a flow cytometer, depending on the ratio of the cross-sectional areas of the cell and beam, it will receive approximately $10^{10}$–$10^{11}$ photons or quanta.

## 3. Light Absorption Leading to Fluorescence

The various atoms in molecules can exist in a number of discrete energy states, so that to raise an atom from the ground state ($S_0$) to a particular excited state requires the absorption of a quantum with the correct energy. Consequently, the dipole oscillation of electrons and nuclei that accompany the absorption, or emission, of light occurs only at certain frequencies. Moreover, as the energy states of atoms are determined almost completely by the orbits of their valence electrons, and as the energy differences ($\Delta E$) between these states are well defined, there is intense light absorption at the wavelength that will give:

$$\Delta E = h \times v, \text{ or (by substitution) } \Delta E = (h \times c)/\lambda.$$

The absorption spectrum of an atom, therefore, comprises a number of sharp lines at particular wavelengths (e.g., for the hydrogen atom). They occur principally at 102.6, 121.6, 486.9, and 657.3 nm. Within a larger molecule,

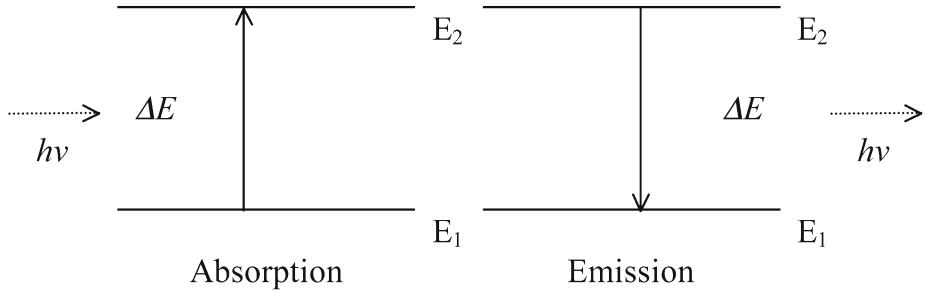


**Fig. 2.** The transition between a higher ($E_2$) and lower ($E_1$) energy state of a valence electron that is associated with the absorption or emission of a photon (or quantum) of light that has the energy $E = hv = E_2–E_1$.

however, the situation is more complex because the electrons of many different atoms occur close together and can interact both with each other and with the nuclei of different atoms. Consequently, although there are a few major electronic singlet states (i.e., ground [$S_0$], first excited [$S_1$], second excited [$S_2$], etc.), there is also a large number of different vibrational energy levels, or substates (denoted $V_0$, $V_1$, $V_2$, etc.) within each major state. In addition, there is a parallel series of triplet energy states (denoted $T_0$, $T_1$, $T_2$, etc.), each of which is at a slightly lower energy level than the corresponding singlet state. When a fluorochrome is in the dark and at ambient temperatures, the majority of molecules are in the ground state, but when it is irradiated, photon absorption typically excites most molecules to a vibrational energy level within the first excited singlet state ($S_1$). However, this energy is lost rapidly (in approx $10^{-12}$ s) by vibrational relaxation (a nonradiative process) so that the molecules drop to the lowest vibrational substate ($V_0$) in the $S_1$ band. All of the energy transitions that lead to photon emission begin at $V_0$ in one of the excited states ($S_1$, $S_2$, etc.) but can terminate in any of the vibrational substates in the electronic ground state ($S_0$). Thus, there are many different possible transitions between the various ground and excited substates, each of which could give rise to an absorption band. However, intermolecular interactions result in a broadening of the bands so that there is a broad band of wavelengths in which light can provide the energy needed to effect all of the various possible transitions. Similarly, fluorescence occurs when light is emitted at wavelengths that correspond to the energy difference between the lowest vibrational substate ($V_0$) of the excited singlet ($S_1$, $S_2$, etc.) and the various vibrational substates in ground ($S_0$) energy levels (**Fig. 3**).

The most likely transitions to occur during light absorption usually span a larger energy difference ($\Delta E$) than those that occur during light emission. Consequently, the photon emitted that produces fluorescence is always of lower

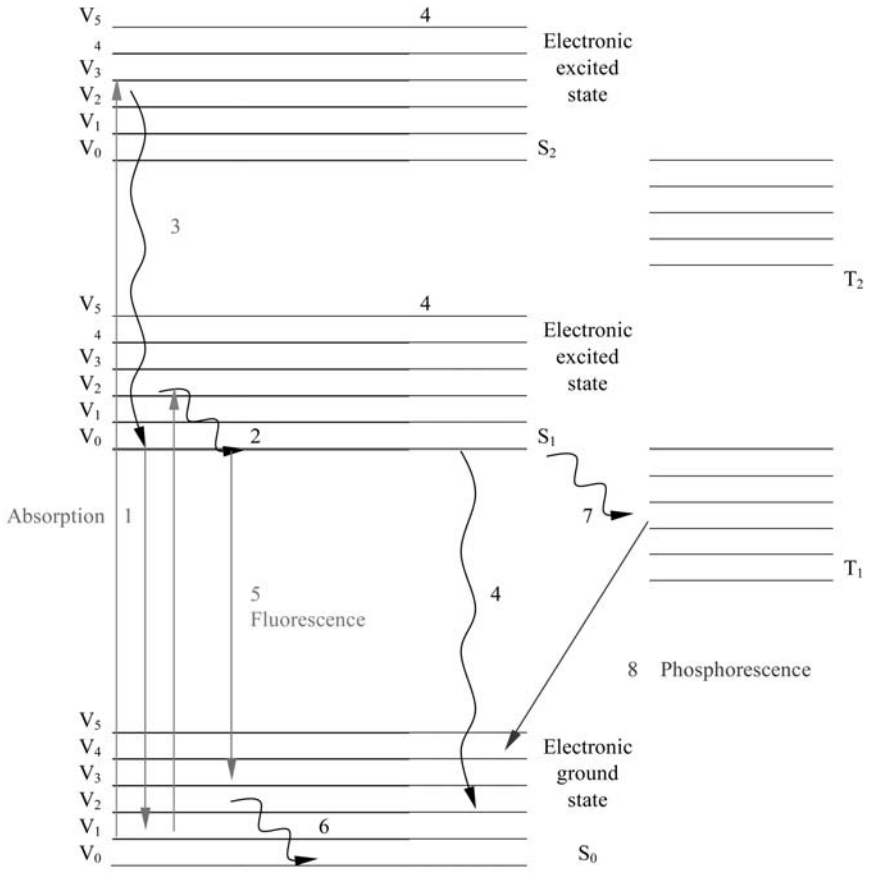


**Fig. 3.** A Jablonski diagram that illustrates the electronic transitions that occur during light absorption (excitation) and emission by a fluorochrome. **(1)** The initial absorption of light energy raises the molecule to an electronic excited state ($S_1$ or $S_2$), from which energy can be lost in nonradiative ways, including **(2)** by vibrational relaxation during a series of transitions between substates in the electronic excited state $S_1$, **(3)** by internal conversion in going from the $S_2$ to the $S_1$ state, or **(4)** by external quenching. **(5)** Molecules with electrons in the lowest excited singlet state ($S_1$) can then undergo the main downward energy transition that produces fluorescence. **(6)** After the emission of fluorescence, there is also the possibility of energy loss by vibrational relaxation as the molecule goes through the ground substates ($S_0$). **(7)** Intersystem crossing from the singlet excited state $S_1$ to the triplet state ($T_1$) does not result in the emission of radiation. **(8)** However, decay (from $T_1$) to the electronic ground state ($S_0$) results in phosphorescence. The various nonradiative vibrational transitions that relax $S_2$ to $S_1$ happen faster than the de-excitation processes between $S_1$ and the ground state $S_0$. The most probable upward energy transition will correspond to the energy of quanta at the peak wavelength of the absorption spectrum and the most probable downward transition to the peak wavelength of the emission spectrum. (After **ref. *4***.)

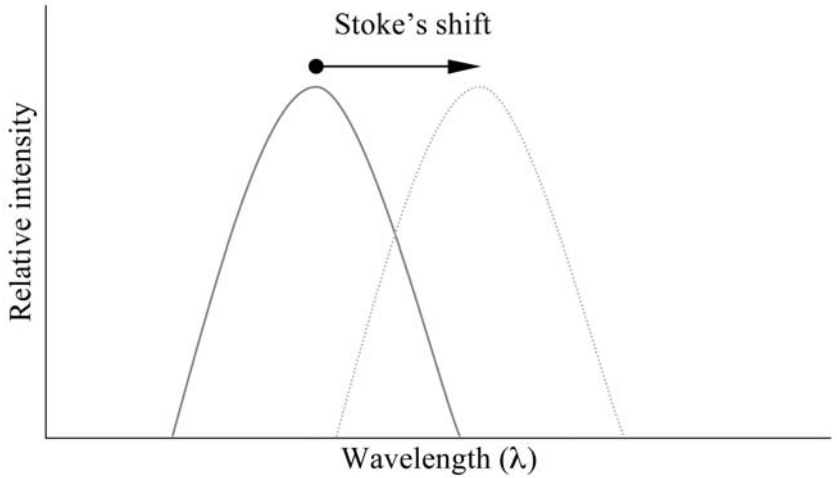


**Fig. 4.** The absorption (or excitation) (solid line) and emission (dotted line) spectra of a fluorochrome. The difference between the peak wavelengths of the absorption (or excitation) and emission spectra is known as the Stokes shift and always occurs in the direction of the longer wavelength (smaller energy).

energy (and therefore longer wavelength) than that originally absorbed, as the process is less than 100% efficient and some energy is always lost in other ways, mainly by vibrational relaxation. The most likely transitions correspond to the wavelengths for maximum absorption and emission, and the difference between is them known as the Stokes shift (**Fig. 4**). Fluorescence techniques such as fluorescence microscopy and flow cytometry exploit this characteristic by illuminating cells at one wavelength and detecting the light that is emitted at a longer wavelength; consequently, the larger the Stokes shift, the more easily this can be done. Because the probability of an electron returning to a given energy level in the ground state after excitation is similar to it occurring in that position before excitation, the absorbance spectra (or excitation spectra) and emission spectra often overlap and are usually mirror images of one another. The probability of light absorption by a substance is measured as its molar extinction coefficient (ε) (conventionally for a 10-mm lightpath) and, of course, varies with wavelength. As an example, the molar extinction coefficient of fluorescein at its wavelength of peak absorbance (495 nm) is 75,000/cm mol/L, but that for PE is $10^6$/cm mol/L. Irrespective of the wavelength used to excite a fluorochrome, its emission spectrum is always constant, but the intensity with which it fluoresce will vary with the excitation wavelength and will reach a maximum when excitation is at the wavelength of maximum absorption. The implications of these points for flow cytometry are, first, that fluorochromes have excitation and emission spectra with quite broad peaks and, second, that

they can be excited suboptimally at wavelengths away from those of their peak absorbance, a feature that can be useful in instruments with a single laser.

Light absorption by a fluorochrome is a very fast process (occurring in approx $10^{-15}$ s), but the emission of light by fluorescence is considerably slower; generally, however, the more strongly that a fluorochrome absorbs light, the faster will be the subsequent light emission (fluorescence). The intensity of the fluorescent light that can be emitted is an important characteristic of a fluorochrome because it affects the sensitivity of detection. It is proportional to the extinction coefficient and a factor known as the quantum yield ($\phi$), which is the ratio of photons (or quanta) emitted to photons (or quanta) absorbed. Numerically, values for $\phi$ can vary from 0 to 1.0 and are a characteristic of the particular fluorochrome. The intensity of fluorescence from a fluorochrome excited in solution is defined by the expression:

$F = I_o \times \varepsilon \times [C] \times x \times \phi$, in which $F$ is the total amount of light emitted, the product $(I_o \varepsilon [C]x)$ is the total amount of light absorbed by the fluorochrome (from Beer's law), $I_o$ is the incident light intensity, $\varepsilon$ is the molar extinction coefficient, $[C]$ is the molar concentration, $x$ is the path length in centimeters, and $\phi$ is the quantum yield.

Quantum yields are always less than 1.0 because other processes, including internal conversion, quenching of various types, and intersystem crossing, compete with fluorescence to dissipate the energy gained by light absorption. All these processes allow electrons to relax from the excited to the ground state without causing a photon to be emitted and consequently reduce the proportion of excited electrons that decays through fluorescence. During internal conversion, energy can be lost within approx $10^{-11}$ s by collision with other (e.g., solvent) molecules or through internal vibrational and rotational modes that result in the production of heat. Quenching is the loss of fluorescence that occurs when energy is transferred by a number of possible mechanisms from a fluorochrome to a nonfluorescent molecule. Most fluorochromes used in flow cytometry are aromatic compounds, which usually have fluorescence lifetimes ($\tau$) of $10^{-8}$ to $10^{-9}$ s, but almost every collision with a quenching molecule such as oxygen results in a loss of the excited singlet state and hence the abrogation of fluorescence. In general, quenching molecules are present in great excess over fluorochrome molecules and collision rates between the two can reach approx $10^{-8}$/s when the quencher is present in millimolar concentrations. Intersystem crossing occurs when the excited singlet state is converted into the excited triplet state because the excited electron has changed its spin. The excited triplet state, which is at a lower energy than the excited singlet state, can then decay to the ground state either by phosphorescence (at a longer wavelength than fluorescence) or by internal conversion. In practice, phosphorescence is rarely seen in solution because its lifetime is several seconds and the energy that could

give rise to it is more readily lost by collisions with quenchers and by internal conversion. The final process that may diminish the quantum yield is photo-oxidation, which happens when an electron is transferred from a donor molecule that has been excited by absorbing light, to an acceptor molecule that in turn becomes reduced. It occurs because the transferred electron is less tightly bound to the nucleus when in its excited than in its ground state.

The key difference between chemiluminescence and fluorescence is that during chemiluminescence the substrate is irreversibly altered during the light-generating reaction, and consequently each molecule of the substrate produces a photon only once. In fluorescence, the process of photon absorption and emission by each molecule involved can occur many times, before the oxidation favored by the excited state results in photobleaching. For example, fluorescein isothiocyanate (FITC) will go through approx 35,000 cycles in which the fluorescence (or excited state) lifetime lasts on average 4 ns ($4 \times 10^{-9}$ s), whereas molecules of Hoecsht 33258 and propidium iodide (PI), when bound to DNA, can go through approx 100 and approx 200 cycles, respectively. Provided that excitation is at an optimal (or near optimal) wavelength, several hundred photons will be emitted from each cell-bound fluorochrome molecule in the time (5–50 μs) that it takes a cell to pass through the analyzing point of a flow cytometer, but less than 20% of the total will eventually reach the appropriate detector. The intensity of fluorescence is also determined by the lifetime of the excited state (i.e., the time it takes for an excited molecule to decay to the ground state from which further cycles of excitation and, perhaps, emission are possible), which is a characteristic of each fluorochrome and its environment. Many factors that affect the molecular environment (e.g., ionic strength, pH, and solvent polarity) can markedly influence the quantum yield. Thus, it is best that the solution parameters of ionic strength and pH be kept constant and usually within the physiological range, unless they need to be altered for experimental reasons. Fluorochromes can suffer "photobleaching" under high-intensity illumination when highly reactive singlet oxygen is produced by reactions between molecular oxygen and fluorochrome molecules in the excited singlet or triplet states. Photobleaching can occur during flow cytometry, but the resultant fading of the fluorescence is not as serious a problem as in fluorescence microscopy, because the cells are usually illuminated just once, for a very short period, and the signal is collected at the same time.

## 4. Mechanisms of Fluorescent Staining

There are several different mechanisms by which fluorescent staining can occur. Some dyes have an affinity for certain substances and accumulate in high concentrations in particular locations or organelles within cells (e.g., lipohilic

dyes such as merocyanine 540 accumulate in membranes and reactive dyes such as the succiidimyl ester of carboxyfluorescein diacetate [CFDA] bind covalently, predominantly to proteins). Fluorochromes are frequently conjugated to protein ligands (e.g., antibodies), and these conjugates bind specifically and/or in higher amounts to cells expressing the appropriate receptor (or antigen) than to others. Other fluorescent probes may show a marked increase in their quantum efficiency when bound to a particular substance or when they are in a particular environment, a mechanism that was originally termed "fluorochroming" or "hyperchroming." For instance, there are not only localized increases in concentration when ethidium bromide (EB), PI, or the Hoechst dyes bind to DNA, but as a result of the change in their environment their fluorescence can increase by up to 100-fold. Similarly, that of the cyanine dyes (e.g., thiazole orange and TO-PRO-1®) can increase by up to 1000-fold when bound to DNA.

## 5. Cellular Autofluorescence

Mammalian cells contain substances that will fluorescence when illuminated during flow cytometry. These include pryidines (e.g., in NAD [nicotinamide adenine dinucleotide]/NADH [reduced nicotinamide adenine dinucleotide] and NADP [nicotinamide adenine dinucleotide phosphate]/nicotinamide adenine dinucleotide phosphate hydrogen [NADPH]), the flavins (e.g., in flavin adenine dinucleotide [FAD] and FMN [flavin mononucleotide] nucleotides), and the porphyrins (e.g., in protoporphyrin), which are excitable in the UV and/or blue wavelengths and which fluoresce in the green, green-orange, and yellow-red regions of the spectrum. There is little difference in the intrinsic excitation and emission spectra of the various blood leukocytes, but there are marked differences in their relative fluorescence intensities ***(5)***. Autofluorescence from lymphocytes excited at 488 nm is low and is equivalent to that of approx 660 fluorescein MESFs (molecules of equivalent soluble fluorochrome) or approx 50 PE MESFs when measured in the fluorescence channels used for those fluorochromes. That from neutrophils is somewhat higher, and fluorescence signals from reduced NADPH have been used to monitor the respiratory burst. However, that from eosinophils is higher still (2000–3000 fluorescein MESFs), and much has been attributed to FAD present in granules. Interestingly, in HL60 cells, autofluorescence decreases significantly between 2 and 24 h after the induction of apoptosis by any one of a number of different stimuli. Probably the most important consequence of autofluorescence is that it limits the sensitivity of detection, particularly of fluorochromes that emit in the green region of the spectrum (e.g., fluorescein). If autofluorescence causes a severe problem, it is probably best to avoid it by using fluorochromes that can be excited at longer (e.g., >600 nm) wavelengths.

## 6. Fluorescence Resonance Energy Transfer

If two different fluorochrome molecules are in close proximity, their electronic orbitals can interact by exciton or coupled oscillator mechanisms (resonance). Consequently, energy absorbed by the one with the shorter excitation wavelength and therefore the higher energy absorption (the donor) can be passed to the other (the acceptor), which fluoresces. The process is usually called fluorescence resonance energy transfer (FRET) but is also known as singlet–singlet or exciton resonance energy transfer because after the donor fluorochrome has been excited, it rapidly loses energy by internal conversion until it reaches the first excited singlet state. Then, if the energy difference from the ground state matches that of the acceptor absorption, energy transfer will occur by resonance between the donor and acceptor. Consequently, the donor is quenched (does not fluoresce), and the acceptor is excited and subsequently fluoresces, but for this to happen, the wavelength at which the donor would normally fluoresce must overlap with the wavelength at which the acceptor would normally absorb light. Although it is theoretically possible for the donor to emit photons (light) and for the acceptor to absorb the emitted photons (**Fig. 5**), this does not usually occur in cytometry, because it requires more than millimolar concentrations of fluorochromes ***(6)***. The efficiency with which energy is transferred during FRET is proportional to the inverse of the sixth power of the distance separating the two fluorochromes. Consequently, it occurs best when the two fluorochromes are separated by approx 2–10 nm but can occur at distances of up to 100 nm. Fluorescein and the rhodamines commonly have been used as the donor-acceptor pairs in FRET studies. However, there is some advantage to using the carbocyanine dyes, Cy3™ and Cy5™, as a donor-acceptor pair because their spectral characteristics and long wavelength emissions provide greater sensitivity and need less correction for autofluorescence ***(7)***.

One important application of FRET has been in the development of "tandem dyes," in which one fluorochrome (the donor) that can be excited at a convenient wavelength (e.g., 488 nm) is covalently coupled to a second (the acceptor). When the donor is excited, much of the absorbed energy is transferred to the acceptor, which then fluoresces at a longer wavelength than the donor would normally fluoresce. Another application has been to determine whether two different antigens co-localize on the surface of a cell. To do so, cells are labeled with antibodies specific for the antigens, one of which has been conjugated to a potential donor fluorochrome and the other to a potential acceptor fluorochrome. If the two antibodies bind to sites that are close together, FRET will occur and can be seen as decreased fluorescence from the donor and increased fluorescence from the acceptor fluorochrome, in comparison with cells that have been labeled separately with the fluorochrome-conjugated antibodies. Conversely, if the two antibodies bound to antigenic sites that were present in similar densities on

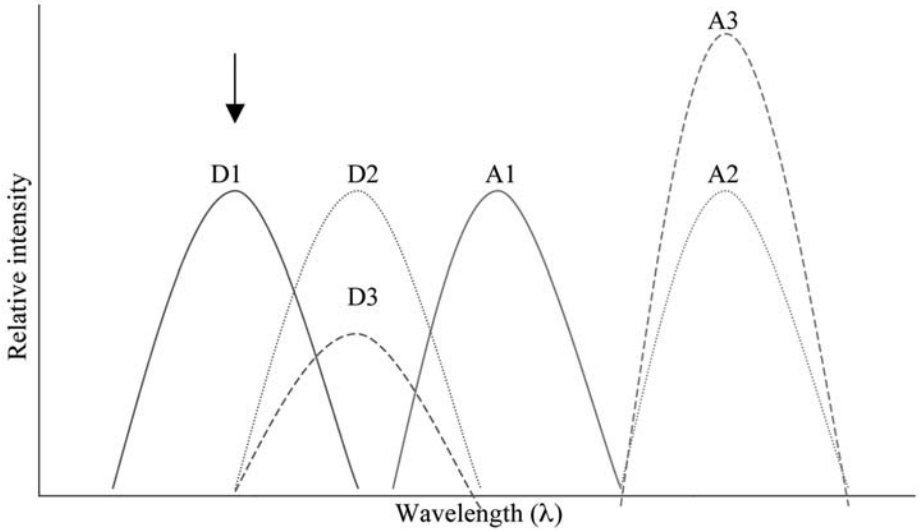


**Fig. 5.** Hypothetical spectra from a pair of fluorochromes (D = donor, A = acceptor) that are suitable for fluorescence resonance energy transfer (FRET). The absorption (or excitation) spectra of the donor and acceptor are shown by the solid lines, D1 and A1, respectively, and their emission spectra are shown by the dotted lines, D2 and A2, respectively. If the donor and acceptor are in close proximity, and are illuminated by a wavelength (indicated by the vertical arrow) at which the donor but not the acceptor can absorb light, there can be nonradiative energy transfer from the donor to the acceptor which results in the acceptor fluorescencing. Under these circumstances, there will be decreased fluorescence emission intensity from the donor (shown by the dotted line, D3) and fluorescence from the acceptor (shown by the dotted line, A3) that would not normally occur if the acceptor alone were illuminated at this wavelength. Note that for FRET to occur, the emission of the donor must overlap with the absorption of the acceptor.

the cell surface and were randomly distributed, FRET would not occur. Although, in these situations, energy transfer between the acceptor and donor is generally approx 90%, there is always some background fluorescence from the donor. Software to help determine separation distances between cell surface antigens by flow cytometry is available from Soft Flow, Inc. (Burnsville, MN).

A further application of FRET has been in the construction of substrate analogs (e.g., for proteases), in which a donor and an acceptor dye are covalently linked to amino acids in the peptide on either side of a potential cleavage site. They are, therefore, close enough for FRET to occur between them in the uncleaved substrate, but if the specific protease is present and the peptide is cleaved, further FRET between the fluorochromes is prevented and there is a

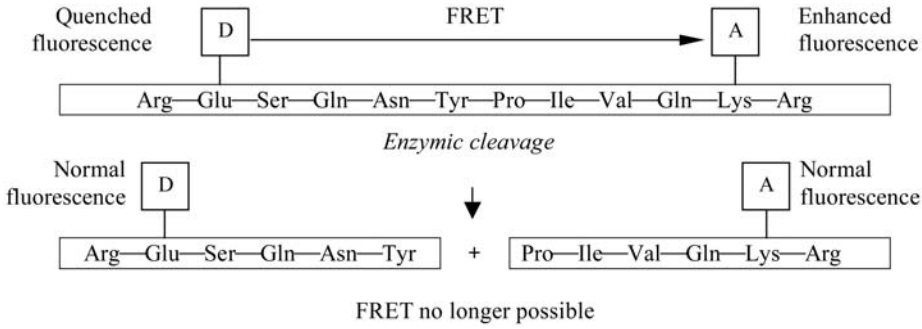


**Fig. 6.** Fluorescence resonance energy transfer (FRET) in a substrate analog used for assaying intracellular proteases. In the uncleaved substrate mimic, the fluorescence intensity of the donor fluorochrome **(D)** that is attached to the peptide is quenched by FRET to the acceptor fluorochrome **(A)**, the fluorescence intensity of which is enhanced because the two are held in close proximity **(upper diagram)**. When the peptide is cleaved, the fragments are released and the normal fluorescence intensity of both donor and acceptor fluorochrome is restored. Illumination at a wavelength at which the donor, but not the acceptor, absorbs light will then no longer give rise to fluorescence from the acceptor **(lower diagram)**.

consequent increase in donor fluorescence and decrease in acceptor fluorescence (**Fig. 6**).

## 7. Fluorochromes Used for Labeling Antibodies, Proteins, and Ligands

### *7.1. Fluorescein and Other Green-Fluorescent Fluorochromes*

The two earliest and still most widely used fluorochromes for labeling antibodies are fluorescein (FITC) and PE (excitation/emission maxima approx 495/520 nm and 480,545/575 nm, respectively); they were useful because they provided a first and second color that could be excited by an argon ion laser at 488 nm. Now more recently, antibodies labeled with the tandem dyes based on PE and another photosynthetic protein, peridinin–chlorophyll a complex (PerCP), have been used to provide a third and/or fourth color that can be excited by light of this wavelength. Neither tetramethylrhodamine nor Texas Red® (excitation/emission maxima 350–355, 548–552/576–577 nm and 560/731 nm, respectively), which are widely used in immunofluorescence microscopy, has been used widely in flow cytometry, because they absorb poorly at 488 nm. Although in those cytometers fitted with a laser emitting in the 550- to 650-nm range, antibodies labeled with allophycocyanin (APC), APC–Cy™7, Cy™5, or Texas Red® can be used to provide an extra color for immunofluorescence analysis. Fluorochromes that could be used for antibody labeling and that can be excited in

the UV region include Alexa™ 350, derivatives of 7-aminocoumarin (e.g., 7-amino-4-methylcoumarin-3-acetic acid [AMCA]), and Cascade Blue®. Because of high nonspecific binding, the latter is not recommended for flow cytometry.

FITC (molecular weight 389) was one of the most widely used labeling reagents (**Fig. 7A**) because it will readily fluoresceinate proteins to produce moderately stable conjugates that have a high quantum yield (approx 0.5). The absorption peak of fluorescein is close to 488 nm, which makes it good for single-color staining, but the trail of its emissions into longer wavelengths is a disadvantage for multicolor applications. Moreover, its fluorescence is pH-sensitive (maximal at pH 8–9) and it suffers from a high rate of photobleaching. Consequently, several fluorescein derivatives, or replacements, that have similar spectral characteristics but give antibody and protein conjugates that have greater stability and higher fluorescence intensities (e.g., DTAF [dichlorotriazinylamino fluorescein], carboxyfluorescein and 2′,7′-difluorofluorescein (Oregon Green™ 488)] have been developed. Another example, BODIPY® FL (BODIPY-3-propionic acid) (excitation/emission maxima approx 503/512 nm), differs structurally from fluorescein and has a smaller Stokes shift, but it has a higher fluorescence intensity and its fluorescence is less pH-sensitive than fluorescein (**Fig. 7B**). Alexa™ 488 (excitation/emission maxima 494/519 nm; Invitrogen, Carlsbad, CA) is another more photostable and brighter alternative to FITC and belongs to the Alexa™ series of dyes. There are approx 20 dyes in the series; they are numbered (Alexa™ 350 to Alexa™ 750) by the laser lines at which they are optimally excited, and they are characterized by having a narrow emission spectrum. The Oyster® cyanine-based dyes (Denovo Biolabels GmbH, Münster, Germany) are yet a further series of recently synthesized dyes that have absorption peaks (500–656 nm) falling in the visible spectrum, have narrow emission spectra, and are numbered on the same basis. The structures of some small fluorochromes are illustrated in **Figure 7**.

### *7.2. Phycobiliproteins (Phycoerythrin, Allophycocyanin, and CryptoFluor™ Dyes) and PerCP*

Phycobiliproteins are components of photosynthetic systems, which comprise multiple tetrapyrole groups covalently attached to a protein which absorb light maximally between 470 and 650 nm and pass on the energy by resonance energy transfer to chlorophyll, which absorbs at 670 nm. They have high extinction coefficients (e.g., that of bacterial PE [B–PE] is $2.4 \times 10^6$/cm mol/L) and high quantum yields (e.g., B-PE, 0.98), which confer good light absorption and high fluorescence intensities, making their conjugates some of the most sensitive probes available. On a molar basis, their fluorescence is approx 30-fold greater than that of fluorescein, but in practice, cells labeled with phycobiliprotein antibodies have fluorescence intensities between five- and tenfold greater than

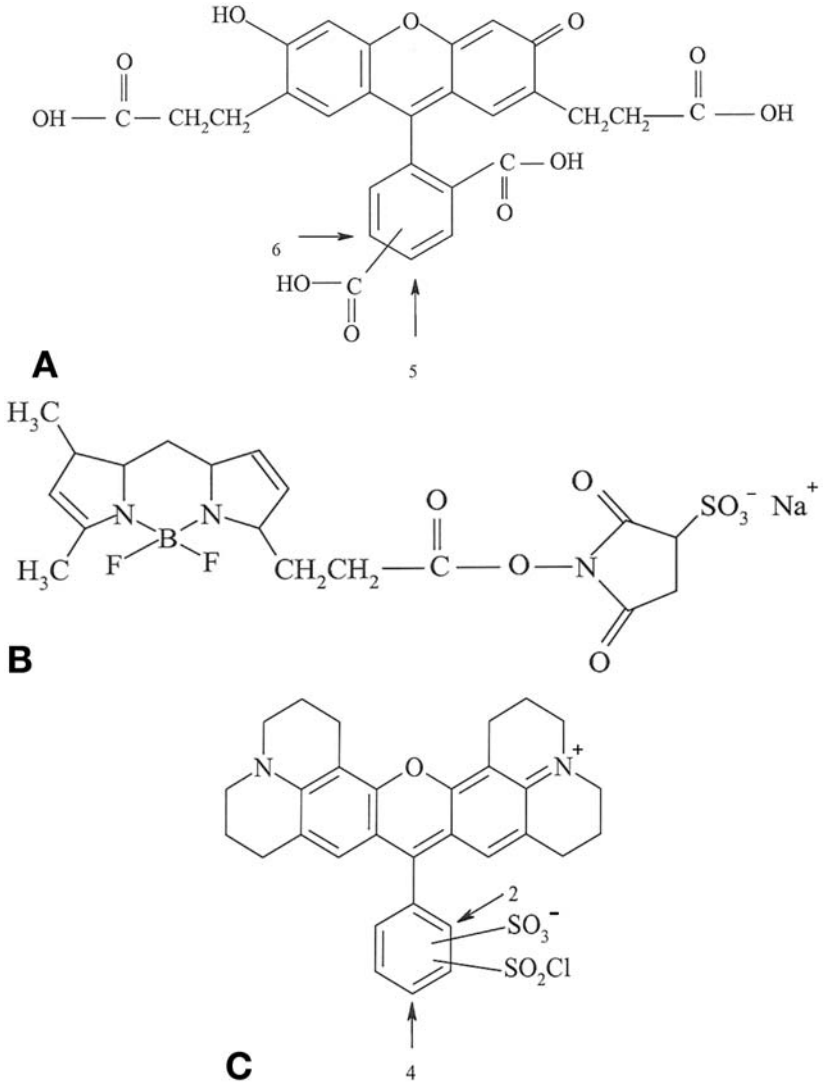


**Fig. 7.** The structures of some small fluorochromes that are used for labeling proteins. **(A)** Fluorescein: $R^1$ = –N=C=S and $R^2$ = H in fluorescein 5-isothiocyanate (FITC isomer I, the more commonly used isomer), and $R^1$ = H and $R^2$ = –N=C=S in fluorescein 6-isothiocyanate (FITC isomer II). **(B)** 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid sulphosuccimidyl ester (BODIPY® FL, SSE). **(C)** Texas Red® sulphonyl chloride (mixed 2- and 4- isomers). *See* **Figure 8** for the details of conjugation reactions.

those labeled with the corresponding fluoresceinated antibody. Although single molecules of PE have been detected in a specialized instrument ***(8)***, the detection limit in an ordinary flow cytometer is between 500 and 1000 fluorochrome molecules per cell. Because the phycobiloproteins have a broad absorption spectrum, they can be excited at several wavelengths, making them useful for both single- (488 nm) and multiple-laser applications. Although all phycobiliproteins have only a small Stokes shift, their fluorescence can be readily distinguished from that of FITC. The most commonly encountered are B-PE, a 241-kDa water-soluble protein from cyanobacteria (excitation/emission maxima 546, 565/575 nm); R-PE, a 196-kDa protein from eukaryotic red algae (excitation/emission maxima 480,546,565/578 nm); and APC, a 104-kDa protein from a cyanobacterium (excitation/emission maxima 650/660 nm). The absorption maximum of APC at 650 nm enables it to be excited strongly by illumination at 633 nm in those flow cytometers that are equipped with a helium–neon laser. The disadvantage of using phycobiloproteins as fluorescent labels is that their large size can cause steric hindrance when they are conjugated to proteins and can also produce high backgrounds if fixed and/or permeabilized cells are not well washed to remove unbound PE- or APC-labeled antibodies. The molar ratio of fluorochrome to protein in commercially available PE-conjugated antibodies is usually approx 1, and these reagents should not be frozen but stored with 0.1% sodium azide at 4°C.

A series of phycobiliproteins (approx 30–43 kDa), called the CryptoFluor™ dyes, with absorption/emission maxima in the range of 545–645/576–658 nm isolated from cryptomonads are available commercially (Martek Biosciences Corporation, Columbia, MD) and can provide an alternative to PE if the large size of the latter is a problem. PerCP (excitation/emission maxima 488/677 nm) is another water-soluble, 35-kDa, photosynthetic pigment that is obtained from dinoflagellates. Like the phycobiloproteins, it has a high quantum yield (approx 1), can be conjugated to antibodies, and provides a useful third color or fourth color because its emission spectrum overlaps little with PE. Unfortunately, it is particularly light-sensitive; it must, therefore, be kept in the dark to minimize photodecomposition and consequently is not recommended for use with stream-in-air flow cytometers.

### *7.3. Tandem Dyes*

Tandem dyes are conjugates of two fluorochromes, one of which is often PE (e.g., PE–APC, PE–TR, and APC–Cy™7). One of the earliest tandem dyes was PE–TR (also called ECD™ and Coulter Red613; emission maximum 613 nm), but it has now been superseded by PE–Cy™5 (also called CyChrome™, Tri-Color™, Red670, or Quantum Red™; emission maximum 667 nm). Because PE emissions trail considerably into the longer wavelengths, there is significant spectral overlap between the emissions from PE and those from all of the

PE-based tandem dyes. Greater care than usual is needed, therefore, when setting the compensation for multicolor work involving PE and PE-based tandem dyes, and the amount of correction needed varies from lot to lot, depending on the efficiency of resonance transfer in that particular tandem conjugate. It is also particularly important to keep these dyes in the dark and to avoid conditions that would break the covalent bonds between the two dyes in the tandem. For if this happens, the liberated PE component will emit over its usual wavelength range and there will be no emission at the longer wavelength expected for the tandem, leading to false results. PE–Cy™5 is one of the most successful tandem dyes, because Cy™5 has a high extinction coefficient (approx $2 \times 10^5$/cm mol/L) and its peak absorption at 652 nm is optimal for FRET from PE. Maximum brightness in the tandem is achieved when between five and eight Cy™5 molecules are coupled to each PE molecule, and commercially available antibody conjugates normally have a fluorochrome-to-antibody molar ratio of 1:1. PE–Cy™5 has a greater Stokes shift than does PE–TR and consequently requires less compensation from PE. It is generally regarded as the best third color for immunolabeling, but its disadvantages are that its conjugates must be kept in the dark because it is irreversibly degraded in ambient light levels in less than 1 h and it binds nonspecifically to monocytes. PE–Cy™5 is not recommended for multicolor work in which dual excitation by both a 488-nm and 635-nm laser is possible, unless there is a facility for interlaser compensation. The main problem with the PE-based tandem dyes is that the efficiency of FRET decreases as the emission wavelength of the acceptor gets further away from that of the donor, so the tandems that emit at the longer wavelengths also emit at the usual PE emission wavelength. Alexa™ 488–APC (green-fluorescent Alexa™ crosslinked to APC; Invitrogen) is a recently produced tandem dye based on APC which can be excited at 488 nm, leading to emission at approx 660 nm and giving an alternative third color for use in cytometers equipped with a single 488-nm laser. The PBXL™ fluors (Martek Biosciences Corporation) are supramolecular tandem dye complexes containing many crosslinked phycobiliprotein subunits and are the brightest labels currently available. For example, PBXL™-1, which comprises B-PE, R-phycocyanin, and APC, can be excited at 488 nm and emits at 660 nm. Their large size precludes their use in some situations, but they can be conjugated to antibodies, streptavidin, biotin, etc., and have potential in the study of weakly expressed cell surface antigens. The characteristics of a number of tandem dyes are given in **Table 2**.

### *7.4. Quantum Dots*

Quantum dots (Qdot®; Quantum Dot Corp., Hayward, CA) are recently developed fluorescent nanoparticles with properties that differ significantly from those of the small dyes and fluorescent biomolecules considered so far. They comprise three layers, a semiconductor core that is surrounded by a shell, which is then

**Table 2**
**Characteristics of Some Tandem Dyes**

| Donor Name | Absorption maximum nm (laser line nm) | Acceptor Name | Commercial name (acronym) | Tandem dye Emission maximum (nm) | Synonym (acronym) |
|---|---|---|---|---|---|
| Alexa™ 488 | 495 (488) | Allophycocyanin (APC) | | 660 | Alexa™ 488–APC |
| Allophycocyanin | 650 (635, 647) | | Alexa™ fluor dyes | e.g., 702; 719; 779 | APC–A680; APC–A700; APC–A750 |
| Allophycocyanin | 650 (635, 647) | Indodi-carbocyanine | Cyanin 5.5 (Cy™5) | 694 | Allophycocyanin–cyanin5.5 (APC– Cy™5.5) |
| Allophycocyanin | 650 (635, 647) | Indodi-carbocyanine | Cyanin 7 (Cy™7) | 767-780 | Allophycocyanin–cyanin7 (APC– Cy™7), PharRed |
| Peridinin–chlorophyll a complex (PerCP) | 490 (488) | Indodi-carbocyanine | Cyanin 5.5 | 695 | PerCP–cyanin5 (APC– Cy™5.5), TrueRed |
| Phycoerythrin (PE) | 496,546,565 (488) | | Alexa™ fluor dyes | e.g., 627; 667; 702 | PE–A610; PE–A647; APC–A680 |
| Phycoerythrin | 496,546,565 (488) | Allophycocyanin | | 660 | PE-APC |

| | | | | | |
|---|---|---|---|---|---|
| Phycoerythrin | 496,546,565 (488) | Sulphonylchloride rhodamine | Texas Red® (TR) | 613 | Phycoerythrin–Texas Red® (PE–TR), Energy Coupled Dye (ECD™), Red613 |
| Phycoerythrin | 496,546,565 (488) | Indodi-carbocyanine | Cyanin 5.5 | 670 | Phycoerythrin–cyanin5 (PE– Cy™5), Cy™-Chrome, Tri-Color®, Red670 and Quantum Red™ |
| Phycoerythrin | 496,546,565 (488) | Indodi-carbocyanine | Cyanin 5.5 | 694 | Phycoerythrin–cyanin5.5 (PE–Cy™5.5) |
| Phycoerythrin | 496,546,565 (488) | Indodi-carbocyanine | Cyanin 7 | 780 | Phycoerythrin–cyanin7 (PE–Cy™7), |

covered by a two-layer coating. Depending on which color fluorescence is required, the cores are made from cadmium sulphide, cadmium selenide (CdSe), or cadmium telluride for fluorescence in the UV-blue, most of the visible range, or the far red-infrared, respectively. Spherical or ellipsoidal in shape, they are selected for size in order to "fine-tune" the emission wavelength; for example, particles of CdSe greater than 6 nm in diameter are used for 655 nm and less than 3 nm in diameter for 525-nm emission. The shell is a largely inert, thin, insulating, transparent layer of zinc sulphide which is surrounded by the inner layer of the coating, which comprises organic ligands that are attached covalently to the shell. The outer layer of the coating is a mixed polymer with a hydrophobic facet interacting with the inner coating and a hydrophilic one interacting with the exterior in order to confer solubility in aqueous media. The outer layer also provides a suitable surface for the attachment of antibodies, streptavidin, lectins, nucleic acids, etc. Quantum dots do not have an absorption spectrum like fluorescein or PE but instead absorb light of all wavelengths up to that of their emission. They have molar extinction coefficients of $10^6$ –$10^7$ at 300–400 nm and narrow emission spectra that are typically 30 nm wide at the wavelengths that are half of the maximum emission intensity. Quantum dots are extremely photostable at wavelengths greater than 300 nm, and most important, those of different colors can be excited simultaneously by the same source.

Physical measurements indicate that most blue (the smallest) and most red (the largest) quantum dot particles have sizes of 3–10 nm (average spherical diameter), but the organic coating probably adds another 1 nm (and proteins another 2–4 nm) to the diameter. Thus, the overall size of Qdot® particles is similar to that of medium-sized proteins and somewhat smaller than that of PE. At present, Qdot® particles with emissions at 525, 565, 605, 655, 705, and 800 nm are available already conjugated to protein A or streptavidin or as secondary antibody conjugates; they are also available in kits with the reagents necessary for conjugating to purified antibodies. Antibody-conjugated Qdot® particles have been used successfully to stain both surface antigens and intracellular antigens in cells that have first been permeabilized.

### *7.5. Conjugation of Fluorochromes to Antibodies, Proteins, and Small Ligands*

Fluorochrome-conjugated antibodies, proteins, and small ligands provide powerful tools for detecting antigens and receptors, for detecting or sorting cells, or for probing aspects of cell function ***(9)***. Unfortunately, however, conjugation of a fluorochrome usually leads to quenching of fluorescence and a decreased quantum yield (e.g., when fluorescein is coupled to an antibody, its quantum yield decreases from 0.85 to between 0.3 and 0.5). In general, fluorochrome-labeled antibodies need to have a quantum yield greater than 0.4 when bound to cells

to be useful. It is possible up to a point to increase the fluorescence intensity by increasing the fluorochrome/protein ratio, but high fluorochrome/protein ratios result in decreased specific binding and increased nonspecific binding, and the optimum ratio for producing maximum brightness with fluorescein is between two and four fluorochrome molecules per molecule of protein. Fluorochrome-labeled versions of many cytokines (e.g., as Fluorokine™; R&D Systems, Inc., Minneapolis, MN) and of some small ligands for surface receptors (e.g., fMLP [*N*-formylmethionyl leucine phenylalanine]) are obtainable commercially (e.g., from Invitrogen) and can be used to determine the relative affinity and number of receptors per cell under different conditions. For instance, PE-labeled (and biotin-labeled) cytokines can be used to assess the distribution of cytokine receptors in combination with simultaneous immunofluorescence staining for surface antigens such as CD4 or CD8. In addition, fluorescent ligands (e.g., phalloidin and phallacidin) for components of the cytoskeleton can be obtained already conjugated to coumarin, fluorescein, BODIPY® FL, Oregon Green™ 488, and rhodamine, as well as to biotin (Invitrogen), from commercial sources. The fluorescent phallotoxins can be used to report filamentous actin (F-actin) formation, as they bind well to F-actin polymers (large and small) but not to monomeric G-actin, and the fluorescence intensity of cells reflects their content of F-actin.

Most of the antibodies that are needed in biomedical studies or immunohematology can also be obtained from commercial sources already conjugated to FITC, PE, APC, PerCP, or to a tandem dye (e.g., PE–Cy™5), and increasing numbers are becoming available conjugated to BODIPY® FL or to the Alexa™ dyes. Relatively few, however, can be obtained already conjugated to Texas Red®, the Alexa 350, AMCA, or Cascade Blue®, but anti-species immunoglobulins, avidin, or streptavidin conjugated to these fluorochromes can be purchased for use in indirect labeling procedures if required. If a fluorochrome-labeled antibody (or other protein) cannot be obtained commercially, it can be produced fairly quickly on a small scale in the laboratory ***(10)***. However, a purified antibody (or protein) must be used; otherwise, other components present in serum, culture supernatant, or ascites fluid (or protein preparation) may also become labeled with the fluorochrome and give high nonspecific binding; if necessary, antibodies can be isolated using commercially obtainable reagents (e.g., ImmunoPure® Plus; Pierce Biotechnology, Inc., Rockford, IL). If the purified antibody (or protein) is to be conjugated to a relatively small fluorochrome (e.g., fluorescein), it is mixed with a reactive form of the dye which is usually the isothiocyanate, the succinimidyl ester, or (less frequently) the sulphonyl chloride (**Fig. 8**). All of these derivatives react with the amine groups on the N-terminus or with ε-amino group of lysine residues in proteins; reactions with isothiocyanates and succinimidyl esters are usually carried out at a pH

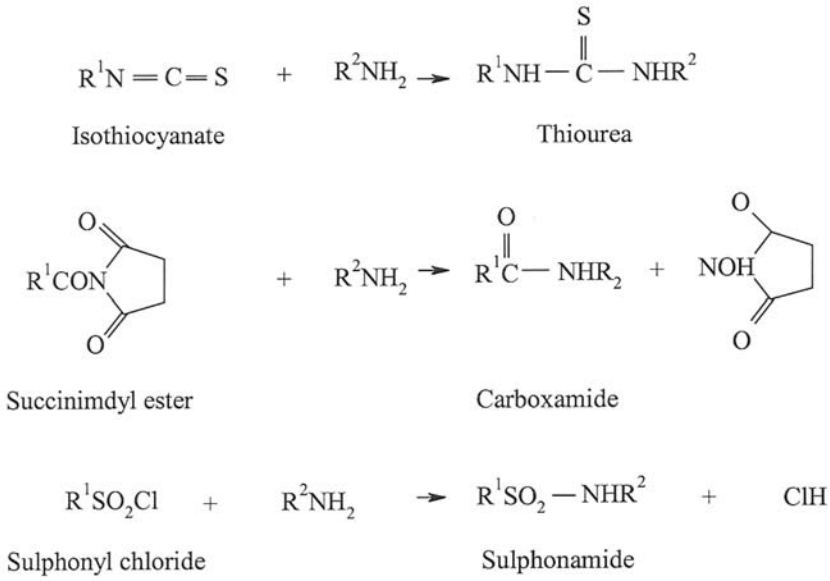


**Fig. 8.** Primary amines in antibodies or other ligands are often the target group for reaction with the isothiocyanate–, succimidyl ester–, and sulphonyl chloride–reactive derivatives of fluorochromes, which would be attached to these molecules at the position $R^1$.

of more than 9 and approx 8.5, respectively. Reactive fluorochrome derivatives can also be obtained that have aliphatic spacers between the fluorochrome and the reactive group, thereby allowing bond formation with less accessible amine groups as well as with those that are on the surface of the protein. Reagent kits (e.g., QuickTag Conjugation Kit; Roche Diagnostics, Mannheim, Germany, and Alexa™ protein labeling kits; Invitrogen) as well as the individual reactive fluorochrome derivatives are available commercially; some enable labeled antibodies to be produced within 1 h and without requiring gel filtration or dialysis. There are a range of optimum fluorochrome/protein ratios for each combination, with too-low ratios giving insufficient sensitivity and too-high ratios giving decreased affinity and higher nonspecific binding. These ratios can conveniently be determined by absorbance spectroscopy, but some trial and error may be needed to determine the optimum for sensitivity and specificity. Because of the nature of the fluorochrome, PE-labeled antibodies (or other proteins) are best produced using the reactive pyridyldisulphide derivative of PE or by crosslinking (e.g., using the Protein–Protein Crosslinking kit; Invitrogen). Roederer ***(11)*** provides detailed protocols for conjugating several different fluorochromes to mAbs.

### 7.6. Indirect Immunolabeling with Fluorochromes Conjugated to Protein A or G, Avidin, or Streptavidin

When a fluorochrome-conjugated antibody is not available, an indirect labeling procedure can be used in which an unlabeled primary antibody is revealed using a fluorochrome-conjugated anti-species immunoglobulin or a fluorochrome-conjugated form of protein A or G. Alternatively, a biotinylated primary antibody can be revealed with a fluorochrome–avidin or fluorochrome–streptavidin conjugate, or an unlabeled primary antibody can be revealed using biotin-labeled protein G to form a bridge to the same conjugates. Avidin and streptavidin fluorochrome conjugates usually provide a high sensitivity of detection because both avidin and streptavidin bind four molecules of biotin per molecule with high affinity ($K_a$ approx $10^{14}$ mol/L). However, avidin can bind nonspecifically to negatively charged cell components (e.g., nucleic acids and polysaccharides) and streptavidin can bind to integrins, giving a high background under certain circumstances. If required, biotinylated antibodies can be produced in the laboratory by using one of several different reactive derivatives of biotin which will attach to amine, sulphydryl, carboxyl, or carbohydrate groups on the protein ***(12)***. And just as with the reactive derivatives of fluorochromes, some can be obtained with an aliphatic spacer between biotin and the reactive group, to permit reaction with less-accessible sites.

## 8. Fluorochromes for Labeling Nucleic Acids

There are three common uses in flow cytometry for fluorochromes that stain nucleic acids: DNA quantification, the assessment of membrane integrity, and the rapid discrimination of nucleated from non-nucleated cells or cell fragments. None of the dyes that are currently available binds specifically to just RNA or DNA. Pyronin-Y and thiazole orange (absorption/emission maxima, 555/580 nm and 509/525 nm, respectively) will stain RNA and can be excited by the 488-nm line from an argon ion laser. Pyronin-Y also binds to DNA and glycosaminoglycans. Thiazole orange undergoes a fluorescence enhancement on binding nucleic acids (e.g., approx 3000-fold on binding DNA) but fluoresces more strongly (because of a higher quantum yield) when bound to RNA than to DNA where it binds preferentially to AT-rich regions. Many more dyes are available for staining DNA (reviewed in **refs. *13*,*14***) than for staining RNA, but these dyes also bind to RNA; therefore, when quantifying DNA, samples are usually fixed and treated with RNAase (50–100 µg/mL). If working with EB or PI, it should be remembered that they are potent mutagens that must be treated with great care. They can be purchased as solutions to avoid contact with the powders, and after use, solutions can be decontaminated by filtration through activated charcoal, which is then incinerated or completely degraded in buffer by reaction

with sodium nitrite and hypophosphorous acid. Although the mutagenic/carcinogenic potential of many other nucleic acid dyes is not known, it would be prudent to treat all compounds that are capable of binding to DNA to treat with the same degree of caution that is afforded to EB and PI. The properties of some dyes that bind to nucleic acid are described below, and the structures of some are illustrated in **Figure 9**. It is worth bearing in mind that flow cytometry can be used not only to assess the total nucleic acid content of cells, but also to detect and quantify particular nucleic acid sequences. For example, polymerase chain and *in situ* hybridization reactions can be run using fluorochrome-labeled nucleotides with cell suspensions in vitro and the results analyzed (perhaps in conjunction with immunophenotyping) by flow cytometry ***(15)***. Some applications of this principle include the detection of HIV sequences in latently and actively infected lymphocytes, the estimation of teleomere length ***(16)***, and the evaluation of allogeneic engraftment in the recipients of gender-mismatched bone marrow transplants.

*Acridine orange* binds to both types of nucleic acid, but its spectral characteristics are different when bound to RNA and DNA (absorption/emission maximum 460/650 nm and 502/536 nm, respectively). It has not been widely used in flow cytometry, partly because its sensitivity is markedly affected by even slight variations in operating parameters.

*7-Aminoactinomycin D* (7-ADD) (excitation/emission maxima approx 500, approx 580/approx 660 nm) is a dye (molecular weight 1270) that preferentially binds to GC-rich regions of DNA. It is usually excluded from live cells and can be used to label the DNA of dead cells. It can be excited by a 488-nm laser, but its quantum efficiency is low (0.035). Its main virtue is that it can be used simultaneously with FITC- and PE-labeled antibodies when a measurement of permeability or DNA content is required in the context of two-color immunofluorescence; however, its disadvantage is that it produces DNA distributions that are broader than those given by the Hoechst dyes.

*DAPI* (4′,6-Diamidino-2-phenylindole) (excitation/emission maximum 350/470 nm) is an AT-specific DNA dye that has properties similar to the Hoechst dyes (*see* subsequently in this subheading).

*DRAQ5* (1,5-bis{[2-(methylamino)ethyl]amino}-4,8-dihydroxyanthracene-9, 10-dione) (excitation maximum approx 650 nm) is a membrane-permeant synthetic anthroquinone that enters cells and intercalates strongly into DNA. It is optimally excited by red light but can also be excited suboptimally by the 488-nm line of an argon ion laser and emits in the far-red to short infrared (from approx 665 to greater than 780 nm). It has several advantages over LDS-751 and is now available commercially (Cambridge BioScience Ltd, Cambridge, UK). In particular, it can be used as a quantitative DNA stain and can be used in conjunction with FITC- and PE-conjugated antibodies without the need for compensation ***(17)***.

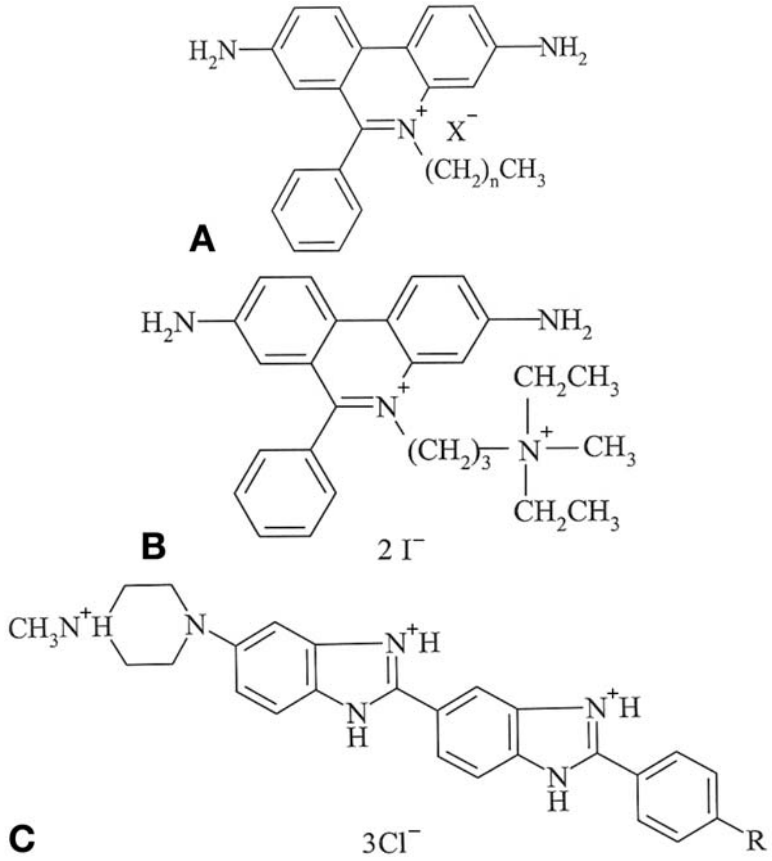


**Fig. 9.** The structures of some dyes that bind to nucleic acids. **(A)** Ethidium bromide ($n$ = 1 and X = Br) and the cell-permeant dye hexidium iodide ($n$ = 5 and X = I). **(B)** Propidium iodide. **(C)** Hoechst dyes, R = OH in Hoechst 33258 and R = $OCH_2CH_3$ in Hoechst 33342.

*EB* and *PI* are structurally similar dyes, but they cannot be used interchangeably. EB (excitation/emission maxima, 518/605 nm; **Fig. 9A**) enters intact cells slowly, but PI (excitation/emission maxima 535/617 nm; **Fig. 9B**), which has a higher water solubility and two positive charges (rather than one), is excluded from cells with an intact membrane. Both EB and PI intercalate between the bases of nucleic acids and show little preference for nucleotide sequence. There is a change in their absorption and emission maxima when bound to nucleic acids, and they also undergo an enhancement in fluorescence. The advantages of PI over EB for staining DNA are that it gives lower coefficients of variation in ploidy studies and that its emission is 10–15 nm further into red than that

of EB, which allows fluorescein labels to be used more easily for simultaneously staining other components. PI is generally used in preference to EB for testing membrane integrity, although EB has been used to investigate the slight increases in permeability that occur during the early stages of apoptosis. When using PI to quantify DNA, cells are fixed and the RNA digested with RNAase before staining, to avoid binding to double-stranded regions of RNA. Reagent kits containing PI and stable RNAase can be obtained commercially (e.g., DNA stain; Beckman Coulter, Fullerton, CA, and Cellular DNA Flow Cytometric Analysis Kit; Roche Applied Science, Indianapolis, IN).

*Hoechst 33258* and *Hoechst 33342* (excitation/emission maxima approx 350/460 nm) (**Fig. 9C**) bind preferentially to the minor outer groove of the helix in AT-rich regions of DNA and can be excited with the 325-nm line of a 1–10 mW helium–cadmium laser. They are membrane-permeant dyes, but apoptotic cells (which have permeable membranes) take up dye faster than live cells. The Hoecsht dyes can be used to quantify DNA, but higher concentrations of Hoechst 33342 are required for vital DNA staining than are needed for staining fixed or permeabilized cells (5–10 μmol/*L* for at least 30 min, compared with ≤ 3 μmol/*L*).

*LDS-751* is a cell-permeant stain that binds to RNA (excitation/emission maxima approx 590/607 nm) and to DNA (excitation/emission maxima approx 543/approx 712 nm) with an approx 20-fold increase in fluorescence on binding to double-stranded DNA ***(18)***. It can be excited by the 488-nm line of an argon ion laser, and its emission in the red/long red wavelengths makes it particularly useful for multicolor analysis in instruments equipped with just a single 488-nm laser. It is useful for distinguishing leukocytes from non-nucleated cells and platelets in whole blood.

*Mithramycin* (excitation/emission maxima 440/575 nm) is an antitumor antibiotic that forms fluorescent complexes with GC-rich regions of DNA but does not bind to RNA. It has a relatively low quantum efficiency but can be excited by the 441-nm line from a helium–cadmium laser or the 457-nm line from an argon ion laser. Ethanol-treated cells are permeable to the mithramycin and are optimally stained using 50–100 μg/mL of mithramycin in a $Mg^{2+}$ concentration of 15–200 mmol/L. If cells are stained with combination of EB and mithramycin and excited by blue–violet light, energy trapped by the mithramycin can induce EB fluorescence by FRET, and in these circumstances the fluorescence is DNA-specific because mithramycin (unlike EB) binds only to DNA.

*SYTO®*, *SYTOX®*, *TOTO®*, and *TO-PRO®* dye series (Invitrogen) are strongly absorbing cyanine dyes with extinction coefficients (ε) that are typically more than 50,000/cm mol/L at visible wavelengths. They have moderate-to-very high affinity for nucleic acids to which they bind by intercalation (and at high dye/base ratios also by external binding) and little, or no, affinity for other biopolymers. Their quantum yields are remarkably low (usually <0.01) when unbound

but increase (often more than 1000-fold) to as high as 0.9 upon binding to nucleic acids. Each member in the series has different spectral characteristics, but representatives in the series can be found that have fluorescence excitation and emission wavelengths that span the entire range of the visible spectrum, making them suitable for use in many different instruments. It is their differences in permeability to cell membranes and specificity in binding to nucleic acids that allow their assignment to different series. The SYTO® dyes are cell-permeant, lower-affinity nucleic acid stains that will bind to RNA and DNA in both live and dead eukaryotic cells as well as both Gram-positive and -negative bacteria. There are many members in the series, some of which have spectral characteristics that overlap with fluorescein, but they cannot be used for DNA quantification, because they also stain other components. The SYTOX® dye family comprises three cell-impermeant cyanine dyes that are particularly good dead-cell stains. SYTOX® Green is a high-affinity nucleic acid stain with little base selectivity which easily enters cells with permeable plasma membranes, but is excluded from live cells. It has the advantages that no wash steps are required when it used as a viability stain, because all of the SYTOX® dyes are essentially nonfluorescent in aqueous medium, and that it can be used with blue-, orange-, and red-fluorescent labels for multiparameter analyses. SYTOX® Blue and SYTOX® Orange are stains with similar physical properties but dissimilar spectral properties. The TOTO® and TO-PRO® dye series are derivatives of thiazole orange (a cyanine dye) which are cell-impermeant as a result of their positive charge and which bind to double-stranded DNA with high affinity and also to single-stranded DNA and RNA. Members of the TO-PRO® series (e.g., TO-PRO-1®, excitation/emission maxima 515/531 nm) are monomeric and have two positive charges, whereas those of the TOTO® series (e.g., TOTO-1®, excitation/emission maxima 514/533 nm) are dimeric versions of these dyes and therefore have four positive charges. The TOTO-1® dye, which is a dimeric version of TO-PRO-1®, has an affinity for double-stranded DNA which is higher than that of the ethidium homodimers and approx 100-fold greater than that of the monomeric TO-PRO-1®.

*Vybrant® DyeCycle™* stains (Invitrogen) are permeant DNA-selective quantitative stains that can be used for DNA content analysis in living cells. The Vybrant® DyeCycle™ Violet stain (excitation/emission maxima 366/437 nm) is excitable by the 405-nm line of the recently developed violet diode laser. There is also a Green stain excitable at 488 nm and an Orange stain that can be excited using either a 488-nm or 532-nm laser line.

## 9. Probes for Cell Viability and Apoptosis

Cells die by ways that are characterized in their extremes as necrosis or apoptosis, and when dying, they undergo many changes. The earliest changes

may be damaging but reversible, and it may be difficult to define the point when recovery is no longer possible. A wide range of different criteria have been used to distinguish "live" from "dead" cells and to determine, using multiparameter flow cytometry, whether cells are undergoing changes typical of necrosis or apoptosis. Criteria used for monitoring viability include ATP generation, esterase activity, loss of membrane integrity, and maintenance of the transmembrane potential (which requires good membrane integrity). Those used for monitoring apoptosis include DNA strand breakage, exposure of phosphatidylserine on the plasma membrane, increased membrane permeability, induction of caspase activity, and loss of mitochondrial transmembrane potential. It is important to exclude dead cells from the analysis when immunophenotyping because they bind antibodies nonspecifically, and membrane integrity revealed by DNA staining (e.g., with PI) is a reliable single-parameter method that can be used for this purpose. However, if the nature of cell death is being studied *per se*, it is best to monitor several different criteria simultaneously. The structures of some of the probes used for monitoring viability, oxidative metabolism, and intracellular ion concentrations are given in **Fig. 10**. Many reagent kits based on different principles and incorporating different fluorochromes are available from Invitrogen and other suppliers for monitoring viability and apoptosis; some of these kits are listed in **Tables 3** and **4** (reviewed in **refs. *19–21***).

### *9.1. Membrane Integrity*

Although it is possible to assess membrane integrity using fluorochrome-labeled antibodies that will react only with biomolecules confined to the cytoplasm or organelles, it is generally done using dyes that react with internal components (e.g., DNA) but that are normally excluded from cells with an intact plasma membrane. The DNA-binding dyes, 7-AAD (7-aminoactinomycin D) and PI, have long been used to identify cells with permeable membranes, but the newer DNA stains such as SYBR®-14, SYTOX GREEN®, YOYO®-1, and YO-PRO™-1 (Invitrogen) can also be used. Alternatively, cells can be stained with dyes that are membrane-permeant and that are retained inside live, but not dead, cells. These dyes are often used as their acetoxy-methyl (AM) esters, which diffuse rapidly into cells (at 37°C), where they can be hydrolyzed quickly by ubiquitous nonspecific esterases to yield intracellular fluorescent dye concentrations of more than 25 µmol/L within 10 to 60 min ***(22)***. Because the AM esters are poorly soluble in water, they are normally dissolved first in dimethylsulphoxide as a 1–10 mmol/L stock solution and then added to the medium at concentrations in the range of 1–25 µmol/L. Examples of dyes that are retained after hydrolysis, and that can be monitored in the fluorescence channel used for fluorescein, include fluorescein diacetate, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein AM ester (BCECF-AM) (**Fig. 10A**), CFDA, and calcein-AM, which

is better retained than the others. Fura-Red is a similar dye that can be detected in the fluorescence channel used routinely for PE. Target cells can also be loaded with these dyes for use in lymphocyte cytotoxicity assays.

On occasion, it may be necessary both to assess the viability of cells in a sample and to fix them for subsequent analysis. This can be done if the cells are incubated with ethidium monoazide before fixation. Ethidium monoazide cannot enter cells with intact plasma membranes but will enter permeable cells and attach covalently to any nucleic acid if photoactivated. Consequently, after deliberate photoactivation in vitro, only the dead cells with damaged membranes (and not the viable cells) will be labeled and fluoresce. Alternatively, cells can be incubated with Tri-Color®-labeled streptavidin (streptavidin coupled to the tandem dye PE–Cy™5), which will specifically and irreversibly enter dead cells and cannot be removed by washing, permeabilization, or fixation ***(23)***.

### *9.2. Transmembrane Potential*

There is a small (approx 70 mV) but measurable difference in electrical potential (the transmembrane potential) between the interior of cells, which is electronegative, and the external solution. It exists because there are concentration gradients of $K^+$, $Na^+$, and $Cl^-$ across the cell membrane which arise in part from the impermeability of the plasma membrane to ions and in part from the action of ATP-dependent ion pumps present in the membrane. The transmembrane potential can be monitored by charged dyes that redistribute across the membrane and is a useful indicator of the status of cells. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$[3]) is a positively charged (cationic) carbocyanine dye that binds readily to negatively charged cells and to negatively charged mitochondria. It can be excited at 488 nm and its fluorescence intensity, which is detectable in FL1, can be used as an indication of the transmembrane potential. Bis(1,3-diethyl barbituric acid trimethine oxonol) or ($DiBAC_4$[3]) (frequently called "bis-oxonol") is a anionic lipophilic dye that can be used instead of $DiOC_6$(3), but as a result of its negative charge, it does not bind to mitochondria and is a better dye for measuring plasma membrane potential. Rhodamine 123 and $DiBAC_4$(3) have been widely used for measuring transmembrane potential in bacteria, but $DiBAC_4$(3) is considered better than $DiOC_6$(3) for use in mammalian cells ***(3)***. When measuring the transmembrane potential, the cells must be in protein-free media, the optimum conditions for dye equilibration determined, and hyperpolarizing and depolarizing controls incorporated. Different membrane potentials are created using valincomycin (a potassium ionophore) and buffers of varying $K^+$ concentrations in order to construct a calibration curve that relates fluorescence intensity in an approximate way to the transmembrane potential ***(24)***.

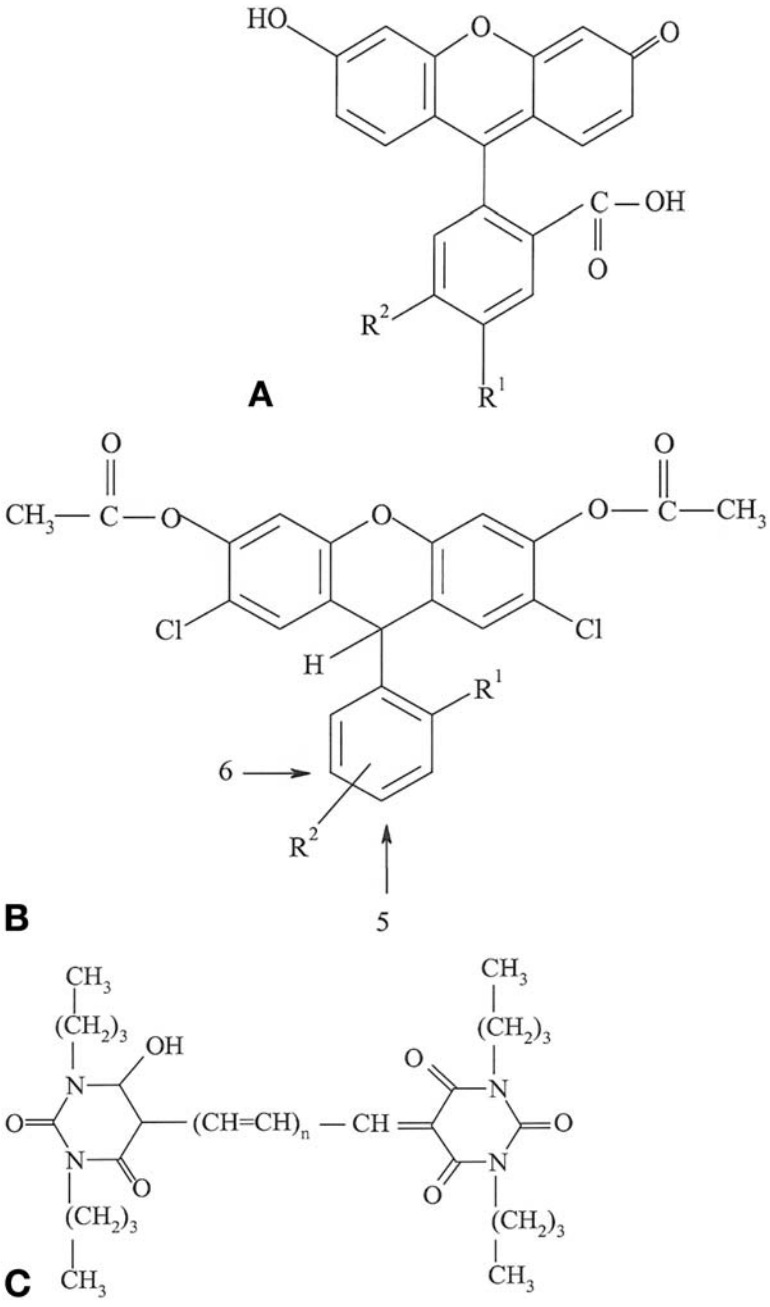


**Fig. 10.** (*Continued*)

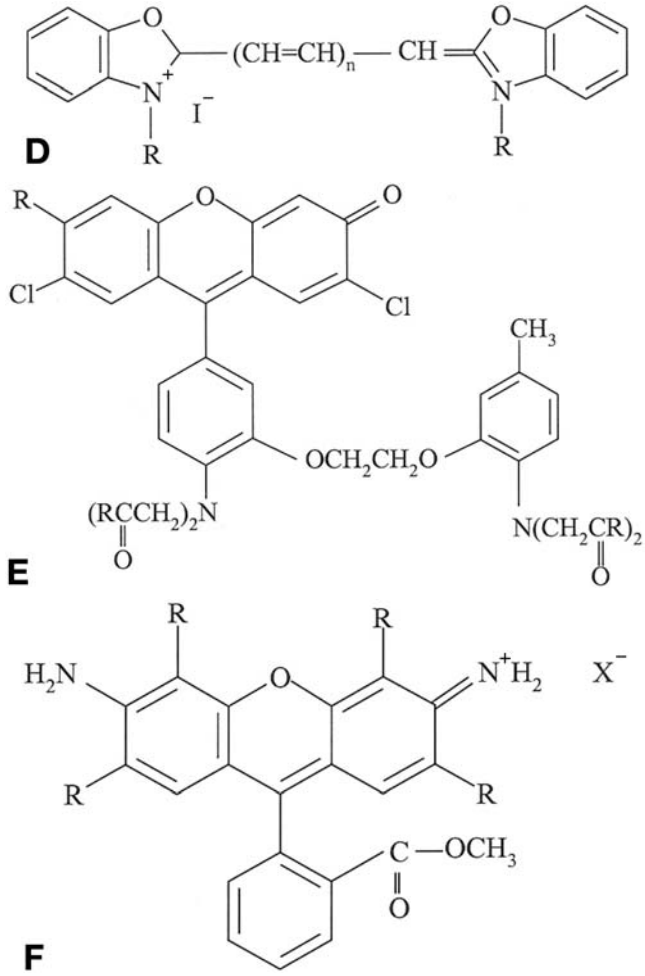


**Fig. 10.** The structures of some fluorescent probes that are used for functional studies. **(A)** The mixed isomers of 2′,7′-bis(carboxyethyl)-5-(and -6-)-carboxyfluorescein (BCECF), a pH-sensitive probe. **(B)** The mixed isomers of 5- (and 6-)-carboxy-2′,7′-diclorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA), a probe for the estimation of intracellular oxidants. **(C)** The bis-barbituric acid oxonols (DiBAC), which are slow-response membrane-potential dyes: $n = 1$ in bis(1,3-dibutylbarbituric acid)trimethine oxonol (DiBA$C_4$[3]), and $n = 2$ in bis(1,3-dibutylbarbituric acid)pentamethine oxonol DiBA$C_4$(5). **(D)** The oxa carbocyanines (DiOC), which are slow-response membrane-potential dyes: $n = 1$ and R = $–(CH_2)_4CH_3$ in 3,3′-dipentyloxacarbocyanine iodide (DiO$C_5$[3]), and $n = 1$ and R = $–(CH_2)_5CH_3$ in 3,3′-dihexyloxacarbocyanine iodide (DiO$C_6$[3]). **(E)** The cell-permeant $Ca^{2+}$ indicators Fluo-3, in which R = $O^–$, and Fluo-3 AM (Fluo-3 acetoxy-methyl ester), in which R = $–OCH_2COOCH_3$. **(F)** Rhodamine 123, a mitochondrial transmembrane probe.

**Table 3**
**Some Reagent Kits for Assessing Cell Viability**

| Commercial name | Supplier | Principle of the assay |
|---|---|---|
| LIVE/DEAD® *Bac*Light Bacterial Viability Kit | Invitrogen | SYTO® 9 dye and propidium iodide label live and dead bacteria, respectively |
| LIVE/DEAD® Fixable Violet Dead Cell Stain Kit | Invitrogen | A violet-fluorescent reactive dye (excitable at approx 405 nm) reacts with the surface of live and internal contents of dead cells; subsequent fixation is possible |
| LIVE/DEAD® Viability/ Cytotoxicity Kit | Invitrogen | Esterase action on calcein AM and exclusion of ethidium homodimer I (membrane integrity) |

### *9.3. Apoptosis*

*Phosphatidylserine* occurs almost exclusively on the inner leaflet of the plasma membrane in viable cells but becomes exposed on the outer leaflet usually during the early stages of apoptosis and before the loss of membrane integrity. The loss of lipid asymmetry and exposure of phosphatidylserine can be detected using fluorochrome-conjugated annexin V (a $Ca^{2+}$-dependent phospholipid-binding protein), but because phosphatidylserine exposure also occurs during necrosis, simultaneous staining for membrane permeability (e.g., with PI) enables the two states to be distinguished.

*Mitochondrial transmembrane potential* is maintained in viable but not apoptotic or necrotic cells and can be probed using dyes such as JC1 and rhodamine 123. The latter is a cationic dye that concentrates in live cells and membrane-bounded organelles (e.g., mitochondria) to produce a strong green fluorescence. Cells undergoing the early stages of apoptosis can lose their mitochondrial transmembrane potential while maintaining their plasma membrane integrity and therefore exhibit decreased rhodamine 123 fluorescence while still excluding PI; later on, however, they exhibit no rhodamine 123 fluorescence and become permeable to PI. There may be some advantage in using EB instead of PI, in conjunction with rhodamine 123, to detect these early apoptotic changes in cells given that EB seems to be a more sensitive indicator of membrane permeability than does PI ***(25)***. However, rhodamine 123 has not been found to be as reliable for measuring mitochondrial transmembrane potential as the symmetric cyanine dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC1), which forms fluorescent aggregates that reflect the transmembrane potential. The JC1 monomers emit at 527 nm, and the JC1 aggregates emit at 590 nm; consequently, the ratio of the fluorescence at these

two wavelengths gives a measure of transmembrane potential which is independent of dye concentration (uptake).

*DNA strand breakage* caused by an endonuclease cleaving chromosomal DNA at linker regions between nucleosomes is a characteristic feature of apoptosis induced by many stimuli in most cell types and can be detected in several ways. For example, if cells are fixed in alcohol or acetone, the small DNA fragments produced by cleavage will diffuse out when the cells are washed, resulting in reduced DNA content (staining) in apoptotic cells. However, cells should not be fixed with formaldehyde, because its action in crosslinking proteins and nucleic acids will cause DNA fragments to be retained. Alternatively, fluorochrome-, biotin-, or digoxigenin-labeled dUTP nucleotides can be added to the 3′-OH ends of broken DNA strands using the enzyme TdT (terminal deoxynucleotidyl transferase) (terminal deoxynucleotidyl transferase dUTP nick-end labeling, or TUNEL, assay) and visualized directly or with a fluorochrome conjugated to avidin/streptavidin or anti-digoxigenin, respectively.

*Caspase Activation* The activity of certain caspases (cysteine-aspartyl–specific peptidases) and other enzymes associated with the apoptotic pathway can be measured with substrate analogs (e.g., the CellProbe™ [Beckman Coulter] and PhiPhiLux [OncoImmunin, Inc., Gaithersburg, MD] reagents), which produce a fluorescent signal after enzymic conversion. Caspases can also be detected using fluorochrome-labeled inhibitors of caspases (FLICAs) that bind irreversibly to the active site of caspases. The FLICA ligands are peptide-fluoromethyl ketones that have been conjugated to fluorescein or sulphorhodamine, with the specificity to particular caspases being provided by the peptide. Alternatively, the carboxyfluorescein derivative (FAM) of benzoxycarbonyl-valinealanine-aspartic acid-fluoromethylketone (zVAD-FMK) (shortened to FAM-VAD-FMK) can be used as a reactive broad-spectrum caspase inhibitor.

## 10. Fluorescent Probes for Determining Intracellular Ion Concentrations

Fluorochromes are available that can be used to estimate the concentration of several biologically important ions, including $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, $K^{+}$, $Cl^{-}$, and $H^{+}$ (or pH). These fluorochromes all bind their target ions stoichometrically with a dissociation constant that approximates to the usual intracellular concentration of the ion and, on binding it, undergo some detectable change in their fluorescence properties, which is often an increase or decrease in quantum yield or a wavelength shift in the absorbance and emission spectra. The optimum amount of dye to be used is a compromise between the most that can be used without buffering the ion and perturbing normal cell function, and the least that will give easily detectable fluorescence. Most of the ion-selective dyes contain

**Table 4**
**Reagents and Reagent Kits for Detecting Changes Characteristic of Apoptosis**

| Commercial name | Supplier | Principle of assay[a] |
|---|---|---|
| Annexin-V FLUOS Staining Kit | Roche Diagnostics (Basel, Switzerland) | Annexin V binding and/or exclusion of propidium iodide |
| ApoAlert™ Annexin V Assay | BD Biosciences (San Jose, CA) | Annexin V binding |
| ApoAlert™ Mitochondrial Membrane Sensor Kit | BD Biosciences (Clontech) | BD MitoSensor™ Dye senses changes in mitochondrial transmembrane potential. |
| APO-BRDU™ and APO-DIRECT™ kits | BD Biosciences (PharMingen) | TUNEL assay |
| Apo-BRDU™ and Apo-Direct™ TUNEL Assay Kits | Chemicon International Inc., (Temecula, CA) | TUNEL assay |
| ApopNexin™ Apoptosis Detection Kits | Chemicon | Annexin V (various conjugates) and propidium iodide |
| ApopTag® Plus In Situ Apoptosis Detection Kit | Oncor (Gaithersburg, MD) | TUNEL assay |
| Apoptosis detection kit | | |
| Apoptosis detection kit | R&D Systems, Inc. | Annexin V binding |

| | | |
|---|---|---|
| BD™ Anti-Active Caspase-3 | BD Biosciences | Antibody specific for the active form of caspase 3 |
| Caspase Flow Cytometric Assay Kit | Calbiochem Merck KGaA; Darmstadt, Germany | Cleavage of $(aspartyl)_2$-Rhodamine 110 (substrate analog) |
| CaspSCREEN™ Apoptosis Detection Kit | BioCat GmbH (Heidelberg, Germany) | Cleavage of $(aspartyl)_2$-Rhodamine 110 (substrate analog) |
| DeadEnd™ Fluorometric TUNEL System | Promega (Madison, WI) | TUNEL assay |
| DePsipher™ | R&D Systems, Inc./Trevigen | Mitochondrial transmembrane potential |
| FlowTACS™ | R&D Systems, Inc. | TUNEL assay |
| In Situ Cell Death Detection Kit | Roche Diagnostics | TUNEL assay |
| PhiPhiLux® | OncoImmunin, Inc. (Gaithersburg, MD) | Profluorescent green and red caspase substrate analogs |
| Vybrant Apoptosis Assay Kits #1 to #14 | Invitrogen | A series of kits comprising different reagents/ fluorochromes for the multiparameter characterization of cell death |
| YO-PRO™-1 | Invitrogen | Selective uptake of DNA stain by apoptotic cells |

[a]TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

one or more carboxyl groups that are charged at pH 7.3, which cause(s) them to be excluded from cells, so they are most often used as uncharged (and, therefore, cell-permeant) lipophilic esters, particularly as the AM esters (as described in **Subheading 9.1.**). The ion-sensitive dye is produced from these precursors following hydrolysis by nonspecific esterases and remains trapped within the cell.

### *10.1. Calcium Ions*

Calcium ions are key regulators of several processes in eukaryotic cells and despite much higher external concentrations (1.3 mmol/L or approx $10^{-3}$ mol/L), cytoplasmic $Ca^{2+}$ concentrations are maintained at very low levels (100–150 nmol/L or approx $10^{-7}$ mol/L). One key function of $Ca^{2+}$ is to act as the second messenger when signals from the exterior of the cell are transmitted to the interior. During the response to certain external stimuli, there is a rapid rise in $Ca^{2+}$ concentration, which is achieved by an influx from the exterior, or by a release from internal stores (e.g., from calciosomes or the sarcoplasmic reticulum). The increased $Ca^{2+}$ concentration can regulate a number of processes directly or by binding to calmodulin (a ubiquitous eukaryotic $Ca^{2+}$-binding protein), in which it induces a conformational change that enables calmodulin to participate in many different regulatory processes. Increases in cytoplasmic $Ca^{2+}$ concentration are, therefore, one of the earliest indicators of cellular activation after signal transduction. There are two types of dye useful for measuring intracellular $Ca^{2+}$ concentrations by flow cytometry. One (e.g., INDO-1) undergoes a change in excitation/emission wavelength maxima on binding $Ca^{2+}$ (346/495 nm in low [$Ca^{2+}$] and 330/408 nm in high [$Ca^{2+}$]), whereas the other (e.g., Fluo-3) (excitation/emission maxima 506/526 nm) and its analogs undergo an increase in fluorescence intensity on binding $Ca^{2+}$. Binding of $Ca^{2+}$ by INDO-1 is best assessed by a ratio procedure in which the fluorescence measurements are made on the same cell at the two different emission wavelengths (i.e., in two different channels), whereas the fluorescences of Fluo-3, Fluo-4, and Fluo-5 can be monitored in a single channel. Fluo-4 has a greater absorption at 488 nm than Fluo-3 and as a result gives a stronger fluorescence signal. Fluo-5 is a derivative of Fluo-4 with a lower affinity for $Ca^{2+}$, making it suitable for detecting $Ca^{2+}$ concentrations between 1 μmol/L and 1 mmol/L, which would saturate both Fluo-3 and Fluo-4. All dyes are best loaded into cells as their AM ester. Fluorescence intensity results can be interpolated with a calibration curve that has been constructed in the same cells using a $Ca^{2+}$ ionophore and set $Ca^{2+}$ concentrations determined by a $Ca^{2+}$ ethyleneglycoltetraacetic acid (EGTA) solution. Because EGTA is a $Ca^{2+}$ chelator, which does not cross the plasma membrane, it can also be used to determine whether ingress of $Ca^{2+}$ from the external solution is involved in the response to a stimulus.

### *10.2. pH Values*

The large negative transmembrane potential of eukaryotic cells leads to the passive inward diffusion of $H^+$, which if not controlled, would result in acidification of the cytoplasm. However, to permit the cell to function, its cytoplasmic pH ($pH_i$) is usually maintained near to neutral (pH approx 7.2) by one or more $Na^+$-driven antiporters ($Na^+$–$H^+$ ion exchangers). These ion pumps are proteins located in the membrane which use energy stored in the $Na^+$ gradient to export $H^+$ that has entered by inward diffusion or that has been produced by metabolism. In many cell types, there is a strong correlation between $pH_i$ and cell proliferation, with changes in $pH_i$ detectable by flow cytometry being associated with progression through the cell cycle in cells as diverse as yeasts and lymphocytes. In cultured mammalian cells, increases in $pH_i$ values to 7.4–7.5 are associated with proliferation, whereas decreases in $pH_i$ to 7.1–7.2 are associated with quiescence and to $pH_i$ 6.8 with decline. Observations on mammalian cells in both batch and continuous cultures indicate that there is an approximately linear relationship between the $pH_i$ and growth rate, at least over the $pH_i$ range 7.4–7.6.

Three main pH indicator dyes are available for use in flow cytometry: BCECF, carboxy-seminaphthorhodafluor (SNARF®-1), and 2,3-dicyanohydroquinone (DCH). All are weak acids, and therefore their $pK_a$ must be matched to the expected range of pH values to be measured. BCECF, which is loaded into cells in the form of its AM ester, has a $pK_a$ of 7, making it useful for measurements in the physiological pH range. It is retained well by cells and is confined mostly to cytosol, enabling measurements to be made over periods of 1–2 h, if required. Although its quantum yield is relatively low, its pH-dependent absorption maximum at 500 nm makes it easily excitable by a 488-nm argon laser, and its fluorescence at 525 nm increases with pH. To avoid potential variability resulting from differences in dye loading or cell size, it is preferable to measure the fluorescence intensity ratio at approx 525 nm to more than 610 nm, rather than just the intensity at approx 525 nm. SNARF®-1, which is also loaded in its AM form, is now probably the most frequently used pH probe because it has several advantages compared with BCECF. Like BCECF, it can be excited at 488 nm, but it undergoes greater pH-dependent changes, resulting in a decrease in fluorescence emission intensity at approx 575 nm and a concomitant increase at approx 625 nm. Consequently, ratio measurements at these wavelengths give better sensitivity in the pH range 7.0–8.0 than can be obtained with BCECF. DCH, which has a $pK_a$ of 8, is not much used, because it absorbs in the UV and has a high leakage rate from cells. When using any of these probes, the optimum conditions for dye loading must be established, and a pH calibration curve must be constructed using cells treated with the ionophore

nigericin (which exchanges $K^+$ for $H^+$ across membranes) held in buffers of different pH values containing high $K^+$ concentrations.

## 11. Fluorescent Probes for Phagocytosis and Oxidative Metabolism

### *11.1. Phagocytosis*

Several different strategies can be used to assess the phagocytic activity of cells (e.g., neutrophils) in vitro, either alone or together with simultaneous measurements of the oxidative burst (reviewed by Plested and Coull ***[26]***). Generally, fluorochrome-labeled latex microspheres or fluorescent particles (e.g., zymosan, formaldehyde-fixed bacteria, or yeasts) that have been opsonized (usually with serum) are added to phagocytic cells at particle/cell ratios ranging from 5:1 to 100:1 and incubated at 37°C with intermittent, or continuous, shaking. At a defined time(s), samples are removed, the cells are washed to remove unattached particles and resuspended, and the fluorescence from surface-bound and engulfed particles determined. For example, if fluorescein-labeled formaldehyde-fixed bacteria have been used, EB (which binds to DNA) can be added (50 µg/mL) to the cells after they have been washed. Because it is unable to pass through the plasma membrane of viable phagocytic cells, it will stain only the adherent bacteria. Consequently, those phagocytic cells that have already internalized bacteria will be green-fluorescent and those that have adherent bacteria will be red-fluorescent. It is also possible to distinguish internalized from adherent fluorescein-labeled particles by lowering the pH of the external medium or by adding a dye (e.g., trypan blue or crystal violet) that will quench fluorescence from the adherent, but not from the internalized, particles. However, the reproducibility of these procedures can sometimes be less than satisfactory. When these techniques are being used only to distinguish phagocytic from nonphagocytic cells, the size of the ingested particle is of little importance. However, when attempting to quantify phagocytic activity, it is best to use small particles such as fluorescent microspheres because in histograms of fluorescence intensity, these give sharper peaks corresponding to cells that have ingested one, two, three, etc. particles, than if larger particles such as yeasts are used. Fluorochrome-labeled microspheres (0.5–2.0 µm), bacteria (e.g., *Escherichia coli* and *Staphylococcus aureus*), and zymosan are commercially available from several sources (e.g., Invitrogen), and diagnostic reagent kits using fluoresceinated *E. coli* are also available (e.g., Phagotest®; Orpegen Pharma, Heidelberg, Germany, and Vibrant™; Invitrogen).

### *11.2. Oxidative Metabolism*

Several different dyes for detecting the production of intracellular oxidants are available, including carboxy-$H_2$DFCDA, 2′,7′-dichlorodihydrofluorescin

diacetate ($H_2$DCFH-DA), dihydroethidium (Hydroethidine™, a trademark of Prescott Labs; Invitrogen), and dihydrorhodamine 123, all of which are cell-permeant nonfluorescent precursors that on oxidation are converted to a fluorescent form. Esterases first convert $H_2$DCFH-DA to a nonfluorescent intermediate, 2′,7′-dichlorofluorescin (DCFH), that is retained in the cell. Then during the respiratory burst, the intermediate is further converted by $H_2O_2$ and superoxide ($O^-_2$) together with peroxidase action to fluorescent 2′,7′-dichlorofluorescein (DCF), which is also well retained in cells (**Fig. 11**). DCFH can be oxidized not only by $H_2O_2$ and $O^-_2$, but also by nitric oxide. This is not usually a problem if it is being used to measure the oxidative burst in neutrophils, because they have little L-arginine. However, if the cells are treated with an oxidative burst inhibitor (e.g., diphenylene iodinium), it can be used to study nitric oxide production in monocytes. Nitric oxide production can also be detected using 1,2-diaminoanthroquinone (Invitrogen), which is nonfluorescent until it reacts with nitric oxide, when a red-fluorescent precipitate is produced.

If EB is reduced with sodium borohydride, it yields hydroethidine, which is cell-permeant and which on oxidation gives EB that will intercalate into DNA and make the cell red-fluorescent. In practice, cells are usually incubated with hydroethidine for 10 min at 37°C and then stimulated to undergo a respiratory burst while the red fluorescence (i.e., of EB) is being measured; if required, $H_2$DCFH-DA and hydroethidine assays can be run simultaneously. Currently, the most sensitive technique for detecting the respiratory burst is probably the oxidation of nonfluorescent dihydrorhodamine 123 to rhodamine 123 by $H_2O_2$ and peroxidases, because the product is positively charged and accumulates in mitochondria. A reagent kit containing unlabeled *E. coli* and dihydrorhodamine 123 (Phagoburst®; Orpegen Pharma) is available for diagnostic monitoring of the oxidative burst stimulated by bacterial phagocytosis. The Fc OxyBURST® reagents (Invitrogen), which comprise BSA–anti-BSA immune complexes coupled covalently to $H_2$DCFH, are useful for monitoring phagocytosis by monocytes and other cells that have only low esterase activity. These immune complexes are internalized after binding to cellular Fc receptors, and the nonfluorescent DCFH (which unlike $H_2$DCFH-DA does not need to be processed by intracellular esterases) is oxidized by the respiratory burst to fluorescent DCF.

## 12. Fluorochrome-Labeled Substrate Analogs for Measuring Enzyme Activity

Enzymes are essential components that regulate cell properties and function. Moreover, the presence and/or activity of a particular enzyme is often related to the cell type and/or its differentiation state, a principle that is exploited in hematology analyzers by using esterase and/or peroxidase activity to distinguish leukocyte classes. An extension of this principle is to incubate cells

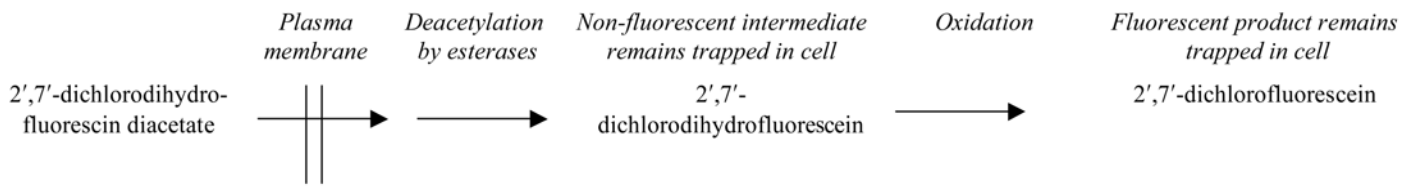


**Fig. 11.** The uptake and conversion of 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFH-DA) to the fluorescent product 2′,7′-didichlorofluorescein (DCF) that is retained within the cell.

with nonfluorescent cell-permeant substrate analogs that are converted by the enzyme of interest into a fluorescent form that is retained in the cell and can be monitored by flow cytometry. The technique, which has been termed "cytoenzymology" (Beckman Coulter), is applicable to live cells in whole blood, isolated cells, and microorganisms; among the enzymes that can be assayed using its series of 36 different CellProbe™ substrate analog reagents are β-galactosidase, β-glucuronidase, caspases, glycosidases, microsomal dealkylases, peptidases, and peroxidases. Haugland ***(2)*** has discussed in detail the detection of enzyme activity by means of fluorogenic substrates, and a wider range of products for this purpose are available from Invitrogen. If these fluorogenic substrate analogs are used in conjunction with immunophenotyping, the enzyme activities of various cell types and subsets can be more fully characterized. The FRET-based PhiPhiLux series of peptide analogs for detecting caspases is also available from OncoImmunin, Inc.

## 13. Fluorescent Dyes for Measuring Total Protein

The total protein content of cells can be estimated using a number of acidic dyes that bind ionically or using reactive dyes that bind covalently to proteins. Unfortunately, the latter dyes react mainly with positively charged amino groups and consequently can bind to materials other than proteins. For example, FITC will bind covalently to proteins and the fluorescein moiety remains attached after washing, whereas sulphorhodamine 101, available as FluoReporter® in the Fluorometric Cell Protein Assay Kit (Invitrogen), forms electrostatically stable complexes at low pH values. Estimates of total protein content that are made in conjunction with quantitative DNA measurements can be useful for measuring growth and metabolism in heterogeneous populations. An interesting recent development of this principle is an assay of (antigen-) peptide-specific T-cell proliferation that measures the decrease in fluorescence when antigen-stimulated cells that have been stained with carboxyfluorescein diacetate succidimyl ester proliferate.

## 14. Fluorochrome Combinations Suitable for Use in Instruments Equipped with a Single Laser Emitting at 488 nm

The choice of fluorochromes for use in flow cytometry studies is crucially determined by the illumination source(s) available in the instrument; however, in the vast majority of flow cytometers in current use, there is (at least) an air-cooled argon ion laser usually providing 15–25 mW at 488 nm. Fluorescent light is then passed through a number of optical filters before reaching the photomultiplier that initiates the signal amplification in each fluorescence channel, and these filters are usually numbered sequentially (FL1 through FL3 or FL4) starting from the lowest wavelength. Older instruments such as the EPICS® XL (Beckman Coulter) and the FACScan™, Research FACScan™,

and FACStar™ (BD Biosciences) that are powered by a single laser emitting at 488 nm usually have three or four fluorescence detectors. For example, a FACScan™ with three fluorescence channels detects light at 530 ± 15 nm (FL1), 585 ± 21 nm (FL2), and >650 nm or >670 nm (FL3), using band-pass (FL1, FL2) and long-pass (FL3) filters, whereas the EPICS® XL detects fluorescence at 525 ± 12.5 nm (FL1), 575 ± 12.5 nm (FL2), 620 ± 12.5 nm (FL3), and 675 ± 12.5 nm (FL4) using band-pass filters. One problem encountered when working with instruments powered by a single 488-nm argon laser is to find different fluorochromes that can be optimally excited at this wavelength yet that fluoresce at significantly different wavelengths. Because there are relatively few dyes with these characteristics, fluorochromes that have absorption (excitation) maximum wavelengths away from 488 nm are often used, but excitation at suboptimal wavelengths limits the intensity of fluorescence that can be emitted. Another problem is that most fluorochromes emit light over a wider range of wavelengths than is selected by the band-pass and/or long-pass filters. Consequently, although most of the fluorescence from fluorescein is collected in the FL1 channel of instruments such as the EPICS® XL and FACScan™, some also enters the channel (FL2) that is often used to monitor emissions from PE. Likewise, if PE is being used, some fluorescence from PE also enters both the FL3 and FL1 channels. Therefore, whenever cells have been labeled with two (or more) fluorochromes, it is necessary to correct the signal in each fluorescence channel for the "spillover" of the fluorescence from other fluorochromes that are being detected primarily in the other channels. Compensation for spillover may be done either during or after data acquisition ***(27)***. Traditionally, it has been done by using the analog circuitry, available in many cytometers, to subtract from the number of events recorded in one channel, a percentage of those that are being recorded primarily in another channel. The percentage of events to be subtracted is most easily set by using beads that contain the fluorochromes being used (e.g., Calibrite or Quantibrite [BD Biosciences], CompenFlow Flow Cytometry Compensation Kit [Invitrogen], QIFI kit [Dako, Glostrop, Denmark], or Quantum Series [Flow Cytometry Standards Corporation, now part of Bangs Laboratories, Inc., Fishers, IN]). However, fine adjustments need to be made using cells (e.g., lymphocytes) that have been labeled using antibodies (e.g., CD3, CD4, and CD8) that have been conjugated to the appropriate fluorochromes. In some cytometers (e.g., the Epics XL [Beckman Coulter]), the process can be done automatically using software (the XL SYSTEM II™) supplied with the instrument. Ortolani ***(28)*** and Roederer ***(29,30)*** provide a fuller discussion of the principles and practice involved in correctly setting compensation for multicolor analysis. Just recently, alternative approaches were developed that transform raw data to avoid the production of zero or negative values

(HyperLog™; ***[31]***) and that scale the logarithmic fluorescence intensity axes so that the signals from all cells are properly represented (the "Logicle" visualization; ***[32]***).

A good combination of fluorochromes for use in two-color immunolabeling is FITC and PE. Similarly, a good combination for use in three-color immunolabeling is FITC (FL1), PE (FL2), and PerCP or a tandem conjugate such as PE–indodicarbocyanine (PE–Cy™5) (FL3). Four-color immunolabeling can be readily done using FITC (FL1), PE (FL2), PE–Texas Red® (FL3), and PE–Cy™5 or PerCP or, preferably, PE–indotricarbocyanine (PE–Cy™7) (FL4) because its emission goes further into the long red region than the others. It is, of course, necessary to compensate for spectral overlap with all fluorochrome combinations, and this gets more difficult as the number of fluorochromes being used increases; consequently, fluorochromes with narrower bandwidths, such as the BODIPY® dyes, are particularly useful for multicolor immunolabeling. However, because of spectral overlap, the maximum number of different fluorochromes that can satisfactorily be detected simultaneously by instruments powered by a single laser emitting at 488 nm is, at present, four/five. Before attempting to use fluorochromes that emit in the long red region of the spectrum, it is worth checking that the cytometer is fitted with a photomultiplier (e.g., R3696; Hamamatsu Photonics K.K., Hamamatsu City, Shizuoka Pref., Japan) that has good sensitivity at these wavelengths. Also, before undertaking four-color analysis, it is worth checking that the instrument hardware/software allows compensation to be set adequately for spectral overlap between the channels used for detecting the various fluorochromes. Premixed reagents are commercially available to facilitate multicolor analysis of lymphocyte antigens (e.g., Simultest™, comprising FITC–CD3 and PE–CD4; TriTEST™, comprising FITC–CD3/PE–CD8/PerCP–CD45; and MultiTEST™, comprising FITC–CD3, APC–CD4, PE–CD8, and PerCP–CD45 [BD Biosciences]). When choosing antibody conjugates for multicolor staining, the fluorescence intensities and signal-to-noise ratios of each should be taken into account. In general, PE-labeled antibodies give a five- to tenfold greater sensitivity than comparable FITC-labeled antibodies, which are more sensitive than antibodies labeled with tandem dyes (e.g., PE–Cy™3, PE–Cy™5) or PerCP. Consequently, it is best to stain the more highly expressed antigens with tandem dyes or PerCP, and the more weakly expressed ones with PE. The selection of fluorochrome conjugates for maximum sensitivity has been discussed by Maecker et al. ***(33)***. A selection of the commonly used fluorochromes that can be excited at 488 nm, that can be conjugated to antibodies for immunolabeling or used directly in functional studies, and that can be detected in flow cytometers with three or four fluorescence channels are listed in **Tables 5** and **6**, respectively.

## 15. Fluorochrome Options When Using Instruments Equipped with Light Sources Additional to a 488-nm Laser

The addition of a laser(s) that emit at a wavelength(s) other than 488 nm makes the instrument more versatile because it extends the range of fluorochromes that can be used and therefore increases the number of cellular parameters that can be measured simultaneously. Several flow cytometers (e.g., the Cytomics FC 500, EPICS® XL, and EPICS® XL-MCL [Beckman Coulter], the FACS Aria™, FACS Calibur™ I and II, FACScan™, FACS Canto™, FACStar™ PLUS, and FACS Vantage™ SE I, II, and LSRII [BD Biosciences]) can have two, three, and four lasers. The MoFlo® flow sorter (Cytomation Inc, Fort Collins, CO) has four lasers. A wide variety of different additional light sources have been used in specialized research cytometers. However, those used most frequently in commercial instruments are helium–neon and helium–cadmium lasers emitting at 633 nm and 325 nm, respectively, and (more recently) krypton ion lasers emitting at wavelengths in the range of 407–415 nm. Some instruments (e.g., the FACS Vantage™ with the TurboSort® facility) have been fitted with dye-tunable (560–640 nm) lasers. A few older BD Biosciences and Ortho Chemical Diagnostics (Raritan, NJ) flow cytometers and the Bio-Rad BRYTE HS (Bio-Rad, Hercules, CA) were equipped with mercury or xenon arc lamps that emit over a wide range of wavelengths, the former with additional peaks at 366, 405, 436, 546, and 578 nm. In some instruments with multiple lasers, the beams are focussed at the same point, whereas in others, the beams are parallel and focused at different levels in the flow stream, with an electronic time delay (typically, 10–15 μs) used to correlate emissions from a single cell. The fluorescence detectors are usually arranged so that four receive signals associated with the primary 488-nm beam and two receive signals associated with each additional laser. It is also usually possible to change the optical filters on each fluorescence detector in order to select the range of emitted wavelengths for analysis. However, for various practical reasons (e.g., electronic limitations and energy transfer between fluorochromes), it is often impossible to use all of the theoretically possible combinations of illumination and fluorochromes simultaneously. Four-color analysis is generally straightforward, and five- or six-color analysis can often be readily achieved, but it gets increasingly difficult to incorporate greater numbers of colors into the analysis. Moreover, it may be necessary to use filters with much narrower bandwidths than usual (which diminishes the amount of light reaching the photomultipliers), and interlaser compensation may be needed if using tandem dyes that can be excited by both lasers. Fluorescent stains that can be excited at these additional wavelengths are listed in **Tables 7** and **8**.

Some possible fluorochrome combinations, which might be detected in the different channels of cytometers such as the FACScan™ or FACS Calibur™

**Table 5**
**Fluorochromes That Can Be Conjugated to Antibodies, or Other Ligands, for Use in Flow Cytometers Equipped with a 488-nm Laser and Three or Four Fluorescence Detection Channels[a]**

| Channel | Filter peak transmission (typical wavelengths [nm]) | Fluorochrome | Wavelength (nm)[b] | |
|---|---|---|---|---|
| | | | Excitation | Emission |
| FL1 (green) | 530 BP | Alexa™ 488 | 494 | 519 |
| | | BODIPY® FL | 503 | 512 |
| | | Cy™2 | 492 | 510 |
| | | FITC (fluorescein) | 496 | 516–525 |
| FL2 (orange) | 575–585 BP | B-PE | 480, 565 | 578 |
| | | R-PE | 480, 545 | 575 |
| | | Cy™3 | 512, 552 | 565, 615 |
| FL3 (red)[a] | 620 BP or >650 LP | PE–TR | 480, 565 | 613 |
| FL4 (long red) | 675 BP or >675 LP | PE–Cy™5 | 480, 565, 650 | 667 |
| | | PE–Cy™7 | 480, 565, 743 | 767 |
| | | PerCP | 490 | 675 |

[a]In instruments with three fluorescence detectors, the FL3 channel is usually fitted with a long-pass (e.g., >650 nm or >670 nm) filter and will normally detect fluorochromes that emit in either the red and/or the long red wavelengths.

[b]Values given for peak excitation (absorbance) and emission wavelengths will vary slightly according to the solvent conditions under which they are measured.

BP, band-pass; B-PE, bacterial phycoerythrin; FITC, fluorescein isothiocyanate; LP, long-pass; PE–Cy™5, phycoerythrin–indodicarbocyanine (also called CyChrome™, Tri-Color®, Red670 and Quantum Red™); PE–Cy™7, phycoerythrin–indotricarbocyanine; PE–TR, phycoerythrin–Texas Red® (also called ECD™, Coulter Red613); PerCP, peridinin–chlorophyll *a* complex; R-PE, Rhodophycae phycoerythrin.

equipped with 488-nm argon and 633-nm helium–neon lasers, four-color detectors, and interlaser compensation, are listed in **Table 9**. Some commonly used four-color combinations are FITC/PE/Quantum Red™ (QR)/allophycocyanin (APC), FITC/PE/PerCp/Cy™5, or FITC/PE/PI/APC. Five-color (e.g., FITC/PE/PE-Texas Red [also called ECD]/PE-Cy5™ or APC/PE-Cy7™) analysis using a single 488-nm laser is possible in the Cytomics™ FC 500 (Beckman Coulter), but the use of a second laser (e.g., emitting at 635 nm) allows greater freedom of choice in fluorochromes. Because multicolor fluorescence is such a powerful tool for the analysis of hemopoietic cell populations, it is inevitable that the number of colors used in routine diagnostic work will continue to increase further and will probably do so at a faster rate than in previous years. The practical aspects of multicolor fluorescence analysis for immunophenotyping have been discussed by Baumgarth and Roederer ***(34)***.

**Table 6**
**Fluorescent Probes for Functional Studies That Can Be Used in Cytometers with a Single 488-nm Laser and Three or Four Fluorescence Detectors[a]**

| Channel | Fluorochrome | Use | Excitation (nm) | Emission (nm) |
|---|---|---|---|---|
| FL1 (green) | Acridine orange | DNA | 500 | 526 |
| | | RNA | 460 | 650 |
| | BCECF | Membrane integrity; pH (load as AM ester) | Approx 505 | 525:640 |
| | Calcein | Membrane integrity (load as AM ester) | 494 | 517 |
| | Carboxyfluorescein diacetate | Membrane integrity (load as AM ester) | 511 | 534 |
| | 2′,7′-dichlorofluorescein diacetate | Metabolic burst, oxidative metabolism | 510 | 532 |
| | $DiBAC_4(3)$ | Transmembrane potential | 493 | 516 |
| | $DiOC_6(3)$ | Transmembrane potential | 484 | 501 |
| | Fluorescein diacetate | Membrane integrity (load as AM ester) | 475 | 530 |
| | JC1 | Mitochondrial trans-membrane potential- | 485–585 | 590[b] |
| | PicoGreen®[c] | Highly selective for DNA | 502 | 523 |
| | Rhodamine 123 | Mitochondrial trans-membrane potential | 507 | 529 |
| | SYBR® Green I[c] | High-sensitivity DNA stain | 494 | 521 |
| | SYTOX® Green | Cell-impermeant nucleic acid stain | 504 | 523 |
| | Thiazole orange | Nucleic acid stain | 453, 515 | 530 |
| | TO-PRO®-1 | Moderately high-affinity DNA stain | 515 | 531 |
| | TOTO®-1 | High-affinity DNA stain | 514 | 533 |
| | YOYO®-1 | High-affinity DNA stain | 491 | 509 |

| | | | | |
|---|---|---|---|---|
| FL2 (orange) | Dihydroethidium (Hydroethidine™)[d] | Metabolic burst, oxidative metabolism | 518 | 605 |
| | Ethidium bromide | Cell-impermeant nucleic acid stain; apoptosis | 518 | 610 |
| | Ethidium monoazide | Fluorescent photoaffinity nucleic acid label compatible with fixation | 464 | 625 |
| | Fluo-3, Fluo-4, Fluo-5 | Calcium (load as AM ester) | 464 | 526 |
| | Propidium iodide | Cell impermeant nucleic acid stain; viability | 520 | 610 |
| | SNARF®-1 | Intracellular pH (load as AM ester) | 488–530 | 580:640 |
| FL3 (red) | 7-AAD | Generally cell-impermeant nucleic acid stain | 546 | 647 |
| | Fura Red™ | Loaded as AM ester; fluorescence decreases on binding calcium | 450–500 | Approx 660 |
| FL4 (long red) | LDS-751 | Nuclear DNA | 543 | 712 |
| | DRAQ5[e] | Nuclear DNA | 488, 514 | 665–780 |

[a]Where there are only three fluorescence detectors, FL3 will normally detect fluorochromes emitting in the red and the long red wavelengths.

7-AAD, 7-aminoactinomycin D; AM, acetoxy-methyl; BCECF, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein; $DiBAC_4(3)$, bis-(1,3-dibutyl-barbituric acid) trimethine oxonol; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; SNARF®-1, a seminaphthorhodafluor dye.

[b]As aggregates.

[c]Originally intended for use in solution assays but has been successfully used in flow cytometric assays.

[d]After oxidation to ethidium.

[e]DRAQ5 can be excited in the UV and at 488, 514, 568, 633, or 647 nm with increasing efficiency as the wavelengths increase.

**Table 7**
**Fluorochromes That Can Be Conjugated to Antibodies, or Other Ligands, for Use in Flow Cytometers Equipped with Light Sources Providing Excitation at Wavelengths Other than 488 nm[a]**

| Fluorochrome | Excitation (nm) | Emission (nm) |
|---|---|---|
| Europium–APC chelate | 340 | 660–670 |
| Europium–APC–Cy™7 chelate | 340 | 750–790 |
| Terbium–PE chelate | 340 | 570–590 |
| Alexa™ 350 | 346 | 442 |
| AMCA | 353 | 442 |
| Cascade Blue® | 400 | 420 |
| Alexa™ 430 | 431 | 541 |
| PE–Cy™5 | 480, 565, 650 | 670 |
| PE–Cy™7 | 480, 565, 743 | 767 |
| PerCP | 490 | 675 |
| Alexa™ 488 | 491 | 515 |
| Alexa™ 488 APC | 491 | 660 |
| Alexa™ 750–PE | 496 | 775 |
| Cy™5 | 500–570, 625, 650 | 682 |
| Cy™3 | 512, 532 | 565, 615 |
| Alexa™ 532 | 531 | 554 |
| Texas Red®-X | 543 | 571 |
| Alexa™ 546 | 556 | 573 |
| Alexa™ 568 | 578 | 603 |
| Texas Red® | 587 | 602 |
| Alexa™ 594 | 590 | 617 |
| APC | 650 | 660 |
| APC–Cy™7 (PharRed) | 650, 755 | 767 |
| Cy™7 | 743 | 767 |
| PerCP–Cy™5.5 (TruRed) | 490, 675 | 695 |

[a]7-AAD, 7-aminoactinomycin D; ADB, 1,4,-diacetoxy-2,3,-dicyanobenzene; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; APC, allophycocyanin; APC–Cy™7, allophycocyanin–cyanin7; DAPI, 4′, 6-diamidino-2-phenylindole; DCH, 2,3-dicyanohydroquinone; $DiSC_2(5)$, a thia carbocyanine dye; PE–Cy™5, phycoerythrin–cyanin5; PE–Cy™7, phycoerythrin–cyanin7; PerCP, peridinin chlorophyll a complex protein; SNARF®-1, a seminaphthorhodafluor dye; SYTO® 17, a cell-permeant cyanine dye; SYTOX® Blue, a cell-impermeant cyanine dye; TO-PRO™-3, a monomeric cyanine dye; TOTO®-3, a dimeric cyanine dye.

**Table 8**
**Functional Stains That Can Be Used in Cytometers with Lasers Providing Excitation at Wavelengths Other than 488 nm[a]**

| Fluorochrome | Use | Excitation (nm) | Emission (nm) |
|---|---|---|---|
| Indo-1 AM | Calcium concentrations (ratiometric procedure) | 346 | 401:475 |
| Hoechst 33342 | Vital DNA stain; AT-selective | 350 | 461 |
| Hoechst 33258 | Vital DNA stain; AT-selective | 352 | 461 |
| DAPI | Vital DNA stain; AT-selective | 358 | 461 |
| DCH loaded as ADB | pH measurement (ratiometric procedure) | 365 | 420–440 and 500–580 |
| Dihydroethidium (Hydroethidine™) | Metabolic burst, oxidative metabolism | 365, 518 | 605 |
| Propidium iodide | Membrane-impermeant DNA stain; viability | 365, 535 | 617 |
| SYTOX® Blue | DNA stain | 431 | 480 |
| Chromomycin A3 | DNA stain; GC-selective | 445 | 575 |
| Mithramycin | DNA stain; GC-selective | 445 | 575 |
| SYTOX® Green | Membrane-impermeant DNA stain | 504 | 523 |
| LDS-751 | As a vital DNA stain | 543 | 712 |
| 7-AAD | Impermeant DNA stain; GC-selective | 546 | 647 |
| SYTOX® Orange | Membrane-impermeant DNA stain | 547 | 570 |
| SNARF®-1 | pH measurement | 548 | 579 |
| LDS-751 | As a vital RNA stain | 590 | 607 |
| Oxonol-V | Transmembrane potential | 610 | 639 |
| SYTO® 17 | Membrane-permeant DNA stain | 621 | 634 |
| TOTO®-3 | High-affinity DNA stain | 642 | 660 |
| TO-PRO™-3 | Moderately high-affinity DNA stain | 642 | 661 |
| $DiSC_2(5)$ | Transmembrane potential | 649 | 671 |
| DRAQ5 | As a vital DNA stain | Approx 650 | Approx 665–780 |

[a]7-AAD, 7-aminoactinomycin D; ADB, 1,4,-diacetoxy-2,3,-dicyanobenzene; DAPI, 4′, 6-diamidino-2-phenylindole; DCH, 2,3-dicyanohydroquinone; $DiSC_2(5)$, a thia carbocyanine dye; SNARF®-1, a seminaphthorhodafluor dye; SYTO® 17, a cell-permeant cyanine dye; SYTOX® Blue, a cell-impermeant cyanine dye; TO-PRO™-3, a monomeric cyanine dye; TOTO®-3, a dimeric cyanine dye.

**Table 9**
**Fluorochromes That Could Be Used for Four-Color Fluorescence in Cytometers (e.g., FACScan™ or FACS Calibur™) Equipped with 488- and 633-nm Excitation[a]**

| Channel | Filter wavelengths (nm) | Fluorochrome |
|---|---|---|
| FL1 | 530 ± 15 | Alexa™ 488, BODIPY® FL, calcein, DTAF, FITC, Fluo-3, rhodamine 123, TOTO®-1, TO-PRO™-1 |
| FL2 | 585 ± 21 | Cy™3, PE, PI |
| FL3 | >670 | PerCP, PerCP–Cy™5, PE–Cy™5, PE–Cy™7, PE–TR, APC[b], APC–Cy™7[b] |
| FL4 | 660 ± 15 | APC[b], Cy™5[b], TOTO®-3[b], TO-PRO™-3[b] |

[a]BODIPY-FL, 4,4-difluor-4-bora-3a,4a-diaza-s-indacene-3-propionic acid; Cy™3, indocarbocyanine; DTAF, fluorescein dichlorotriazine; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE–TR, phycoerythrin-Texas Red®; PI, propidium iodide; TO-PRO™-1 and TO-PRO™-3, are monomeric cyanine dyes with different spectral properties; TOTO®-1 and TOTO®-3 are dimeric cyanine dyes with different spectral properties; other abbreviations as in **Table 2**.

[b]Excited by 633-nm laser, others excited at 488 nm.

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# 4

# Quality Control in Flow Cytometry

**David Barnett and John T. Reilly**

**Summary**

An important requirement for all flow cytometry is to have instrument and methodological quality control procedures in place. These procedures are used to ensure the quality of results generated. They must also be performed frequently enough to facilitate the identification of problem areas. This requires both internal quality control and external quality assessment procedures. The current issues of internal and external quality control and problems that may manifest during flow cytometric procedures are discussed here.

**Key Words:** Cell enumeration; control beads; internal and external quality assurance.

## 1. Introduction

Flow cytometry, during the last 20 yr, has become an integral part of clinical and diagnostic pathology. Indeed, many pathology laboratories use flow cytometry to provide diagnostic and therapeutic support for clinicians treating a wide variety of malignant and nonmalignant disorders. Its uses include leukemia immunophenotyping, monitoring of lymphocyte subset, optimum time for peripheral blood stem cell (PBSC) harvesting, leukocyte and reticulocyte counting, platelet analysis (e.g., Bernard-Soulier and Glanzman's syndrome), and red cell analysis (e.g., paroxysmal nocturnal hemoglobinuria and feto-maternal hemorrhage). In the next few years, the flow cytometer is poised to revolutionize DNA and RNA molecular analysis through technologies such as multiplexing.

Due to the extensive repertoire for clinical flow cytometry, there is an important requirement to have instrument and methodological quality control procedures in place. These procedures must be used in such a manner so as to underpin the quality of results generated. They must also be performed frequently enough to facilitate the identification of problem areas. It is also essential to have both internal quality control (IQC) and external quality assessment (EQA) procedures

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in place. This chapter will focus on the current issues of IQC and EQA and highlight problems that may manifest during flow cytometric procedures.

## 2. Internal Quality Control

IQC can be defined as a set of procedures that monitor the instrument, analytical method, and operator performance and that validate the reports generated. Such procedures should be performed on a frequent enough basis to ensure that drift, or bias, can be detected and be supported by fully documented standard operating procedures. IQC should be one of the first areas to be investigated once problems arise and may provide vital clues regarding the underlying problem. All staff should be made familiar with the procedures involved for IQC. The following section highlights key areas of IQC.

## 3. Instrument Quality Control

Flow cytometry in the past generally required highly trained individuals. However, the introduction of benchtop flow cytometers into clinical laboratories has resulted in the involvement of more junior staff while many hospitals rotate laboratory staff on a regular basis. Thus, the use of a properly controlled flow cytometer will provide additional confidence to individuals undergoing training. Instrument control procedures should involve daily calibration or be involved, at the very least, every time the instrument is switched on. Simple protocols are available to assist the monitoring of the laser, fluidics, and optics.

Daily calibration is usually performed using commercially available latex beads and biological controls that allow the operator to monitor: (1) light scatter and fluorescence peak channel coefficients of variation (CVs), (2) light and fluorescence peak channel drift, and (3) instrument sensitivity and the facilitation of compensation setup to adjust for spectral overlap. Furthermore, biological controls, particularly those that are "full process" controls, will assist both the training of staff and the monitoring of the staining process. For instrument control, several types of bead standard are available: (1) blank beads (type 0), (2) alignment (type I) beads, (3) reference beads (type II), (4) compensation beads (type II/III), and (5) calibration and antibody-binding beads (type III) ***(1)***.

### *3.1. Type 0 Beads*

These beads are certified as blank and have a size similar to that of lymphocytes but with a broad CV (>2%). The fluorescence intensity is lower than the autofluorescence of labeled cells and so they are used to establish the "noise level" in conjunction with antibody-binding or calibration standards.

### *3.2. Alignment Standards (Type IA and B)*

Alignment bead standards are either the same size or smaller than cells and facilitate the verification of fluidic and optical alignment. These beads are of a

particular value for the larger cell sorters. They are a single uniform population and generate CVs of less than 2%. The fluorescence intensity is usually very bright and generally does not match that of the tested samples. The value of the type I bead is limited for the benchtop flow cytometer because the manufacturer will have set the alignment of such instruments. When the instrument is used on a daily basis to monitor the peak channel of detection, as well as the CVs of light scatter and fluorescence parameters, specific information can be obtained that will alert the operator of potential problems in these areas.

### *3.3. Reference Standards (Type IIA–C)*

These beads are usually used to establish photomultiplier tube (PMT) settings and can also be used to establish compensation. Daily monitoring of the bead target channel value facilitates a consistency check. However, type II beads have recently been used to establish unified instrument setup across different platforms and, in theory, facilitate direct data comparison. Reference beads are homogeneous, with a fluorescence intensity similar to cells. However, they have broad CVs, a fact that precludes their use for alignment purposes. It should be noted that, as with alignment beads, the emission spectrum does not usually match that of the samples.

### *3.4. Calibration Standards (Type IIIA–C)*

These beads are required to check the linearity, sensitivity, and detection levels of each PMT and generally have a size and fluorescence intensity similar to stained cells. However, the emission spectra may not necessarily match those of samples and may not respond to microenvironmental changes. Type III beads can also be used to determine antigen density by using either (1) beads with predefined amounts of fluorochrome, enabling the fluorescence scale to be appropriately calibrated, or (2) beads that specifically bind mAb that can then be used to calculate the amount of antibody bound to cells.

Once the instrument performance has been verified using the bead outlined above, the final optimization of the flow cytometer should be undertaken. This is best achieved using biological procedural controls. The use of assay calibration provides an important component of IQC and facilitates the monitoring of the relationship between measurement response and the “known” value. Unfortunately, however, no universally accepted reference material exists, although the Eurostandards FP5 project recently has made some progress in this area. Several commercial products are also available that provide the operator with target values to validate the operating system and methodology. It is important that such products be stable over time, be transportable, and have matrix properties that resemble, as closely as possible, the samples under test. An important factor in quantitative assays is that procedural controls reflect levels of the cell population under study. For example, if the laboratory routinely

counts $CD34^+$ cells, the procedural control should have $CD34^+$ counts that reflect those usually encountered in clinical practice. In qualitative assays, the use of procedural controls should reflect the level of cellular antigen expression normally encountered. Most biological controls have an antigen density similar to that found on fresh specimens, although no procedural controls exist for leukemia/lymphoma immunophenotyping, because the diversity of such disorders makes the selection and preparation of such controls difficult. However, within a given pathological specimen, several normal cell phenotypes may exist that could act as internal controls for the staining technique ***(2)***. Finally, the increasing importance of minimal residual disease monitoring in hematological malignancies has presented a new challenge for IQC. It has been suggested that this may be overcome by spiking malignant cells, with a unique phenotype (either antigen expression or antigen density) into a normal blood sample ***(3)***, although the concentration should reflect the detection limit (e.g., one malignant cell per $10^4$ leukocytes). Work currently being undertaken by the United Kingdom National External Quality Assessment Scheme (UK NEQAS) for Leukocyte Immunophenotyping in conjunction with the United Kingdom Acute Lymphoblastic Leukemia Minimal Residual Disease (UK ALL) MRD Flow Group is attempting to address this issue.

Since the development of the first stable whole blood preparation in the mid-1990s ***(4,5)***, a number are now commercially available. Several can be used as full process controls, permitting the use of lysing reagents and multiple-color staining. It is important to emphasize that, due to individual variability, fresh normal specimens or cryopreserved cells are not ideal as process controls but may be used for instrument setup and for optimizing compensation. However, even if stable biological controls are used, the final optimization of the flow cytometer is best achieved using freshly stained biological material. A review of some of those currently available can be found in Bergeron et al. ***(6)***.

It is essential that maximal use be made of the data generated by the above quality control materials. Beads and biological control data should be logged daily, together with instrument settings. It is important that all settings be re-established after a change in bead or biological control batch or after an instrument service. The use of Levy-Jennings-type plots will help highlight any potential instrument or methodological problem.

Time versus count and time versus fluorescence plots provide useful information when acquiring bead data, and it is important to compare histograms on successive days. Such data can assist in identifying errors that result from fluidic and/or optical problems, although because these generate individual histograms, no Levy-Jennings-type plots can be generated. Examples of using time as a parameter are shown in **Figure 1**.

It is important, when performing IQC, to have procedures in place that facilitate the interpretation and troubleshooting of collected data. It is vital to establish

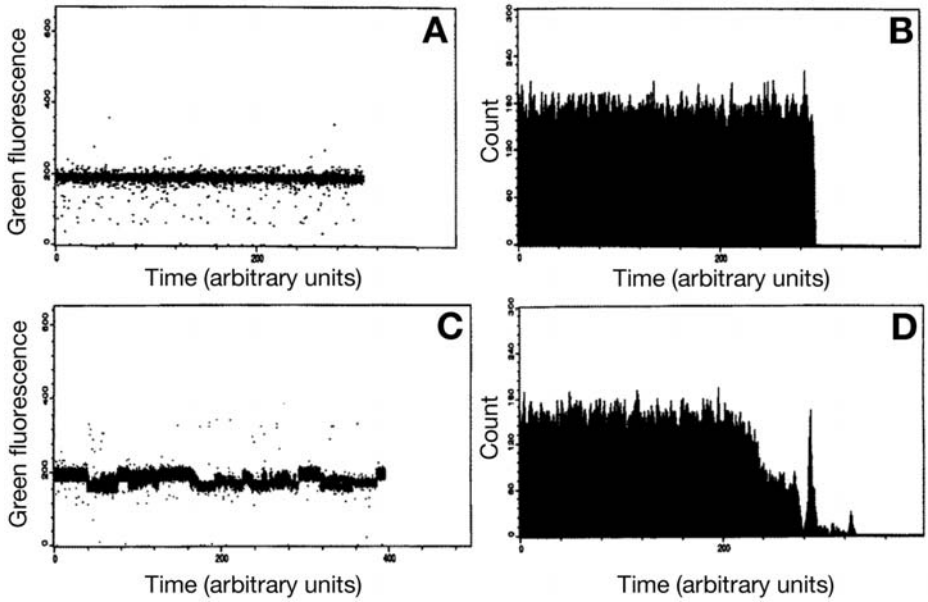


**Fig. 1.** Using time (in seconds) as a quality control parameter on a FACSCalibur. The data were acquired using CellQuest (BD Biosciences, San Jose, CA) and monitoring was performed by Coulter Flow Check Fluorophore (Beckman Coulter, Fullerton, CA). **(A)** Normal laser pattern when bead fluorescence was plotted versus time. Note the low CV. **(B)** Normal fluidic pattern observed for beads acquired in **(A)** over the same time period. **(C)** Abnormal laser pattern using fluorescent beads. **(D)** Abnormal fluidic observed when flow cell is blocked.

boundaries that will help in identifying unacceptability criteria that will enable remedial action to be taken if values lie outside established ranges. One approach is to use Levy-Jennings-type plots, which facilitate the visual identification of trends, particularly if run on a daily basis. Furthermore, it is important that there be procedures in place that allow the constant review of patient data (e.g., comparison of the previous CD4 counts) and that, within any immunophenotyping procedure, a given panel allow consistency checks (e.g., comparison of replicate CD3 values).

## 4. Quality Control Issues and Pitfalls

Three areas in which problems occur in immunophenotyping are (1) specimen processing, (2) data acquisition, and (3) data analysis/reporting.

### *4.1. Specimen Processing*

It is important that specimens be as fresh as possible *(7)*, especially when undertaking antigen-density quantification or cytoplasmic staining. Several guidelines

have been published that address the issues of specimen integrity ***(8–11)***. Correct handling and transportation procedures need to be established, and the choice of anticoagulant is important. $K_3$EDTA, for example, is preferred because it maintains the morphology and the flow cytometric profile of the cellular components ***(8,10)***. Red cell lysis and cell separation procedures are frequent causes of analytical variation ***(12)***. Overlysis may result in changes in forward scatter (FSC) and side scatter (SSC) patterns, quenching of fluorochromes (particularly if aldehyde-based lysis reagents are used), and selective cell loss. In addition, it has been reported that if bead-based technologies are used, excessive vortexing may induce an electrostatic charge on the tube, resulting in the beads sticking to the tube and thus affecting the absolute counting ***(13)***. In contrast, poorly lysed specimens retain red cells that may impair the identification of specific cell populations and produce tube-to-tube variation in FSC and SSC patterns, making consistent gate placement difficult. Inappropriate vortexing conditions may similarly lead to tube-to-tube variation. For example, samples treated too vigorously exhibit excessive cell debris or develop separation of granulocyte populations. Furthermore, undervortexing may result in cell doublets, termed "escapees," that will be excluded from routine gating strategies ***(14)*** (**Fig. 2**).

Centrifugation can be a major cause of cell loss and poor final sample preparation. Excessive centrifugation causes cell damage and alters the FSC and SSC characteristics, as well as increasing the level of cell debris, and cell loss may occur during the washing step if centrifugation is too gentle (Peel and Barnett, *unpublished observation*).

### 4.2. Data Acquisition

The number of events acquired and the configuration of the instrument are two important variables influencing the reproducibility of results. The configuration and optimization of the instrument have already been discussed, although final optimization of the light scatter histograms may be required on an individual-sample basis. The FSC versus 90° (SSC) histogram should adjusted to facilitate the visualization of all cell populations, with the lymphocyte population placed approximately midway along the *x*-axis. In addition, the negative cell population should be located within the first log decade when analysed on the fluorescence channel.

It is important that sufficient events be acquired to facilitate accurate identification of individual cell populations; for example, if a lymphoproliferative disorder is being analyzed, at least 5,000 lymphocyte events should be acquired, whereas $CD34^+$ stem cell enumeration requires a minimum of 100 $CD34^+$ events to ensure robust statistical analysis ***(11)***. In addition, recent $CD4^+$ T-lymphocyte enumeration guidelines recommend the collection of at least 2,500 lymphocyte

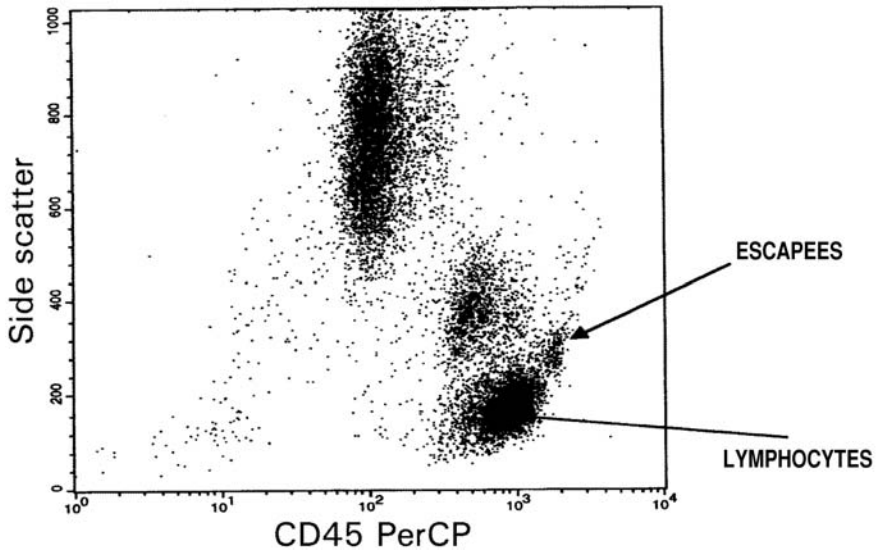


**Fig. 2.** CD45 versus side angle light scatter dot plot, showing lymphocyte escapees.

events ***(8)***, whereas 100,000 events should be analyzed when detecting minimal residual disease.

### *4.3. Data Analysis*

A number of variables may affect data analysis: (1) the gating strategy and gate placement/analysis region used ***(15)***, (2) the establishment of acceptability criteria, (3) the use of automated gating, (4) the use of quality control checks (within-assay replicates), (5) the cursor placement for isotype controls, and (6) the development of artifactual staining patterns.

In the late-1980s, the improvement in software, coupled with the discovery of additional fluorochromes, enabled $CD4^+$ T-lymphocyte identification and characterization by either light scatter gating procedures or differential staining with CD45 and CD14 ***(16)***. These approaches are now recognized to have several disadvantages; first, an FSC/SSC gate approach does not identify gate contaminants and may result in falsely low percentage values, whereas the need for larger panels (i.e., up to six tubes) increases analysis time, specimen handling, and ultimately cost. Second, it is not possible to detect tube-to-tube variation when a light scatter gate is derived from CD45/CD14 "back-gating." In addition, the isotype control fails to control for CD45/CD14 staining. Therefore, it is recommended that $CD4^+$ T = lymphocyte enumeration be performed using three-color immunophenotyping using one of the three gating strategies highlighted below. A summary of the evolution of current gating techniques has been published ***(17)***,

and the advantages and disadvantages of each technique are detailed in the published British Committee for Standards in Haematology (BCSH) guideline for $CD4^+$ T-lymphocyte enumeration ***(8)***.

The results issued from a laboratory will depend on the gating strategy employed. For example, when using the T-gating approach ***(18)***, it is important that values for $CD4^+$ and $CD8^+$ T-lymphocytes be expressed as a percentage of the total T-lymphocyte population and not of the total lymphocyte population. The placement of the analysis region is a further factor for consideration. If the gate is too tight, there is a possibility of excluding relevant cells, whereas an overgenerous gate will include contaminating cells and result in falsely low results ***(15)***. Failure to establish acceptability criteria can lead to the release of erroneous results. Recent guidelines for $CD4^+$ T-lymphocyte enumeration have suggested that tube-to-tube variation for replicate antigens be less than 3%, with monocyte contamination within a CD45/SSC gate being less than 5% and a lymphosum (CD3 + CD19 + NK cells (CD16 and/or CD56) being equal to 100% ± 3% ***(8,10)***. Recently, Glencross et al. ***(19)*** described a simple two-color approach termed "panleucogating" in which only CD45/CD4 are used. This protocol was developed due to the increasing requirement to develop affordable, accessible, and robust CD4-counting techniques that can be used in countries with limited resources. The approach offers a practical and robust dual-platform alternative to single-platform techniques in such countries.

A similar evolution of gating strategies has occurred for $CD34^+$ stem cell enumeration, the most important being the strategies of Milan ***(20)***, Mulhouse ***(21)***, the Dutch Foundation for Immunophenotyping of Hematological Malignancies (SIHON) ***(22)***, and the International Society of Hematotherapy and Graft Engineering (ISHAGE) ***(11)***.

The Milan protocol is the simplest and involves the use of FSC and SSC to gate out red cells, debris, and cell aggregates. The gated, nucleated cell population is then plotted on a histogram of CD34 fluorescence intensity versus SSC. Only $CD34^+$ events with low SSC are used to calculate the number of PBSCs. The Mulhouse protocol is a logical development of the Milan protocol and uses $CD45^+$ events to identify the leukocytes. The latter are then plotted on CD34 versus SSC. Only $CD34^+$ events with low SSC are used to calculate the number of PBSCs, expressed as a percentage of $CD45^+$ cells. The SIHON approach incorporates the DNA/RNA laser-excited dye (LDS-751) to identify nucleated cells during data collection. In addition, by incorporating antibodies directed against CD14 and CD66e, monocytoid ($CD14^+$) and mature myeloid ($CD66e^+$) cells are excluded during list-mode data analysis given that false-positive staining due to Fc receptor-mediated mAb binding will be eliminated. Finally, the ISHAGE approach takes advantage of the dim CD45 expression of $CD34^+$ stem cells with low SSC and incorporates a sequential gating strategy. Such a

strategy has been adopted by the Stem Cell Enumeration Committee of the ISHAGE ***(11)*** and is currently the most popular gating strategy in the UK ***(23)***. Subsequently, this strategy was further developed to assess sample viability and obtain absolute counts in a single-platform format ***(24,25)***.

A single-platform version of the ISHAGE protocol, termed "Stem-Kit" (Beckman Coulter, Inc., Fullerton, CA), has been developed, in which a known concentration of microbeads is added to a known amount of stained, lysed whole blood ***(24)***. The absolute $CD34^+$ cell count is calculated from the observed ratio between the number of counted beads and $CD34^+$ cells. A single-platform, software-driven method using a proprietary DNA/RNA stain to threshold on nucleated cells (ProCount kit; BD Biosciences) has been developed ***(26)***.

In contrast to $CD4^+$ T-lymphocyte enumeration, in which it is accepted that CD45/SSC is the best gating strategy, it is unlikely that standardization of $CD34^+$ PBSC enumeration will be achieved in the foreseeable future. As a result, it has been suggested that "the most effective approach to reducing interlaboratory variation in CD34 enumeration consists of the adherence to consensus protocols formulated in general terms" ***(27)***. These protocols, combined with real-time evaluation of performance by the organizations for external quality assurance, will assist in ensuring that site-to-site interlaboratory variation be reduced. An Australian study reported the results of a multicenter study to address these issues ***(28)*** and identified that major methodological variations were the parameters used for gating $CD34^+$ cells and the denominator used for calculating the percentage of $CD34^+$ cells (i.e., percentage of $CD45^+$ events or percentage of total nucleated cells). The study concluded that without standardization, less than half of participating centers obtained reproducible results and that the ISHAGE gating strategy was the most reproducible. The findings from a study involving 24 European sites have further shown that if targeted training is implemented, it is possible to achieve interlaboratory CVs of less than 10% for single-platform techniques ***(29,30)***.

An additional factor that may influence data analysis is the isotype control. The value of isotype controls, however, has been questioned ***(2,31)*** and guidelines state that $CD4^+$ T-lymphocyte and $CD34^+$ cell enumeration may be undertaken without isotype controls ***(8,32)***. The use of isotype controls in leukemia/lymphoma immunophenotyping is conflicting. However, it should always be recognized that in the majority of leukemias, normal counterpart cells exist that can be used to determine the level of background staining ***(33)***. However, it is important that if isotype controls are used, they be matched to the test antibody, both with respect to fluorochrome and (more importantly) antibody concentration, and that the cursor be correctly placed during data analysis.

Finally, operators should be aware of the nature of false-positive staining reactions. These may occur due to the nonspecific binding of antibody through

Fc receptors, often observed with acute monoblastic leukemias and from technical errors (e.g., the use of inappropriate antibody) or the inclusion of dead cells in the analysis. Blocking the binding site, with a preincubation step using rabbit serum, can reduce nonspecific antibody binding via the Fc receptor. In addition, the identification of clonal B lymphocyte populations, by the demonstration of light chain restriction, may be masked by cytophilic antibody binding. This problem can be overcome if the immunoglobulin is removed by suspending and subsequent washing of the cells in phosphate-buffered saline (PBS) at 37°C for 30 min. More recently, this approach has been applied to whole blood techniques with good effect. Briefly, 0.5 mL of whole blood is suspended in 9.5 mL of PBS at 37°C for 30 min, with gentle inversion of the tube every 10 min, followed by washing three times in PBS. Care is needed when removing the supernatant so as not to disturb the cell pellet, which is then resuspended in 0.5 mL of PBS before analysis as for whole blood.

Finally, inappropriate instrument setup can be a source of erroneous results and is usually attributed to inappropriate flow cytometer compensation (for further details, *see* **ref. *34***).

## 5. External Quality Assessment (EQA)

Despite the general acceptance and routine use of immunophenotypic analysis, EQA has only recently begun to address the important issues of quality control and standardization in flow cytometry. Local, regional, national, and international EQA schemes (EQASs) have now been established ***(4,14,22,23,35–38)***. In principle, EQA is designed to test the whole analytical and reporting procedure and should be used by the laboratory to complement their IQC activities. The nature of EQA means that, in most instances, the sample distribution is restricted to four to six times per annum and, therefore, provides only a "snapshot" of laboratory performance. Nevertheless, because EQA involves many laboratories, significant information can be obtained about the performance of specific instrumentation as well as the effectiveness of specific reagents and methodological approaches. In addition, comparison of an individual laboratory performance with a group using the same technique can be determined over a period of time and, in conjunction with IQC procedures, can be used to help identify specific problem areas.

Participation in an EQAS is currently compulsory in some countries (e.g., US and Canada) although it remains voluntary in most European countries. In the UK, the implementation of an accreditation process for clinical pathology laboratories (operated by Clinical Pathology Accreditation [CPA] [UK] Ltd) has made participation in an EQAS desirable. Such accreditation processes are also being applied to EQASs within the UK by CPA (UK) Ltd following the establishment of a CPA (EQA) committee.

One of the prerequisites for an EQAS is that it reflect, as closely as possible, the procedures and tests employed in the clinical laboratory. The materials issued should meet the same exacting standards encountered in IQC. Furthermore, while an EQAS should have the ability to identify specific problem areas, it should also provide a strong educational element.

The following section highlights three key problem areas supported by data from UK NEQAS for Leukocyte Immunophenotyping Scheme ***(4,23,39)***, namely (1) reagent selection, (2) definition of the "positive" value, and more recently (3) absolute count enumeration.

## 6. Reagent Selection

The commercial availability of a large number of different specific mAb reagents has made interlaboratory reproducibility, and hence external quality assurance, difficult. A survey of routine UK laboratories in 1989 revealed a total of 86 antibodies being used as front-line reagents for leukemia diagnosis. Furthermore, the use of monoclonal antimyeloperoxidase antibody, one of the most informative reagents ***(40)***, was frequently omitted. The publication of the BCSH guidelines, which include recommended minimum antibody panels for acute and chronic leukemia immunophenotyping (BCSH ***[41–43]***), has resulted in a greater degree of standardization within the UK. Many problems still remain, however, including the lack of standardization of analysis techniques, the use of different antibody sources, antibody dilutions, fluorochrome conjugates, and lysing and fixation reagents.

### *6.1. Reagent Selection (Clones)*

Different clones of mAbs, directed against the same cluster of differentiation, may vary in their ability to detect the corresponding antigen on leukemic cells. For example, the expression of CD34, a membrane-associated glycoprotein found on pluripotential stem cells, lineage-committed hematopoietic progenitors, and some mature populations of both endothelial and stromal lineage ***(44–46)***, may be dependent on the choice of reagent. It has been found that CD34 mAbs react with different sites on the CD34 antigen (at least three as defined by their sensitivity to neurominidase) and, as a result, have different binding properties ***(44–48)***. The epitope recognized by B13C5 is sialic acid-dependent, in contrast to that recognized by ICH3. Therefore, it is not surprising that different antibodies can produce variation both in CD34 estimation and sensitivity in identifying circulating hematopoietic progenitors ***(20,49)***.

The use of different panels and even clones of mAbs may account for the wide variation in incidence reported for myeloid antigen expression in acute and chronic lymphoproliferative disorders. The coexpression of myeloid- and lymphoid-associated antigens in childhood acute lymphoblastic leukemia is

well described, although the incidence of aberrant expression ranges from less than 5% to more than 30% ***(50,51)***. This discrepancy can be partly explained by the use of different panels of mAbs, but significant differences still occur when comparing antibodies with the same CD antigen. Thus, in two studies, the incidence of cases expressing CD13 and/or CD33 was 4% and 16%, respectively ***(52,53)***. In addition, the expression of myeloid antigens has been reported to be of prognostic importance by some ***(50)***, but not by all ***(54)***, groups. Interestingly, a further study ***(55)*** noted that the detection of myeloid antigens in childhood acute lymphoblastic leukemia is dependent, in part, on the commercial source of antibody. Similar conflicting data have been reported for CD13 and CD33 expression in B-cell chronic lymphocytic leukemia ***(56,57)***. Furthermore, CD14 was not detected by Polliack and colleagues ***(57)***, whereas others have described between 29% and 84% of cases that were positive for this antigen ***(56,58)***. Interestingly, Pinto and colleagues ***(59)*** demonstrated that the CD14 epitope is detected only with the My-4 antibody and not by other CD14 antibodies. We have reported similar findings in chronic lymphatic leukemia, in which CD13 and CD33 were detected using only a particular reagent ***(60)***.

### *6.2. Reagent Selection (Conjugates)*

The increasing use of multiparameter analysis has moved flow cytometry to a new level. It is important that the correct choice of fluorochrome-conjugated antibody be made. For example, it has been reported that cells labeled directly with fluorescein isothiocyanate (FITC)-conjugated antibodies will not be as bright as those stained using indirect FITC methods and that this decreased sensitivity may be in the order fivefold to sixfold ***(60)***. This is of practical importance when antigen expression is low. Phycoerythrin (PE) and the newer fluorochromes (e.g., tandem-color fluorochromes) have a much higher quantum yield than FITC, thus increasing sensitivity. As a result, statistically significant differences have been documented between samples analyzed with FITC- and PE-conjugated antibodies for the following antigens: CD3, CD5, CD13, CD14, and CD33 ***(61,62)***. In a UK NEQAS survey investigating CD13 detection, for example, eight of 24 laboratories that used FITC-conjugated antibodies obtained values of less than 50% (overall 58%), and three of these eight recorded negative results. In contrast, all 12 laboratories using PE-conjugated reagents obtained values greater than 50% (mean 77%). Therefore, PE conjugates, or tandem-color fluorochromes (e.g., PE/Cy5), should be used for single-color analysis. In the case of multicolor analysis, the more sensitive fluorochrome-conjugated antibody should be used for detecting the weaker antigen, typically CD13, CD19, and CD33, whereas strongly expressed antigens (e.g., CD45 or HLA-DR) can be detected using FITC or PerCP (peridinin chlorophyll protein). In addition, stearic hindrance, resulting from the simultaneous binding of different

mAbs should always be considered when selecting reagent combinations (e.g., CD3 and the T-cell receptor complex). Recently, we showed that when performing antigen-density measurements, the use of single-color staining will give markedly different results when compared with the data derived using multicolor staining ***(63)***.

The developments of additional fluorochromes that can be detected with a single laser, and the availability of computer software capable of rapid data analysis, have enabled the incorporation of multicolor analysis in the routine diagnostic laboratory ***(64)***. It is now commonplace to undertake leukemia diagnosis with as many as six colors. As a result, greater care and understanding are required by the operator on how certain combinations of antibody and/or fluorochrome will impact the results obtained. UK NEQAS for Leukocyte Immunophenotyping is currently undertaking data collection to identify the impact of various conditions such as antibody combinations, instrument setup, and data collection and analysis.

## 7. Definition of Positive Values

Data from UK NEQAS, together with a review of the literature, have demonstrated considerable differences in the definition of antigen positivity. The development of newer and more sensitive fluorochromes, coupled with multiparametric technology, will further increase this dilemma. The simplistic approach using an arbitrary cutoff point (e.g., >10% for immunocytochemistry, >20% for immunofluorescence analyses), as suggested in BCSH guidelines ***(41)***, will probably not be applicable in the future. Data analysis procedures that currently employ the placement of a cursor at the boundary of the negative population are likely to be inappropriate ***(33)***. Procedures that are more biologically relevant, such as antigen-density quantitation, may yield more meaningful information. Such evaluations would require the production of reference materials that express the antigens of clinical importance ***(4,63)***, although the technical difficulties should not be underestimated, especially for pathological preparations.

## 8. Absolute Count Enumeration

The enumeration of absolute $CD4^+$ T-lymphocyte and $CD34^+$ hematopoietic stem cells is important for the clinical management of HIV-infected individuals and for patients with cancer who are undergoing PBSC transplantation, respectively. The need for an accurate and reproducible method for absolute cell counting may take on even more importance following the National Blood Authority strategy to issue blood products with an absolute white blood cell count (WBC) of less than $5 \times 10^6$ leukocytes per blood product unit ***(65)***.

Currently, peripheral stem cell and lymphocyte subset analysis is routinely undertaken by flow cytometry, employing either a dual- or, less frequently, a

single-platform approach. The dual-platform technique uses immunophenotypic data derived from the flow cytometer together with the total WBC, or total absolute lymphocyte count, obtained from a hematology analyzer. It is recognized, however, that a major factor contributing to the high interlaboratory CVs reported for absolute $CD4^+$ lymphocyte counts is the WBC generated by different hematology analyzers ***(66)***. In addition, the intermachine variance for WBC increases significantly for values below $0.1 \times 10^9$/L, precluding this approach for the quality control of leukocyte-depleted blood products. In contrast, single-platform technology derives the absolute cell count directly from the flow cytometer, using either precision fluidics or microbead technology ***(24,26,67)***.

One important aspect that needs to be considered when introducing new and potentially more accurate technologies is the impact that this will have upon clinical decision-making. UK NEQAS for Leukocyte Immunophenotyping have examined the variability that single-platform and dual-platform approaches have upon $CD4^+$ T-lymphocyte counts that are used to determine disease progression of HIV. A previous study by Kunkl et al. ***(68)*** of 18 laboratories using the dual-platform approach to determine CD4 counts highlighted that there was as much as 16% variability at a count at 200 $CD4^+$ cells per μL. This is reduced to as low as approx 6% with a single-platform approach (Barnett, *unpublished observations*), thus giving greater confidence when making clinical decisions based upon absolute counts.

Data from UK NEQAS for Leukocyte Immunophenotyping showed that interlaboratory CVs for $CD4^+$ T-lymphocyte counts were consistently lower for single-platform (mean 13.7%; range 10%–18.3%) compared with dual-platform methodology (mean 23.4%; range 14.5%–43.7%). Subgroup analysis of single-platform users demonstrated mean overall interlaboratory CVs of 17.2%, 13%, and 7.1% for the FlowCount, TruCount, and volumetric approach, respectively. The lowest interlaboratory CVs obtained for a single sample according to each single-platform approach were 4% (TruCount), 4.4% (volumetric), 4.6% (FACSCount), and 12.7% (FlowCount). Similarly, the mean interlaboratory CV for $CD34^+$ stem cell enumeration using nonstandardized single-platform approaches was 18.6% (range 3.1%–36.9%) compared with 28.6% (range 19%–44.2%) for the dual-platform technology. Our results suggest that absolute cell subset enumeration should be performed by single-platform technology and that such an approach should improve the quality control of multicenter clinical trial data for $CD4^+$ T-lymphocyte and $CD34^+$ stem cells ***(69)***.

## 9. Conclusion

The implementation of quality control procedures, both internal and external, should be considered as good laboratory practice (GLP). This chapter has outlined several measures that can provide the laboratory the basis for the use of such

procedures. Further measures of GLP also extend to the logging of reagent use, monitoring refrigerator and freezer temperatures, and ensuring that staff members have an excellent career development program. However, the most important factor when performing any flow cytometric procedure is IF IN DOUBT, REPEAT!!!

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# 5

# Experimental Design, Data Analysis, and Fluorescence Quantitation

**Mark W. Lowdell**

**Summary**

Errors in flow cytometry can arise from two discrete processes: data acquisition and data analysis. Data acquisition errors can be avoided by good sample preparation and accurate instrument setup. Instrument standards, in the form of fluorescent beads, can be used to confirm instrument performance in both settings. The only aspect of data acquisition for which controls are unavailable is the ability of the operator to design the correct experiment. Help with this is described in this chapter.

**Key Words:** Cluster analysis; fluorescent beads; fluorescence intensity.

## 1. Introduction

The likelihood of success of any experiment is largely determined at the design stage and that is especially true of the sort of complex, multiparametric experiments that are usually analyzed by flow cytometry. There is a need for a basic understanding of the biological system being investigated before starting the experiment and certainly before acquiring a single event on the flow cytometer. The number of cells needed to be acquired from each sample is determined by the frequency of the cell of interest. The rate of sample acquisition is determined by the electronics of the cytometer and the hydrodynamics of the sheath and sample stream. The optimal fluorochrome for the analyte of interest is determined by the flow cytometer setup, the availability of reagents, and the density of expression of the analyte. In some cases, the antibody clone will be important because some clones give a higher signal-to-noise ratio than others for a specific antigen. Sometimes, the answers to these questions require one or more preliminary experiment, but more often than not, it is possible to predict the answers and take them into account at the planning stage of the experiment.

From: *Flow Cytometry: Principles and Applications*
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## 2. How Many Events Should Be Acquired?

In the early years of flow cytometry, the instruments were predominantly used for the analysis of DNA content of isolated cell nuclei and it was common practice to acquire 10,000 nuclei (data events) into each file. This value seems to have acquired a mystical significance within the "flow community," with every paper stating "10,000 events were acquired as list mode data." Unfortunately, quite often in the data seen in papers, these files contain 500 cells of interest and 9500 events of no significance to the question asked. Even worse, in the case of rare-event analyses (detection of stem cells or subsets thereof), a file containing 10,000 events may contain fewer than 20 cells of interest. Thus, before starting an experiment, it is necessary to determine the likely frequency of the cell of interest and the information to be obtained. For example, if the frequency of $CD34^+$ hematopoietic progenitors within a blood sample is to be determined, the list mode data file must contain all of the nucleated cells so that the $CD34^+$ fraction can be determined as a percentage of the nucleated cells. If the blood sample is from a patient undergoing stem cell mobilization, this might require a data set of 60,000–100,000 nucleated cells. If the sample is from a normal individual who has not undergone stem cell mobilization, the likely frequency of $CD34^+$ events will be 5- to 10-fold lower so it would be necessary to acquire 250,000 events and to store the entire set of data. In contrast, if the frequency of a subset of $CD34^+$ cells (e.g., $CD117^+/CD133^+$) is of interest within the total progenitor cell population, a fixed number of $CD34^+$/side scatter $(SSC)^{low}$ cells should be acquired and all of the CD34-ve events gated out to keep the size of the list mode data file manageable and to speed the analysis.

It is often good practice to predetermine the rarest cell population of interest and then to set a counting region around this population (e.g., if there is interest in the expression of surface antigens on both lymphoid and myeloid cells or even of T cells and B cells in the same sample). Given that there will be many more lymphocytes than monocytes and T cells than B cells, it is inappropriate simply to acquire 10,000 total events, because it is likely that the monocyte or B-cell populations will be too small for reliable analysis. It would be better in this instance to set up the acquisition parameters to accept all cells of interest but to acquire a fixed number of the rarest population. This fixed number can be determined by the question being asked of the rare population (e.g., if the experiment is to measure natural killer [NK] cell activation in response to tumor cells and to cytokines). Physical selection of NK cells (or any other cell type) may initiate cell activation, so stimulation of the NK cells within a nonsorted cell suspension is attractive. It is possible to mix isolated lymphocytes with irradiated tumor cells for fixed time periods and then label with mAbs to activation antigens and to antigens specific to NK cell subsets. **Figure 1** shows data from a typical experiment. Human lymphocytes have been preincubated with an

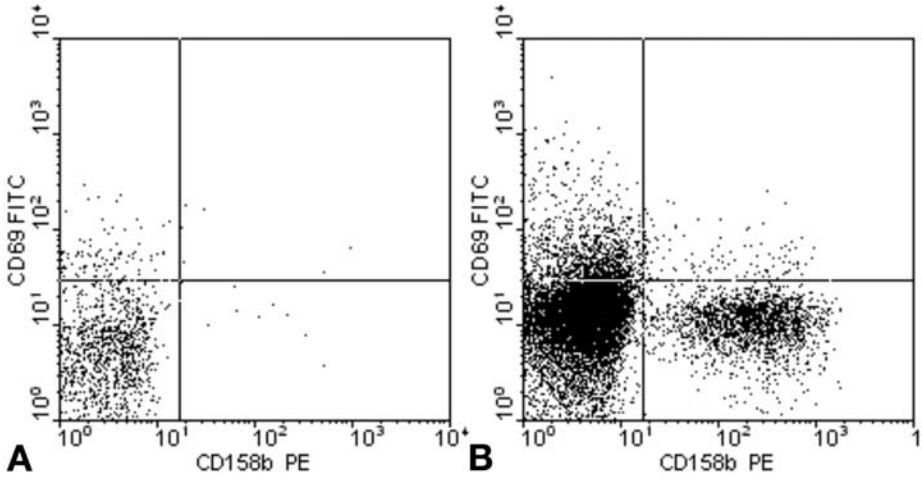


**Fig. 1.** Isolated lymphocytes were mixed with irradiated tumor cells for set time periods and then labeled with mAbs to activation antigens and to antigens specific to NK cell subsets.

irradiated tumor cell line for 2 h and then labeled with CD3 allophycocyanin (APC), CD56 PC5, CD158a fluorescein isothiocyanate (FITC), and CD69 phycoerythrin (PE). NK cells have been gated as $CD56^+/CD3$-ve cells with forward scatter (FSC)/SSC characteristics of lymphocytes. The data have then been arrayed as CD158a vs CD69 to identify the proportion of activated NK cells within the $CD158a^+$ vs CD158a-ve subsets. Plot A shows the result of analyzing a list mode data file containing 10,000 lymphocytes. Plot B shows the effect of setting a 2000-event counting gate on $CD56^+/CD3^-/CD158a^+$ events and of storing the entire data set. The very low frequency of $CD158a^+$ NK cells in the sample results in plot A containing too few events for reliable analysis of the proportion of activated cells.

### 3. The Use of "Thresholding" to Enhance Data Acquisition Rates

Regardless of the flow cytometer being used, it cannot distinguish a signal of interest from noise. All instruments require the operator to set an electronic threshold below which all signals are not stored by the computer, and events below the threshold are commonly termed "noise." Most instruments default the threshold to the forward angle light scatter detector because it is an indicator of cell size and most experimenters know the likely size of the particles of interest at the outset of their experiment. A typical analog benchtop flow cytometer will be able to acquire 2000–3000 events per second above the threshold. Events above this frequency will not be processed. This is because they will pass

through the optics while the electronic circuitry is resetting; this is called the "dead time" of the instrument.

In regard to the previous example of studying a rare progenitor cell subpopulation as a proportion of $CD34^+$ events, it would be perfectly justifiable to increase the concentration of mononuclear cells in the sample tubes such that the rate of acquisition exceeds the maximum threshold of the instrument. Then, by changing the threshold parameter to the fluorescence channel in which the $CD34^+$ events fall, theoretically the cells could be analyzed at a frequency of 2000 $CD34^+$ events per second, allowing the CD34-ve cells to be lost below the threshold and not even trigger the electronics. In the analysis of clinical blood samples processed by lysis without washing, it is common to set the instrument threshold on CD45 expression so that all red cell debris is excluded from the acquisition and only leukocytes expressing CD45 are gated for analysis.

## 4. The Choice of Fluorochrome

The principal choice of the fluorochromes available at the experimental planning stage is determined by the optical setup of the flow cytometer. Four-color instruments capable of measuring four colors of fluorescence are now almost the lowest specification, and many laboratories have access to five- and six-color machines. The improvement in optical systems has allowed the use of low-power solid-state lasers that are cheap to buy and run and small enough to fit into benchtop cytometers so that it is possible to have four laser lines and 16 optical channels in a machine that occupies less bench space than a single-laser, three-color cytometer did in the 1980s.

Given this enormous heterogeneity in equipment specification and the speed with which the field advances year after year, it is impossible to give definitive advice on fluorochrome choice. Nonetheless, there are some basic rules that should always be followed if possible (i.e., whenever the appropriate fluorochrome conjugate is available) and problems to be aware of when these rules cannot be followed.

FITC was the first fluorochrome to be conjugated to a protein and is currently the most widely available mAb conjugate. However, it is less than ideal in multiparametric flow cytometry because it is relatively poorly excited by 488-nm lasers, is rapidly quenched when internalized into low-pH vesicles (unless that is a feature you wish to use), and has a long emission spectrum, which contaminates the emission of other fluorochromes excited at 488 nm, such as PE. This makes it the fluorochrome of second choice if studying weakly expressed antigens, such as activation-associated molecules, in which it is likely to underestimate the positive fraction.

PE is an excellent fluorochrome for flow cytometry because it is extremely stable and has a high quantum efficiency resulting in a high signal-to-noise ratio

and so enhances the detection of weakly expressed antigens. It is, however, a very large molecule and may not be suitable for the detection of intracellular antigens in some permeabilization protocols. Although adequately excited by a 488-nm laser line, PE is optimally excited by green lasers, and suitable solid-state lasers of appropriate wavelength are available. At least one manufacturer (Miltenyi Biotec Inc., Auburn, CA) has designed a flow cytometer that uses such a laser in place of the conventional 488-nm argon ion and that uses PE and PE-based tandem reagents in place of FITC.

PE is an excellent fluorochrome in its own right but is also an excellent donor molecule for red-emitting fluorochromes such as cyanine (Cy) 5 (*see* **Chapter 3**). These PE-tandem dyes make excellent "third" colors for benchtop flow cytometers, but it is necessary to be aware that some tandem conjugates are better than others. In ideal conditions, the accepter fluorochrome absorbs all of the emitted light from the donor PE molecule and no PE signal can be detected. When using PE-tandem conjugates for the first time, it is worth checking for PE emission. Signal-to-noise ratios from these tandem reagents are generally good but poorer than from PE alone, so they should not be chosen for detection of the most weakly expressed antigens. This is particularly true of the only naturally occurring tandem fluorochrome, PerCP (peridinin chlorophyll protein), which has a particularly low quantum efficiency and is a poor reagent for detection of low-density antigens.

Flow cytometers are increasingly being supplied with multiple lasers, most commonly with a red diode laser emitting at 633 nm. This is an excellent laser line for the excitation of APC and for tandems dyes thereof such as APC/Cy7. APC is a stable fluorochrome with good quantum efficiency and is relatively easy to conjugate to proteins. APC/Cy7 is an unstable reagent, and labeled cells must be kept on ice prior to analysis.

The Alexa dyes from Invitrogen (Carlsbad, CA) cover a very wide spectrum of excitation and emission, and a suitable reagent can be found for most applications.

## 5. The Choice of mAb Clone

Apart from choosing an appropriate fluorochrome for the detection of individual antigens, the particular clone of antibody may impact upon the results. Not all mAbs specific for a given antigen will bind to the same epitope. There are many cases in which this has no impact on the detection of the antigen, but in some cases the results can be epitope-specific. A good example is the detection of $CD34^+$ cells by flow cytometry. Anti-CD34 mAbs have been produced which bind to three different epitopes on the CD34 molecule, some of which are fixation-sensitive and all of which have different avidities for their respective mAb. Class III anti-CD34 mAbs have been shown to be the most reliable for

detection of circulating mobilized hematopoietic stem cells, but even with class III mAbs, the use of lysing reagents that contain paraformaldehyde reduces the fluorescence intensity and leads to the underestimation of the proportion of $CD34^+$ cells.

## 6. The Choice of "Negative" Control

This is one of the most controversial issues in flow cytometry and one that has inspired many hours of discussion and exchange of e-mails. For decades, nascent flow cytometrists have been taught to include isotype-matched controls in all experiments to determine the background level of mAb binding and thus the threshold level for a "positive" event. This approach seems inherently sensible and attractive. However, the use of an isotype-matched control requires that one make many assumptions, most of which are simply not justified. First, the degree of nonspecific binding of a mAb is not solely dependent upon its isotype; plainly protein concentration is important as is the concentration of immunoglobulin of that isotype. In a panel of mAbs, it is likely that all will be at different specific concentrations and thus each should have an isotype control of matching isotype concentration. Second, the fluorochrome/protein ratio of your isotype control must be matched to that of your mAb. This is difficult to achieve when using a panel of mAbs from a single supplier but impossible when combining mAbs from two or more suppliers in a single experiment. There are additional, more esoteric concerns about the appropriateness of isotype controls which need not bother us here, but all are eloquently discussed by O'Gorman and Thomas ***(1)***. The mythical status of isotype-matched controls leads to their completely inappropriate use. At least one publication in a high-impact journal states that an isotype-matched control was used to set the cutoff for positivity in a sample labeled with an HLA-peptide tetramer; a reagent further removed from an immunoglobulin is hard to conceive!

So what can be gleaned from the use of an isotype-matched sample (note the removal of the term "control" here), and how can the proportion of "positive" events in a sample without a control of some sort be reliably determined? First, matched-isotype labeled samples can show whether nonspecific binding is likely to be an issue with the cells being analyzed and is probably the only justified use of such reagents. Second, in most experiments, the aim is to determine the proportion of positive events in a mixed sample. Inherent in this concept is that some cells will be "negative," and where the two populations are distinct with respect to their fluorescence intensity, the positive fraction can be readily identify. This is called "cluster" analysis (or "cloud" analysis in the United States) and is the recommended method for enumeration of $CD4^+$ T cells and $CD34^+$ progenitor cells in clinical laboratory protocols. It can be used to analyze data presented in 1D histograms but is much more easily applied to data arrayed as 2D dot plots.

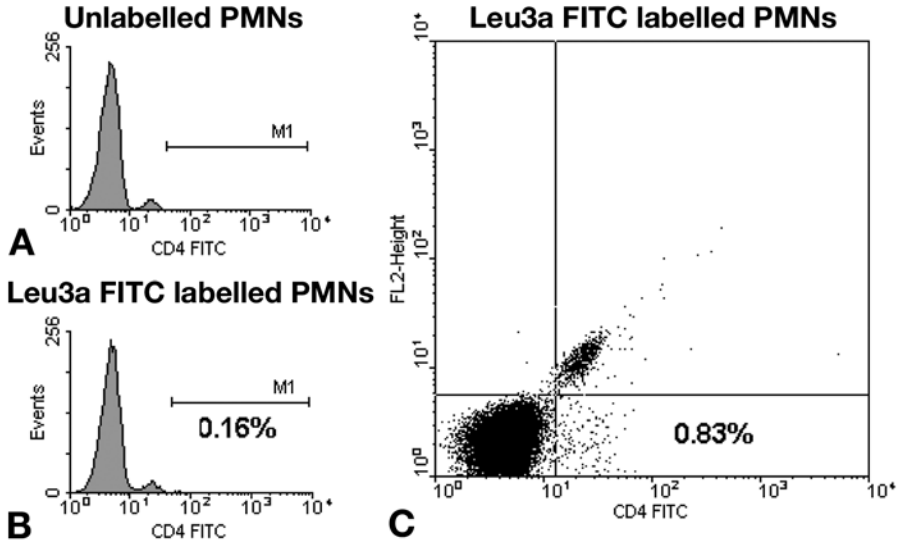


**Fig. 2.** The benefit of dual-parameter analysis of single fluorochrome-labeled cells. **(A)** Background nonspecific binding and autofluorescence are shown. **(B)** Binding of CD4. Because of the high autoflourescence, the weak binding of CD4 is masked. **(C)** The use of dual-parameter analysis allows the distinction between the autofluorescence and true CD4 binding and so facilitates the determination of a more accurate measure of $CD4^+$ cells.

In fact, the use of histograms for data analysis is actually bad practice and can conceal data rather than enlighten. Two examples are presented below.

### *6.1. Analysis of CD4 Expression on Granulocytes*

A whole-blood sample was labeled with FITC-conjugated anti-CD4 (Leu3a; Becton Dickinson Immunochemistry System, San Jose, CA), washed, and analyzed by flow cytometry. A "control" tube contained the same preparation which was unlabeled. After acquisition, an analysis gate was established around the granulocytes as determined by FSC and SSC signals and the data were presented as a 1D histogram. **Figure 2A** shows the background autofluorescence of the cells. There is a clearly "negative" peak and smaller peak of autofluorescent cells that are eosinophils. A marker has been set to the right of the autofluorescent population to determine the "positive" events. **Figure 2B** shows the same cells labeled with anti-CD4 FITC. The same two populations are observed but there is a very small tail of events to the right of the autofluorescent eosinophils which one might suppose are $CD4^+$ basophils and represent 0.16% of the granulocytes. However, a hematology analyzer reported the basophil population as 0.9% of the granulocytes. **Figure 2C** shows the same data from

**Figure 2B** as a 2D dot plot with CD4 FITC arrayed on the *x*-axis and FL2 fluorescence on the *y*-axis. These cells were not labeled with a PE-conjugated mAb, so this purely represents the autofluorescence of the cells. It is now readily apparent that the autofluorescent eosinophils appear in the upper-right quadrant. In contrast, the $CD4^{+}$, nonautofluorescent basophils are found in the lower-right quadrant and a population of $CD4^{wk+}$ cells are resolved from within the autofluorescent cluster, giving the proportion of basophils as 0.83% of total granulocytes, which much better approximates that reported from the hematology analyzer.

### *6.2. Analysis of "Necrotic" and "Apoptotic" Cells in a Culture Using Propidium Iodide*

The enumeration of dead or nonviable cells in a population is a common use of flow cytometry. It is another contentious issue which has led to much discussion. A typical approach to the assessment of dead cells is addition of a solution of propidium iodide (PI) or other membrane-impermeable DNA dye to a cell suspension. It is believed that cells with an intact cell membrane will remain nonfluorescent while those with a damaged membrane will fluoresce. This is partly true. Most cells are impermeable to low concentrations of PI for short periods of time, but prolonged exposure to PI leads to false-positive events. Whether this is through slow ingress, failed egress, or simply nonspecific binding to the cell membrane is unclear, but certainly a weak PI solution (1–5 μg/mL is ideal) should be used and the samples assayed within minutes of adding the PI solution. PI positivity is only ever a surrogate marker of viability, it is a marker of membrane integrity and gives little indication of the cells' ability to recover.

PI is an unusual dye. Once it enters through the cell membrane into the cytoplasm, the cells will fluoresce weakly. In cells with a damaged nuclear membrane, the dye will access the DNA whereupon it will intercalate and fluoresce much more intensely. This is a valuable tool because it allows the discrimination of cells at different stages of death. Nonetheless, it should be used with caution. **Figure 3A** shows the PI fluorescence of a population of myeloid leukemia cells after 48 h in culture. At least three subpopulations are apparent: a small proportion of $PI^{-}$ cells, a large population of $PI^{wk+}$ cells, and a third population of $PI^{+}$ cells. It might be concluded that the majority of cells are nonviable. However, displaying the data as a 2D dot plot with FSC on the *x*-axis and PI on the *y*-axis (**Fig. 3B**) resolves four subpopulations and identifies a large group of cells with low red fluorescence but high FSC. These are autofluorescent myeloid blasts. Beneath them on the plot is a smaller population of normal lymphocytes that are contaminants of the sample. It is important to note that during the early stages of apoptosis and necrosis, the FSC signal decreases, either through change in the refractive index of the cell

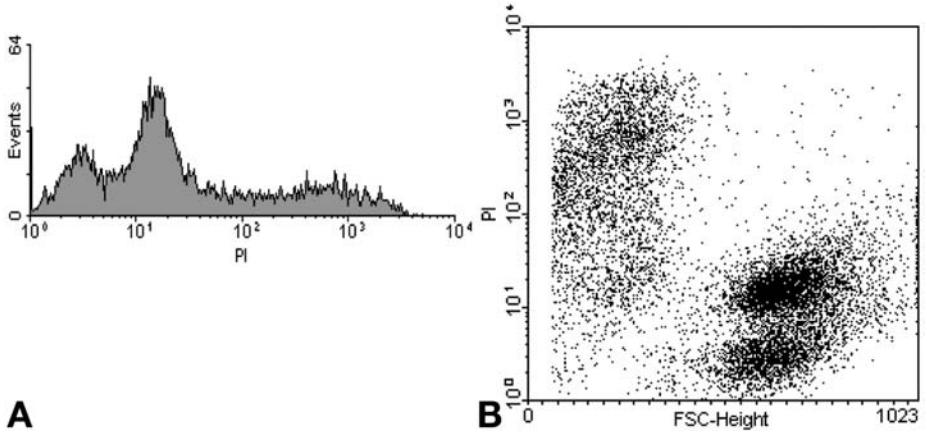


**Fig. 3.** The use of forward light scattering and propidium iodide labeling for the identification of apoptotic and necrotic cells (3) from live (1) and autofluorescent (2) cells.

(apoptosis) or through cell shrinkage (necrosis), and thus combined analysis of FSC versus PI uptake is a powerful tool for the analysis of cell death ***(2)***. It is at least as good as PI versus annexin V expression in most cases.

## 7. The Measurement of Fluorescence Intensity

A regular use of flow cytometers is the determination of the density of specific molecules on the surface of one or more cells in a population. These measurements may be relative, semiquantitative, or quantitative depending upon the question asked and the reagents available.

In most cases, the measurement of "relative fluorescence intensity" is adequate, where the fluorescent channel number that best approximates the average fluorescence of one population is compared with the same value from a second population labeled with the same reagent. The only difficulty with such a comparison is determining the average level of fluorescence. In most cases, when fluorescent signals derived from mAb binding are measured, the data are log-transformed to provide sufficient resolution of the cells. This means that the distributions, although appearing Gaussian, are actually "log normal" distributions in which the mean fluorescence intensity will be skewed to the right; that is, the mean will overestimate the true average fluorescence intensity. The best estimate of the average of log-arrayed fluorescent signals is the median or geometric mean, so always assess "median fluorescence intensity" rather than mean.

Median fluorescence intensity is a value that should be taken from a single log-normal distribution. Taking a single median value from a fluorescence signal that contains multiple log-normal distributions should be avoided (e.g., the PI signal in **Figure 3A**, which shows at least three subpopulations).

A typical experiment in which one might want to measure relative fluorescence intensity is shown here, where the effect of tumor cells on the expression of CD69 on resting NK cells and T cells from the same donor was investigated. Mononuclear cells were incubated overnight in the presence or absence of a tumor cell lysate and labeled with anti-CD3, anti-CD69, anti-CD16, and anti-CD56. **Figure 4** histograms A and B show the background expression of CD69 on NK and T cells, respectively, incubated in the absence of the tumor cell lysate. NK cells show a higher proportion of $CD69^+$ cells than do the matched T-cell population, and the intensity of expression of CD69 is higher on the NK cells as measured by the median channel fluorescence intensity (MedCF). It is not possible to conclude that the CD69 expression is more dense on the NK cells, because there is no certain measurement of relative cell size; all that can be determined is that there are more CD69 molecules on the NK cells than their matched T cells. A relative fluorescence intensity may be calculated by expressing the increased CD69 expression as a percentage of the level of expression on the T cells as follows:

$$\frac{\text{MedCF CD69 NK - MedCF CD69 T}}{\text{MedCF CD69 T}} \times 100$$

Plainly, the NK cells have shown considerably greater activation in response to the tumor cell lysate than the T cells and the relative fluorescence intensities of the two populations can be compared with each other or with their nonstimulated state.

The measurement of relative fluorescence intensity in this case is quite adequate for the required purpose, but to compare experiments run on different days or between collaborating centers, it is valuable to have some form of semiquantitative measurement. For this, there are fluorescent standards and these are most commonly and reproducibly provided by latex beads. A huge variety of latex beads are available commercially, but for fluorescent semiquantitation purposes, a kit containing a minimum of five populations of beads with increasing fluorescence intensities should be used. These beads each have a nominal value of fluorescence intensity, termed molecules of soluble fluorochrome/bead, ascribed to them so that individual median channel values can be converted into a standardized numerical value. These values are only semiquantitative but can be used to standardize experiments between runs or between cytometers. An example is presented below:

A bead preparation containing unlabeled beads plus seven bead populations expressing increasing amounts of fluorochrome was analyzed by flow cytometry, and the resultant fluorescent peaks were arrayed as log-amplified data (**Fig. 5A**).

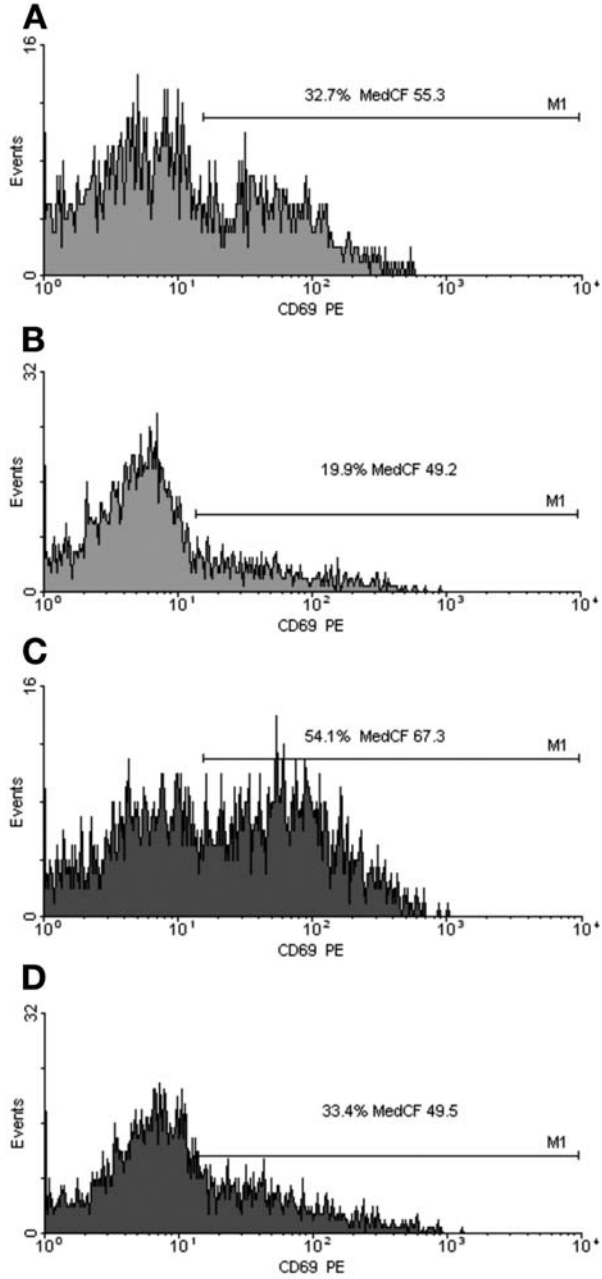


**Fig. 4.** Histograms **A** and **B** show the background expression of CD69 on NK and T cells, respectively, incubated in the absence of the tumor cell lysate. Histograms **C** and **D** show the effect of stimulation with the tumor cell lysate on the same cells.

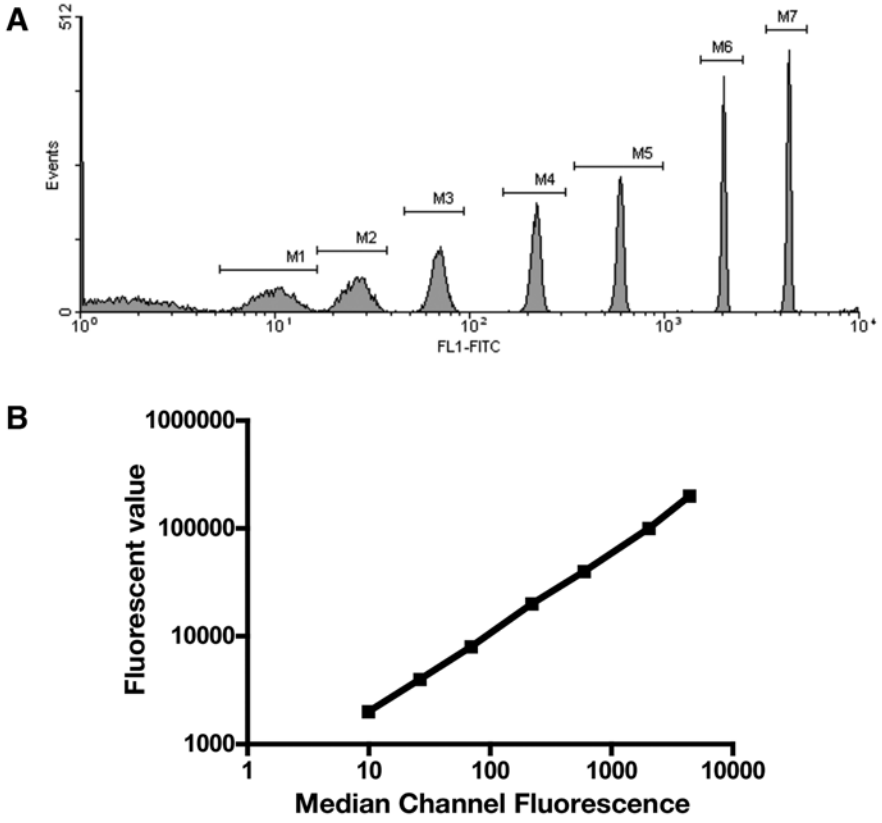


**Fig. 5.** **(A)** Multiparameter fluorescent beads are analyzed with logarithmic acquisition and linear data array. **(B)** The correlation between median channel linear data array of logarithmically amplified fluorescence and molecules of soluble fluorochrome/bead as defined by the manufacturer.

The median channel fluorescence of each fluorescent peak (not the unlabeled beads) was determined and plotted against the stated fluorescence intensity of each bead population as given by the manufacturers on a log-log display (**Fig. 5B**).

The median channel fluorescence value of a cell population can be resolved to a standardized fluorescence intensity by interpolation onto this straight line. It must be noted that the values used for fluorescence intensity are not standardized between manufacturers, so this is not a quantitative value in the true sense of the word.

An alternative approach that is closer to absolute quantitation of binding sites for individual mAbs is use of the so-called "antigen-binding capacity" (or ABC) beads. These are available from at least two different manufacturers and are

based on the provision of beads that are coated with known numbers of molecules of antibody to murine immunoglobulin Fc. By incubating beads with the fluorochrome-conjugated antibody of choice, the median channel fluorescence value associated with a specific number of molecules of bound mAb can be determined using a plot similar to the one shown above (**Fig. 5**). This statement has at least two caveats, however. First, the beads measure binding of the mAb of interest via its Fc, which is determined by the affinity of the anti-Fc mAb on the beads. In contrast, experimentally, the binding of the mAb of interest to the cells of interest is determined by the affinity of the mAb of interest for its specific antigen. Second, it is assumed that the mAb of interest can bind its specific antigen in a monovalent fashion only and this is by no means certain unless a monovalent antibody can be purchased. Nonetheless, such beads have been used to semiquantitate the expression of specific antigens on the surface of leukemic cells at the time of disease presentation and to then demonstrate that the level of expression of these antigens is specific to the malignant clone when compared with normal immature bone marrow mononuclear cells that arise in patients after chemotherapy ***(3)***.

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# 6

# Apoptosis Detection by Flow Cytometry

**Paul Allen and Derek Davies**

**Summary**

Apoptosis is a biologically important form of cell death and therefore detection and measurement of apoptosis have become imperative. Apoptotic cells have many characteristics that can be measured by flow cytometry. These include cell plasma membrane changes, changes in plasma membrane permeability, changes in mitochondrial membrane permeability, caspase activation, and DNA cleavage. Determination of any one or a combination of these changes by flow cytometry allows the identification and quantification of apoptotic cells in a mixed population. It can also give valuable information about the molecular pathways that cells take during cell death. The advantages and limitations of each procedure designed to measure any of these characteristics are discussed, and some illustrated protocols are described.

**Key Words:** Apoptosis; caspases; DNA cleavage; mitochondria; plasma membrane; viability.

## 1. Introduction

Apoptosis is a form of cell death that has evolved to counter cell proliferation. Examples of apoptosis are seen during fetal development (e.g., loss of webbing between digits), in the deletion of autoreactive immune cells in the neonate and during the maintenance of tissue and organ homeostasis in the adult. Aberrations in apoptosis control result in disease; cancer and autoimmune diseases can originate from the inhibition of apoptosis; and degenerative diseases (e.g., Parkinson's disease) can be a consequence of unwanted activation of apoptosis. Apoptosis contrasts with necrosis, often referred to as "accidental cell death," in that the plasma cell membrane remains intact. The classical signs of *rubor*, *calor*, *tumor*, and *dolor* (redness, heat, swelling, and pain, respectively), synonymous with necrosis, are not seen, because cellular contents of apoptotic cells are never released into interstitial spaces to cause inflammation. Rather, apoptotic cells

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are phagocytosed by surrounding cells. Estimates suggest that a typical adult consisting of approx $10^{14}$ cells loses $10^7$ cells per day through apoptosis, in a painless and unnoticed fashion.

Apoptosis can also be induced, or activated directly, rather than result from programmed events such as those cited above. For example, therapeutic agents used in the management of neoplastic disease induce apoptotic cell death in target cells at physiologically tolerable concentrations. Thus, in cancer, blockade of apoptotic pathways contributes not only to oncogenesis but also to drug resistance ***(1)***.

## 2. Apoptotic Pathways and the Role of the Mitochondrion

Apoptotic cells exhibit specific characteristics. These include chromatin condensation, cell shrinkage, and membrane blebbing. This latter characteristic is responsible for the "pinching-off" of apoptotic bodies from the parent cell. Each apoptotic body contains a random assortment of nuclear and cytoplasmic material, but crucially the cell membrane remains intact, and *in vivo*, these bodies are removed by phagocytosis. Another marker of apoptosis is activation of the caspase-activated deoxyribonuclease, an endonuclease that cuts DNA at sites exposed by histones. These sites are at regular intervals (every 182 base pairs [bp]) and if the cleavage is complete will produce DNA fragments consisting of multimers of 182 bp (i.e., 182, 364, 728, 1456, etc.). When these fragments are run on agarose gel electrophoresis, they separate out to form a characteristic "DNA ladder," a hallmark of apoptosis. DNA fragmentation to this degree is preceded by DNA cleavage into much larger fragments of 300 kbp to 50 kbp. If the final stages of DNA cleavage do not take place, conventional electrophoresis would not resolve these fragments into a ladder. In fact, pulsed field electrophoresis is required to identify this level of DNA fragmentation ***(1)***.

It is generally accepted that most, but not all, forms of apoptosis are the end result of caspase activation. Caspases are cysteinyl aspartate–specific proteinases that recognize a tetrapeptide sequence on target proteins. Caspase activation can be autocatalytic (e.g., caspase 8) or the result of holoenzyme generation due to cleavage and an association with cofactors (e.g., caspase 9) or to cleavage from upstream caspases (e.g., caspases 3, 6, and 7). Caspases have been arbitrarily divided into initiator caspases (e.g., caspases 8 or 9), which are those that activate directly to the induction of apoptosis (resulting in further downstream caspase cleavage), or effector caspases (e.g., caspases 3, 6, and 7), which are those that once activated by initiator caspases, cleave caspase substrates that are responsible for creating the characteristic phenotype of apoptosis ***(2)***.

Induction of apoptosis can be brought about by proteins that are extrinsic to the cell; that is, they are ligands (e.g., fas-L, tumor necrosis factor [TNF]-α, and TNF-related apoptosis-inducing ligand [TRAIL]) that bind to ligand-specific

cell surface receptors that have transmembrane and cytosolic domains. The resultant conformational change of the intracellular domain binds adaptor proteins (e.g., FADD [Fas-associated death domain]) that in turn recruit caspase 8 molecules. This autocatalytic caspase initiates downstream events of the extrinsic pathway. The large majority of other apoptosis-inducing stimuli activate the intrinsic pathway. This pathway can be induced, for example, by changes in the cellular environment such as loss of growth factor(s), changes in temperature or pH, hypoxia, or other forms of "stress" such as DNA damage. The key player in this pathway is the mitochondrion ***(2–4)***. Mitochondria are the sources of ATP production for the cell. For ATP production to occur, there must be a net negative charge on the inner mitochondrial membrane. However, on the induction of apoptosis, a key mitochondrial event takes place which is known as mitochondrial outer membrane permeabilization, or MOMP. Proteins that are normally found in the mitochondrial intermembrane space are released into the cytosol, and the inner transmembrane electrical potential ($\Delta\psi m$) is lost. One protein released is cytochrome C. This triggers assembly of the cytochrome C/caspase 9/Apaf 1 complex, which is also known as the apoptosome. This is a caspase-active complex that subsequently activates downstream effector caspases that drive apoptosis onward ***(4–6)***. MOMP is a point of no return for the cell in that, once induced, the cell is effectively on an irreversible course to cell death. MOMP is brought about by the insertion of pores into the outer mitochondrial membrane. One mechanism may involve the assembly of the permeability transition pore consisting of the adenine nucleotide transporter plus the voltage-dependent anion channel stretching from the inner to the outer mitochondrial membranes, respectively. The result is a collapse of the $\Delta\psi m$ and an uptake of water molecules ***(3,5)***. The mitochondrial membrane therefore swells, rupturing the outer mitochondrial membrane. Another model based on experimentation shows a role for members of the Bcl-2 family of proteins. These proteins share regions of homology known as the BH (Bcl-2 homology) domains; some of the proteins promote MOMP and hence apoptosis, and others prevent MOMP and hence inhibit apoptosis. Two Bcl-2 proteins (Bax and Bak) that each contain three BH domains (BH1, BH2, and BH3) oligomerize and form pores in the outer mitochondrial membrane, thus generating MOMP. Oligomerization is a result of activation by a subfamily of the Bcl-2 proteins that contain only the BH3 domain (e.g., tBid). The eponymous Bcl-2 protein plus other multidomain antiapoptotic proteins can block Bax and Bak by binding to them and preventing their activation ***(4,5,7)***.

## 3. Apoptosis and Necrosis

Necrosis is often referred to as "accidental cell death." This is because cells are killed by rupture of the plasma membrane, reminiscent of events seen in

burns and cuts. The resultant leakage of cellular contents into the surrounding environment activates inflammation. However, necrosis can be brought about by less severe insults that nevertheless cause membrane rupture. One example is a poison like sodium azide, which blocks the activity of energy-dependent ion pumps. The decline of $Ca^{2+}$, $Mg^{2+}$, K, and $Na^{2+}$ down their respective concentration gradients results in a compensatory intake of water by the cells. Mitochondrial membranes also swell, anaerobic glycolysis occurs, and there is a decrease in pH. The plasma cell membrane ruptures and lysomal enzymes and degrading phospholipase are released, resulting in characteristic inflammation ***(1)***.

Therefore, there is an apparent dichotomy in cell death represented by apoptosis on the one hand and necrosis on the other. Apoptosis is a preprogrammed event that maintains tissue homeostasis. It is also a response to mild stimuli from the environment or from physiological inducers like TNF-α, Fas-ligand, or TRAIL. Necrosis is induced by prolonged or extreme insult or is a result of mechanical rupture of the cell membrane. However, are these processes entirely separate events? Evidence that they are not comes from several sources. Necrosis is often seen after an initial apoptotic response; the same stimulus can induce either apoptosis or necrosis, depending on the concentration and/or the length of time of insult; caspase inhibition will block apoptosis, but the cell can still die through necrosis and MOMP plus a loss of $\Delta\psi m$, and cytochrome C release occurs in both apoptosis and necrosis. Thus, apoptosis and necrosis could be two extremes of a continuum. This implies that taking any one single parameter of cell death in isolation may not define the form of cell death under examination ***(1)***.

Many of the characteristics of apoptosis described above are amenable to identification and quantification by flow cytometry. Some of the techniques are described later in this chapter.

## 4. Viability and Necrosis

Light scatter as measured by a flow cytometer is a complex amalgam of the way particles reflect, refract, and diffract light. The amount of scattered light will depend on several factors, notably cell size, nuclear/cytoplasmic ratio, granularity of the cytoplasm, surface topography, and the difference in the refractive index between the intra- and extracellular media. It will also depend at which angle the scattered light is collected. However, a dot plot of forward versus 90° side scatter will often allow the difference between light scatter properties of live, dead, and apoptotic cells to be seen.

Cell condensation during early apoptosis is seen as a decrease in forward light scatter only. As apoptosis proceeds, both forward and 90° side scatter diminish. In necrotic death, cells swell initially and then cellular contents are rapidly released. This is seen on the flow cytometer as an initial increase in

forward light scatter followed by a rapid decrease in both forward and 90° side scatter. Changes in cell size can be used in conjunction with surface phenotypic markers to identify the dying population.

Changes in plasma membrane permeability represent a more discriminatory feature of dying cells. Cationic dyes (e.g., propidium iodide [PI] or 7-amino-actinomycin D [7-AAD]) are not permeant for viable cells. In necrosis, or late apoptosis, plasma membrane rupture allows uptake of these dyes. However, during apoptosis, changes in energy-dependent ionic membrane pumps permit cellular entry of 7-AAD to some degree. Thus, necrosis and late apoptosis cells fluoresce brightly, whereas apoptotic cells fluoresce less brightly and viable cells exclude the dye altogether and are therefore negative for fluorescence.

Another example is Hoechst 33342. This dye is taken up much more slowly in viable cells than in either apoptotic cells or dead cells. Hoechst 33342 and the vital dye PI prove a useful combination. After a short staining period, viable cells take up neither Hoechst nor PI; apoptotic cells take up only Hoechst (blue), and both Hoechst and PI are found in necrotic cells. Moreover, these dyes permit use of a third fluorochrome like fluorescein isothiocyanate (FITC) for further characterization of the dying cells.

The monomeric cyanine dyes are alternatives to these dyes and also capitalize on the altered membrane permeability of apoptotic cells. A commonly used example is the green fluorescent nucleic acid dye, YO-PRO-1. Changes in apoptotic cell plasma membranes allow the normally cell-impermeant YO-PRO-1 to enter apoptotic cells and bind to DNA. These cells will also exclude PI, thus discriminating among viable cells, apoptotic cells, and dead cells.

### *4.1. Detection of Apoptosis Using Uptake of 7-AAD*

1. Wash cells in PBS and resuspend at $1 \times 10^6$/mL in PBS.
2. Add 7-AAD to 1 mL of cells (i.e., $10^6$ cells) to give a final 7-AAD concentration of 20 µg/mL.
3. Incubate the cells at 4°C for 20 min.
4. Pellet the cells and resuspend in 500 µL of 2% paraformaldehyde.
5. Analyze the cells *within 30 min* of fixation with a dot plot using forward scatter on the *x*-axis and 7-AAD fluorescence on the *y*-axis.
6. Three distinct populations should be observed: a population with 7-AAD fluorescence equivalent to the negative control (nonstained paraformaldehyde fixed cells, which are the viable cells), 7-AAD-bright cells that are the dead or late apoptotic cells, and 7-AAD-dim cells that are the apoptotic population.

Note: Cells do not have to be fixed in this protocol, so **step 4** can be omitted. However, if fixation is preferred, it can be performed either prior to or after staining with 7-AAD. It is important that the flow cytometry be performed within 30 min of staining cells after fixation.

### 4.2. Detection of Apoptosis and Necrosis Using Hoechst 33342 and PI

1. Harvest $1 \times 10^6$ cells and resuspend in 1 mL of PBS.
2. Add Hoechst 33342 to a final concentration of 1 µg/mL (e.g., add 100 µL of a stock Hoechst solution of 10 µg/mL to 900 µL of cell suspension).
3. Add PI to a final concentration of 5 µg/mL (e.g., one drop of PI from a stock of 50 µg/mL using a pastette).
4. Incubate for 5 min and analyze using a dot blot of PI on the *x*-axis and Hoechst on the *y*-axis.

Note: Hoechst 33342 emission spectrum shifts toward the red in apoptotic cells. To avoid loss of Hoechst fluorescence, use a 400 long-pass filter instead of the usual Hoechst DNA filter combination of 400 long-pass and a 480 short-pass filters.

### 4.3. Detection of Membrane Changes Using YO-PRO-1 (Fig. 1)

1. Harvest $1 \times 10^6$ cells and wash once in PBS.
2. Resuspend cells in 1 mL of medium with 1 µ*M* YO-PRO-1.
3. Incubate cells at room temperature for 20 min.
4. Wash once in PBS and analyze immediately (keep cells on ice until analysis).
5. YO-PRO-1 fluorescence, after 488-nm excitation, is measured between 515 and 545 nm.

Addition of a dead cell discriminator such as PI will enable live cells (YO-PRO-1- and PI-negative) to be separated from apoptotic (YO-PRO-1-positive, PI-negative) and dead (YO-PRO-1-positive, PI-positive) cells. In addition, as cells die and become progressively necrotic, they follow a parabolic course on a dot plot, being initially bright for both dyes and then progressively less so.

## 5. Apoptosis

The many characteristics of apoptosis can be exploited to identify apoptotic cells amongst viable cell populations. The loss of DNA from cells as a result of DNA fragmentation is technically the easiest to detect. Small fragments consisting of 182-bp DNA multimers can be eluted from apoptotic cells after permeabilization with 70% ethanol and washing in phosphate buffer. The permeabilization also allows entry of the dye PI into cells which intercalates with DNA. The resulting DNA profile for proliferating viable cells shows a DNA distribution for cells in $G_0/G_1$ phase, S-phase, and $G_2$/M phase of the cell cycle, but the elution of DNA from apoptotic cells gives a population of cells with a DNA content less than that of cells in $G_0/G_1$.

The nucleosomal fragmentation of DNA starts with the initial formation of 300-kbp and/or 55-kbp fragments. It is only after this primary stage that the 182-bp fragments are generated. In some cell types, this latter fragmentation does not occur. These large fragments are too big to be eluted from cells, so on

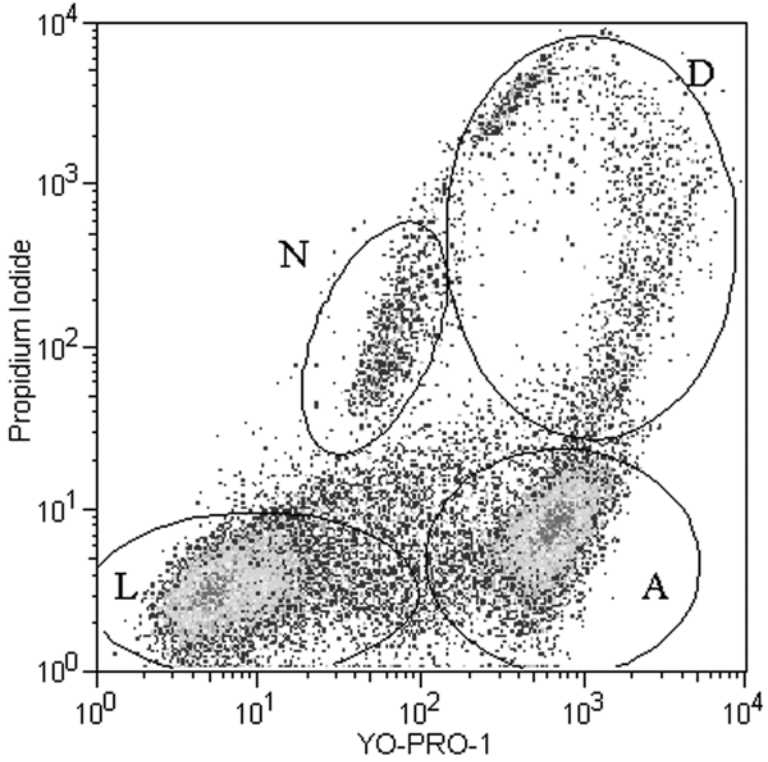



**Fig. 1.** Apoptosis detection by YO-PRO-1 (alterations in cell plasma membrane). Live cells (L) are negative for both YO-PRO-1 and PI. Early apoptosis (A) shows increased YO-PRO-1 fluorescence but remains PI-negative. Dead cells (D) are YO- PRO-1- and PI-bright, but as the cells become necrotic, DNA degrades and the fluorescence of both dyes is reduced (N).

a DNA content assay, there would be no apparent loss of DNA in apoptotic cells. The researcher would have to resort to pulsed field gel electrophoresis to resolve, and hence identify, fragmentation of this nature. However, the breaks in the DNA strands which are essential for DNA fragmentation can be detected using a technique called TUNEL (terminal deoxynucleotidyl transferase [TdT] deoxyuridine triphosphate [dUTP] nick end labeling). The nicks, or breaks, in the DNA are caused by the activation of the caspase-activated deoxyribonuclease. The technique depends on the ability of TdT to end-label the nicks with the nucleotide dUTP independently of any template from the opposing strand.

Biotinylation or conjugation of dUTP with digoxygenin can be used to identify the newly incorporated nucleotide in the DNA. For flow cytometry, biotin can be detected using FITC-conjugated avidin and digoxygenin can be

detected with antibodies carrying a fluorochrome. Better still is to use a protocol incorporating conjugated dUTP directly. No secondary reagents are required and this eliminates the possibility of nonspecific binding of secondary reagents. This technique allows for the discrimination of apoptosis, necrosis, and viability because nicks occur with a higher frequency in apoptotic than in necrotic cells. (*See* **Subheadings 5.1.** and **5.2.**).

Changes in the distribution of membrane proteins, glycoproteins, and phospholipids during apoptosis can be used to detect apoptosis as an alternative to DNA fragmentation. One change in particular that is easy to exploit is that of phosphatydylserine (PS) distribution. In normal viable cells, the inner plasma membrane layer contains anionic phospholipids and the outer layer the neutral lipids. Therefore, the distribution of PS is asymmetrical and is found primarily on the inner leaflet of the membrane. However, during apoptosis, the distribution of anionic lipids flips and PS is then found primarily on the outer leaflet of the membrane. Annexin V is a calcium-dependent phospholipid-binding protein with a high affinity for PS and therefore can be used to detect the externalized PS. Annexin V is available conjugated to a range of fluorochromes and therefore is suitable for use in multiparameter analyses. (*See* **Subheading 5.3.**).

The mitochondria can also be targeted for apoptosis detection because the generation of MOMP and the subsequent drop in $\Delta\psi m$ can be measured on the flow cytometer. The asymmetrical distribution of $H^+$ ions across the mitochondrial inner membrane maintains the internal net negative charge that is required for normal mitochondrial function. The cationic lipophilic fluorescent dyes CMXRos (chloromethyl-X-rosamine), TMRE (tetramethylrhodamine ethyl ester), JC-1 (5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbo-cyanide iodide), $DiOC_6(3)$ (dihexyloxacarbo-cyanide iodide), and rhodamine 123 are taken up and distributed across the inner membrane in a way that is dependent on the $H^+$ distribution (i.e., to the mitochondrial inner membrane matrix in normal viable cells). This occurs only because the effect of $\Delta\psi m$ is far greater in terms of cationic dye distribution than that of the plasma membrane. This is illustrated by the application of 137 m*M* KCl, which disrupts the plasma membrane potential and causes only a small drop in cellular fluorescence. The protonophore carbonylcyanide m-chlorophenyl hydrozone (mClCCP) disrupts the $\Delta\psi m$ and results in a much larger decrease in fluorescence.

The dye $DiOC_6$ is taken up into the mitochondria of healthy cells and reflects the $H^+$ ion distribution in these cells. The collapse of $\Delta\psi m$ in apoptosis, resulting in the redistribution of $H^+$ ions, also results in the concomitant loss of $DiOC_6$ from mitochondria and hence a decrease in fluorescence on the flow cytometer. JC-1, however, is a little more informative. JC-1 forms aggregates in the presence of a high membrane potential and fluoresces in the red region at 590 nm. In apoptosis, the $\Delta\psi m$ collapses and the JC-1 aggregates

dissociate to form monomers that fluoresce in the green region at 527 nm. Therefore, it is possible to plot green fluorescence against red fluorescence for a population of cells. Even in healthy cells, it will be possible to discern both JC-1 aggregates and JC-1 monomers, both of which are an indication of the degree of heterogeneity of mitochondria in individual cells. Thus, the majority of mitochondria will have high $\Delta\psi m$ but some will be low. However, in an apoptotic population, the majority of cells will have lost any $\Delta\psi m$ and this loss will be reflected in a loss of JC-1 aggregates and hence of red fluorescence. (*See* **Subheadings 5.4.–5.6.**).

Caspase activation in apoptotic cells may also be detected. This can be achieved by using an antibody directed against the catalytic site of specific caspases or by using a nonfluorescent substrate that is cleared by the enzyme to yield a detectable fluorescent product. (*See* **Subheading 5.7.**).

### *5.1. Detection of Apoptosis Using a PI DNA Content Assay (Fig. 2)*

1. Harvest approx $2 \times 10^5$ cells and wash once in phosphate-buffered saline (PBS).
2. Resuspend the cells in 0.5 mL of ice-cold 70% ethanol and incubate on ice for more than 2 h.
3. Wash cells once in PBS and resuspend in a 1-mL mix of 50 µg/mL PI and 200 µg/mL RNAse in PBS for 30 min at 37°C.
4. Do NOT wash cells prior to analysis on the flow cytometer. PI is an intercalating agent and will leak out of the cell if the population is resuspended in buffer not containing PI.
5. Analyze using pulse processing to gate out aggregated cells and cell debris. On a histogram plot of red fluorescence against cell numbers, the apoptotic cells fall to the left of the $G_0/G_1$ peak.

### *5.2. Detection of Apoptosis by a TUNEL Technique*

1. Harvest $1.5 \times 10^6$ cells and wash in PBS.
2. Resuspend in 1 mL of PBS, add 1 mL 2% w/v paraformaldehyde, and place on ice for 15 min.
3. Wash twice in PBS and resuspend the pellet in 2 mL of 70% ethanol.
4. Place at –20°C for at least 30 min, but overnight (approx 16 h) may give better results. Cells can be stored for up to 3 d at this stage.
5. Rehydrate the cells in PBS by pelleting the cells, aspirating the ethanol, and resuspending the cells in 1 mL of PBS.
6. Pellet and resuspend the cells in 50 µL of cacodylate buffer (0.2 *M* potassium cacodylate, 2.5 m*M* Tris-HCL, pH 6.6, 2.5 m*M* $CoCl_2$, 0.25 mg/mL bovine serum albumin, 5 units of TdT, and 0.5 n*M* dUTP-FITC) and incubate for 60 min at 37°C.
7. Wash twice in PBS.
8. Analyze on a histogram plot of green fluorescence against cell numbers.

Note: A control sample should omit Tdt in **Step 6**.

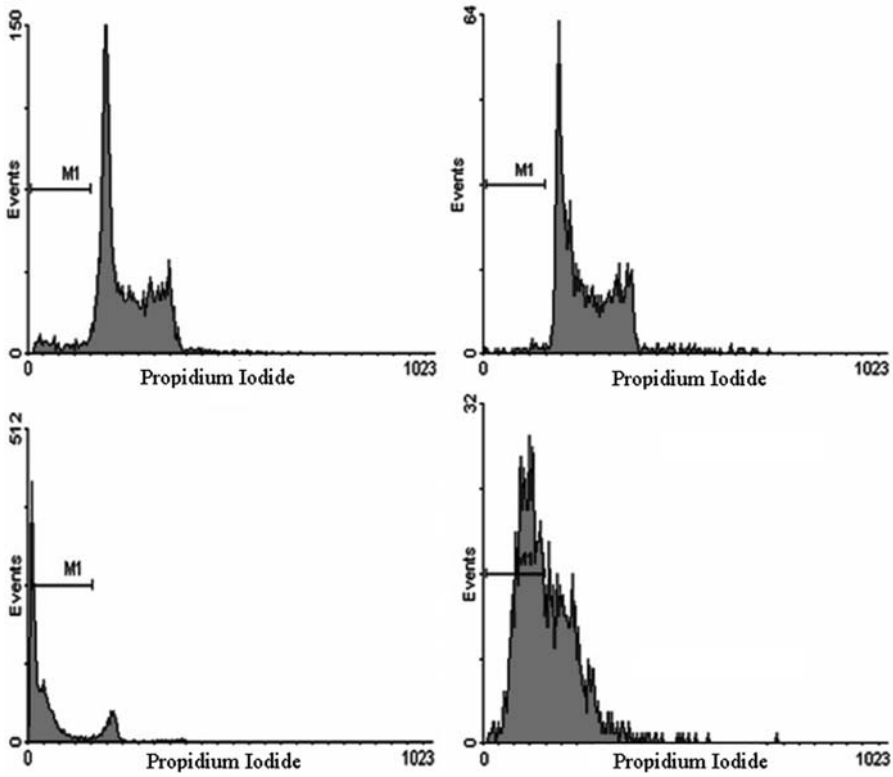



**Fig. 2.** Propidium iodide/RNAse-stained cell lines showing **(upper panels)** unperturbed cell cycle distributions and **(lower panels)** sub $G_0/G_1$ apoptotic populations in the $M_1$ interval gate. HL-60 cells are shown on the **left panels**, and K562 cells are shown on the **right**.

### *5.3. Detection of PS as a Marker of Apoptosis Using Annexin V (Fig. 3)*

1. Wash $1 \times 10^6$ cells in PBS and resuspend in 1 mL of incubation buffer (10 m*M* HEPES, pH 7.4, 150 m*M* NaCl, and 5 m*M* $CaCl_2$).
2. Add FITC-labeled annexin V to a final concentration of 2.5 µg/mL.
3. Wash once in incubation buffer, resuspend in 1 mL of incubation buffer, and analyze cells using a histogram of green fluorescence on the *x*-axis and cell numbers on the *y*-axis.
4. Use nonstained cells and untreated cells as negative controls.
5. Alternatively, if PI (5 µg/mL) is incorporated in the incubation buffer at **step 4**, a dot plot of PI on the *x*-axis against annexin V on the *y*-axis can be used to distinguish viable cells (which are negative for both PI and annexin V), apoptotic cells (which are annexin V-positive but exclude PI and are therefore PI-negative), and late apoptotic or necrotic cells (which are double-positive for PI uptake and annexin V staining).

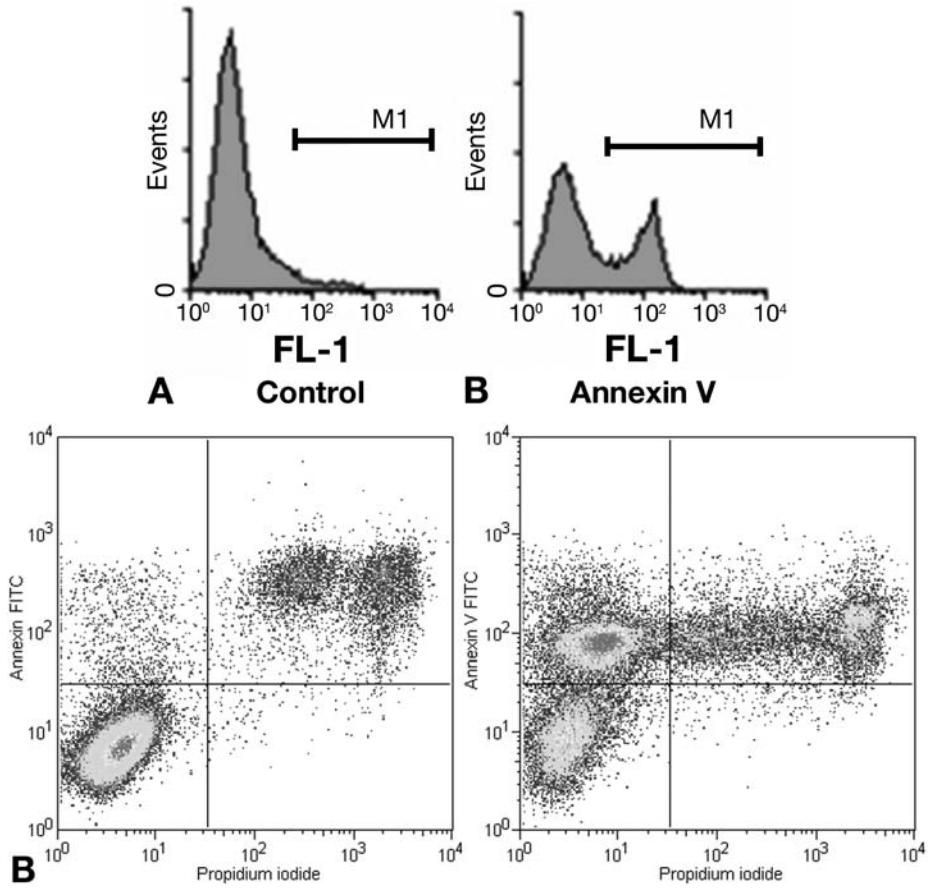
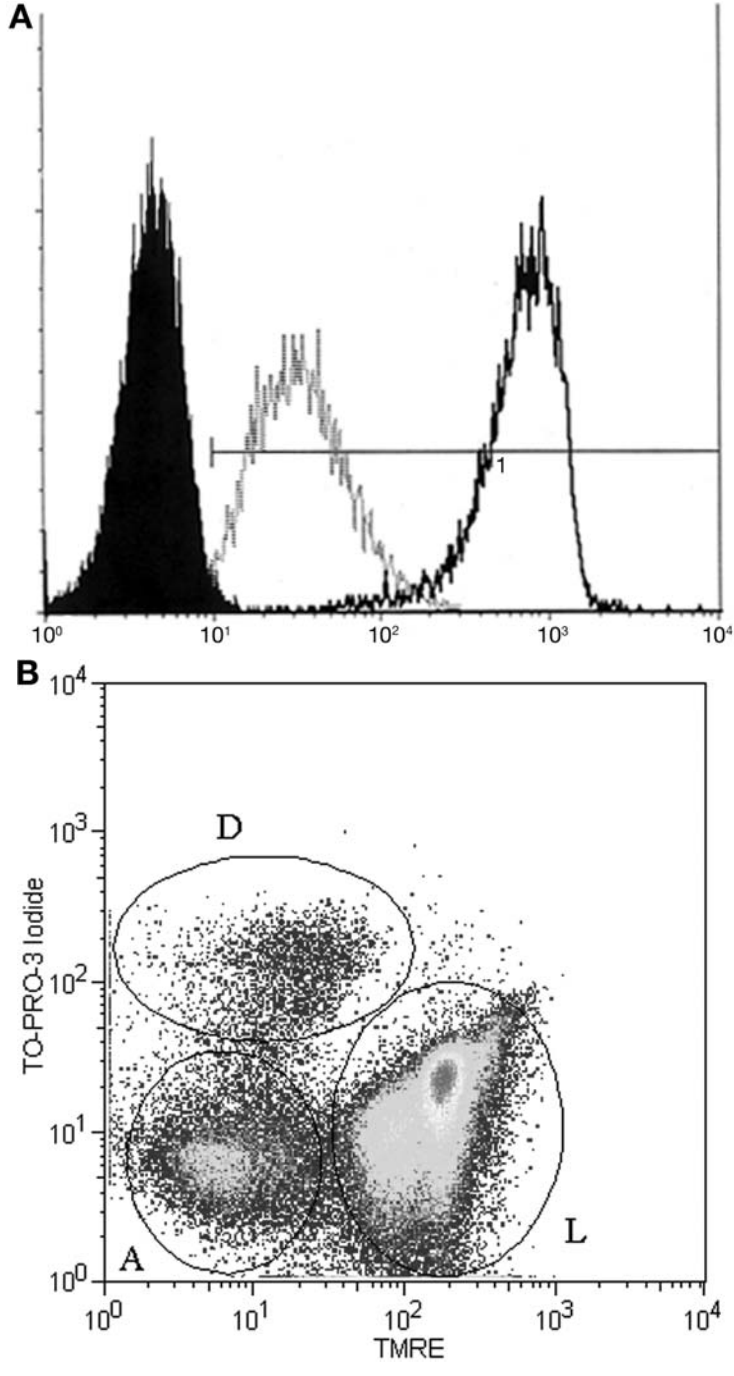



**Fig. 3. (A)** FITC-conjugated annexin V staining for phosphatidylserine exposure on apoptotic cells **(right)** compared with viable control cells **(left)**. (Images were kindly provided by Ulrike Jahnke, Barts and The London School of Medicine and Dentistry.) **(B)** Dual-staining of control cells **(left)** and apoptotic cells **(right)** stained with propidium iodide (PI) (*x*-axis) and annexin V (*y*-axis). The quadrant analysis shows viable cells negative for annexin V and excluding PI **(lower left)**. Apoptotic cells stain with annexin V but excluding PI **(upper left)**. Secondary necrotic cells (i.e., necrosis after apoptosis) are positive for both PI and annexin V **(upper right)**. Necrotic or mechanically damaged cells positive for PI only are shown in the **lower right** quadrant (very few).

### *5.4. Detection of Changes in Δψm Using Nonfixable Dyes (Fig. 4A)*

1. Harvest $1 \times 10^5$ cells and wash once in PBS.
2. Resuspend cells in 1 mL medium containing 40 n*M* $DiOC_6$ or alternatively 1 μ*M* JC-1.
3. Incubate cells for 15 min at 37°C.


A
1
$10^0$
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B
D
A
L
TO-PRO-3 Iodide
TMRE
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$

4. Wash once in PBS.
5. Resuspend in 1 mL PBS and analyze immediately.
6. $DiOC_6$-stained cells can be analyzed using green fluorescence on the *x*-axis against cell numbers on the *y*-axis. A decrease in green fluorescence indicates a loss of $\Delta\psi m$.
7. JC-1-stained cells can be analyzed using red fluorescence (590 nm) on the *x*-axis against cell numbers on the *y*-axis. A decrease in red fluorescence indicates a loss of $\Delta\psi m$. Alternatively, two-color fluorescence can be used with a dot plot of green fluorescence on the *x*-axis and red fluorescence on the *y*-axis.

### *5.5. Detection of Changes in Δψm Using CMXRos (Fixable)*

1. Harvest $1 \times 10^5$ cells and wash once in PBS.
2. Resuspend cells in 1 mL medium containing 30 m*M* CMXRos. (Stock solutions of CMXRos can be made in dimethyl sulfoxide at concentrations 100- to 200-fold more concentrated than required and stored at –20°C.)
3. Incubate cells at 37°C for 15 min.
4. Analyze cells using green fluorescence against cell numbers.

Alternatively, cells can be resuspended in medium containing 150 m*M* CMXRos, and after incubation they can be fixed by resuspending the CMXRos-stained cell pellet in 4% paraformaldehyde in PBS for 15 min at room temperature.

Cells can be stored at 4°C and/or stained for a second antigen of interest using membrane permeabilization procedures such as acetone, ethanol, or detergents.

Note: A positive control for these experiments is to treat cells with an uncoupling agent (e.g., 50 μ*M* mClCCP), which completely abolishes the transmembrane potential.

### *5.6. Detection of Changes in Δψm Using TMRE (Fig. 4B)*

1. Harvest $1 \times 10^6$ cells and wash once in PBS.
2. Resuspend cells in 1 mL of medium with 40 n*M* TMRE. (Stock solution is 1 m*M* in ethanol; working solution is 40 μ*M*.)
3. Incubate cells at 37°C for 20 min.
4. Wash once in PBS and analyze immediately (keep cells on ice until analysis).
5. TMRE fluorescence is measured, after 488-nm excitation, between 560 and 600 nm.

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**Fig. 4. (A)** Mitochondrial membrane potential ($\Delta\psi m$) measured by $DiOC_6$. An unstained cell population is shown on the **left**, and a control population of healthy $DiOC_6$-positive cells is shown on the **right**. The population in the **middle** consists of cells with a reduced $\Delta\psi m$. **(B)** Apoptosis detection by TMRE ($\Delta\psi m$) and TO-PRO-3 (changes in plasma membrane permeability). Live cells are bright for TMRE but are TO-PRO-3-negative (L). Early apoptotic cells have reduced TMRE staining but remain negative for TO-PRO-3 uptake (A). Dead cells have lost $\Delta\psi m$ and are positive for TO-PRO-3 uptake (D).

Addition of a dead cell discriminator such as 7-AAD (488-nm excitation) or TO-PRO-3 (633-nm excitation) will enable live cells (TMRE-bright, 7-AAD-negative) to be separated from apoptotic (TMRE-dim, 7-AAD-negative) and dead (TMRE-dim, 7-AAD-positive) cells.

### *5.7. Detection of Caspase Activity Using Antibodies to Activated Caspases (Fig. 5)*

1. Harvest $1 \times 10^6$ cells and wash once in PBS.
2. Resuspend cells in 1 mL of 1% paraformaldehyde.
3. Incubate cells at room temperature for 10 min.
4. Spin cells, remove supernatant, and add 0.5 mL of 0.1% saponin.
5. Incubate at room temperature for 20 min.
6. Add appropriate amount (0.25 μg) of fluorescently labeled primary antibody and incubate at 4°C for 30 min.
7. Wash twice in 0.1% saponin and then resuspend in 0.5 mL PBS.
8. Analyze cells, plotting fluorescence versus cell count.

Antibodies to several activated caspases are available commercially, notably caspases 3, 8, and 9. The usual precautions of antibody staining need to be taken (i.e., titration of the antibody using a positive control and the use of appropriate controls when assessing positivity).

## 6. Methodological Advantages and Disadvantages

Generally, the great advantage of flow cytometry over other techniques is the rapidity in which large numbers of cells can be analyzed. However, the flow cytometer is also well suited to analyzing cell death, and techniques have capitalized on the biological characteristics of apoptosis and necrosis to identify and quantify cell death and to distinguish between the two forms. This has been enhanced by the advent of new fluorochromes that enable the measurement of more than one parameter simultaneously for the identification of cell processes. Examples would include (1) Hoechst staining with annexin V to determine which phase of the cell cycle is producing apoptotic cells and (2) coupling apoptotic markers with antigen expression to determine which cell types are apoptotic in mixed populations, and (3) linking mitochondrial studies of apoptosis to phenotypic marker expression. Also, multiparameter analysis of apoptotic characteristics (e.g., TMRE and annexin V) may help to elucidate the apoptotic pathway in question.

The principal disadvantage of flow cytometry methods for cell death is over interpretation of the data. What the flow cytometer tells us is the amount of apoptosis that is detectable at the time of sampling. It tells us nothing about the rate of apoptosis nor anything about the total amount of cell death that may be present in the cell population as a whole. It does not give us a cumulative overview of the

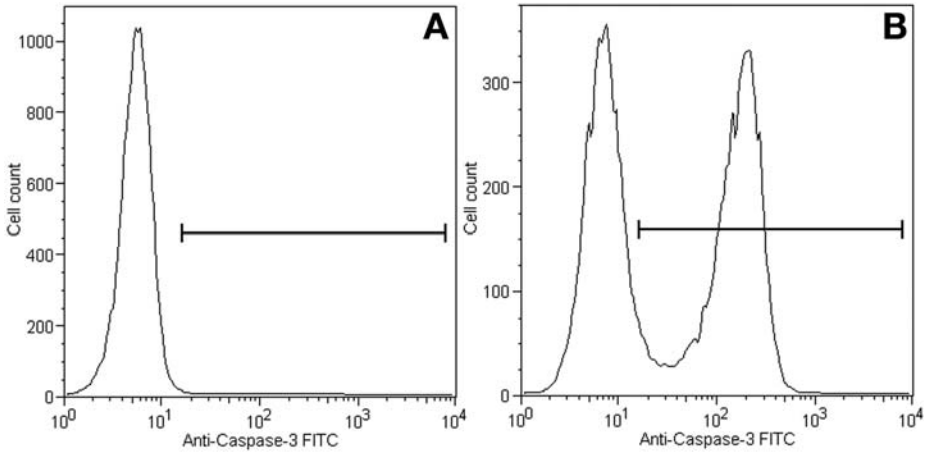


**Fig. 5.** Detection of activated caspase 3 in staurosporine-treated Jurkat cells. **(A)** Untreated control cells with a marker indicating positivity. **(B)** Treated cells that show both negative and positive (activated caspase 3) populations.

amount of death being induced or being observed. For example, 10% cell death every 4 h is more cell death than 20% every 24 h, yet the result from the cytometer is 20% each time. These problems may be resolved using technology other than flow cytometry (e.g., time-lapse video). Apoptosis in an individual cell is a rapid event, so as in all live cell assays, it is important to sample at different time points to monitor the progression of apoptosis on a population basis.

## 7. Problems Associated with DNA Content Assays

Reproducibility can be a problem in DNA content assays, and each step must be standardized to reduce this problem to a minimum. Variables include cell type, time of treatment with ethanol to permeabilize cells, and the washing (or elution) procedure. The observation that washing cells in 0.2 *M* phospho-citrate buffer at pH 7.8 greatly enhances extraction of fragmented DNA illustrates the latter point. It is possible to overestimate the amount of apoptosis when using loss of DNA as a parameter. The apoptotic population is often called a sub $G_0/G_1$ population because it is found to the left of the $G_0/G_1$ peak in a DNA profile of the viable cells. However, mechanically damaged cells would fall here as would normal hypodiploid cells. Furthermore, this region would also include poorly stained material if the primary gate does not exclude cell debris from the analysis. It is also possible to underestimate the amount of apoptosis with this method, particularly if the apoptotic cells are being generated from cells in the second gap phase of the cell cycle ($G_2$) or mitosis, because loss of DNA from these cells would give a DNA content that would put cells in either the S-phase or the $G_0/G_1$

phase of the cell cycle on the flow cytometer, rather than giving a sub $G_0/G_1$ population. Also, because the cells have been permeabilized to extract the DNA, there is no discrimination between apoptosis and necrosis, because both forms of cell death will take up PI when permeabilized. In all cases, a logical and consistent gating strategy to assess the level of apoptosis is important.

## 8. Problems Associated with the TUNEL and PS Techniques

The TUNEL technique will detect nicks in DNA associated with DNA cleavage into the 300-kbp and 55-kbp fragments as well as in any subsequent DNA fragmentation down to the 182-bp multimers. However, not all DNA strand breaks are associated with apoptosis. Nicks can be seen in necrotic cells and can also be induced mechanically during sample preparation. The specificity of PS detection for apoptosis is reliant on apoptotic cells retaining plasma membrane integrity. During necrosis, the cell membrane is ruptured, allowing annexin V to enter the cell and bind to PS on the inner leaflet of the plasma cell membrane. Hence, necrotic cells appear positive for PS. This problem can be overcome by counterstaining cells with PI, which is excluded from apoptotic cells. Therefore, necrotic cells are positive for PI and annexin V; apoptotic cells are annexin V-positive but exclude PI. However, normal B cells can also be positive for annexin V *(8)*.

## 9. Problems Associated with the Detection of $\Delta\psi m$

The pitfalls of using cationic dyes to detect loss of $\Delta\psi m$ in mitochondria include the potential to misinterpret data due to artifacts. Examples of artifact are oxidation of fluorochromes, autoquenching of dyes at high intramitochondrial concentrations, and the fact that dye uptake may be a reflection of changes in mitochondrial size rather than transmembrane potential. Also, cells stained with $DiOC_6$ or JC-1 must be analyzed immediately after staining if the cells are actively metabolizing, because fixation is not possible with these dyes. In contrast, it is possible to fix cells stained with CMXRos with paraformaldehyde, which permits storage of cells or subsequent staining of cells for mitochondrial or nuclear proteins.

## 10. Protocols

Note: All these methods give an assessment of the amount of apoptosis, usually as a percentage of the gated population at the time of sampling and not of the cumulative apoptosis over a period of time. The dynamics of apoptosis may be assessed by multiple sampling over time, after induction of apoptosis.

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# 7

# DNA Analysis by Flow Cytometry

**Derek Davies and Paul Allen**

**Summary**

Flow cytometry is well suited to DNA analysis because dyes are available that bind DNA in a proportional and linear fashion. This allows the quantitation of DNA content, enabling the identification of normal diploid cells at rest, those that are actively synthesizing DNA, and those that are either premitotic or actually in mitosis. Once these phases of the cell cycle have been identified, antibody detection of cell cycle-related proteins can be combined with DNA content determination to relate protein expression with stages of the cell cycle. Addition of the thymidine analog, bromodeoxyuridine, during cell growth will also allow the identification of cells that are actively synthesizing DNA. Chromosome numbers, whether aberrant or not, can also be determined using DNA content analyses. Thus, both ploidy (e.g., in megakaryocytes) or aneuploidy (e.g., in neoplastic disease) can be assessed. Protocols, with their advantages and limitations, are described here by.

**Key Words:** Aneuploidy; cell cycle; checkpoints; mitosis; ploidy.

## 1. Introduction

Mammalian cells contain two copies of every autosomal chromosome and two sex chromosomes (XX in females, XY in males). A single copy of every autosome plus one sex chromosome is known as a haploid state for a cell, and the haploid number is n. A complete set of homologous pairs of chromosomes is known as diploid, and the diploid number is 2n. Multiples of entire homologous chromosomal pairs are known as ploidy and are enumerated as 4n, 8n, 16n, 32n, etc. An abnormal number of individual chromosomal pairs is known as aneuploidy and can be a consequence of, for example, only one chromosome for a given pair (monosomy), three copies of a chromosome for a given pair (trisomy), or polysomal copies of particular chromosomes (e.g., chromosomes 1 and 7 in breast cancer) ***(1)***. Thus, by measuring the DNA content of a cell, it is possible to discern whether the cell is a sex cell (i.e., haploid chromosomal

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content); a somatic cell, with a diploid chromosomal content; a cell that has undergone endoreplication and is exhibiting ploidy (DNA replication without cell division); or a cell in which mitosis has been aberrant, resulting in abnormal chromosome numbers, a situation seen in various human syndromes (e.g., Down's syndrome [trisomy 21] and in the neoplastic cells seen in cancer) ***(2)***. Karyotypic abnormalities of two or fewer chromosomes would be difficult, if not impossible, to determine by flow cytometry because cytometers cannot resolve two clonal populations that differ in DNA content by only 4% or less. However, more dramatic karyotypic differences are readily detectable ***(3)***. In addition, if the DNA content of a proliferating cell population is measured, it is possible to track a diploid cell population (2n) precisely duplicating its DNA to attain two copies of every chromosome (4n) through to cell division at mitosis.

## 2. The Cell Cycle

The faithful replication of chromosomes and their equal redistribution to two daughter cells is the *raison d'etre* of cell proliferation and requires strict control of the cell cycle process. Quiescent diploid cells are pushed into the cell cycle by the presence of a mitogenic stimulus (or stimuli) and enter the first gap phase ($G_1$) of the cell cycle. The presence of mitogens induces expression of one or more of the D-type cyclins, which in turn bind to either cyclin-dependent kinase (Cdk) 4 or 6. This complex is kinase-active and as a consequence cells proceed through $G_1$. If mitogenic stimulation persists, cells pass through the restriction point and will continue through the entire cell cycle even if mitogens are withdrawn after the restriction point. If mitogens are withdrawn prior to the restriction point, cyclin D proteins are rapidly degraded and cells return to quiescence ($G_0$). After the restriction point, cyclin D expression is maintained irrespective of mitogenic presence and the cyclin D/Cdk4 or 6 complex promotes cell cycle progression by phosphorylation of proteins required for cell cycle continuity. One such substrate is the retinoblastoma protein, pRb, which when active, is in a hypophosphorylated state. It binds and sequesters the E2F/DP1 heterodimeric transcription factor, which is required for transcription of genes involved in subsequent cell cycle progression. Thus, pRb suppresses the cell cycle and hence is known as a tumor suppressor gene. However, its phosphorylation by cyclin D/Cdk4 or 6 kinase complexes releases E2F/DP1, which is responsible for cyclin E transcription. The cell cycle is now driven by the cyclin E/Cdk2 complex, the kinase activity of which is maximal at the transition from $G_1$ into the DNA synthesis stage of the cell cycle (S-phase). There is additional pRb phosphorylation and continued E2F/DP-1-mediated gene transcription as the cell enters S-phase. The synthesis of cyclin A, which also binds Cdk2 at the protein level, promotes the progression of the cell through S-phase. Ubiquitination and proteosomal degradation of cyclin E take place in early S-phase and are an integral part of S-phase progression.

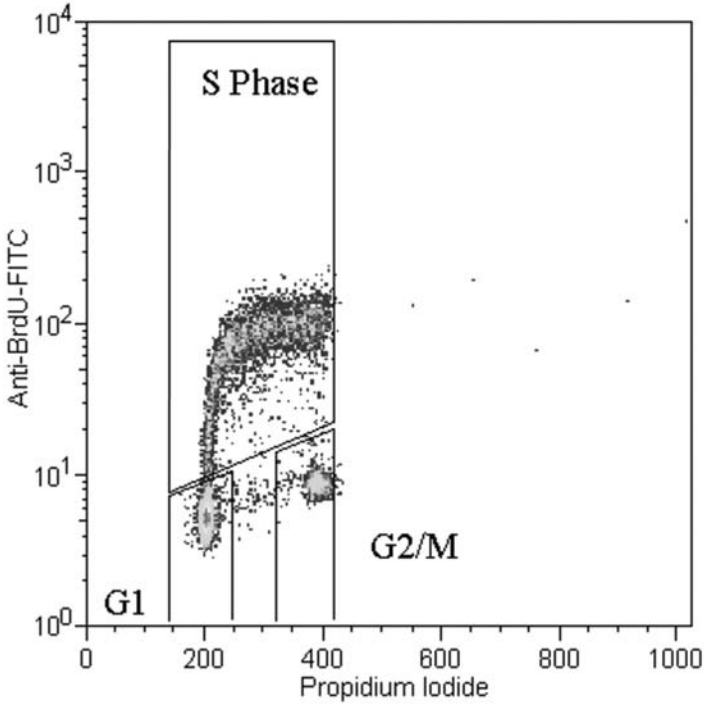



**Fig. 1.** S-phase identification. Dual-staining for DNA content using propidium iodide and antibody-mediated staining for BrdU, which is taken up only by cells synthesizing DNA, shows that cells in either $G_0/G_1$ or $G_2/M$ phases of the cell cycle are negative for BrdU whereas cells in S-phase are positive for BrdU incorporation.

Cyclin A/Cdk2 inactivates E2F by binding to its heterodimer binding partner DP-1 and phosphorylating it, which in part, but not totally, contributes to the inhibition of E2F/DP-1 DNA-binding properties.

As S-phase progresses, cyclin A switches binding partners from Cdk2 to Cdk1. Also, cyclin B accumulates through late S-phase into the second gap phase of the cell cycle ($G_2$). Cdk1 also uses cyclin B as its binding partner to drive cells from $G_2$ into mitosis. Mitotic cell division completes the cell cycle as each daughter cell now comes under the regulation of mitogens in $G_1$ of the next cell cycle. Timely destruction of cyclins A and B occurs in mitosis after their ubiquitination and proteosomal degradation ***(4,5)***.

### *2.1. Protocol for S-Phase Analysis in Proliferating Cells Using Bromodeoxyuridine Uptake (Fig. 1)*

1. Add bromodeoxyuridine (BrdU) to an exponentially growing population of cells to a final concentration of 10 μ*M* and incubate for 1 h at 37°C.

2. Harvest the cells and centrifuge at 400*g* for 5 min.
3. Resuspend the pellet in ice-cold 70% ethanol to a final concentration of $1 \times 10^6$/100 µL volume and incubate on ice for 45 min.
4. Wash $1 \times 10^6$ cells in PBS and resuspend the pellet in 1-mL volume of a denaturing buffer (2 *M* HCl plus 0.5% bovine serum albumin) and incubate for 20 min at room temperature.
5. Wash once in PBS and resuspend the pellet in a 1-mL volume of a neutralizing buffer of 0.1 *M* sodium borate, pH 8.5, for 2 min at room temperature.
6. Wash once in PBS and resuspend the pellet in 100 µL PBS.
7. Add 90 µL of cells to 10 µL of anti-BrdU mAb diluted to a predetermined optimal concentration and incubate for 1 h at room temperature.
8. Wash in PBS. If the anti-BrdU antibody was not directly conjugated, add 100 µL of a 1:10 or 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse $F(ab)_2$ fragments for 1 h at room temperature.
9. Wash once in PBS and resuspend in a 1-mL volume of 50 µg/mL PI and 200 µg/mL RNAse for 30 min at 37°C.
10. Do NOT wash prior to analysis.

## 3. Checkpoints

Cells have evolved cell cycle checkpoints that will stop cell cycle progression at key stages of the cell cycle so that repair mechanisms can become activated to remedy both errors of replication or induced damage that would otherwise compromise fidelity of chromosome duplication and segregation. The predominant checkpoints occur at the transition from $G_1$ to S phase, the transition from $G_2$ into mitosis, and in mitosis itself. Thus, the $G_1$ checkpoint prevents entry into S-phase and precludes DNA synthesis after an aberrant mitosis, the $G_2$ checkpoint prevents entry into mitosis if DNA synthesis is not complete or has been terminated prematurely, and the mitotic spindle checkpoint halts exit from mitosis if chromosome alignment on the mitotic spindle is not correct or if spindle formation has been compromised ***(6,7)***.

Cdk inhibitors (Cdkis) are important proteins in prompting cell cycle arrest. They bind to and inhibit the kinase activity of Cdk/cyclin complexes. One family of Cdki is the INK4 family. The most characterized family members are p15, p16, p18, and p19 (INK4b, INK4a, INK4c, and INK4d, respectively) and they bind to Cdk4 and Cdk6. Thus, cyclin D/Cdk4 and cyclin D/Cdk6 kinase activity is inhibited by this family. Hence, pRb usually remains in a hypophosphorylated (and therefore in an actively suppressive) mode in the presence of these Cdki given that pRb is a substrate for the cyclin D/kinase complexes. Cells do not therefore pass the restriction point and cell cycle arrest in $G_1$. A second family of Cdkis includes $p21^{waf1/sip1/sdi}$, $p27^{kip1}$, $p57^{kip2}$, and p107, which bind predominantly to Cdk2 and hence inhibit cyclin E/Cdk2 and cyclin A/Cdk2 kinase activity. Therefore, in this situation, cells are restrained

during late $G_1$ or at the $G_1$-to-S-phase transition. $p27^{kip1}$ expression is often high in quiescent cells and then decreases in a nontranscriptional manner on the application of mitogens, implying a role for $p27^{kip1}$ in the passage of cells through the restriction point ***(8)***.

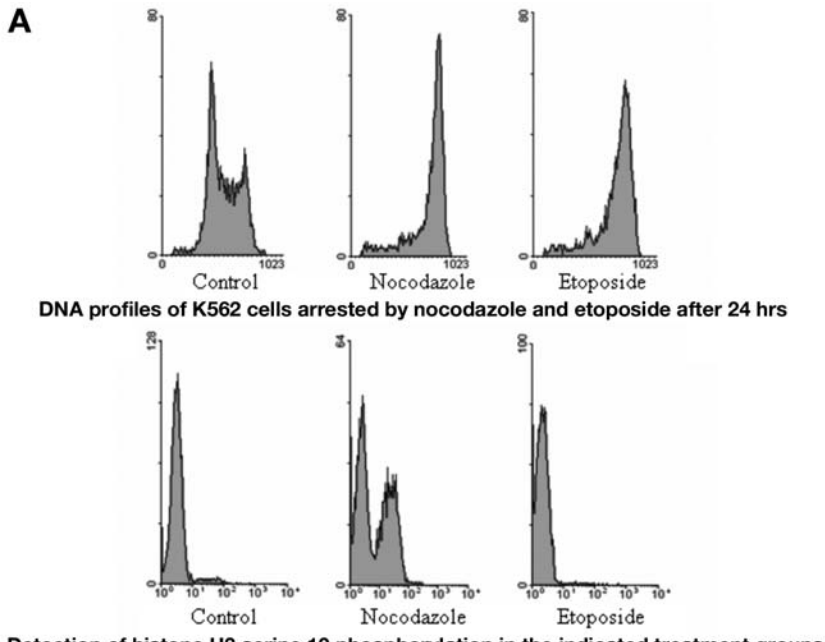
The p53 tumor suppressor gene is also a key player of events at $G_1$. It is a nuclear phosphoprotein that regulates the transcription of proapoptotic genes (e.g., *Bax* and *PUMA*) and cell cycle regulatory genes. An example of the latter is $p21^{waf1/sip1/sdi}$. p53 expression is stabilized upon the detection of DNA damage and can transactivate $p21^{waf1/sip1/sdi}$ expression, causing cell cycle arrest at $G_1$ as described above. Thus, mutations in p53 compromise induction of apoptosis and the $G_1$ cell cycle checkpoint ***(9)***.

The cell cycle can also be inhibited at the transition from $G_2$ to mitosis, often as a result of incomplete DNA synthesis or the induction of DNA damage during S-phase. Thus, cells can be prevented from entering mitosis after compromised DNA replication. Entry into mitosis requires an active form of the cyclin B/Cdk1 kinase complex and this activity must be nuclear. Once Cdk1 has bound cyclin B, kinase activity requires phosphorylation of threonine 161 (T161) on Cdk1 and dephosphorylation of threonine 14 (T14) and tyrosine 15 (Y15) in the ATP-binding site of Cdk1. The balance between phosphorylation and dephosphorylation of these latter two residues is maintained by the wee 1 kinase and the Cdc25 family of phosphatases. DNA damage can provoke sequestration of Cdc25, thus preventing it from dephosphorylating T14 and Y15 and hence retaining cyclin B/Cdk1 in the inactive form. Cell cycle arrest is the result. Other models show cytoplasmic sequestration of the cyclin B/Cdk1 complex, again resulting in arrest of the cell cycle at $G_2$ ***(4,10)***.

The mitotic checkpoint, or the spindle assembly checkpoint, stops the cell cycle in mitosis by preventing cells from advancing into anaphase and has evolved to prevent aneuploidy. Proper chromosomal segregation is achieved by organizing the sister chromatids on the bipolar mitotic spindle. The chromatids bind to the spindle via their kinetochores, and unattached kinetochores generate a stop signal that inhibits the anaphase-promoting complex (APC). The stop signal is created through recruitment of checkpoint proteins Bub1, Bub R1, Mad1, and Mad2 to the unattached kinetochores, which combine to inhibit one of the APC adaptor proteins, Cdc20. The cell cycle stops in metaphase, and cells will not enter anaphase while this checkpoint is intact ***(6)***.

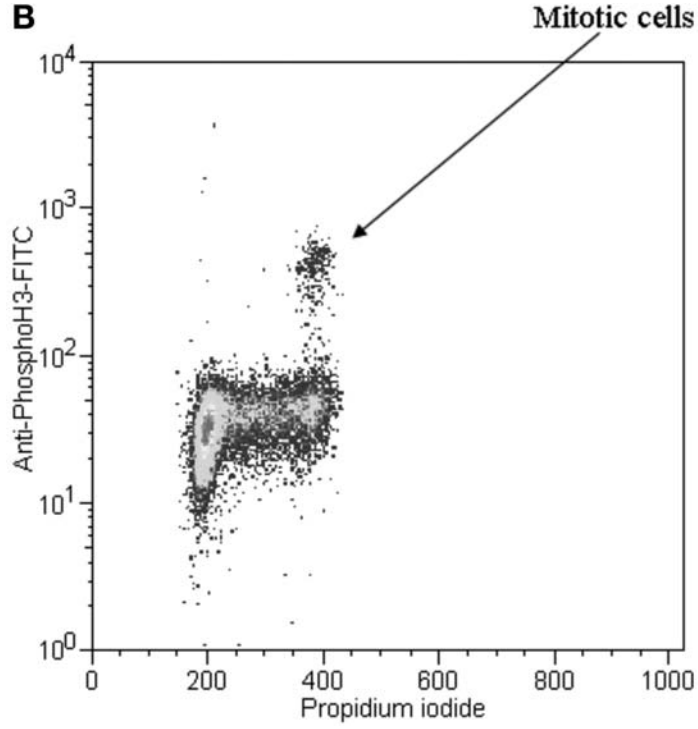
### 3.1. Detection of Cyclins and Other Nuclear Proteins (Fig. 2 and 3)

1. Harvest approx $1 \times 10^6$ and wash once in PBS.
2. Resuspend the cells in 0.5 mL of ice-cold 70% ethanol and incubate on ice for 45 min. Substitute ethanol for ice-cold pure methanol for cyclin D studies.
3. Wash once in PBS.




**DNA profiles of K562 cells arrested by nocodazole and etoposide after 24 hrs**

**Detection of histone H3 serine 10 phosphorylation in the indicated treatment groups**



4. Add a 1-mL volume of 0.9% Triton X-100 in 150 m*M* sodium chloride, 1 m*M* HEPES (pH 7.4), and 4% fetal calf serum. Incubate for 10 min at room temperature.
5. Wash once in PBS and resuspend the pellet in 100 µL of PBS.
6. Add 90 µL of the suspension to 10 µL of a 1:20 dilution of primary antibody (i.e., a final dilution of 1:200). Incubate for 2 h at room temperature or overnight at 4°C.
7. Wash once in PBS. If the primary antibody was not directly conjugated, add 100 µL of 1:10 or 1:20 dilution of FITC-conjugated goat anti-mouse $F(ab)_2$ fragments and incubate for 1 h at room temperature.
8. Wash once in PBS and analyze.
9. If simultaneous bivariate analysis of cell cycle distribution is required, counterstain with a 1-mL volume of 50 µg/mL PI and 200 µg/mL RNAse incubated for 30 min at 37°C. Do NOT wash the cells prior to analysis.

Notes: This protocol cites 70% ethanol as the permeabilization/fixation agent in conjunction with a TX-100 step. In certain circumstances, the TX-100 stage may not be required and therefore may be omitted from the protocol. Also, 1% paraformaldehyde for 2 h at 4°C represents an alternative for cyclin studies although it is not recommended if DNA staining for bivariate cyclin/ DNA content is to be performed, because paraformaldehyde impairs PI staining of DNA.

Cyclin D studies should be carried out using pure methanol rather than 70% ethanol although 1% paraformaldehyde can again be used if there is to be no simultaneous DNA staining.

Variation from the predicted cyclin distribution about the cell cycle may be observed under certain circumstances, particularly as a result of cell cycle pertubation (e.g., after drug treatment and in various tumor cell lines).

## 4. Numerical Chromosomal Aberrations

In the past, determining the absolute DNA content of cells has proven to be useful in the prognosis of hematological malignancy. A DNA index (DI) is established to assess abnormal DNA content. Cells are stained with propidium iodide (PI), and the DI is an expression of the ratio of DNA content of $G_0/G_1$ cells in the abnormal population to that of a normal population. It is possible to identify the abnormal peak by comparing its position with nucleated chicken

**Fig. 2.** Cell cycle analysis. **(A)** Cell cycle distribution of cells stained with the DNA dye propidium iodide **(top)**. **Left column** is of control cells and shows a normal cell cycle distribution, **center column** is of cells treated with the mitotic inhibitor nocodazole and shows cells accumulating in mitosis, and **right column** is of cells treated with a DNA damaging agent and shows cells accumulating in $G_2$. From these DNA profiles, it is difficult to distinguish between mitotic arrest and $G_2$ arrest. The **bottom panel** of histograms shows the usefulness of staining for a mitotic protein (histone H3 serine 10 phosphorylation) in making the distinction possible. **(B)** Propidium iodide staining and staining for histone H3 serine 10 phosphorylation can be combined on a dot plot that shows positive antigen staining of mitotic cells.

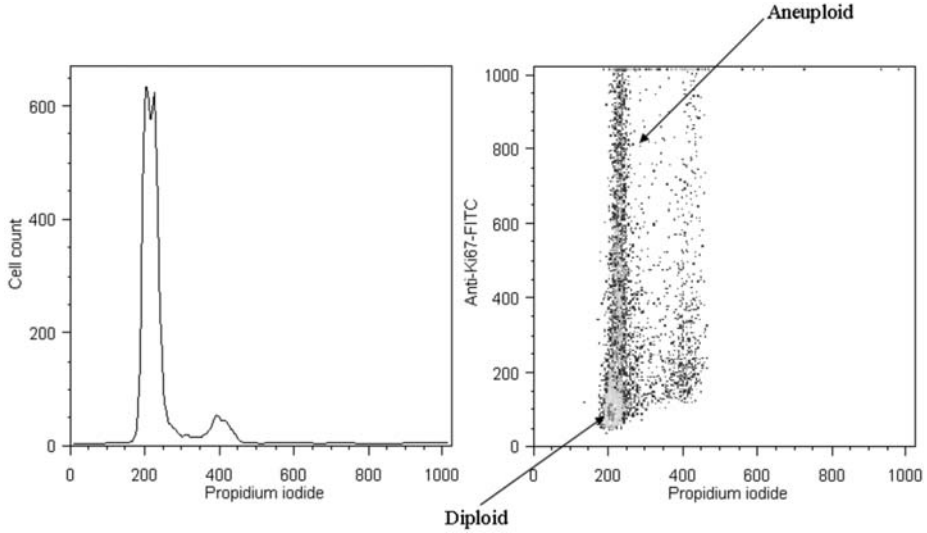



**Fig. 3.** Ki67 staining of proliferating cells in an aneuploid population. **Left panel** shows a DNA profile of an aneuploid colorectal polyp, which also contains normal cells. The DI confirms that the aneuploid population is close to being diploid. The **right panel** shows the same sample stained at the same time with an antibody against the proliferation antigen, Ki67. The normal diploid cells are negative for Ki67, but the aneuploid cells are Ki67-positive.

erythrocytes or normal lymphocytes. (In fresh blood samples [e.g., from leukemia patients], these can be mixed in with the test sample and analyzed together.) In a disease like acute lymphocytic leukemia, a residual normal population is often present to verify the position of the normal population. The DI for a normal diploid population is 1.00, hyperdiploid cells have a DI of more than 1.00, and a hypodiploid population has a DI of less than 1.00. Tetraploid cells have a DI of 2.00. (It is not within the scope of this chapter to outline a protocol for diagnostic purposes. The reader is referred to published standard operating procedures from recognized service centers where this is a requirement.)

The resolution of the flow cytometer is paramount in resolving abnormal peaks from normal peaks or in identifying more than one abnormal clone. This technique therefore requires optimization of the cytometer because large coefficients of variance (CVs) would mask the identification of individual populations should they exist. Merkel et al. ***(3)*** quote CVs of less than 5.00 as being essential for accurate data interpretation. In addition, there are published guidelines that define acceptable standards of sample preparation for clinical samples ***(11)***. In hematological malignancies, the DI has been used to predict high- and low-risk groups. For example, in childhood acute

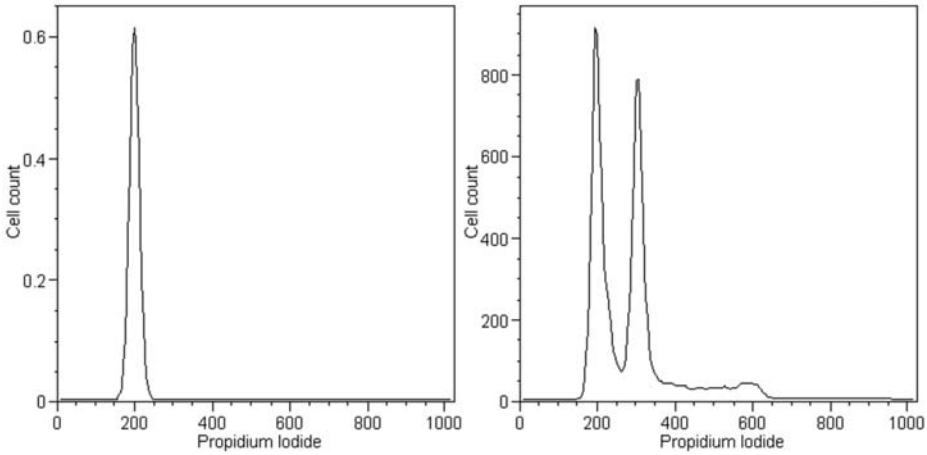



**Fig. 4.** DNA analysis showing aneuploidy. Nuclei were extracted from paraffin-embedded tissue and stained with propidium iodide. **Left panel** shows normal biopsy tissue, and the **right panel** shows biopsy from tumor tissue of the same patient, exhibiting a clear example of aneuploidy.

lymphocytic leukemia, hyperploidy as defined by a DI of more than 1.16 and a modal chromosome number of more than 50 gave a favorable prognosis, whereas a DI of less than 1.16 identified those patients with a less favorable outcome. Age and white cell count were also part of the assessment of these patients *(12)* (**Fig. 4**).

## 5. Dyes Used to Determine DNA Content

Commonly used dyes for DNA content assays are the phenanthridinium nucleic acid intercalators, PI and ethidium bromide (EtBr). Both excite on the 488-nm blue laser, but photobleaching of EtBr/DNA complexes means that PI is the practical option. Cell permeabilization is required because both of these dyes are impermeant for viable cells. These dyes intercalate with all nucleic acids so RNAse is incorporated in most protocols and the fluorescence detected reflects DNA content only. Both dyes are routinely used with antibody conjugates in multiparameter analyses.

Alternative dyes for DNA content determination include Hoechst 33258, Hoechst 33342, TO-PRO-3 iodide, DRAQ5, and 7-aminoactinomycin D. The Hoechst dyes bind to the minor groove of DNA, are cell-permeant (the latter has a slightly higher permeability than the former), can be used on nonfixed cells, and are excited by the UV end of an argon (488-nm) laser. The Stokes shift is large enough for use in multiparameter analyses with other fluorochromes. These dyes can displace nucleic acid intercalators because of their

**Table 1**
**Examples of Nucleic Acid Binding Dyes**

| Dye | Excitation maximum (nm) | Excitation minimum (nm) | Binding mode |
|---|---|---|---|
| Acridine orange (DNA) | 500 | 526 | Intercalation |
| Acridine orange (RNA) | 460 | 650 | Electrostatic attraction |
| 7-Aminoactinomycin D | 545 | 650 | G-C pairs |
| Chromomycin A3 | 445 | 475 | G-C pairs |
| DAPI (4′,6-Diamidino-2-phenylindole) | 359 | 461 | A-T pairs |
| DRAQ5 | 630 | 660 | Intercalation |
| Ethidium bromide | 520 | 600 | Intercalation |
| Hoechst 33258 | 352 | 455 | A-T pairs |
| Hoechst 33342 | 352 | 455 | A-T pairs |
| Propidium iodide | 538 | 617 | Intercalation |
| Sytox green | 503 | 524 | Intercalation |
| TO-PRO-3 iodide | 644 | 657 | Intercalation |
| YO-PRO-1 | 491 | 509 | Intercalation |

high affinity for poly d(A-T) sequences. The cyanine dyes have also turned out to be useful in DNA-based analysis and include the SYTO dyes. They are cell-permeant (and thus stain viable cells), exhibit large fluorescent enhancement on DNA binding, and have a low intrinsic fluorescence (**Table 1** and **Fig. 5**).

### *5.1. Protocol for Cell Cycle Distribution by DNA Content (Fig. 2A)*

1. Harvest approx $2 \times 10^5$ cells and wash once in phosphate-buffered saline (PBS).
2. Resuspend the cells in 0.5 mL of ice-cold 70% ethanol and incubate on ice for more than 2 h.
3. Wash cells three times in PBS and resuspend in a 1-mL mix of 50 µg/mL PI and 200 µg/mL RNAse in PBS for 30 min at 37°C.
4. Do NOT wash cells prior to analysis on the flow cytometer. PI is an intercalating agent and will leak out of the cell if the population is resuspended in buffer not containing PI.

**Fig. 5.** Cell cycle analysis. **(A)** Cell cycle distribution of cells in a proliferating population of cells stained with the DNA dye Hoechst 33342. The interval gates a, b, and c label the $G_0/G_1$, the S-phase, and the $G_2$/M populations, respectively. **(B)** This is an example of cells transfected with a GFP-tagged protein. Cells have been separated into GFP-positive and -negative populations, and the cell cycle has been determined for each population by staining with Hoechst 33342.

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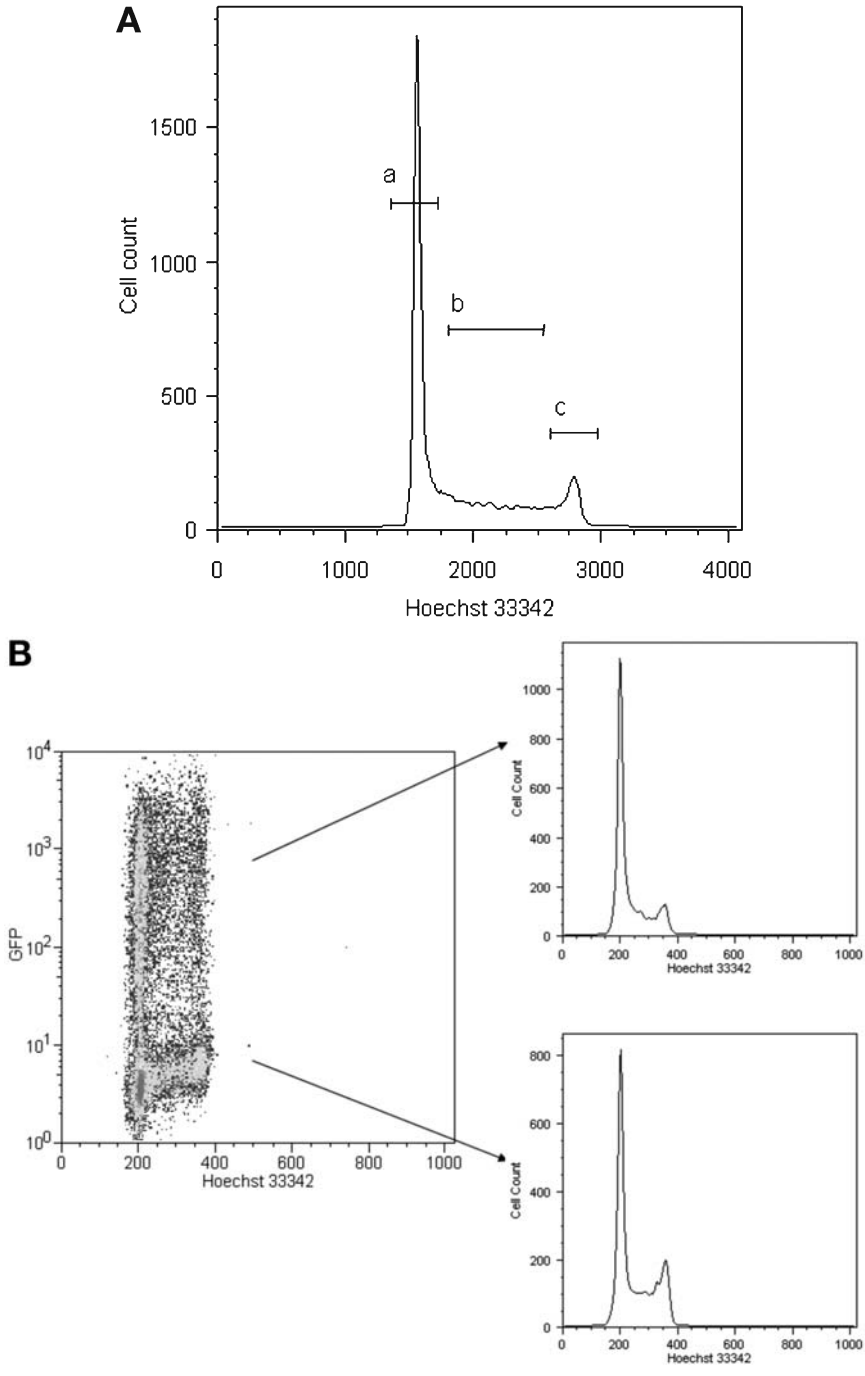
A
Cell count
1500
1000
500
0
a
b
c
0
1000
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Hoechst 33342
B
GFP
$10^4$
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$10^1$
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Hoechst 33342

**Fig. 5.**

Notes: DNA content alone will not distinguish between $G_0$ and $G_1$ nor between $G_2$ and premitotic cells.

### *5.2. DNA Staining of Live Cells Using Hoechst 33342 (Fig. 5A)*

1. Harvest $1 \times 10^6$ cells and wash once in PBS.
2. Resuspend cells in 1 mL of medium 10 µg/mL Hoechst 33342. (Stock is 1 mg/mL in distilled water.) The exact concentration of Hoechst that will optimally stain DNA will need to be determined for each cell type but is generally in the range of 5–20 µg/mL.
3. Incubate at 37°C for 30–120 min; again, the precise time will need to be determined for each cell type.
4. Add 50 µL of PI (50 µg/mL) and keep on ice until analysis.

Hoechst is excited by a range of wavelengths from UV (approx 325 nm) to violet (407 nm). Fluorescence emission is measured with a 440/40 band-pass filter. Dead cells are excluded on the basis of their PI fluorescence, and emission can be collected above 670 nm after UV, violet, or 488-nm excitation.

## 6. Pulse Processing (Figs. 6 and 7)

Pulse processing refers to the ability of flow cytometers to analyze the pulse generated by a cell passing the laser so that three parameters relating to the pulse can be derived from the same signal. This is especially important in DNA analysis because two out of the three parameters when examined at any one time can be used to eliminate cellular aggregation and even to distinguish doublets from single cells.

Every cell that passes the laser is seen as an event by the cytometer, but clearly the DNA content of a clump of cells is different from that of a single cell and this difference must be discriminated against (e.g., a single diploid cell has a 2n DNA content and an aggregate or a doublet consisting of 2 diploid cells will be seen as a single event, but clearly the DNA content is $2 \times 2n$ [i.e., 4n, which is exactly the same as a cycling cell prior to mitosis]). Thus, if we were conducting cell cycle studies, an aggregate of two cells in $G_0/G_1$ would be confused as a single cell in either $G_2$ or mitosis.

The pulse processor allows measurement of the height of a pulse, which is related to the intensity of fluorescence; the width of the pulse, which is related to the time that the cell is passing through the laser (and therefore the size of the event); and the area of the pulse, which is related to the total fluorescence generated by the cell passing the laser (**Fig. 6**). Doublet discrimination can be achieved in two ways. One way is to create a dot plot of pulse area against pulse width, which for a population of singlet cells increase simultaneously as DNA synthesis occurs through S-phase. However, a doublet of cells in $G_1$ will have the same pulse area as a single cell in $G_2$/M but the pulse width will be much greater, allowing a distinction to be made on the dot plot. An alternative is to

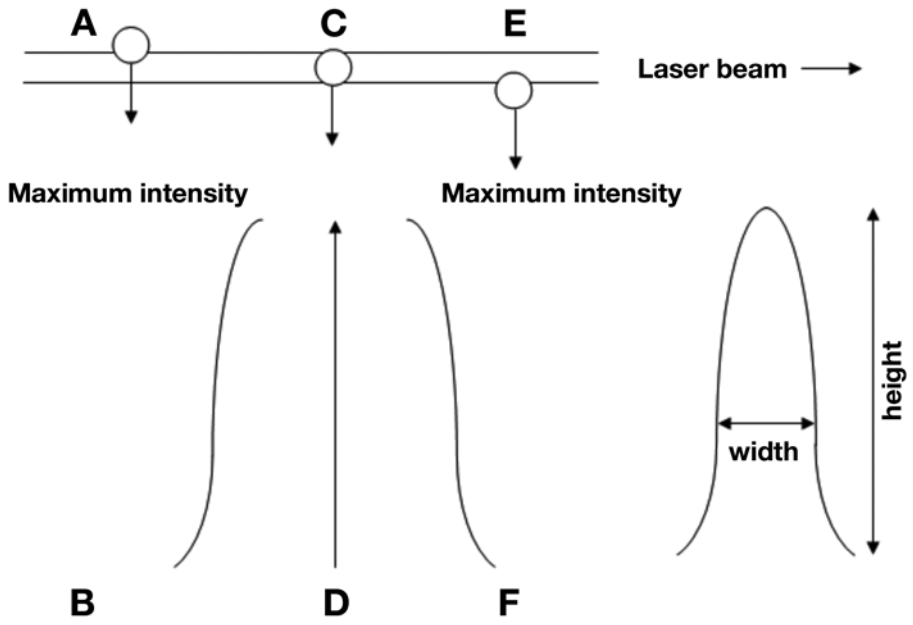


**Fig. 6.** Pulse processing. As a cell enters the laser beam **(A)**, a signal is generated **(B)**. This increases as the cell penetrates further and further into the beam. When the cell is in the center of the beam **(C)**, the signal reaches maximum intensity **(D)**. As the cell leaves the beam **(E)**, the signal falls back to zero **(F)**. This enables the measurement of signal intensity (height), signal width (which is related to the size of the cell[s] passing through the laser), and the area under the curve (which provides a measure of the total signal derived from the cell passing through the beam).

plot pulse area against pulse height on a dot plot. In singlet cells, an increase in pulse intensity is proportional to the size of the cell (pulse area). This is not true of doublet or aggregates of more than two cells. The gains are adjusted so that a relationship of 1:1 is attained for pulse height against pulse area. Singlet cells from a proliferating population of cells will form a 45° line of cells with $G_0/G_1$ cells toward the bottom left of the screen and $G_2$/M cells in the top right. Cells in S-phase bridge the two populations. Cells outside this relationship of 1:1 will be doublets or aggregates of cells. (*See* **Fig. 7**)

## 7. Fixation and Permeabilization of Cells for Analysis

For the majority of DNA-binding dyes to be able to quantitatively stain the DNA of cells, they must first be made permeable. This may be achieved either by detergent treatment (e.g., 0.1% Triton X-100) or by fixation. The advantage of fixation is that cells may be kept at 4°C for several days or weeks prior to

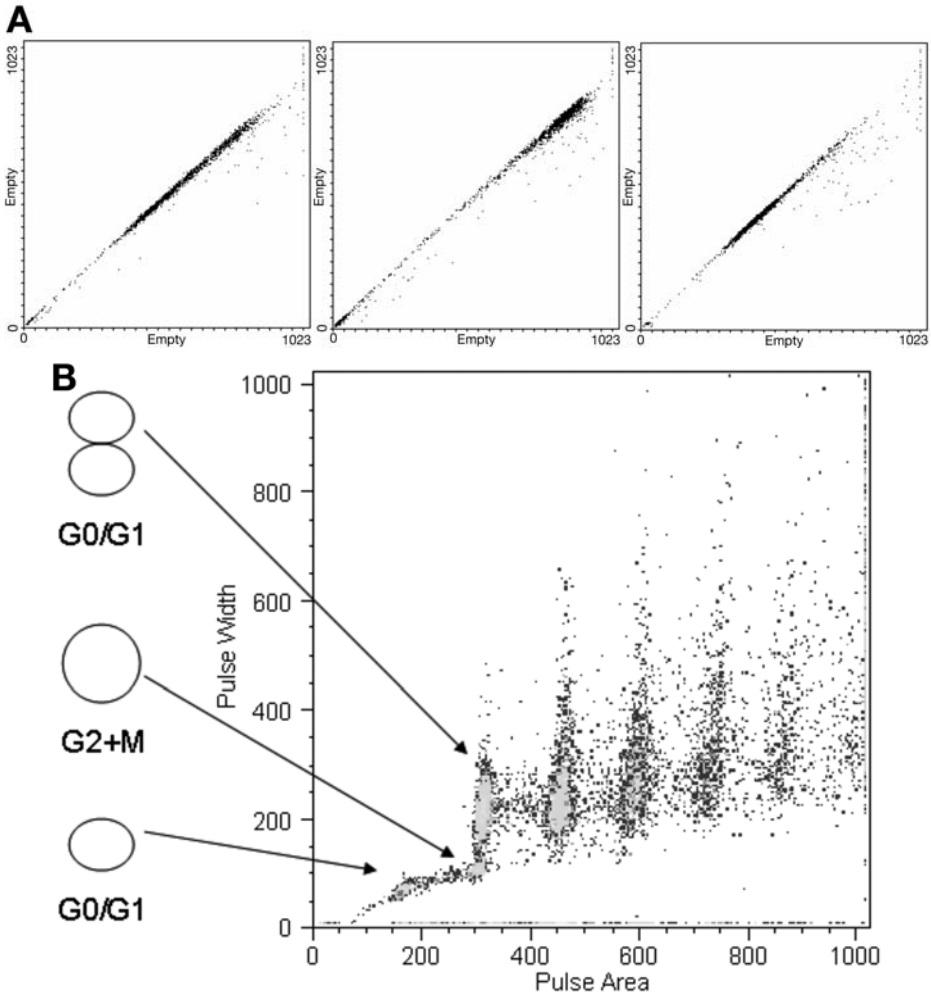


**Fig. 7.** Doublet discrimination. **(A)** Signal area and signal height are directly proportional to each other on flow cytometers for single cells and hence when stained with PI, generate a population that forms a 45° line across a dot plot **(left plot)**. $G_2$/M cells are at the top right **(center plot)**, and $G_0/G_1$ cells are toward the bottom left of the dot plot **(right plot)**. Cells that fall outside of this relationship are cell aggregates. **(B)** An alternative is to plot signal area against signal width for a given fluorochrome. This visualizes cell cycle distribution of doublet populations and larger aggregates (as shown).

analysis. There are two principal types of fixatives used: alcohols (generally ethanol or methanol) or aldehydes (generally paraformaldehyde). Alcohol, usually 70% ethanol, is preferred because this is a dehydrating fixative that denatures nuclear proteins and allows better access of the DNA-binding dye to the DNA.

Paraformaldehyde is a crosslinking fixative and, as this locks proteins in position, can lead to suboptimal DNA CVs. However, in some situations (e.g., in which GFP fluorescence needs to be preserved), 1% paraformaldehyde may be used.

## 8. Protocols

The methods described herein represent basic starter protocols for the analysis of the cell cycle and some of the components that drive it. Each method should be adapted to requirements. Flow cytometric analysis requires fixation and permeabilization of the cell. The fixation process should not destroy antigen epitopes, and permeabilization should be sufficient to enable antibody penetration, particularly when staining for nuclear antigens such as the cyclins. Optimal conditions and antibody concentrations should be assessed for each experimental situation and, likewise, for the permeabilization/fixation procedures.

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# 8

# Immunological Studies of Human Cells

**Ulrika Johansson**

**Summary**

With an ever increasing availability of antibodies, tetramers, and dyes for the analysis of cell function, flow cytometry remains an important tool for the study of cells of the immune system. Multicolor flow cytometry offers the advantage of swift analysis of distinct cell types in whole-blood or mixed cell cultures. The cells of interest can be identified using a lineage-specific antibody and their functional properties analyzed using, for example, cytokine-specific antibodies or cell viability dyes. Combining the analysis of cell function with the use of a cell sorter allows for further studies and culture of phenotypically and functionally defined cell populations.

**Key Words:** Cell proliferation; dump channel; intracellular cytokines; protein transport; rare event analysis.

## 1. The Identification of Cells in Human Blood

Cells are usually defined according to their expression of cell surface antigen (e.g., T cells are $CD3^+$ and these may further be divided into $CD4^+$ T cells and $CD8^+$ T cells). Most of the major cell lineages in human blood express certain surface antigens exclusively. For example, CD19 is found only on B cells, and CD56 is expressed solely by natural killer (NK) cells. The monocyte and myeloid dendritic cell (DC) compartment, on the other hand, is less easily defined. Indeed, for monocytes, the best characteristic that will label the entire monocyte population in peripheral blood is probably still considered to be nonspecific esterase staining ***(1)***. For practical purposes, the CD14 antigen is used as a "marker" for monocytes but is in fact expressed only weakly on some monocytes. Most of these $CD14^{dim}$ monocytes express the Fc gamma receptor III, CD16. If $CD16^+$ monocytes were investigated, it would be necessary to include an antibody for CD56 because NK cells also express CD16. Granulocytes, too, are $CD16^+$ but are easily distinguished from monocytes in a forward scatter

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(FSC)/side scatter (SSC) plot. Alternatively, CD4 is expressed on all human monocytes and can be used because the cells are quite easily distinguished from $CD4^+$ T cells in a FSC/SSC dot plot, and CD3, for example, may be used to ensure that all $CD4^+$ T cells be excluded. However, the CD4 molecule is also expressed on the majority of DC subsets and so it would be difficult to distinguish the DCs from the monocytes without using a number of additional antibodies.

Certain cell subsets are identified using functional analysis alongside phenotyping. The T-regulatory cells, for example, may be defined by their production of interleukin (IL)-10 and/or transforming growth factor-β, alongside the expression of certain surface antigens, including CD25 or IL-2 receptor ***(2,3)***. Another example of functional identification is the use of antigen–antigen-presenting molecule complexes. These will bind to the corresponding specific T-cell receptor (TCR). Thus, peptide-major histocompatibility complex (MHC) tetramers (or pentamers) may be used to specifically label antigen-specific T cells ***(4)***, and glycolipid antigen-CD1d tetramers are now also available for the identification of NK T cells (NKT cells) ***(5,6)***.

**Table 1** shows the main cells found in human peripheral blood and how they generally are characterized using flow cytometry. For an extensive list of the expression pattern of cell surface antigens, there are numerous useful websites that may be consulted. Finally, it is important to be aware of the possible up- and downmodulation of surface antigens if phenotyping for the identification of cells. In the case of the T-regulatory cells, for example, the use of CD25 together with CD4 or CD8 during an ongoing immune response is very limited because all activated T cells transiently upregulate CD25.

## 2. Rare Event Analysis

The immune system contains several cell subsets that, although low in number, carry out functions that are of research interest. For instance, DCs as a group constitute no more than 2–3% of mononuclear cells in whole blood and the different DC subsets are present at even lower frequency. The plasmacytoid DCs, for example, may be detected in the region of 10,000 cells per milliliter whole blood and thus constitute less than 1% of peripheral blood leukocytes ***(7)***. Other examples of rare immune cells that are the focus for studies today include NKT cells, stem cells, and endothelial progenitor cells. As an example, take the acquisition of 10,000 mononuclear events. If $CD14^+$ events are measured, we may expect to find 1000 positive events (assuming a 10% monocyte population). If NKT cells are studied, however, the number of positive events may be less than 10, if the sample is at the lower range of NKT cell prevalence (0.01–1% of total lymphocytes). This low number of positive events increases the probability of variation in the sample and thus reduces the statistical significance of the result. It is also easy to see that if the sample has a lot of

**Table 1**
**Quick Guide for the Identification of the Main Cell Lineages in Human Peripheral Blood**

| Lineage | Positive | Major subsets | | | Negative |
|---|---|---|---|---|---|
| T cells | CD3 | αβ TCR | γδ TCR | | |
| T helper cells | CD4 | | | | |
| T cytotoxic cells | CD8 | | | | |
| T regulatory cells | CD25, FoxP3, IL-10, TGF-b, GITR[a] | CD4 | CD8 | | |
| Natural killer T cells | CD3, αGalCer-CD1d tetramer[b] | CD4 | CD8 | $CD4^-$ $CD8^-$ | |
| Natural killer cells | CD56, various KIR[c] | CD16 | CD57 | | |
| B cells | CD19, CD79, CD24 | CD27 | | | |
| B-la B cells | CD5 | | | | |
| Plasma B cells | CD138 | | | | CD19 |
| Monocytes | CD14, HLA-DR, CD163[d] | CD16 | CD64 | | |
| Plasmacytoid DC | HLA-DR, CD303, $CD123^{high}$ | | | | CD11c CD3, 14, 19, 56 |
| Myeloid DC | HLA-DR | CD1c | CD16 | BDCA-3 | CD3, 14, 19, 56 |
| Myeloid stem cells | CD34, CD117 | | | | |
| Neutrophils | CD15, $CD14^{low}$ | | | | CD3, 19, 56 |
| Eosinophils | CD49d $Autofluorescence^{high}$ | | | | CD16 CD3, 14, 19, 56 |
| Basophils | IgE, $CD45^{low}$, CRTH2[e] | | | | CD3, 14, 19, 56 |

This table is by no means complete but is provided as a guide for the beginner.

[a]Not all of these "markers" are always present ***(37,38)*** (GITR, glucocorticoid-induced TNF receptor-related protein).

[b]alpha-galactosylceramide or other NKT cell specific glycolipid antigen bound to a CD1d tetramer ***(5)***.

[c]Killer cell inhibitory receptors ***(39)***.

[d]CD163 is shedded upon activation ***(40,41)***.

[e]CRTH2: chemoattractant receptor-homologous molecule expressed on Th2 cells ***(42,43)***. Thus, T cells need to be excluded by for example labeling for CD3.

debris or dead cells that cause unspecific binding and false-positive events, the reliability of the result is further reduced. The questions that need to be addressed are, firstly, how many positive events should be acquired and, secondly, how false-positive events can be avoided.

### 2.1. The Statistics of Rare Events: How Many Cells to Acquire?

The counting of populations that are defined as true (positive events) as opposed to false (other events) is governed by the statistics of the binomial distribution. For limiting cases, meaning when only a very small fraction of events is expected to be positive, Poisson distribution is applied. These statistics describe the variance meaning, the variation expected of the number of positive events that would be observed if the same sample were repeated over and over again. From the variance, the standard deviation (SD) and coefficient of variation (CV) are calculated, the latter being the commonly used factor to describe the precision of flow cytometry data.

An in-depth description of these statistics is beyond the scope of this chapter; instead, the way of determining how many events should be acquired to obtain a certain desired reliability will be described in a very simplified but hopefully user-friendly way. For a detailed yet very clear introduction to the statistics relevant for flow cytometric analysis of rare events, the reader is referred to the chapter by Terry Hoy in *Flow Cytometry: Clinical Applications* ***(8)***.

In short, the following approximation of the SD and the CV can be used for cell subsets that constitute 5% or less of total events:

$$SD = \sqrt{r}$$

$$CV = 100/\sqrt{r}$$

Where r is the number of positive events.

The most accurate way of enumerating rare events is to decide on the CV that is acceptable, let's say 5%, and from this to calculate how many positive events should be acquired. This means that the reliability or the precision of the data for each sample is equal.

$$\text{From } CV = 100/\sqrt{r}, \text{ follows } r = [100/CV]^2.$$

$$\text{For } CV = 5\%, r = [100/5]^2 = 400.$$

Thus, 400 positive events should be acquired to obtain a CV of 5%. Clearly, the total number of events that has to be recorded to acquire the 400 positive events will vary between samples. If the rare cell subset in our example is present at approx 5% of total events, approx 8000 total events will have to be acquired: 400/0.05 = 8000. If it is a subpopulation of 0.5%, 80,000 total events need to be acquired, and so on.

In reality, this approach can prove difficult. The prevalence of most cells differs between individuals, and it may not always be possible to obtain a large enough volume of sample from an individual. An example of this is minimal residual disease measurement in bone marrow from treated leukemia patients. Overall cell numbers are often low after the administration of cytotoxic drugs and this

combined with a limited volume of sample available for analysis can make it difficult to achieve the CV desired. If, for example, leukemic cells are present at 0.01% in the marrow, 4 million events must be recorded to achieve a CV of 5% (400/0.0001). This should not pose a problem with regard to time of acquisition, but it is possible that there simply is not enough sample available to reach this number of events, and so data with a higher CV may be considered. It is not uncommon in clinical and research practice to accept CVs of 10–20%.

### *2.2. Other Considerations for Small Populations*

Compared with "normal" analysis, there is an increased importance to ensure that no false-positive events be present when analyzing rare events. In our study of NKT cells in patients with autoimmune thrombocytopenia, for example, less than 50 positive events could be counted in the samples we had available ***(9)***. Clearly then, nonspecific events can skew the data and so results that already have a CV of 20% would become less reliable. As the number of total events acquired increases, so may the number of unspecific events. Again, if a few false-positives were present in a population of 1000 positive events, this may not affect the results very much. But what if only 25 positive events were recorded using the specific antibody and 10 events "appeared" in the gate when the same high number of total events were acquired and those 10 events were either unlabeled or labeled with a well-chosen isotype control (that is, with the same protein/fluorochrome ratio and protein concentration and preferably purchased from the same company as the specific antibody). Should this be the case, the overall labeling procedure and the inclusion of blocking steps probably ought to be looked into. Perhaps a better or an additional marker for the small population would be available or a dump channel could be included.

### *2.3. Excluding Nonspecific Labeled Cells by Using a Dump Channel*

To optimize the analysis, the best possible labeling strategy should be selected. A one-antibody strategy for identification of a certain population may be improved upon by using a second antibody that positively identifies the cells and by using a so-called dump channel. An example of the latter is shown in **Figure 1**. In **Figure 1a**, whole blood was labeled with an anti-blood dendritic cell antigen-2 (BDCA-2 or CD303) monoclonal. This antigen is exclusively expressed by plasmacytoid DCs in human blood, but it is not entirely clear where a gate defining positive cells should be set. In the next plot (**Fig. 1B**), a dump channel was used: a cocktail of antibodies all conjugated to the same fluorochrome and all specific for antigens that are not expressed by plasmacytoid cells. An anti-HLA-DR monoclonal was also included, and it is quite easy to distinguish a DR-positive, phycoerythrin (PE)-negative population. This population could then be gated for BDCA-2-positive and -negative events or,

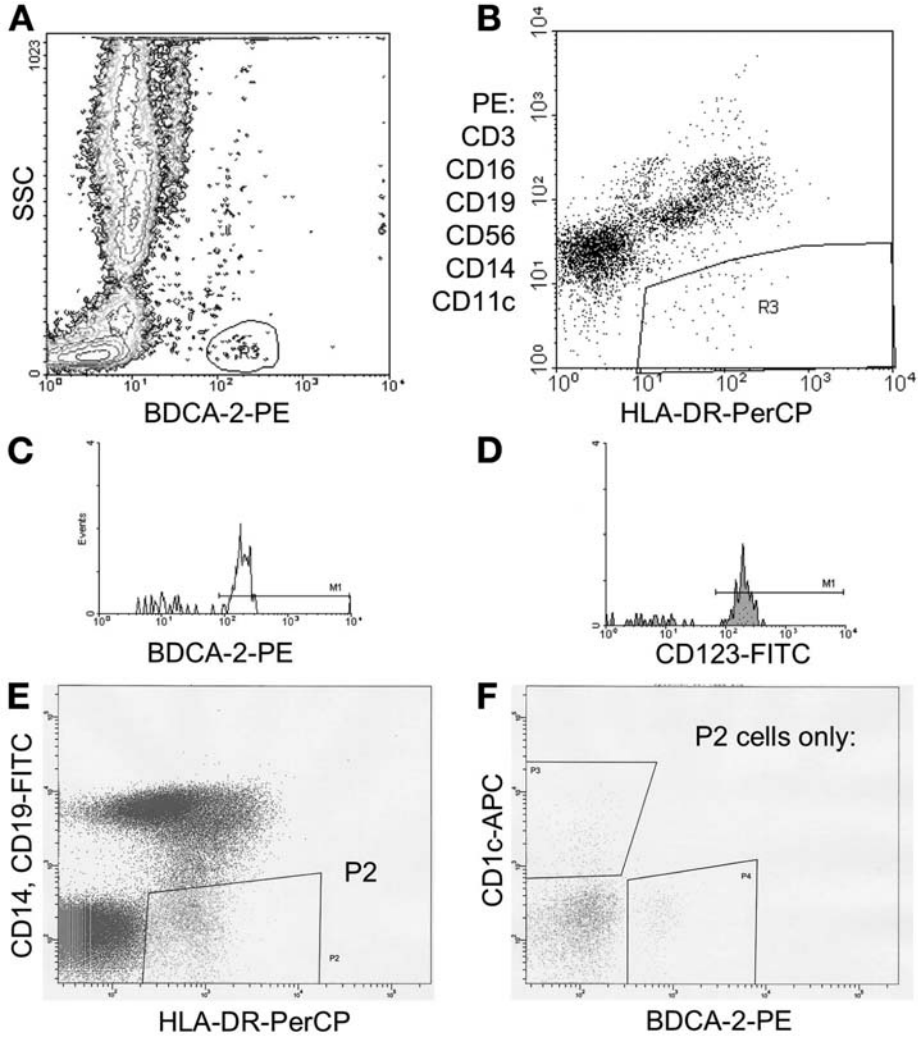


**Fig. 1.** Using a dump channel to identify plasmacytoid dendritic cells (DCs) in peripheral blood. **(A–D)** Whole blood was labeled to identify plasmacytoid DCs. A single antibody specific for these cells BDCA2$^+$ is compared with the use of a dump channel together with anti-CD123, which is expressed on plasmacytoid DLs. After labeling, erythrocytes were lysed and white cells were fixed before analysis on a FACScan. **(A)** This dot plot shows BDCA-2-labeled cells; the side light scatter versus BDCA-2-PE profile is shown. A gate was set around the cluster of BDCA-2-positive cells, but background and/or possibly positive cells made it difficult to define truly positive cells. The histogram in **(C)** shows that this antibody does give a good result; these cells were labeled also with HLA-DR-PerCP (peridinin chlorophyll protein) and were gated on DR$^+$ BDCA$^+$ cells. **(B)** and **(D)** show whole blood labeled with CD123-FITC,

as shown in **Figure 1C**, CD123-positive events, which thus defined the plasmacytoid DC population.

Unspecific labeling is most common for Fc receptor-positive cells, dead and dying cells, and debris. For this reason, CD14, CD16, and CD56 may be useful antigens to target unless, of course, they are expressed by the cells of interest. It may also be desirable to use a dye to exclude dead cells or erythrocytes.

### 3. Dead Cells, Red Cells, and Debris

Dead or dying cells often have an increased ability for nonspecific binding and so could cause some problems. It may therefore be useful to include a dead cell discriminator when analyzing samples that can have relatively large numbers of dead or dying cells present (e.g., cultured cells or tissue-derived cells). This is important to consider not only for rare event analysis but also for functional investigations such as endocytosis and phagocytosis assays. There are many commercially available viability dyes that may be used such as propidium iodide, 7-aminoactinomycin D (7-AAD), and DRAQ5; the use of these dyes is discussed in **Chapters 2** and **6**.

For whole-blood analysis, dead and dying cells are normally not present and so should not pose a problem. The erythrocytes, however, may need to be excluded. Although erythrocytes normally do not bind antibodies unspecifically, their presence makes it impossible to distinguish the mononuclear cell populations in a forward side scatter plot. This problem is often solved by lysing the erythrocytes before or after antibody labeling. Alternatively, CD45 can be included to identify leukocytes or a DNA-binding dye can be used to identify nucleated cells. For rare event analysis, the use of erythrocyte lysis may result in the presence of debris and some erythrocytes may survive the lysis and so interfere with the true event count. This can be corrected for by

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HLA-DR-PerCP, and a PE-conjugated lineage cocktail (CD3, CD11c, CD14, CD16, CD19, and CD56). A gate was set around mononuclear cells in an FSC/SSC dot plot, and this gate was used in **(B)**, which shows the PE dump channel HLA-DR. A gate was set for the PE-negative, HLA-DR-positive cells, and these cells were then plotted in a histogram **(D)**, in which the CD123-positive plasmacytoid DCs could easily be distinguished. **(E)** and **(F)** show an example of mononuclear cell preparations labeled with the BDCA-2-PE and CD1c-APC antibodies to look at plasmacytoid and myeloid DCs, respectively. The cells were also labeled with HLA-DR-PerCP, and the dump channel consisted of CD14 and CD19-FITC. The samples were analyzed on a FACS Aria. **(E)** The FITC dump channel is plotted versus HLA-DR. HLA-DR-positive and CD14- and CD19-negative cells were gated and displayed in the dot plot in **(F)**, in which the CD1c-positive myeloid DC and the BDCA-2-positive plasmacytoid DC populations are identified.

using DNA-binding dyes of which there are several available. Syto-13, for example, fluoresces green ***(10,11)***, whereas LDS-751 and DRAQ5 ***(12–14)*** emit in the far red region of the light spectrum.

### 3.1. Sample Carryover

Presence of debris and dead cells increases the possibility of cell clumping, which may stick to tubing and cause sample carryover or blocking. It is easy, and a good idea, to run a tube of water or buffer after a sample tube to monitor for carryover, and should carryover be present, a thorough clean out of the system might be in order.

## 4. Cell Proliferation

The use of fluorescent dyes that indicate cell cycling together with flow cytometry analysis provides a valuable tool for the study of cell proliferation. This method allows for identification of the cells that proliferate in a mixed cell culture and can give a good estimate of the number of antigen-specific precursors within a mixed population.

### 4.1. Dyes Available for the Analysis of Cell Proliferation

Currently, there are two dyes that are used for flow cytometry analysis of cell proliferation: BrdU (5-bromo-2′-deoxyuridine) and carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE; still commonly known as CFSE). BrdU incorporates into newly synthesized DNA and thus permits indirect detection of proliferating cells. Most protocols use fluorochrome-conjugated anti-BrdU mAbs to detect the BrdU. However, for the antibody to be able to access the BrdU, the technique requires heat or acid treatment of the cells, so that the cells' DNA denatures and exposes the incorporated BrdU. More recent protocols use UV light treatment. This induces apoptosis and DNA strand breaks, and so the anti-BrdU antibodies may gain access to the BrdU.

CFDA, SE, on the other hand, enters the cell and is there converted by esterases into carboxyfluoresceinsuccinimidylester (CFSE). This anionic molecule then irreversibly binds to proteins by coupling to available amines (**Fig. 2**). As a result, for each cell division, the fluorescence is halved. The labeled cells need not be fixed or in any way treated to measure the level of CFSE, and so this dye is an excellent alternative to use to be able to further characterize the population studied. The dye is excited by the 488-nm laser and emits very similar to FITC, with its emission peak at 518 nm.

### 4.2. Using CFDA, SE

Tracking cell proliferation with this dye can be a very powerful technique because it does not require any treatment or fixation of the cells and the

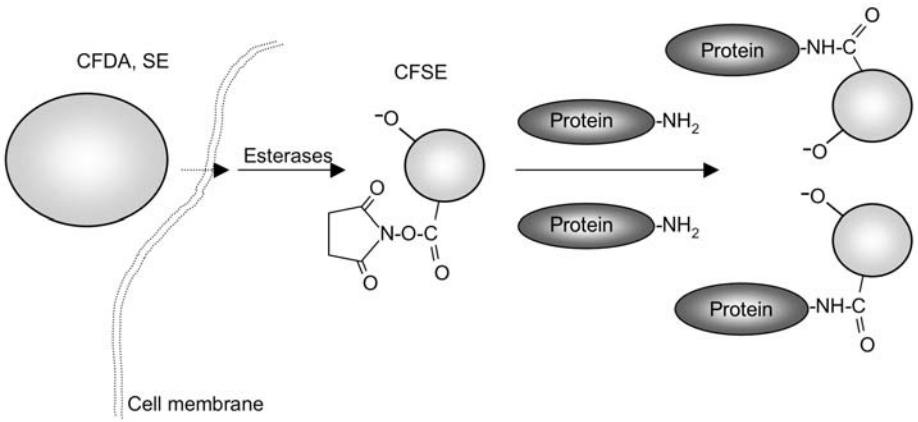



**Fig. 2.** A simplified overview of the mechanisms of CFDA, SE labeling. The nonpolar CFDA, SE spontaneously enters cells, where it is converted into CFSE by esterases. CFSE then couples to available amine side chains of proteins, and so a stable labeling that will be distributed equally between daughter cells has been achieved.

proliferated cells can thus easily be examined for expression of activation and lineage antigens such as CD25, CD4, and CD56 at the time of harvest. Combining CFDA, SE proliferation analysis with peptide-MHC tetramer labeling in particular would make it possible to distinguish the specific, antigen-induced proliferation from any bystander activation-induced proliferation. Bystander activation simply means the indirect activation of non-antigen-specific cells, usually by the activated antigen-specific T cells ***(15)***. Thus, both NK cells and non-antigen-specific memory T cells can proliferate in antigen-stimulated cultures, and the researcher may want to monitor the degree of bystander activation occurring or simply make sure that antigen-specific T-cell proliferation only be taken into account.

It is also possible to study intracellular cytokine levels of the CFSE-labeled proliferated cells ***(16)***. If this assay is performed, it might be of interest to keep in mind that a protein transport blocker may have to be included during the last 4–20 h of culture. This will likely affect the proliferating capacity, and it may be useful to first set up an experiment that shows which cells proliferate. Once this has been established, the intracellular cytokine content could be investigated at different time points throughout the culture period, with or without the addition of a protein transport blocker. Thus, quite a few experiments may be required to fully map the proliferative and cytokine secretion response from a certain antigenic stimulation ***(16)***.

### 4.3. Labeling Cells with CFDA, SE

The labeling technique is relatively straightforward, and a general protocol is outlined below and is to be used as a guideline. Perhaps the most important thing to consider for CFDA, SE labeling and analysis is the concentration of dye used. Too high a dose can make it difficult to distinguish the cycles of proliferation. It may also be problematic to achieve good compensation if too much dye is used. Of course, this is a potential problem mainly if fluorochromes with emission spectra close to CFDA, SE are used, such as PE. In most circumstances, a final concentration of approx 5 µg/mL should be fine, but a titration is always recommended.

After the cells have been labeled, they may be cultured according to standard protocol (the cells should be kept in the dark). The usual rules for when to harvest the cells apply (e.g., human memory T cells could be studied after 3 d and naïve T cells after 7 or even 10 d).

### 4.4. CFDA, SE Labeling Protocol

1. CFDA-SE is dissolved at a concentration of 25 mg/mL in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and stored at –20°C.
2. Wash the cells once or twice and resuspend the pellet in phosphate-buffered saline (PBS) at $1 \times 10^7$ cells per mL.
3. Add 2–5 µ*M* final concentration of CFDA-SE.
4. Mix well and incubate at room temperature in the dark for 15 min with gentle shaking.
5. Add equal volume of fetal calf serum and leave for 1 min to stop the dye uptake.
6. Wash twice in warm complete media.

### 4.5. Controls for Proliferation and for Antigen Labeling

As with any other proliferation assay, it is good practice to include a positive control (e.g., IL-2 or a mitogen such as phytohaemagluttinin [PHA]). This should confirm culture condition and overall viability and "health status" of the cells. The negative control normally consists of the population to be tested together with antigen-presenting cells but without antigen. When, for example, irradiated B cells or total mononuclear cells are used as antigen-presenting cells, some researchers also include the antigen-presenting cells alone or together with PHA, as a control to confirm that there is no proliferation occurring within this population. However, this should not normally be required when using CFDA, SE and flow cytometric analysis; the test population could be labeled with peptide-tetramer, CD3, or another suitable antigen to identify the population of interest.

In the case of CFDA, SE labeling, a positive control is useful for easily identifying the fluorescence peaks that represent the cell division cycles. For this

**Table 2**
**Inducers of Cell Proliferation**

| Product | Final concentration |
|---|---|
| IL-2 | 10–50 units/mL |
| Phytohaemagluttinin | 1–10 µg/mL |
| Tetanus toxoid | 10 µg/mL |
| Purified protein derivative | 50–100 units/mL |

purpose, however, PHA may not be the best choice. PHA is a strong mitogen and so all T cells will be induced to proliferate. Instead, a common recall antigen such as tetanus toxin or PPD (purified protein derivative from *Mycobacterium tuberculosis* or *M. Bovis*) could be used. In such cultures, one should normally be able to find T cells that have proliferated with various numbers of cell cycles, some NK cell proliferation, and also T cells that have not proliferated at all. If the researcher is new to proliferation assays in general, commonly used doses of these antigens and PHA and IL-2 for the induction of proliferation in human freshly isolated peripheral blood mononuclear cells are shown in **Table 2**.

At the time of harvest and cell surface labeling before analysis, the normal cell labeling controls should be used. Thus, the appropriate controls for compensation setup, including CFDA, SE-labeled cells, alone should be used. Because the original CFDA, SE labeling of the cells may differ from experiment to experiment, the settings should be confirmed each time that analysis is performed.

### *4.6. Analysis of CSFE-Labeled Cells*

After harvesting, the cells may be labeled as desired and according to standard protocol. For the acquisition and analysis of data, cells could be looked at firstly in a FSC/SSC plot and a gate set around the cells of interest. Depending on the experiment setup, it may be useful to include a dead cell exclusion dye. The viable gate can then be used in a dot plot showing CFDA, SE versus, for example, side scatter or CD3. For the beginner, it may at this point be desirable to have quite a large number of positive control cells so that adequate time can be spent to find the different peaks and to establish the relevant compensation settings. If a large number of cells have proliferated, it may not be completely straightforward to find the typical gradual decrease in fluorescence that is so often shown in CFDA, SE plots. Adjusting the setting to view only 20–50% or so of the total cell number could be helpful in these cases. Also, if cells have been treated with a powerful antigen for 7 d and the original culture contained

only T cells, or even a primary antigen-specific T-cell line, it is not unlikely that all cells have proliferated at least once. Thus, there may not be any "zero cycle" point present if only $CD3^+$ or $CD4^+$ T cells are assessed. The zero cycle point is perhaps not always of interest for the researcher; however, a zero cycle of proliferation is required if the precursor frequency is to be estimated (*see* **Subheading 4.7.** for precursor frequency calculation). Here, the problem could be solved by including the antigen-presenting cell population; these cells do not proliferate and therefore can serve to represent the original, zero cycle peak. As always in flow cytometry and labeling of cells with different sizes and intracellular content, larger cells such as monocytes or Epstein-Barr-transformed B-cell lines will have higher CFDA, SE fluorescence intensity than the smaller T cells. Thus, the first cell proliferation cycle after a monocyte-indicated zero cycle point may not occur at exactly half the fluorescence intensity of the monocyte peak but rather a bit further toward the intercept. Another option is to use the negative control (that is, tested cells and antigen-presenting cells cultured without antigen). For the beginner, it could be helpful to combine a few cells from the nonantigen and the antigen culture in one tube, to very easily compare the peaks between these two T-cell populations.

It is difficult to say where exactly the detection limit of proliferation occurs. With regard to cycles of division, eight to 10 successive generations of lymphocytes have been demonstrated to be resolved ***(17,18)***. There could also be limits with regard to how many total events are available if cell cultures of quite precious material are studied, and so even if 1–5% of the total survived population are cells that have proliferated, it may prove difficult to record a high enough number of total events to reach a good cluster of proliferated events. As previously mentioned in the rare event analysis section, if only a few events can be identified, the CV will be high and ultimately it is up to the researcher to determine what an acceptable CV is. **Figure 3** shows an example of a relatively minor population of proliferating T cells. The experiment was part of a study investigating autoantigen-induced proliferation of $CD4^+$ T cells, and a minimum of 100 events were collected in the proliferation-positive $CD4^+$ gate, giving a CV of 10%. If a small population is followed, it is recommended to use the appropriate controls as outlined earlier and, whenever possible, to repeat the experiments. The section on rare event analysis deals further with some of the issues related to the analysis of small populations.

### *4.7. How to Calculate Proliferation and Graphically Display Data*

Clearly, the aim of an experiment must dictate how the obtained data should be presented. If the point to be made or proved/disproved is that a certain cell subset proliferates while another one does not, a simple dot plot with CFDA, SE on the *x*-axis and one of the relevant surface antigen on the *y*-axis could be

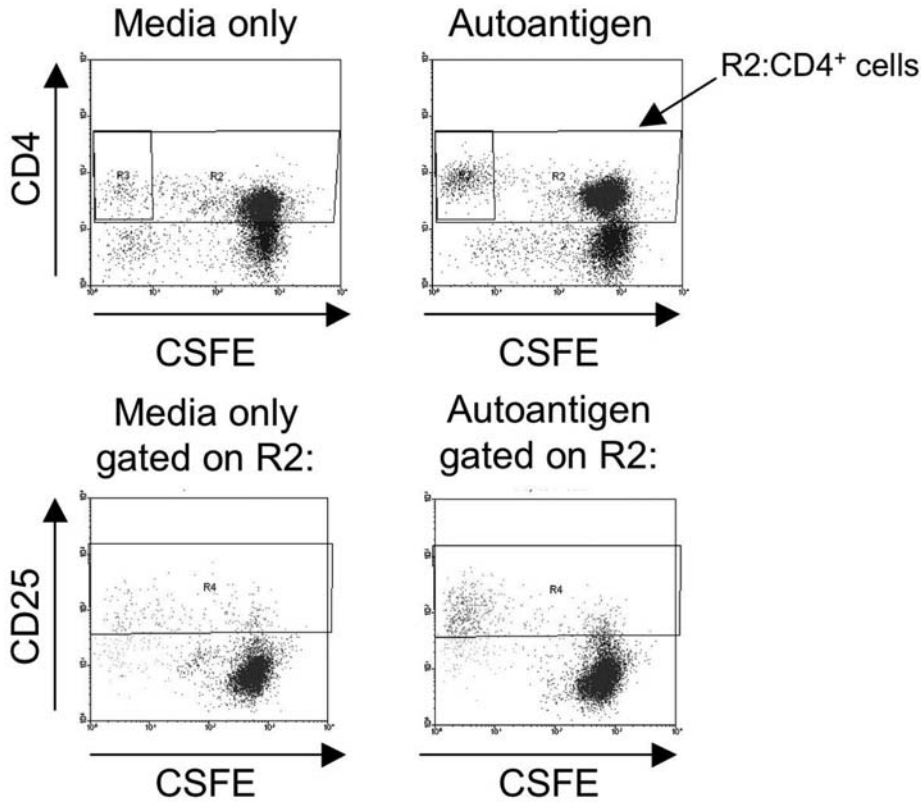



**Fig. 3.** Using CFDA, SE to monitor autoantigen-specific $CD4^+$ T-cell proliferation. Peripheral blood mononuclear cells were labeled with 1 μ*M* CFDA, SE and cultured for 7 d with media alone or the autoantigen at a concentration known to induce T-cell proliferation. After 7 d, cells were labeled with mouse anti-human mAbs for CD4 (PerCP) and CD25 (PE) and analyzed on a FACScan.

used to good effect. An example can be seen in **Figure 3**; here, the aim was to confirm that the $CD4^+$ T cells that had proliferated in response to antigen stimulation expressed CD25. Therefore, a first gate was set around viable mononuclear cells (R1 [not shown]), and a second gate for the $CD4^+$ cells, avoiding $CD4^{low}$ monocytes (R2). R1xR2 cells were then displayed in a dot plot of CFDA, SE and CD25. Antigen-treated cultures were then compared with the culture treated with vehicle alone.

Often, a number or the specific amount of proliferation is wanted. For this purpose, the cell division index could be calculated. Described well in a report by Mannering et al. ***(19)***, the cell division index is a straightforward figure, derived by dividing the total number of proliferated cells in cultures with

antigen by the total number of proliferated cells in cultures with media alone. For this approach to be used, it is important to acquire an equal number of viable events for both antigen-treated and nontreated cultures. The data are then analyzed by setting one large gate around all cells that have divided, avoiding only the antigen-presenting cell/zero cycle peak. The number of proliferated cells in the antigen-stimulated culture is then divided by the number of proliferated cells in the media alone/vehicle culture:

$$\text{Cell division index} = \frac{\text{Number of CFSE}^{\text{dim}} \text{ cells with antigen}}{\text{Number of CFSE}^{\text{dim}} \text{ cells without antigen}}$$

CFDA, SE is often selected as a method when the precursor cell frequency is wanted ***(20,21)***. Here, a gate is set around each of the peaks representing a division, and the number of cells in each peak (or division cycle) is recorded. For each peak, this number is divided by a factor of two times the division cycle number. Once the number of precursor cells has been established for each peak, the total number can be summarized:

$$\text{Precursor cell frequency} = \frac{\text{Number of cells in division cycle}}{2n}$$

where n = division cycle number.
For example:

100 cells in third peak
=> third peak equals two rounds of divisions
=> 100 cells / (2 × 2 rounds of division) = 100 ÷ 4 = 25

The same calculation is performed for each peak, let's say,
second peak: 10
third peak: 25
fourth peak: 30
fifth peak: 15
No sixth peak was observed, and so the precursor frequency would be:
10 + 25 + 30 + 15 = 80 cells.

If this method is used, it is important that the precise number of cells, even T cells, be estimated at the start of culture. Thus, a good estimate of the presence of antigen-specific T cells in, for example, the peripheral blood of an individual before and after vaccination can be determined.

### *4.8. Measuring Cell Subset Expansion in Mixed Cell Cultures*

If for some reason a suitable labeling cannot be found, there is still the possibility of mapping the expansion of a certain population; perform a straightforward labeling of the cell subset at relevant time points during culture and

compare antigen-stimulated with unstimulated (vehicle only) cultures. This approach was used to study NKT cell expansion; an example is shown in **Figure 4**. Data were calculated as the percentage of $Va24^+Vb11^+$ NKT cells of total viable mononuclear cells. In this study, these experiments served to prove that the addition of the steroid predisolone hampers the antigen-specific expansion of NKT cells ***(9)***. It is important to remember, however, that this approach is not a direct indication of whether a cell population has proliferated or not. The data are to be interpreted as how a certain subset has survived during culture and therefore now makes for a larger proportion of the total number of survived cells, than at the outset of culture. For example, although it is highly likely that the NKT cells at the outset of culture have proliferated and so the total number of NKT cells is now significantly higher, it is equally likely that a large proportion of, for example, $CD8^+$ T cells have died during culture. Therefore, data cannot be presented as a measurement of proliferation but could be referred to as the expansion of a certain subset under various culture conditions, and the effect of the different conditions could be compared.

## 5. Intracellular Labeling for Cytokines and Chemokines

Cytokines and chemokines play a vital role in the immune system and are therefore often the focus of studies of immune cell function. It is not surprising then that several methods have been developed to investigate the presence of these proteins. In general, neither cytokines nor chemokines are stored preformed in cells (some exceptions are mentioned in **Subheading 5.1.**) but are produced relatively rapidly upon activation and released into the extracellular environment. The majority of assays (e.g., enzyme-linked immunosorbent assay [ELISA], bead arrays, and bioassays) detect soluble cytokines. If using such methods, however, a cytokine profile of a given cell population can only be obtained if the cells have first been sorted or separated. Choosing intracellular labeling technique and flow cytometry, on the other hand, gives the unique opportunity to characterize the cells that produce a given cytokine in a mixed cell population. For example, in mixed cell cultures, the specific cytokine profile of both bystander-activated cells and antigen-specific T cells can be studied using antigen-MHC tetramer labeling of the cell surface followed by intracellular labeling with anti-cytokine antibodies. Perhaps most commonly, researches have investigated T-helper type 1, type 2, and type 0 cytokine profiles. The technique works very well in whole-blood cultures, and this means that it is also very useful for studying monocytes, DCs, granulocytes, and any other easily activated cells because it avoids the unnecessary activation of the cells by purification steps.

The technique involved is similar to other labeling techniques used for flow cytometry. One addition to standard cell surface labeling protocols is the need to permeabilize the cell membrane in a suitable way so that the cytokine/

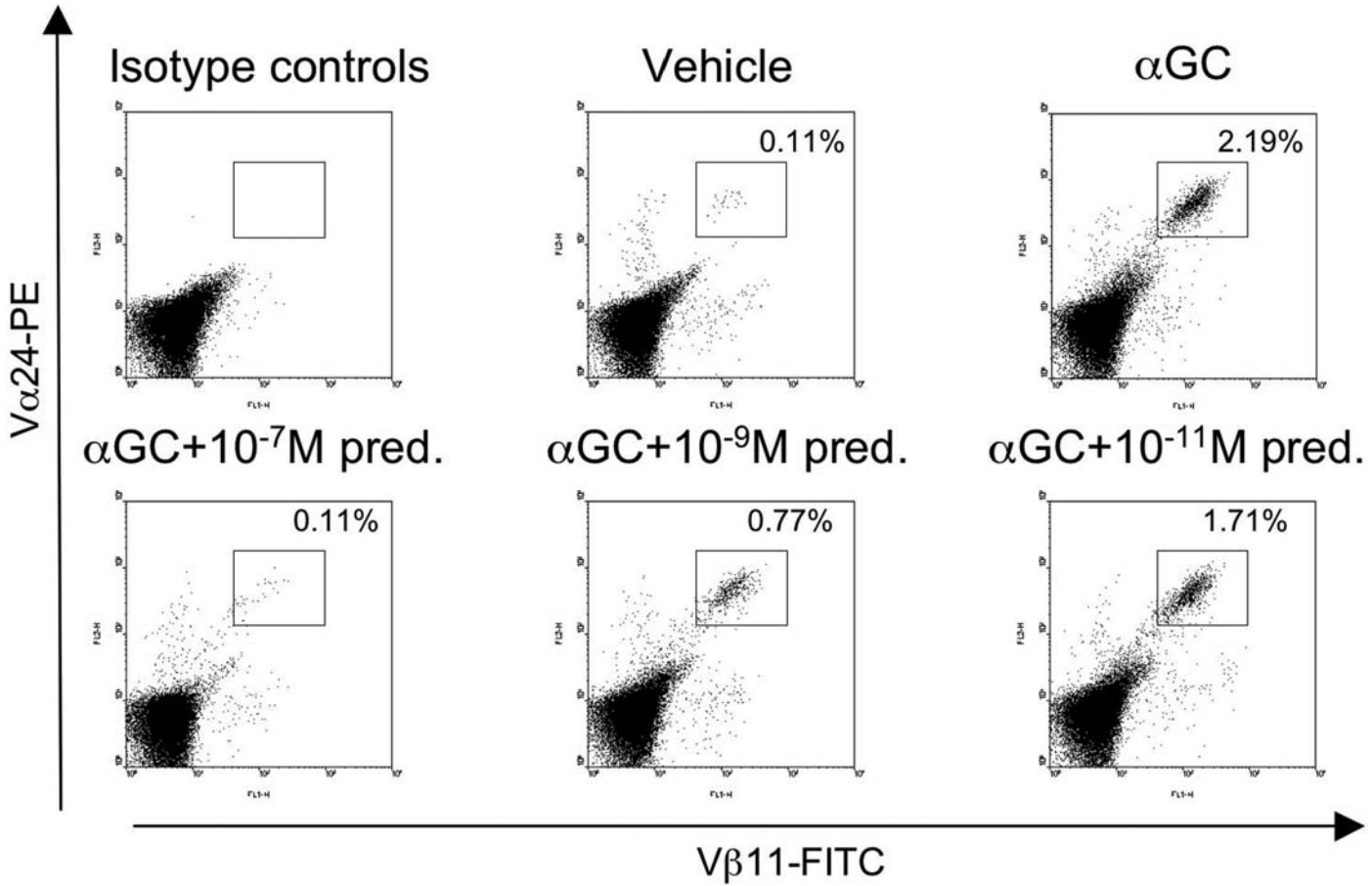

Isotype controls
Vehicle
αGC
0.11%
2.19%
αGC+10-7M pred.
αGC+10-9M pred.
αGC+10-11M pred.
0.11%
0.77%
1.71%
Vα24-PE
Vβ11-FITC

**Fig. 4.**

chemokine-specific mAb can access the proteins. Another difference compared with most other methods is the inclusion of a protein transport blocker, to ensure that the cytokine of interest remains inside the cell. Overall, the methodology can be divided into three steps: (1) the use of a protein transport blocker, (2) cell activation, culture, and time of harvest (this should include a control population), and (3) a labeling procedure.

### *5.1. Blocking Protein Transport*

With only a few exceptions, such as transforming growth factor-β in platelets ***(22)*** or membrane-bound IL-15 on monocytes ***(23,24)***, cytokines are not stored once produced but are released into the extracellular environment. Most secretory proteins typically contain N-terminal signal peptides that direct them to the translocation apparatus of the endoplasmic reticulum. Stored in vesicles, they are transported from the endoplasmic reticulum via the Golgi apparatus to the cell surface and subsequently released ***(25)***. For this reason, it is necessary to use a protein transport blocker during cell activation to "capture" the cytokines inside the cells. The two protein transport blockers used in flow cytometry, monensin and brefeldin A, disrupt protein transport from the Golgi. However, certain proteins follow a nonclassical secretion route that is independent of the Golgi. This unusual group includes IL-1β, which has even been shown to have an increased rate of release in the presence of brefeldin A ***(26)***. Secretion of this cytokine can be induced (by ATP, for example) and, perhaps more interesting for the flow cytometrist, can be blocked by using serum-free medium ***(27)***. In our laboratory, we have looked at lipopolysaccharide (LPS)-induced IL-1β production in monocytes, either purified or as part of mixed cell cultures. Using either serum-free medium or 5% autologous serum, we have had no particular difficulties in detecting monocyte IL-1β (**Fig. 5**).

### *5.2. Monensin and Brefeldin A: Wanted and Unwanted Effects*

The protein transport blockers used in flow cytometry are monensin and brefeldin A. Monensin is a sodium ionophore obtained from *Streptoyces cinnamonensis*, and its use leads to osmotic swelling of post-Golgi endosomal structures and Golgi subcompartments ***(28)***. Apart from cell biologists and flow cytometrists, cattle farmers apparently use this ionophore; its addition to the

**Fig. 4.** Mapping antigen-driven expansion of natural killer T (NKT) cells in PBMC preparations using flow cytometry. Mononuclear cell preparations were cultured for 7 d with 100 ng/mL αGC or vehicle, together with graded doses of the steroid prednisolone, 50 ng/mL human recombinant IL-2, or both. The relative proportions of NKT cells in the resulting cultures were investigated by using antibodies specific for the T-cell receptor expressed by human NKT cells, Vα24 and Vβ11. The plots show percentage of NKT cells of total viable cells.

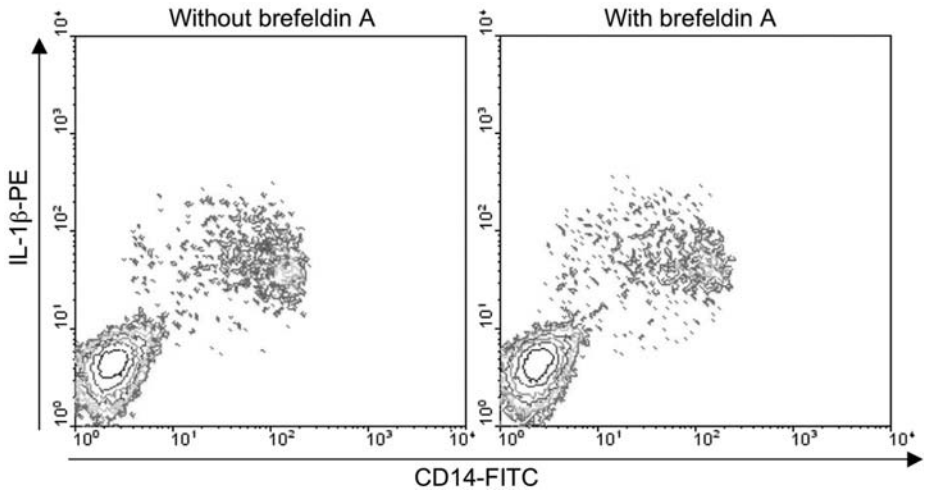



**Fig. 5.** IL-1β-positive monocytes in the absence of protein transport inhibitor. Peripheral blood mononuclear cells were cultured at 37°C, 5% $CO_2$ for 18 h in RPMI-1640 with glutamine, 5% autologous serum, and 100 ng/mL LPS **(right panel)** with or **(left panel)** without brefeldin A (5 μg/mL). The cells were labeled for CD14 (FITC) and IL-1β (PE) and analyzed on a FACScan.

cattle diet increases the ratio of weight gain to food intake ***(29)***. In the laboratory and for humans, however, it is to be considered a toxic substance. This is true also for brefeldin A, which is a metabolite produced by various fungi but which was originally isolated from *Eupenicillium brefeldianum* ***(28,30,31)***. Brefeldin A is a reversible blocker of protein transport from the endoplasmic reticulum to the Golgi, and the function of the Golgi itself is also generally affected. Although brefeldin A is frequently used in cell biology studies, the exact and complete effects of this product on different cell types are still being studied.

Both reagents can alter or affect cell surface expression of certain antigens, and neither can be used in prolonged cultures without inducing significant cell death. There are quite a few publications comparing the use of these two for intracellular cytokine labeling ***(32–34)***. For cell viability, the consensus is that monensin induces more cell death. This may not be of major concern for experiments in which a short incubation time is used; there have been no major differences reported for cell viability between the two reagents for time points up to 6 h. But after longer incubation times (up to 18–24 h), there does seem to be a clear difference between brefeldin A and monensin. For example, O'Neil-Andersen and Lawrence ***(33)*** recorded an average of 69% viable cells in brefeldin A-treated cell cultures, as determined by propidium iodine staining.

The monensin-treated cells had an average of 56% live cells. Thus, for certain cytokines/chemokines (such as IL-12) in which a longer incubation time is often required, it may be preferable to use brefeldin A.

The overall morphology of cells will also be affected by both monensin and brefeldin A and so the scatter characteristics of cells may change. This ought not pose any problem, and it should still be possible to detect, for example, granulocyte, lymphocyte, and monocyte regions in treated whole-blood cultures. However, both monensin and brefeldin A may affect the expression of certain surface molecules and this might have to be considered. Researchers often combine intracellular cytokine analysis with extracellular labeling of activation antigens, and of the more commonly used activation antigens, CD69 can be influenced by the transport blockers. The addition of brefeldin A results in a block of CD69 surface expression by $CD3^+$ cells; however, synthesis of CD69 and thus intracellular expression of CD69 is generally not hindered ***(33)***. It is therefore common to use intracellular labeling also for CD69.

In addition, stimuli that are often used in culture, such as LPS and PMA (phorbol-12-myristate-13-acetate), can also affect cell surface antigen expression. Most notably, T-cell CD4 expression alongside CD3 and CD8 may be downregulated by PMA ***(35)***. This downregulation is to some extent rescued by monensin or brefeldin A; nonetheless, a reduction is still noticeable. For monocytes, CD14 is a receptor for LPS/LPS-binding protein complex and the addition of LPS or other bacterial constituents, including mycobaterial antigens, may induce the downregulation of surface CD14 expression ***(36)***.

Apart from these unwanted changes induced by protein blockers, which one is best to use with regard to labeling efficiency? This may depend on the cell type and protein studied. There is general agreement among reports on proinflammatory cytokine production by human phagocytic cells: brefeldin A results in both a higher percentage of positive cells and a higher fluorescence intensity ***(32–34)***. This may be especially true for tumor necrosis factor-α (TNF-α); Schuerwegh et al. ***(34)*** reported a threefold difference in fluorescence intensity between brefeldin A- and monensin-treated LPS-stimulated monocytes at approx 6 h of incubation. For T-cell cytokines, however, the abovementioned reports found no clear difference. Much less is known about chemokines, and it might be wise to test both monensin and brefeldin A by using a control population of healthy cells stimulated with a known inducer of the protein of interest. If longer incubation times are called for, it would also be advisable to titrate the transport blockers and aim to use the lowest possible concentration.

### *5.3. Cell Activation, Control Populations, and Time of Harvest*

Often, the aim of a study is to determine the cytokine/chemokine profile of a given cell population after exposure to a particular stimuli, perhaps a novel

antigen. For each experimental setup, it is therefore necessary to titrate the antigen concentration and to study the kinetics of the protein production. It is also recommended to include a control population. This should be a known cell population such as peripheral blood mononuclear cells, stimulated with well-known reagents. For example, PMA- and ionomycin-stimulated whole-blood or peripheral blood mononuclear cells would serve as a good control for T-cell IL-2 production. For the most commonly studied cytokines, protocols for how to prepare a useful control population are relatively easy to find on some of the common flow cytometry websites, both academic and commercial. If a protein that is less well studied is to be investigated and no information is available, reverse transcriptase-polymerase chain reaction (PCR) or real-time PCR analysis of cDNA could be relevant, using specific primers and perhaps a suitable general stimuli such as PMA or bacterial and viral products. This approach can be performed using relatively few cells, and cells from different time points and treatments can easily be frozen down and batched up for one single PCR run. Hopefully, the data obtained would shed some light on the stimuli dose that is required as well as the kinetics involved and thereby indicate the relevant time points to investigate for the intracellular analysis.

### 5.4. Labeling Procedure for Intracellular Cytokines

If a cell surface labeling is planned, this must always be carried out first, before fixation and membrane permeabilization. This part of the procedure should follow the standard protocol for the antibody or tetramer that is used. It is worth noting that because the cells will be fixed, it will not be possible to use dyes such as propidium iodide or 7-AAD for dead cell exclusion. It is thus important to use cells in good condition, and this becomes even more pertinent when considering that both monensin and brefeldin A will induce a certain amount of cell death.

The most widely used reagent to permeabilize the cell membrane is saponine; this is a substance derived from the bark of *Quillaja saponaria,* the Soap bark tree (more can be read about this tree and saponins at http://www.ibiblio.org/pfaf/cgi-bin). Saponin will allow antibodies to enter both the cell membrane and membranes of the endoplasmic reticulum and Golgi, and so cytokine/chemokine protein both in the cytosol and the ER/Golgi should be labeled. The permeabilization effect by saponin is reversible. For this reason, it is useful to perform the washing steps after the intracellular labeling in permeabilization buffer, followed by a last wash in ordinary buffer (or PBS).

Treating cells with saponin causes a complete, nonreversible change in cell shape, as can be seen under the microscope. Thus, the scatter characteristics are altered and it can even be impossible to separate lymphocytes from monocytes, for example. By fixing the cells in 1% paraformaldehyde (PFA) before

permeabilization, the morphology is more or less preserved and intracellular antigenicity is conserved also. Of other fixatives, 3% formaldehyde can be used successfully. The protocol below is general and should be used as a guideline.

### *5.4.1. Brefeldin A*

1. Dissolve in 5–20 mg/mL ethanol/DMSO.
2. Store at –20°C (1 yr).
3. Guideline for testing final concentrations: 0.01–0.05 μg/mL.

### *5.4.2. Permeabilization Buffer*

1. PBS without calcium and magnesium.
2. 1% bovine serum albumin.
3. 0.01% sodium azide.
4. 0.1% saponin.

### *5.4.3. Labeling Procedure*

1. Extracellular labeling.
   a. Tetramer/mAb as according to normal protocol.
2. Wash, fix in 1% PFA for 15–20 min at room temperature or at 4°C.
3. Wash, permeabilize (saponin: reversible).
   a. Commercial buffers.
   b. Add 0.1% saponin to your standard labeling buffer.
4. Intracellular labeling (saponin).
   a. Dilute all reagents in saponin buffer.
5. Saponin wash (two times).
6. Wash (one time).
7. Analyze (fix).

For the most commonly studied cytokines and chemokines today, the commercially available antibodies are quite well used and thus well tested. As a rule, they do not bind unspecifically to any considerable extent. If no directly conjugated antibody is available, it is better to use an unconjugated antibody than a biotinylated one. This is because the intracellular content of avidin can be high, resulting in specific binding to avidin by the secondary reagent. It is not a problem to use biotinylated antibodies and a secondary fluorochrome-conjugated antibody in the extracellular labeling step, prior to permeabilization of the cell membrane. If unconjugated antibodies are to be used intracellularly, it is necessary to include an isotype control. The intracellular environment is very "sticky," and it is likely that the background fluorescence will be much higher for the intracellular labeling compared with the extracellular, using the same antibody. As always with isotype controls, it is important to use a concentration of the isotype control which is as nearly identical to that of the specific antibody

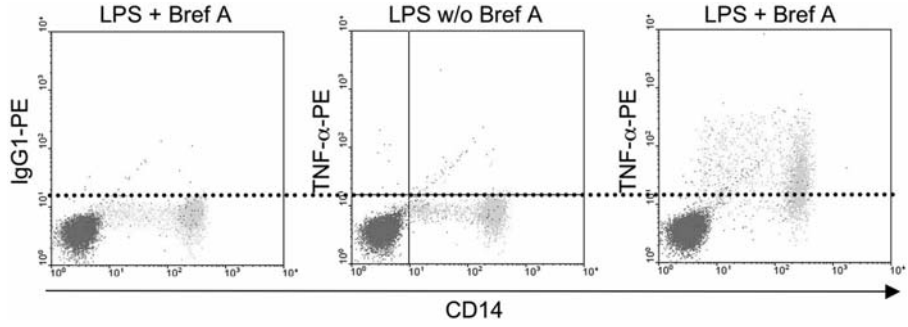


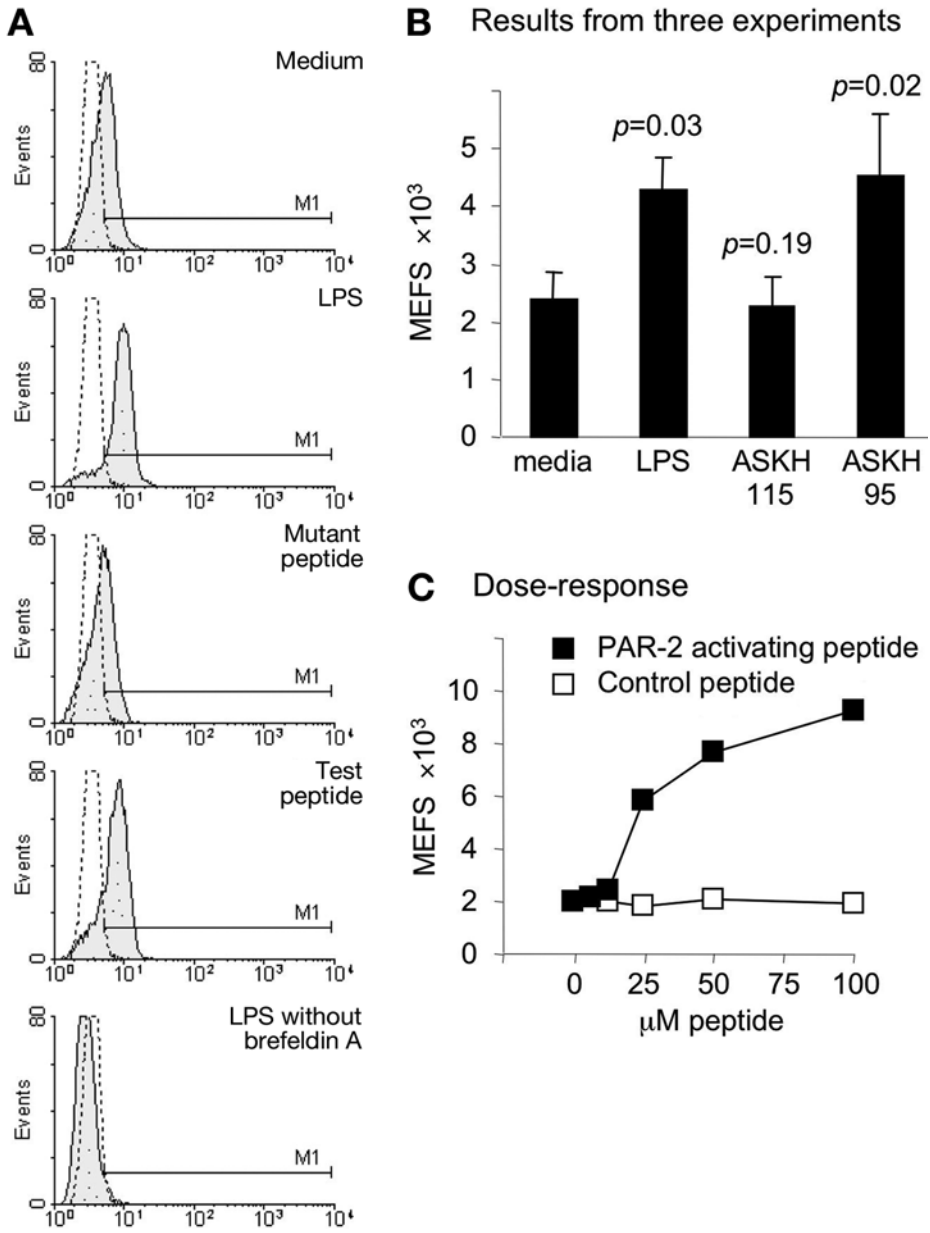

**Fig. 6.** TNF-α labeling in the absence of protein transport inhibitor. Peripheral blood mononuclear cells were cultured overnight with 1 μg/mL LPS with or without 5 μg/mL brefeldin A. The cells were labeled with CD14-FITC and either a PE-conjugated anti-TNF-α mAb or an isotype control and analyzed on a FACScan. The FL1/FL2 profiles of the cells are shown in the dot plots. The broken line is included as a guide. In addition, this figure shows that no clearcut positive and negative population of CD14 TNF-producing cells can be seen and that therefore results would be best summarized as change in MFI.

as possible, so specific and control antibodies are best purchased from the same producer, if it is a commercial antibody that is tested.

However, for directly labeled anti-cytokine antibodies, the control for specific labeling could be the same antibody used on cells that were treated with the same stimuli, but without protein blocker. **Figure 6** shows $CD4^+$ monocytes labeled with an antibody for TNF-α or an isotype control after stimulation with LPS in the presence or absence of brefeldin A. As can be seen, there are no positive cells in the TNF-α-labeled monocytes that were incubated without brefeldin A. Importantly, cells that have been stimulated but not treated with brefeldin A will of course also produce cytokines, and so especially if short incubation

**Fig. 7.** Using molecules of equivalent soluble flourochromes (MESFs) to demonstrate cytokine-labeled data from more than one experiment. Monocytes isolated from peripheral mononuclear cells by magnetic cell sorting were cultured for 12 h with 1 μg/mL LPS, graded doses of a PAR-2 activating peptide, or a mutant peptide for control. After 12 h, cells were labeled for intracellular IL-8 using a PE-conjugated mAb. **(A)** Filled histograms show the IL-8-labeled cells, and broken histograms represent the isotype control-labeled cells. The result, without brefeldin A, for the LPS-treated cells labeled with the IL-8 specific mAb is also shown and is not much different from the isotype control. Given that it is not possible to distinguish a positive and a negative population, it would not be suitable to use the markers shown in the histograms to represent the data as percentage of positive cells. The median fluorescence for each specific labeling was used (importantly, using all cells and not cells within the shown marker only), and the

(*Continued on next page*)




MESFs for these values were determined as described in **Chapter 5**. The value of the isotype control was not taken into account. **(B)** The average of the MESF data from three separate experiments is displayed in a bar histogram; the error bars show the standard deviation of the three experiments. **(C)** The MESFs were used to create a graph for the dose response of the control and PAR-2 activating peptide.

times are used, the non-brefeldin A control may in fact contain cells that still have not secreted the produced cytokines. And most definitely, this strategy would not work for IL-1b, because this protein is not transported via the Golgi.

Generally, it is considered that the most appropriate control is to let the specific antibody compete for the binding site by titrating in the relevant recombinant protein. Of course, if studying a very novel antigen, this could prove difficult.

### *5.5. Quantifying Production of Cytokines*

To assess the absolute concentration of cytokine produced, techniques such as ELISA, bead arrays, or real-time quantitative PCR are necessary. The latter is especially useful when only small numbers of purified cells are available but will of course not confirm actual protein production and so this must be validated. Nonetheless, a "semiquantitative" analysis can be achieved for intracellular cytokine labeling by using fluorescent beads and calculating the MESFs (molecules of equivalent soluble fluorescein) for the cytokine fluorescence data. This method can be helpful when performing repeated experiments that require statistical evaluation. **Figure 7** shows an example of this: IL-8 production by human monocytes treated with media alone, an activator of protease-activated receptor-2 (PAR-2), or control. For all treatments, including the media control, IL-8 was detected, but repeated experiments confirmed that PAR-2-stimulated monocytes consistently produced higher levels of IL-8 in all tested individuals (**Fig. 7**).

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# 9

# Calcium: Cytoplasmic, Mitochondrial, Endoplasmic Reticulum, and Flux Measurements

**Gary Warnes and Marion G. Macey**

**Summary**

Fluorescent dyes that change their spectral properties in the presence of calcium are available. They have been used to measure the changes in calcium levels which occur in cells when stimulated by a variety of agonists. Methods for measuring receptor-mediated calcium flux and for tracking calcium movement within the cell and the movement of internal calcium stores are described.

**Key Words:** Fluo-3; Indo-1; Rhod-2.

## 1. Introduction

The variety of fluorescent calcium probes available permits the investigator to choose a probe that allows immunophenotyping of cell subpopulations and calcium measurements on most flow cytometric analyzers. Indo-1, the UV-excitable ratiometric calcium dye, measures calcium by the nature of its spectral properties when not bound or when bound to calcium, changing from green to violet, respectively ***(1)***. Thus, the ratio of these two spectra can be plotted against time, permitting accurate measurements of calcium changes within cells compared with the less-sensitive argon ion (488 nm)-excitable calcium dyes such as Fluo-3 and Fluo-4, the fluorescence of which increases in intensity only when the dye binds more calcium ***(2,3)***. Also, mitochondria calcium flux can be measured by Rhod-2, which is suboptimally excited at 488 nm and emits at 575 nm, rendering it usable on most commercial flow cytometers ***(4)***.

The tracking of calcium movements within the cell by the use of the ratiometric cytoplasmic calcium probe Indo-1 and mitochondria calcium probe Rhod-2 is facilitated by the use of various agonists ***(5,6)***. These include calcium ionophore, ionomycin, for general cell activation and thapsigargin, an endoplasmic reticulum

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(ER) calcium ATPase pump inhibitor, which releases calcium from the ER, permits the tracking of calcium movements within the cell by the use of ratiometric cytoplasmic probe Indo-1, and mitochondrial calcium probe Rhod-2 (5.6).

## 2. Procedure for Tracking Calcium Movement Within the Cell

A flow cytometer fitted with air/water-cooled argon lasers tuned to 488 nm and 355/360 nm or air-cooled helium–cadmium 325 nm lasers and the following filters will be required: 575/25DF (Rhod-2, orange emission), 405/20DF (Indo-1 bound calcium, violet emission), 530/30DF (Indo-1 unbound calcium, green emission), and dichroic mirror 470LP (Indo-1).

Indo-1 loading. Make a 1 m*M* stock solution of Indo-1 (cat. no. I-1223; Invitrogen, Carlsbad, CA) by dissolving a 50-µg aliquot with 50 µL of dimethyl sulfoxide (DMSO). Resuspend cultured cells in calcium-free phosphate-buffered saline (PBS) at 1–10 $\times$ $10^6$/mL and incubate with 1–2 µ*M* Indo-1 at 37°C for 45–60 min in a water bath (in the dark) or $CO_2$ incubator.

Rhod-2 loading. Make a 0.9 m*M* stock solution of Rhod-2 (cat. no. R-1245; Invitrogen) by dissolving a 50-µg aliquot with 50 µL of DMSO. Resuspend cultured cells in calcium-free PBS at 1–10 $\times$ $10^6$/mL and incubate with 0.5–0.9 µ*M* Rhod-2 at 37°C for 45–60 min in a water bath (in the dark) or $CO_2$ incubator.

### 2.1. Washing Procedure After Loading of Probe

1. Pellet cells at 400*g* and wash twice in calcium-free PBS.
2. Resuspend in calcium-free medium of choice at 1 $\times$ $10^6$/mL.
3. Maintain at the required temperature. (Rhod-2 fluorescence is temperature-dependent.) Keep on ice if required later and readjust to required temperature before use.

### 2.2. Data Acquisition

1. The lasers should be allowed to stabilize, especially UV or helium–cadmium lasers, which take longer to stabilize to normal levels than do air-cooled argon lasers.
2. The cytometer software is adjusted to acquire data in a time-dependent manner (i.e., with time on the *x*-axis), and linear amplification for both the violet and green detectors (on the *y*-axis) is used to give the violet/green ratio for the acquisition of Indo-1 fluorescence. Similarly, adjust the orange (575/25DF) detector to linear for the acquisition of Rhod-2 fluorescence.
3. The voltage of the violet detector of Indo-1 (bound calcium) should be adjusted so that the violet fluorescence falls at approx channel 200 on the linear scale. For the green detector for Indo-1 (unbound calcium), the voltage should be adjusted so that the green fluorescence falls in the middle of the linear scale. Then adjust the linear amplification gains of the instrument to set the Indo-1 ratio function of violet/green fluorescence so that resting cells fall between channels 200 and 300. This procedure should be possible if adequate Indo-1 loading has been achieved and there is an excess of dye that has not bound calcium within the cell population.

4. For the acquisition of Rhod-2 fluorescence, the voltage on the detector is adjusted so that the resting cell baseline fluorescence is set to at least channel 200.
5. Samples should be acquired at a rate of 500–1000 events/s for high-incidence events (e.g., cell lines) or more than 2000 events/s for rare-event analysis.
6. Samples are analyzed for 30–60 s prior to cell activation by the agonist to establish the baseline level of fluorescence for unactivated cells. For most types of calcium flux assays, addition of the agonist is achieved by simply pausing the acquisition, removing the tube from the instrument, adding the agonist followed by rapid mixing, replacing the tube on the instrument, and resuming the acquisition of the sample. For most biological responses at room temperature or 37°C, this procedure is adequate. The flow cytometer is then allowed to acquire data for the activation phase of the calcium flux.

### 3. Indo-1 Measurements of Cytoplasmic Calcium Flux

The use of calcium ionophore, ionomycin, and calcium ATPase pump inhibitor thapsigargin on cells loaded with Indo-1 can be used to monitor the movement of internal calcium stores by the nature of their different mechanisms of action. Ionomycin will only release internal calcium stores within cells (when resuspended in calcium free medium); under such conditions, Indo-1 will detect all stores of calcium released into the cytoplasm (**Fig. 1A**).

Thapsigargin has differential effects on cellular calcium ATPase pumps, irreversibly inhibiting the ER calcium ATPase pump while not affecting those of the plasma membrane. Thus, any stores of ER calcium released are detected by Indo-1 in the cytoplasm on route to the mitochondria (**Fig. 1B**) *(7)*. Indo-1 can thus be used to quantify internal calcium stores other than from the ER by subtraction of the Indo-1 calcium flux profiles generated by ionomycin and thapsigargin shown in an overlay plot (**Fig. 2**).

### 4. Rhod-2 Measurements of Calcium Flux to Mitochondria

Ionomycin will release all internal calcium stores within cells (when resuspended in calcium-free medium), and Rhod-2 will detect all calcium that fluxes to the mitochondria (**Fig. 3A**).

An important event in apoptosis is the movement of calcium from the ER to mitochondria. Thapsigargin, an inhibitor of ER calcium ATPase pumps, mimics this release, which Rhod-2 can detect (**Fig. 3B**) *(5,7)*. Rhod-2 can also be used to quantify internal calcium stores other than from the ER which flux to the mitochondria by subtraction of the Rhod-2 calcium flux profiles generated by ionomycin and thapsigargin shown in an overlay plot (**Fig. 4**).

### 5. Changes in Calcium Within in a Cell After Receptor Activation

Measurement of changes in intracellular ionized calcium concentrations ($[Ca^{2+}]i$) has proven to be of wide use in the study of cellular responses to

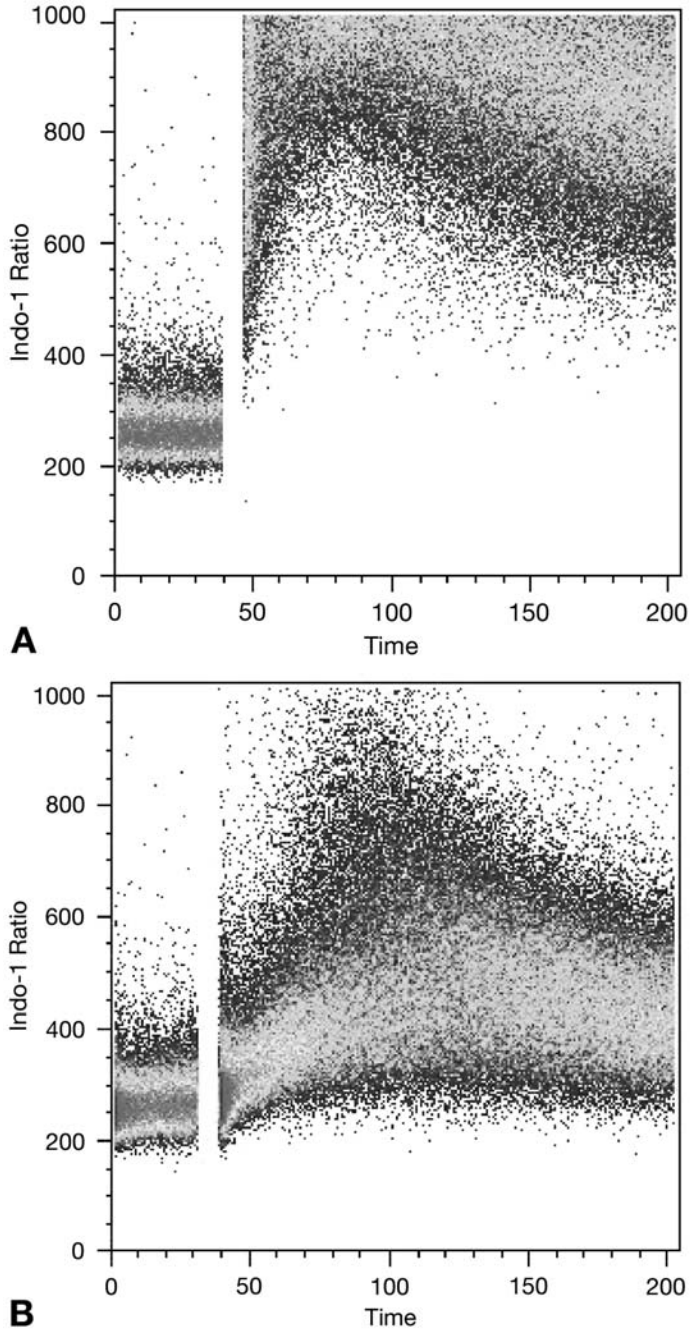


**Fig. 1.** Jurkat cells loaded with 1 µ*M* Indo-1 were activated with (**A**) 10 µg/mL ionomycin and (**B**) 5 µ*M* thapsigargin after 30 s of acquisition on an LSRI. (BD Biosciences, San Jose, CA)

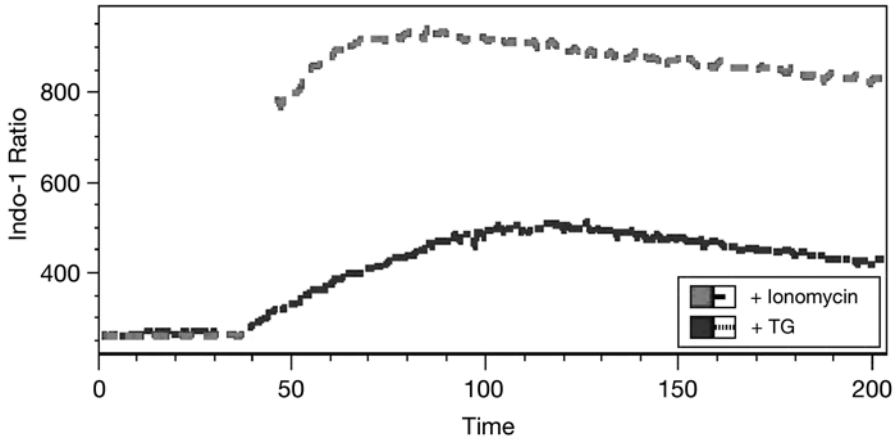


**Fig. 2.** Overlay of ionomycin and thapsigargin responses of Jurkat cells loaded with 1 μ*M* Indo-1. The difference in the responses is a measure of internal stores of calcium released into the cytoplasm other than from the ER.

activating stimuli. Changes in calcium within in a cell after receptor activation may be assessed by flow cytometry. The fluorescent dye Indo-1 has successfully been used in flow cytometry for this purpose, and when used as a ratiometric indicator, it provides optimum sensitivity and accuracy. Unfortunately, this dye requires UV excitation, which is often not available. Fluo-3 acetooxymethyl ester (Fluo-3 AM) is a dye commonly used for measuring changes in calcium in cells; it is excited at 488 nm and emits light at approx 525 nm and so is suitable for use on most flow cytometers. The accuracy of flow cytometric measurement of intracellular calcium with Fluo-3 is compromised by variation in basal fluorescence intensity due to heterogeneity in dye uptake or compartmentalization. This has led to loading cells simultaneously with Fluo-3 and SNARF-1. When SNARF-1 fluorescence is collected at approx 600 nm, its intensity does not change upon cell activation. Furthermore, Fluo-3 and SNARF-1 fluorescence signals exhibit a linear relationship. The ratio of Fluo-3 to SNARF-1 eliminates a significant proportion of variation in fluorescence intensity caused by variation in Fluo-3 uptake and thus can be used as a sensitive parameter for measuring changes in $[Ca^{2+}]I$ ***(8)***. Alternatively, similar results can be obtained using a ratio of green to red fluorescence from the simultaneous loading of the dyes Fura Red and Fluo-3. Both Fura Red and Fluo-3 are excited using the commonly available blue 488-nm laser line. With appropriate concentrations of the two dyes, the magnitude of response with the Fluo-3/Fura Red ratio is greater than that achieved with Indo-1, whereas the intercellular variation in measurement is

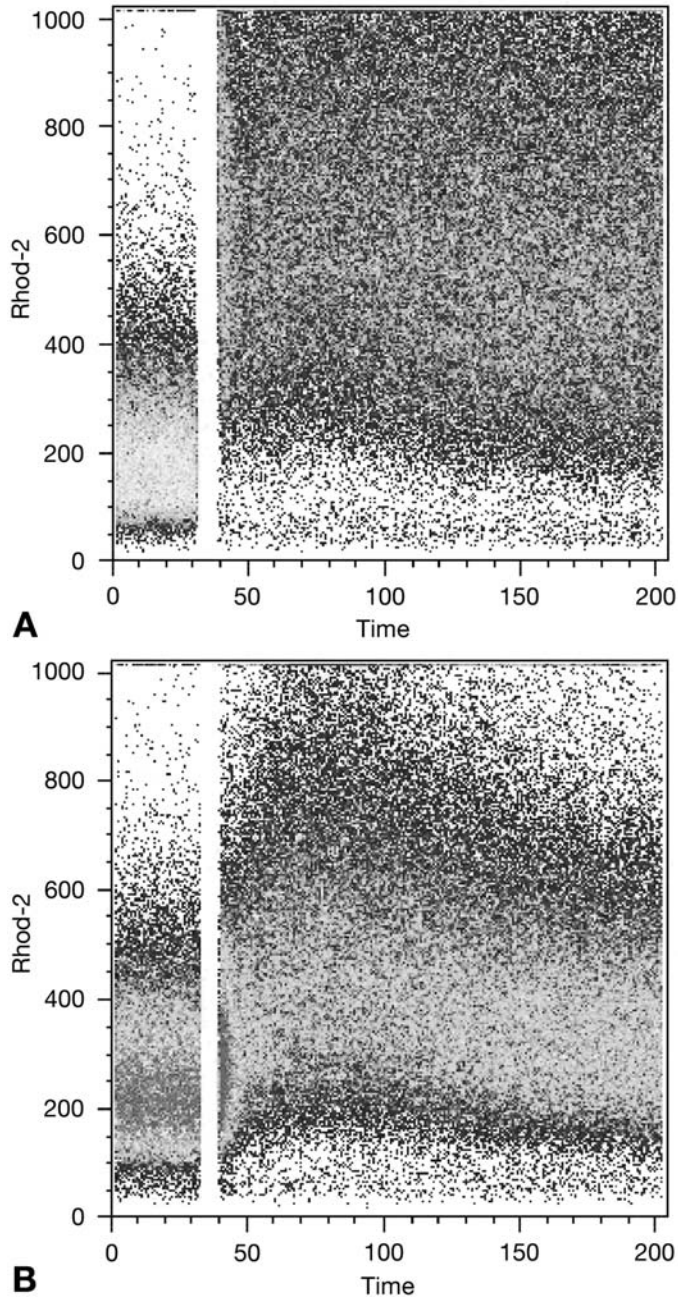


**Fig. 3.** Jurkat cells loaded with 0.5 µ*M* Rhod-2 were activated with **(A)** 10 µg/mL ionomycin and **(B)** 5 µ*M* thapsigargin after 30 s of acquisition on a Becton Dickinson LSRI.

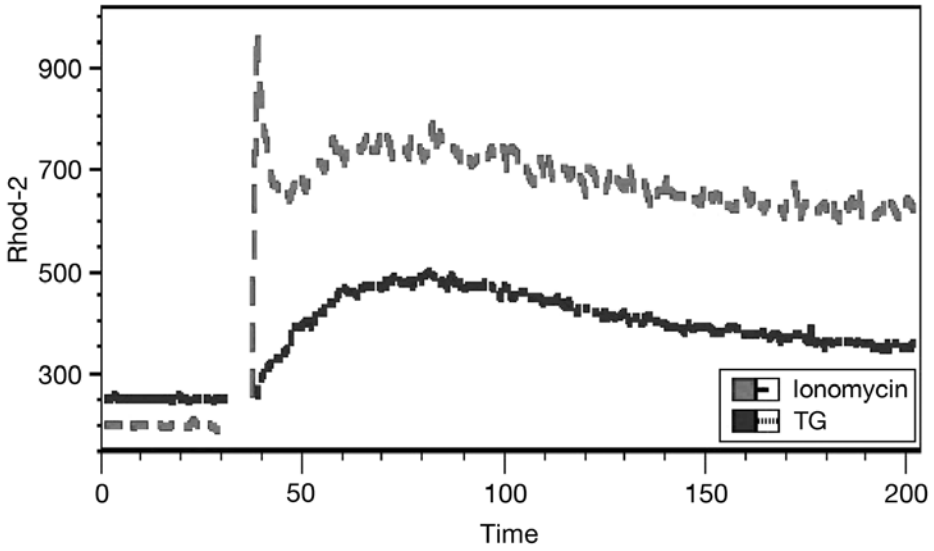


**Fig. 4.** Overlay of ionomycin and thapsigargin responses of Jurkat cells loaded with 0.5 μ*M* Rhod-2. The difference in the responses is a measure of internal stores of calcium released that flux to the mitochondria other than from the ER.

similar to that seen with Indo-1. Analyses can be simultaneously combined with immunofluorescent detection of phycoerythrin-labeled antibodies to enable $[Ca^{2+}]i$ measurement within cell subsets ***(9)***. The method below is a simple procedure that uses Fluo-3 alone but has been shown to be effective ***(10,11)***.

## 6. Calcium Flux Assay Procedure Using Fluo-3

To measure the changes in intracellular calcium in cells in response to activating and control peptides, the calcium indicator Fluo-3 and flow cytometry may be used.

Cells ($2 \times 10^6$/mL) are incubated with Fluo-3 at a final concentration of 2 μ*M* for 30 min at 37°C in Ca- and Mg-free Hanks' balanced salt solution (HBSS) buffered with HEPES (1 m*M*). Cells are then diluted 1:10 in buffered HBSS containing Ca and Mg. The baseline level of Fluo-3 fluorescence is determined in a dot plot of green fluorescence logarithmic scale on the *y*-axis and time in seconds on the *x*-axis. Then, either the test or control peptide is added (100 μ*M* final concentration) and the change in fluorescence recorded. The calcium ionophore A23187 (1–2 μ*M* final concentration) is used as a positive control **(Fig. 5)**.

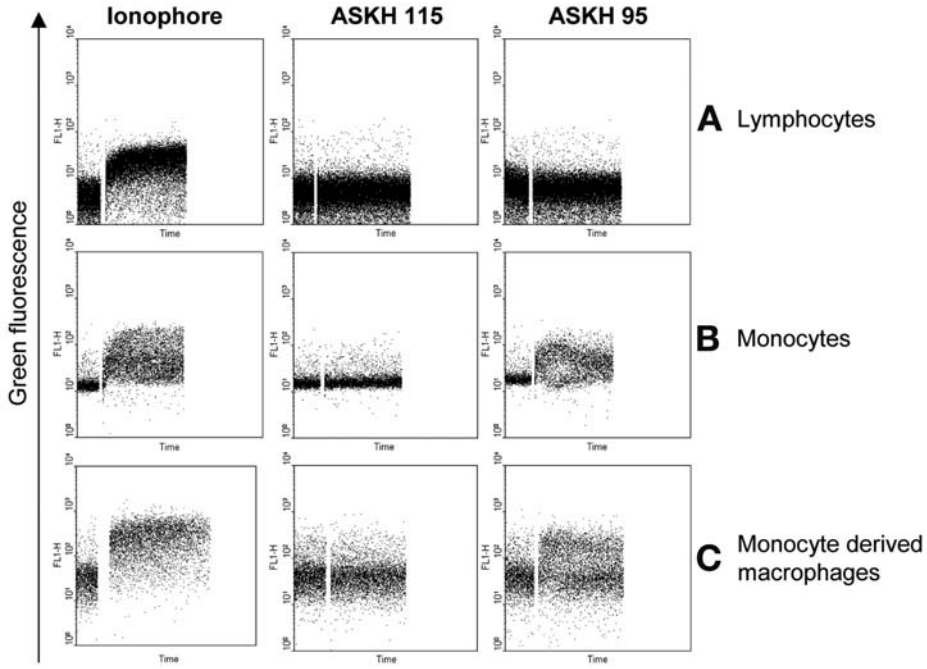


**Fig. 5.** Activation of monocyte and macrophage PAR2 results in calcium flux. Lymphocytes, purified monocytes, and monocyte-derived macrophages were loaded with Fluo 3 and tested for calcium flux generation in response to 1 μ*M* calcium ionophore, the PAR2 activating peptide ASKH 95, or control, ASKH 115 (both at 100 u*M*), using flow cytometry.

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# 10

# Further Functional Studies

**Marion G. Macey**

**Summary**

The assessment of cell surface antigen expression on cells is probably the most common use of flow cytometry. However, cells may be interrogated in many other ways by flow cytometry to measure functional properties. The change in expression of receptors with time may be assessed or the interaction of one cell type with another may be measured. In addition, changes in enzyme activity and membrane potential may be assayed. Experiments to show phagocytosis and release of bioactive molecules may also be performed.

**Key Words:** Cell–cell interactions; functional antigens; receptor signaling.

## 1. Introduction

In this chapter, some of the numerous assays for assessing cell function are described. Lymphocytes, monocytes, neutrophils, dendritic cells, and macrophages are important in the immune surveillance of microorganisms and malignant cells. All cell types are capable of secreting a variety of chemical mediators, and some are able to phagocytose particulate matter. Leukocyte recruitment to sites of infection is guided by complex phenomena that involve microbial emanations, cytokines, interleukins (ILs), chemokines, and microenvironment modifications of the vascular endothelium. The interaction between molecules on the surface of the leukocyte (CD11b and CD62L) with those on the endothelium (ICAM-1, CD62P, and CD62E) is critical for recruitment and attachment. After adherence to endothelium and prior to vascular emigration, monocytes and neutrophils undergo a rapid cytoskeleton rearrangement that is necessary for chemotaxis and for exerting their phagocytic properties against microorganisms. This latter step depends on the expression of specific receptors (FcRs, CD11b, CD14, and CD35) that recognize opsonized (immunoglobulin-coated) microorganisms and particles.

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Micoorganisms and cellular material are phagocytosed into phagolysosomal vacuoles where they are subjected to strong oxygen-dependent micobicidal systems characterized by the so-called reduced nicotinamide adenine denucleotide (NADPH)-dependent respiratory burst and oxygen independent systems, including degrading enzymes, defensins (peptide antibiotics), and cathepsin G.

In addition to the their beneficial role, leukocytes (in particular, monocytes) may be involved in the pathogenesis of several noninfectious diseases such as acute and chronic myocardial ischemia, idiopathic pulmonary fibrosis, emphysema, rheumatoid arthritis, and certain forms of glomerulonephritis. The tissue-damaging properties arise when phagocyte activation mediated by upregulated membrane molecules results in degranulation with release of myeloperoxidase, elastase, and other proteases and by intermediate oxygen radicals.

One of the main obstacles to studying leukocyte function has been the difficulty encountered in (1) the transport of samples that require immediate analysis, (2) rapidly handling cells from peripheral blood and other sources such synovial fluid, bronchiolar lavage, and cerebrospinal fluid, (3) working with small volumes of blood or with blood from leukopenic individuals, (4) the complexity of some tests, and (5) processing several samples in a single work session. Many of these problems may be overcome by bringing the patient to the proximity of the laboratory and by using simple rapid assays to investigate the cells. Flow cytometry allows rapid assessment of the maturational stage of cells; expression of functional antigens, adhesion molecules, and receptors; priming; response to cytokines, chemoattractants, and activators, phagocytosis; and cell–cell interactions.

## 2. Expression of Functional Antigens and Receptors on the Cell Surface

The final array of mature membrane receptors is by no means static on all cells. Many cells can change the expression and number of surface antigens after stimulation. The baseline expression of these antigens and the modifications that they undergo regulate the interaction of the cells with other cells, recruitment to sites of inflammation, chemotaxis, reactivity toward agonists, and degranulation. Monocytes and neutrophils express numerous receptors, including those for growth factors (granulocyte colony-stimulating factor [G-CSF] and granulocyte monocyte colony-stimulating factor [GM-CSF]), ILs (IL-1, IL-6, and IL-8), cytokines (interferon-γ [IFN-γ] and tumor necrosis factor [TNF]), complement components (C5a, C3b, and C3bi), agonists, N-formylmethionyl-leucyl-phenylalanine (fMLP), leukotriene B4 (LTB4), platelet-activating factor (PAF), histamine, adenosine, lipopolysaccharide (LPS), and Fc fragments of immunoglobulin G (IgG) and IgA (CD16, CD32, CD64, and CD89). mAbs are available to most of these molecules and other antigens, such as those associated with adhesion, and allow recognition of the presence and level of expression of these cell surface receptors. Such molecules may be easily identified in whole-blood preparations

by the method first described by McCarthy and Macey ***(1)***. This method is extremely rapid (10–15 min from venesection to analysis) and also avoids the influences of isolation procedures ***(1–9)*** which may result in artifactual changes (in antigens) such as increases in expression of the integrin CD11b or loss of expression of the selectin CD62L.

### *2.1. Whole-Blood Procedure for the Analysis of Leukocytes*

1. Aliquots (5–10 µL) of anticoagulated whole blood are incubated at 4°C with a red fluorescent nuclear dye (LDS-751 or DRAQ5) and either fluorescein- or phycoerythrin (PE)-conjugated mAbs specific for adhesion molecules or receptors.
2. After incubation for 5–10 min, the blood is diluted to 1 mL with HEPES (10 m*M*)-buffered Hanks' balanced salt solution (HBSS) and analyzed by flow cytometry immediately.
3. Leukocytes are identified in a dual-parameter dot plot of side scatter (SSC) (*y*-axis) and log-red fluorescence (*x*-axis). Neutrophils and monocytes may be gated and the expression of the molecule of interest analyzed (**Fig. 1**).

## 3. Receptor Signaling

### *3.1. Signaling Pathways*

Cells express a variety of plasma membrane receptors that allow the recognition of and response to a variety of compounds in an inflammatory environment, including bacterial products such as formyl peptides and LPS, components of the complement and clotting cascades, and soluble factors such as cytokines released by other cells. Receptors can be divided into several groups based on their structure and on the signaling pathways to which they are linked (**Table 1**). Upon ligand binding, the receptor is activated and a signal is transmitted to the cell interior, resulting in the initiation of a cascade of intracellular events.

Receptors for the classical "chemoattractants" (named for their ability to stimulate chemotaxis) such as bacterial products (formyl peptides), products of the complement cascade (C5a), phospholipid metabolites (PAF and LBT4), and cytokines such as IL-8 possess seven membrane-spanning domains and are functionally linked to heterodimeric G proteins. These receptors usually mediate more than chemotaxis, including degranulation and activation of NADPH oxidase. Binding of ligand to this class of receptors leads to activation of the closely associated G-proteins, which in turn activate downstream effector pathways involving an array of intracellular enzymes, including kinases and phosphotases.

In contrast to the seven transmembrane-spanning receptors described above, other receptors such as for growth factors and cytokines and those involved in phagocytosis are linked to the cell interior by pathways primarily involving tyrosine phophorylation. The receptor for platelet-derived growth factor has intrinsic tyrosine kinase activity, and receptors such as the GM-CSF receptor

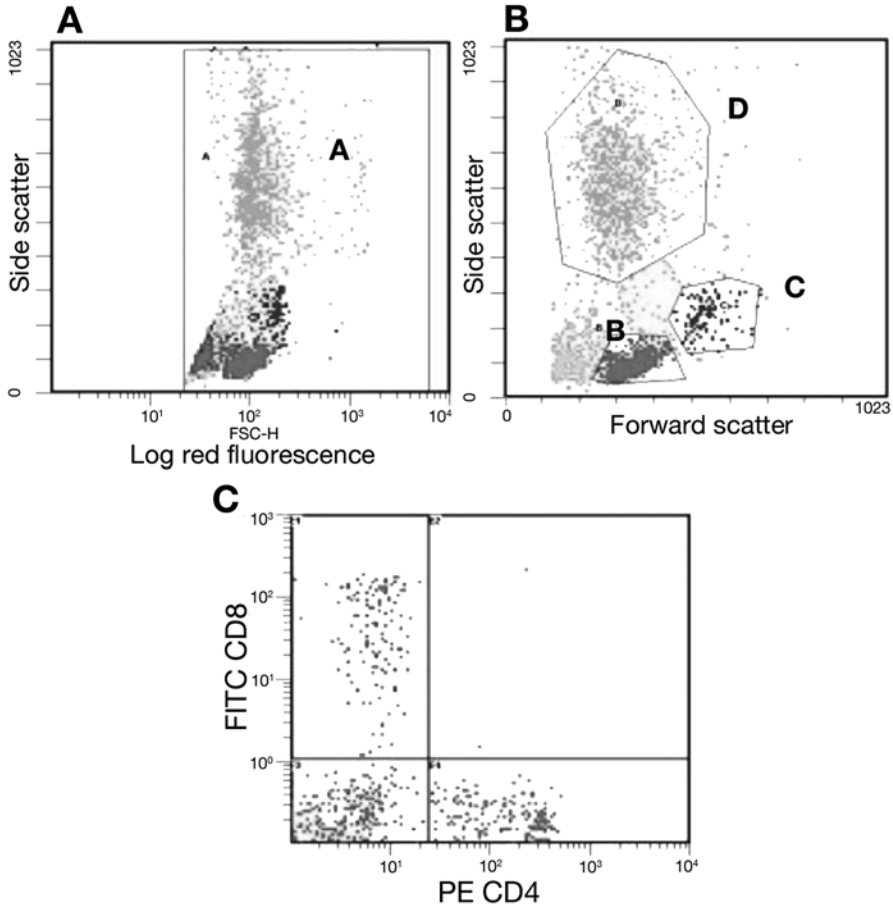


**Fig. 1.** Analysis of cells in whole blood. Nucleated cells in whole blood labeled with the red-fluorescent nuclear dye LDS-751 are identified in a plot of side scatter versus log-red fluorescence region A in histogram **(A)**. Events falling outside A are excluded from analysis. Leukocytes falling within A are backgated to a histogram of forward scatter versus side scatter in which lymphocytes, monocytes, and neutrophils may be distinguished: regions B, C, and D, respectively, in histogram **(B)**. The fluorescence associated with cells in each region may be assessed in dual- or single-parameter histograms. Histogram **(C)** shows the analysis of PE-CD4 and FITC-CD8 on lymphocytes gated in region B.

may be closely associated with cytosolic tyrosine kinases (such as p72syk) that localize to the cytosolic side of the plasma membrane ***(10)***.

Despite the diversity of receptors, the basic activating mechanisms used are limited in number and are shared by various receptors. Often, the binding of

**Table 1**
**Membrane Receptors in Leukocytes That May Participate in Signaling**

| Receptor grouping | Examples | Structural characteristics | Comments |
|---|---|---|---|
| Group 1: G-protein-linked | fMLP, C5a, PAF, LTB4, IL-8, chemokines | Seven transmembrane-spanning domains (serpentine) | Linked to heterodimeric GTP-binding proteins. α and βγ subunits of G-proteins may transduce signals. |
| Group 2: Membrane tyrosine kinase | PDGF | Integral membrane proteins, intrinsic tyrosine kinase activity | Ligation leads to receptor dimerization and cross dimerization and cross (auto) phosphorylation. Adaptor molecules involved via SH2 domains |
| Group 3: Tyrosine-kinase linked | RcγRIIa, GM-CSF | RcγRII is a member of the immunoglobulin family of receptors | Linked to cytosolic tyrosine kinases (src family, including hck, fgr, and perhaps Janus family kinases [JAK]) |
| Group 4: GPI-linked | RcγRIIIb, decay accelerating factor (DAF) | These receptors have no intracellular domain | RcγRIIIb is involved in phagocytosis |
| Group 5: Adhesion molecules selections | β2 integrins (CD11a/CD18, CD11b/CD18 CD11c/CD18) L-selectin | β-integrins have a heterodimeric structure with relatively long cytoplasmic tails L-selectin has an extracellular lectin-binding domain and a very short cytoplasmic tail | Potentiation of the oxidative burst and phagocytosis in adherent cells [$Ca2^{+}$]i, actin cytoskeletal changes, MAP kinase activation, gene expression |
| Group 6: Ceramide-linked | TNF | Two TNF receptors have been cloned, both single membrane-spanning glycoproteins with homology to receptors for nerve growth factor and Fas (CD95) | Tyrosine phosphorylation, activation of membrane-bound sphingo-myelinase with generation of ceramide that in turn activates a 96-kDa protein kinase |

ligands leads to dimerization or clustering of receptors, especially for multivalent ligands on surfaces. Receptor dimerization exposes the receptor or associated proteins to the enzymatic activity of the other receptor (or associated kinase) of the dimer. This is the mechanism involved in growth factor and cytokine receptor signaling. Other receptors undergo a conformational change upon ligand binding which activates enzymatic activity of the receptor or associated proteins (such as heterodimeric G proteins for the fMLP receptor). Other receptors such as the protease-activated receptors (PARs) may be cleaved to expose a tethered ligand that facilitates signaling. Occupation of receptors that are themselves ion channels or are closely associated with ion channels can lead to activation of ion fluxes. This is a common mechanism in excitable tissues such as neurons but may also be relevant to other cells.

### 3.2. Signaling Events Measured by Protein Phosphorylation

Signaling events in single cells may also be monitored by flow cytometry by using intracellular phospho-protein staining techniques. Measurement of protein phosphorylation with phospho-specific antibodies has given insight into kinase signaling cascades within cells after stimulation. Several groups have demonstrated staining of phospho-epitopes for flow cytometric analysis ***(11)***. Among the molecules previously examined are Stat1 ***(12,13)***, Stat4 ***(14)***, Akt ***(15,16)***, ERK and MEK ***(17)***, cJun and p38 ***(18)***, and various others ***(19)***. The methods used to prepare cells for staining with phospho-specific antibodies differed in each case, but they generally employed a fixation step with formaldehyde, followed by permeabilization with alcohols, detergents, or saponin. Because many of the epitopes to be recognized are novel and might be sequestered in protected locales within cells, it remains unclear whether there exists a general method (or set of methods) by which most phospho-epitopes can be stained and analyzed. For the success of such a protocol, two critical parameters must be met: (1) the initial cell-fixation step must be rapid and effective in "freezing" the phosphorylation status of proteins, and (2) permeabilization steps must allow antibody access to their cognate epitopes, in the proper natured or denatured conformation, for binding. Krutzik and Nolan ***(20)*** developed a generally applicable protocol as described below, which benefits multicolor, multiparameter analysis and is applicable for use as new phospho-specific antibodies are created.

### 3.3. Assay for Signaling Events Measured by Protein Phosphorylation

1. Treated and untreated cells are fixed by adding 16% formaldehyde directly into the culture medium to obtain a final concentration of 1.5% formaldehyde.
2. Cells are incubated in fixative for 10 min at room temperature and pelleted. They are then permeabilized by resuspending with vigorous vortexing in 500 µL of ice-cold methanol per $10^6$ cells (this is an approximate number, and more or less

methanol can be used as long as evaporation is not significant) and incubated at 4°C for at least 10 min.
3. At this point, cells can be stored at −20°C for several weeks with minor loss in signal strength. Cells are washed twice in staining media (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA]) and then resuspended in staining media at $0.5–1 \times 10^6$ cells per 100 µL.
4. Optimal concentrations (typically approx 50 ng) of fluorophore-specific mAbs are added and incubated for 15–30 min at room temperature. The cells are washed with 15 volumes of staining media and pelleted. Finally, samples are resuspended in 100 µL of staining media and analyzed.

## 4. Priming and Activation

Priming refers to a process whereby the response of cells to an activating stimulus is potentiated, sometimes greatly, by prior exposure to a priming agent (**Table 2**). Neutrophil and monocyte priming by agents such as TNF-α, G-CSF, GM-CSF, and LPS causes a dramatic increase in the response of these cells to an activating agent. This process has been shown to be critical for phagocyte-mediated tissue damage both in vitro and in vivo. The principal consequence of priming (aside from direct effects on cell polarization, deformability, and integrin/selectin expression) is to permit secretogogue-induced superoxide anion generation, degranulation, and lipid mediator release (LTB4 and arachadonic acid). It is now recognized that most priming agents also serve an additional function of delaying apoptosis and hence increasing the functional longevity of these cells at sites of inflammation. The mechanisms underlying priming include changes in receptor number and/or affinity, G-protein expression, phospholipase C and phospholipase A2 activation, changes in intracellular $Ca2^+$ concentration, protein tyrosine phoshorylation, and enhanced phospholipase D, and phosphoinositide 3-kinase activity. Recent studies have also revealed that it is possible for neutrophils to spontaneously and fully de-prime after an initial challenge with, for example, PAF.

Although priming was initially described as an in vitro phenomenon, many priming agents have clear biological relevance in vivo and are released in response to infection, trauma, and hemorrhage. For example, circulating endotoxin has been associated with the development of adult respiratory distress syndrome ***(21)***, and persistent high levels of plasma TNF-α and IL-6 have been linked to poor outcome in septic shock ***(22)***. Although such cytokines are detectable in the bloodstream only in extreme circumstances, locally generated mediators serve to upregulate the functional responses of extravasated neutrophils or monocytes; indeed, the crosslinking of adhesion molecules is itself a priming stimulus ***(23,24)***, and the process of extravasation per se may result in a degree of priming ***(25)***. Priming may be investigated by flow cytometry by assessing changes in adhesion molecule expression and forward light scatter ***(26,27)***.

**Table 2**
**Priming Agents**

| Priming agent | Time to induce maximal priming | Reference |
|---|---|---|
| ATP | 15 s | Kuhns et al., 1988 |
| Substance P | 1 min | Lloyds et al., 1993 |
| Ionomycin | 2 min | ***(23)*** |
| Inositol hexakisphosphate | 2 min | Eggleton et al., 1991 |
| L-selectin crosslinking | 3 min | Waddell et al., 1994 |
| PAF | 5 min | Vercellotti et al., 1988 |
| CD18 crosslinking | 5 min | ***(24)*** |
| TNF-α | 10 min | Berkow et al., 1987 |
| Interleukin-8 | 10 min | Daniels et al., 1992 |
| Orthovanadate | 10 min | Lloyds et al., 1994 |
| Influenza A virus | 30 min | Busse et al., 1991 |
| LPS | 120 min | Guthrie et al., 1984 |
| GM-CSF | 120 min | Weisbert et al., 1986 |
| IFN-γ | 120 min | Tannenberg et al., 1993 |

ATP, adenosine triphosphate; GM-CSF, granulocyte monocyte colony-stimulating factor; IFN-γ, interferon gamma; LPS, lipopolysaccharide; PAF, platelet-activating factor; TNF-α, tumour necrosis factor-α.

**Figure 2** shows the effect of using fMLP to prime neutrophils prior to the addition of a peptide that activated the PAR-2 that is expressed by neutrophils.

## 5. Prolonged Responses to Cytokines and/or Hormones

Leukocytes produce a number of proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-12, IFN-α, IFN-γ, and IL-6) and anti-inflammatory cytokines (IL-1RA and transforming growth factor-β) in response to various agonists, including some of these cytokines. Studies of how IL-10 and IFN-γ affect cytokine and chemokine production in LPS-stimulated neutrophils have revealed two distinct phases. In the early phase, there is a low level of chemokine release, directly induced by the LPS. This is followed by a second delayed phase, in which endogenous TNF-α and IL-1β synergize with LPS in producing dramatically elevated levels of IL-8, macrophage inhibitory protein-1α (MIP-1α), MIP-1β, and growth-related gene product-α. This sequential production of chemokines by LPS-activated neutrophils, which is regulated by TNF-α and IL-1β, may serve to amplify the recruitment and activation of neutrophils and other leukocytes in vivo during an inflammatory response to LPS. IL-10 has also been shown to be a potent inhibitor of MIG induced by IFN-γ and LPS. By regulating neutrophil-derived cytokine production, IL-10 may have an important regulatory

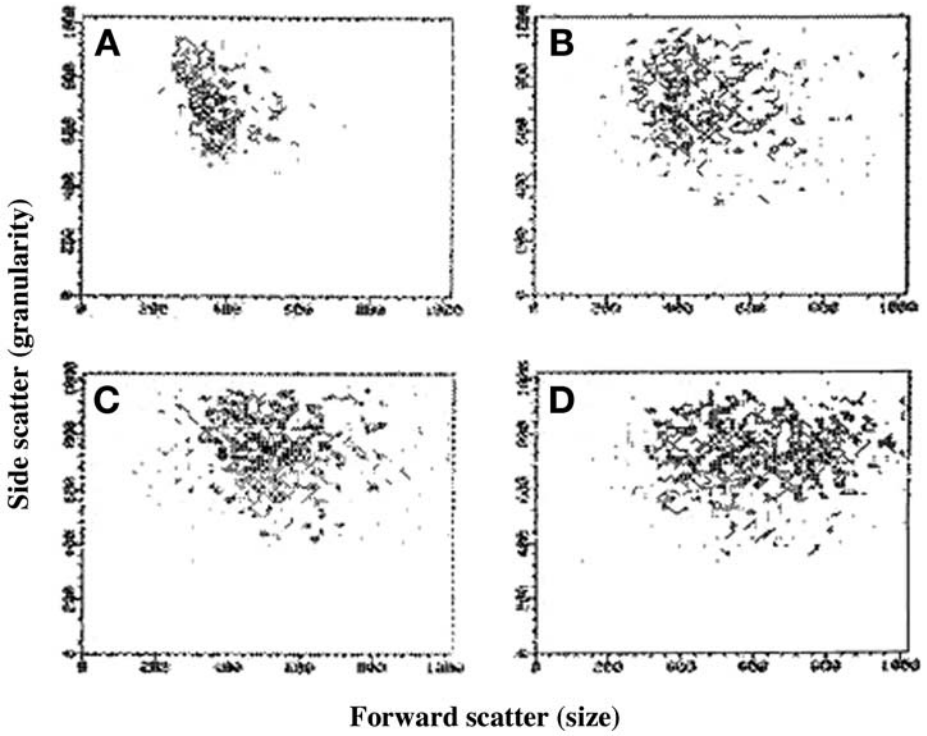


**Fig. 2.** Illustrates the effect on cell size of fMLP priming and PAR-2 activating peptide. Neutrophils were identified as in histogram A of **Figure 1** and backgated to a histogram of forward scatter (FSC) and side scatter (SSC). Histogram A shows the light scattering properties of resting, unstimulated neutrophils. Upon addition of fMLP, the neutrophils swell and have increased forward scatter (histogram B). Addition of peptide that activates PAR-2 also stimulates an increase in size and forward scatter **(histogram C)**. **Histogram D** shows the effect of priming the neutrophils with fMLP before addition of the activating peptide. There is an augmented increase in size and forward light scatter.

role in limiting the duration and extent of acute inflammatory responses (e.g., in lethal endotoxemia). Marie et al. ***(28)*** suggest that IL-10 produced during sepsis ***(29)*** reduces the production of IL-8 and may render neutrophils unresponsive to further stimulation by LPS. IL-4 and IL-13 have also been shown to have an inhibitory affect on neutrophil IL-8 production in the presence of LPS ***(30)***. The intracellular production of cytokines induced by culturing leukocytes with cytokines may be analyzed by flow cytometry and is discussed further in **Chapter 8**. The presence of cytokines both in vitro and in vivo also influences the expression of certain cell surface molecules such as such as CD69 (very early), CD71 (early), CD25 (late), and HLA-DR (very late) on lymphocytes ***(31)***; CD14, CD64, CD83, and chemokine receptors on neutrophils; CD143 and

CD163 on monocytes *(32)*; and CD23, CD25, CD69, CD105, and CD153 on macrophages *(33)*. Recently, cytokine-activated neutrophils with enhanced expression of cell surface molecules have been shown to become as competent as dendritic cells and macrophages in their ability to undertake antigen presentation *(33,34)*.

Hormones such as growth hormone have been shown to reduce phagocytic and metabolic function in neutrophils. This is the result of reduced TNF production *(35)*. Glucocorticoids have also been shown to block PAF-induced downregulation of CD62L and upregulation of CD11/CD18 on neutrophils. This suggests that ligation of glucocorticoid receptors has an anti-inflammatory effect on cells by the inhibition of leukocyte accumulation at sites of tissue injury *(36)*.

### 6. Shape Changes

Several aspects of phagocyte physiology, including motility, phagocytosis, translocation of granules, degranulation and recycling of receptors, and their regulation, are associated with changes in the organization of the cytoskeleton. Upon activation, changes in the cytoskeleton arrangements occur, and actin polymerization (G-actin assembly to form F-actin filaments) represents one of the most dynamic phenomena that characterize phagocyte activation *(37)*. Actin polymerization may be measured by flow cytometry using phalloidin, a fluorescent peptide with a high affinity for polymerized actin *(38,39)*. Both NBD (nitro blue diazonium)-phallacidin and fluorescein isothiocyanate (FITC)-phallacidin facilitate the measurement of the global amount of F-actin with high sensitivity. Flow cytometry, however, does not make a distinction between the two pools of F-actin molecules described as triton-soluble and triton-insoluble *(40)*. Before staining with phalloidin, phagocytes have to be permeabilized.

Forward scatter (FSC) and SSC may also be used to detect shape changes. FSC is proportional to size, and when neutrophils are activated in suspension, they swell and exhibit increased FSC *(41)*. Keller et al. *(42)* showed that there is a direct relationship between light scattering in flow cytometry and changes in shape, volume, and actin polymerization. Shape change has been used to measure the effect of a variety of chemokines on the polarization of eosinophils *(43)*.

### 7. Chemoattractant Binding and Rapid Responses to Chemotaxins/Activators

Monocytes and neutrophils are activated by and respond by moving toward molecules termed chemotactic factors, including the complement component C5a, fMLP, LTB4, PAF, the neuropeptide substance P, and the phorbol ester phorbol myristate acetate (PMA); phagocytic particles; and substances released by microorganisms such as fungi, bacteria, and viruses. The magnitude and

time of response depends on the agonist used, but for most of these, the response time is in minutes rather than hours. Interaction of the cell with these agonists may be investigated through the analysis of the binding of fluorochrome-labeled molecules or by determining features of cellular activation such as cytokine production, surface antigen changes, shape change, or metabolic burst.

Flow cytometry may be used to count or phenotype cells before and after migration through human umbilical vein endothelial cells (HUVECS) grown on membranes. In this assay, the HUVECs are suspended in transwell tissue-culture inserts that are placed into wells in microwell plates. Medium is placed in the well below the insert. Cells are added to the wells and allowed to migrate. The nonmigrated and migrated cells are collected and counted by flow cytometry ***(44)***. The HUVECS may be pretreated with TNF, the migrating cells may be stimulated with agonists, and chemoattractants may be placed in the wells.

### *7.1. Migration Assay*

1. Early passage HUVECs are seeded onto fibronectin-coated 3 μ*M*-pore transwell tissue-culture inserts (Falcon; Marathon Laboratory Supplies, London, UK) at $1 \times 10^5$ cells per well as described previously ***(44)***.
2. HUVECs are allowed to adhere, and control wells are treated for 18 h with 10 ng/mL recombinant human TNF to maximize expression of adhesion molecules and thus leukocyte migration.
3. Control inserts that contain no HUVECs are included. Leukocytes added to these inserts fall through to the lower well and represent maximum migration.
4. TNF is removed, and the monolayers are washed with RPMI/10% fetal calf serum (FCS).
5. Freshly isolated leukocytes are counted, resuspended at $10^7$/mL, and pretreated with 100 μ*M* of stimulant or medium alone.
6. After 1 h, $5 \times 10^5$ leukocytes are added to HUVECs in the top of the transwell in triplicate. Six hundred microliters of RPMI/10% FCS is added to the lower part of the well (underneath the transwell chamber), and leukocytes are allowed to migrate through the HUVEC monolayer for 4 h at 37°C.
7. Supernatants are collected from the upper and lower chambers and placed in separate flow cytometry tubes. The number of migrated leukocytes is counted using a flow cytometer. This may be facilitated by adding beads of a known concentration (Spherotech, Inc., Libertyville, IL) or counting for a defined period of time (**Fig. 3**).

## 8. Membrane Potential and Changes in Ion Permeability

The generation of intermediate oxygen radicals after monocyte and neutrophil stimulation is accompanied by changes in membrane potential. Lipophilic dyes such as the cyanine compounds dipentyloxacarbocyanine ($DiOC_5[3]$) and dipropylthiocarbocyanine ($DiSC_5[3]$) can be used to measure this aspect of cellular activation. These dyes diffuse into the cell with different localization patterns depending on their concentration. Cellular activation is followed by a loss of

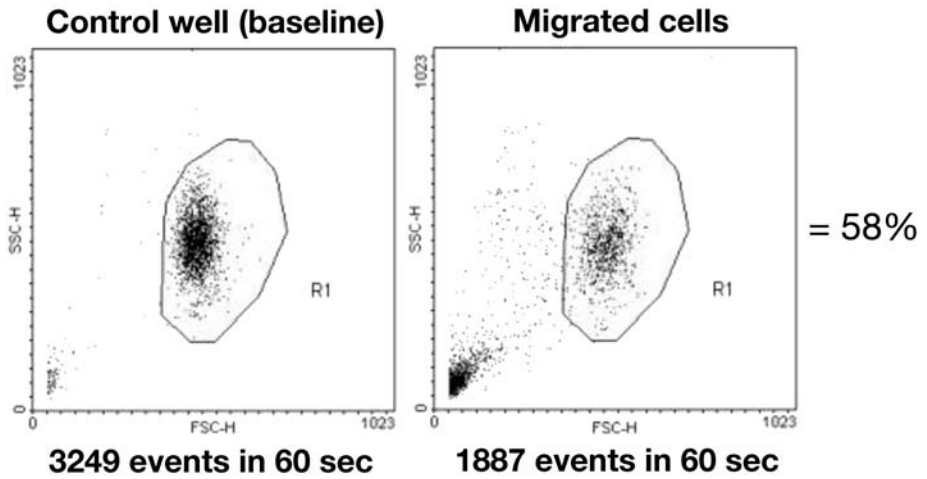


**Fig. 3.** Flow cytometric enumeration of **(left panel)** cells in control wells (no HUVECs) and **(right panel)** cells that have migrated through wells containing HUVECs. The number of events counted in 60 s was recorded. The control count represents the total possible number of migrated cells. The actual number of migrated cells is represented as a percentage of the total possible number.

cell-associated fluorescence, which is related to changes in membrane potential. Stimulation with PMA results in an irreversible loss of fluorescence caused by an irreversible membrane depolarization; however, stimulation with the chemotactic agents fMLP or C5a results in an initial loss of fluorescence with a nadir at approx 2 min, followed by a gradual return to the baseline level. A dose-dependent reduction in fluorescence during the initial phases of stimulation can be observed with increasing concentrations of the chemotactic stimuli. Due to the heterogeneity of the response to fMLP, a small proportion of neutrophils do not respond or do not show the biphasic response to the stimulus when this peptide is used. These potentiometric cyanine dyes have been shown to be independent of the action of oxygen radicals and of mitochondrial activity, although they accumulate into these organelles.

The activation of phagocytic cells leads to an initial cytoplasmic acidification because of the generation of large amounts of protons by NADPH oxidase. As a consequence, proton-conducting channels and the $Na2^{+}$-$K^{+}$ antiport are activated, leading to sustained intracellular acidification. These two aspects of intracellular pH changes may be measured by flow cytometry. Intracellular changes of pH in response to either soluble or particulate stimuli may be monitored by means of the fluorochrome carboxy-seminapthorhodafluor-1-acetomethyl ester (SNARF-1/AM) ***(45)***. This dye, which is modified by elastase, is loaded into the cell and fluoresces in the orange region of the light spectrum. However, with

increasing pH, the fluorescence moves to the red region of the spectrum. A ratio of the red/orange fluorescence is calculated through the calibration of cells with high $K^+$ buffers of defined pH in the presence of nigericin, a polyether ionophore that carries monovalent cations across membranes with high specificity for $K^+$. A 575- to 595-nm band-pass filter and a 620-nm long-pass filter have to be used to evaluate acidic orange SNARF-1/AM and basic SNARF-1, respectively.

### 9. Phagocytosis, Endocytosis, and Oxidative Burst

The phagocytosis of fluorochrome-labeled bacteria, yeast, zymosan, or beads may be readily assessed by flow cytometry. The particles should be opsonized with human serum Igs, and phagocytosis is evaluated by measuring the change in fluorescence of the neutrophil after introduction of the target particle. This evaluation is based on both the calculation of the percentage of neutrophils with intracellular fluorescence and the intensity of fluorescence, which has been shown to correlate with the number of phagocytosed particles.

General variables that can affect this assay are the incubation temperature and the agitation frequency. Different times of incubation can be used to study the kinetics of the phagocytic process and its initial rate (first 10 min). Opsonization with either homologous or heterologous serum may be carried out to distinguish between defects that are cellular and/or plasma-related. FITC-conjugated nonopsonized *Escherichia coli* is now commercially available to facilitate such studies. A specific variable is the bacteria-to-neutrophil ratio because the rate of phagocytosis is dependent on the number of targets available. Quenching performed by trypan blue or other dyes is useful to distinguish between internalized and cell-bound fluorescent particles given that the fluorescence from the latter is quenched in the presence of trypan blue.

Uptake by pinocytosis and trafficking through the endocytic pathways may be facilitated by using fluorescent dextran conjugates that are inert sugar polymers that are readily endocytosed ***(46)***. The localizing of lysosomes in live cells is made possible by staining with dyes termed LysoTrackers™ (Invitrogen, Carlsbad, CA), which have high selectivity for acidic organelles ***(47)***.

An increased oxygen uptake accompanies phagocytosis when stimulated by a number of agonists. The various metabolic changes that oxygen undergoes after reduction by a single electron bring about the so-called respiratory or oxidative burst. The reactions of this oxygen pathway are catalyzed by a membrane-bound NADPH oxidase that is formed from the assembly of membrane (cytochrome) and cytoplasmic components. Several intermediate, highly reactive oxygen radicals such as superoxide anion, hydrogen peroxide ($H_2O_2$), hypochlorous acid, hydroxyl radical, and singlet oxygen are produced ***(48)***. These radicals are important effectors of both the antimicrobial activity and the tissue-damaging

properties displayed by human neutrophils. Therefore, assessing the production of these radicals appears to be important in the diagnosis of chronic granulomatous disease, myeloperoxidase deficiency, sepsis possibly caused by immunosuppression, hematological diseases, and metabolic changes that neutrophils undergo in the course of diseases that are caused by excessive and uncontrolled neutrophil activation. Flow cytometry allows a rapid and sensitive intracellular measurement of some of these components of the oxidative burst, such as superoxide anion and $H_2O_2$. Intracellular superoxide production may be measured by the analysis of the direct action of this radical on hydroethidine (HE) ***(49)***. This blue fluorochrome is oxidized by superoxide anion to red fluorescent ethidium bromide (EB). Substrate loading must be analyzed at 420–460 nm and so requires a laser that emits in the UV region of the spectrum. The resultant EB emits at 600 nm ***(50)***. Upon stimulation and production of superoxide anions, there is an eight- to 20-fold increase in fluorescence in cells from normal individuals. $H_2O_2$ can also oxidize EB, and its activity may therefore interfere with superoxide measurement. Intracellular $H_2O_2$ production has been measured by using the oxidation-dependent fluorescence of several dyes. Brandt and Kerston ***(51)*** first described the formation of highly fluorescent 2′,7′-dichlorofluorescein (DCF) from nonfluorescent 2′,7′-dichlorofluorescin (DCFH), which is the product of the hydolysis of nonfluorescent DCFH diacetate (DCFH-DA). The formation of green fluorescent DCF depends directly on the action of $H_2O_2$ in the presence of peroxidase. Dihydrorodhamine 123 has also been used to analyze $H_2O_2$ generation. Upon oxidation by peroxide, this nonfluorescent dye transforms to the highly fluorescent green rhodamine 123 with maximum emission at 525 nm ***(52)***.

The oxidative burst associated with stimulation by Texas Red-labeled, opsonized bacteria has been described for dual-laser flow cytometry ***(53,54)***. This was modified for use on a single-laser flow cytometer ***(55)***, and two assays were used to measure the functional ability of the isolated neutrophils: (1) the rate of uptake of IgG-opsonized bacteria labeled with propidium iodide (PI) and (2) the oxidative burst associated with stimulation by either opsonized bacteria or the phorbol ester PMA. In the latter assay, the oxidative burst was measured by quantifying the increase in fluorescence associated with the oxidation of nonfluorescent DCFH-DA to the highly fluorescent DCF. The substrate DCFH-DA is a stable nonpolar molecule that readily diffuses through the cell membrane of the neutrophil. Once inside the cell, the acetyl groups are cleaved by enzymes in the cytoplasm to produce a polar molecule (DCFH), which is trapped within the cell. DCFH is nonfluorescent but becomes highly fluorescent when oxidized by $H_2O_2$, which is produced during the oxidative burst in neutrophils. The increase in fluorescence associated with the oxidation of DCFH is therefore a direct measure of the oxidative burst.

### 9.1. Assay Procedure

#### 9.1.1. Bacterial Culture

*Staphylococcus aureus* strain Wood 45 cultures (Protein A-negative; Code NCo7121; Central Public Health Laboratory, National Collection of Type Cultures, London) are maintained on agar slopes and plated on blood agar, and the colonies picked off into peptone water. Cultures are agitated at 37°C overnight. The cells are washed three times in HBSS without phenol red (Invitrogen, Carlsbad, CA, formerly Gibco) and fixed with 70% ethanol at 4°C for 30 min. The fixed cells are then washed twice in PBS containing 1% gelatine and 1% glucose (Sigma-Aldrich, St. Louis, MO) (PBSg) pH 7.2 and resuspended to a final concentration of $1 \times 10^8$ organisms per mL as determined by optical density measurement at 580 nm in a spectrophotometer.

#### 9.1.2. Opsonization of Bacteria

Bacteria $10^8$ in 1 mL of PBSg are mixed with 1 mL of (30 mg/mL) human serum and 1 mL of 0.2 *M* carbonate buffer pH 8.6 and then incubated with vigorous agitation at 37°C for 30 min. The bacteria are washed twice in PBSg and resuspended to a final concentration of $1 \times 10^8$/mL.

#### 9.1.3. PI Labeling of Bacteria

Bacteria $10^8$ in PBSg are incubated with PI (Sigma-Aldrich) at a final concentration of 50 μg/mL at 37°C for 30 min then washed twice with PBSg. The labeled bacteria are resuspended in PBSg to a final concentration of $10^8$/mL.

#### 9.1.4. Preparation of PMA

A stock solution of phorbol 12-myristate 13-acetate (Sigma-Aldrich) 1 mg/mL is made in absolute ethanol, aliquoted, and stored at −20°C. Immediately prior to use, the PMA is diluted in PBSg to a final concentration of 100 ng/mL.

#### 9.1.5. Preparation of DCFH-DA

A stock solution (10 m*M*) of DCFH-DA (Invitrogen, formerly Molecular Probes) is made in absolute ethanol and stored in the dark at 4°C. Immediately prior to use, the DCFH-DA is diluted in PBSg to a final concentration of 5 μM.

#### 9.1.6. Assay of Oxidative Product Formation

1. Neutrophils in whole blood (1 mL) are preincubated for 15 min with 5 μ*M* DCFH-DA in PBSg with agitation at 37°C.
2. After a 15-min incubation, 100-μL aliquots of cells are incubated in sterile round-bottom tubes with 100 μL, PBSg (negative control), opsonized bacteria at

a bacteria/cell ratio of 500:1 (test), nonopsonized bacteria at the same ratio (test control), and PMA at a final concentration of 100 ng/mL (positive control).
3. The tubes are incubated at 37°C with vigorous agitation.
4. The reaction is stopped by the addition of 2 mL of FACS (fluorescence-activated cell sorting)-Lyse for 10 min. The cells are then washed twice in PBSg, resuspended, and analyzed by flow cytometry.
5. Aliquots for each type of stimulation are set up in quadruple such that samples can be removed at times from 0–60 min after commencement of the assay.

### 9.1.7. Assay of Bacterial Uptake

1. Aliquots (100 µL) of blood are incubated in round-bottom tubes with 100 µL of PBSg (negative control), opsonized PI-labeled bacteria at a cell/bacteria ratio of 1:500 (test), and nonopsonized-labeled bacteria (test control).
2. Assays are set up in triplicate, and samples are removed, diluted 1:5 in PBSg, and analyzed by flow cytometry at 10, 20, and 30 min.
3. The reaction is stopped as above.
4. The rate of increase in red fluorescence per minute is taken to be directly related to the rate of uptake of bacteria.

### 9.1.8. Flow Cytometry

All studies are performed on a flow cytometer equipped with an argon laser (488 nm emission). Forward angle light scatter, 90° light scatter, and green (510–550 nm) and red (>580 nm) fluorescence are recorded. The assay for neutrophil stimulation is performed in parallel with that for bacterial uptake. Typical results of a metabolic burst assay are shown in **Fig. 4**.

## 10. Alternative Assays

A number of assays have been described for measurement of neutrophil phagocytosis by other techniques. The important point of distinction between internalized and membrane-bound particles was addressed by Fattossi et al. ***(56)***. In this assay, fluorescein-conjugated heat-killed *Candida albicans* were opsonized by purified antibodies and used as targets for human polymorphonuclear granulocytes (PMNs). The procedure is based on the observation that the target cells lose their green fluorescence upon incubation with EB through the resonance energy transfer phenomenon occurring between the two fluorochromes. PMNs are incubated with the opsonized target for 20 min at 4°C or 37°C in the presence of cytochalsin B, an inhibitor of the phagocytic process that does not affect membrane binding of fluorescein. EB is added, and the green and red fluorescence associated with PMNs is evaluated. EB does not penetrate intact cell membranes, so internalized particles are not affected by EB and remain green, whereas membrane-bound particles assume an intense red stain. By dual-fluorescence analysis, the number of PMNs containing and/or binding fluorescein-labeled targets can be assessed.

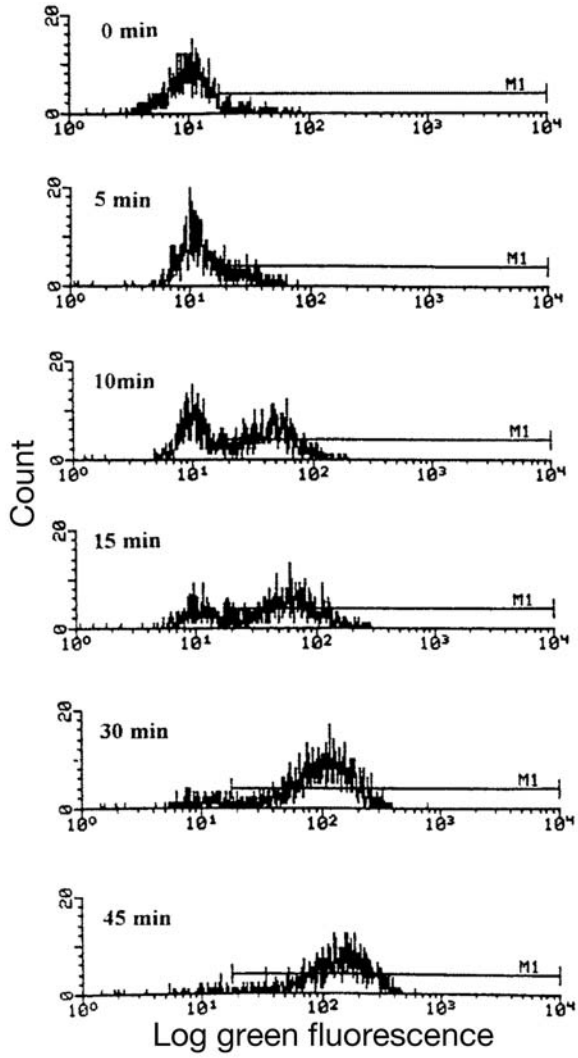


**Fig. 4.** The increase in fluorescence associated with the oxidation of DCFH-DA in neutrophils stimulated with *Staphylococcus aureus* over a 45-min time period.

The oxidation of HE to EB has been used to measure the oxidative metabolic burst in neutrophils stimulated with opsonized fluorescein-labeled bacteria ***(57)***. Dual-fluorescence analysis was used to measure the red EB emission together with the green fluorescence associated with ingested bacteria. In this assay, crystal violet was added to quench the fluorescence associated with membrane-bound bacteria such that only internalized bacteria were assayed.

The relative involvement, in phagocytosis, of receptors for the Fc portion of IgG and the complement components C3b and C4b has also been studied ***(58)***. Fluorescent latex beads coated specifically with IgG or complement components were used in this study. To evaluate bead attachment, the phagocytic assay was performed with 3 μ*M* cytochalasin D-treated cells, which inhibited internalization. The percentage of cells with ingested beads and the number of ingested beads per 100 cells were assessed. This allowed quantitative analysis of the function of cell surface receptors for IgG and complement components.

The fluorescent dye LDS-751 is specific for nucleated cells and has been used in whole-blood assays to eliminate erythrocytes from analysis of granulocyte oxidative metabolic burst ***(41,59)***. The detection of reactive oxygen species is facilitated by oxidation of DCFH-DA (as above). The effect of LPS on phagocytosis and metabolic burst has also been assessed in whole blood ***(60)***.

Rothe and Valet ***(51)*** developed a method based on the evaluation of green fluorescence emitted (1) by acridine orange during phagocytosis as a measure of ingested bacterial DNA and (2) indirectly by the amount of ingested bacteria.

## 11. Nitric Oxide Release

Nitric oxide (NO) plays a critical role as a molecular mediator of a variety of physiological processes, including blood pressure regulation and neurotransmission. However, because free NO is a transient species with a half-life of approx 5 s, investigations of this gaseous molecule have relied largely on studies of NO synthase (NOS). NOS catalyses the NADPH- and $O_2$-dependent oxidation of L-argenine to NO and L-citrulline. Specific inhibitors of NOS isoforms are available which may be used to investigate the biological effects of NO. NO is a highly reactive species that in the presence of oxygen is also reported to release superoxide anion. Under physiological conditions, NO is oxidized to nitrite and nitrate. The dye 2,3-daiminonaphthalene reacts with nitrite to form the fluorescent product 1H-naphthotriazole, and this dye has been used to detect nitrite at concentrations between 10 n*M* and 10 μ*M*. The dihydofluoresceins, dihydrorhodamines, and dihydrorosamines that are used extensively to detect various forms of oxygen are known to react with NO, yielding the same oxidation products. NO also reacts with superoxide or $H_2O_2$ to produce the peroxynitrite anion, which oxidizes dihyrorhodamine 123. Continuous fluorimetric detection of NO in the presence of other reactive oxygen species is also feasible. 1.2-Diaminoanthraquinone is nonfluorescent until it reacts with NO to produce a red fluorescent precipitate ***(62)***.

## 12. Multiparameter Techniques to Assess Function and Phenotype

The simultaneous evaluation of two or more parameters is readily assessed by flow cytometry. Phagocytosis, $H_2O_2$ production, and L-selectin shedding

can be simultaneously measured using PI-labeled bacteria, DCFH-DA, and PE-conjugated CD62L ***(41)***. HE and DCFH-DA can be used together to measure superoxide anion and $H_2O_2$ at the same time ***(49)***, and the simultaneous measurement of intracellular phagosomal pH changes, phagocytosis, ingestion, and degradation of bacteria has also been reported ***(49,63)***. **Figure 5** illustrates the effect of fMLP on the size, metabolic burst, and CD62L expression on neutrophils.

## 13. Adhesion Molecules in Cell–Cell Interactions

Cell–cell interactions are a feature and function of all living cells. Examples include interactions of mobile cells such as ova and sperm and the solid coalescence of skin cells to form a watertight epidermis. Cell–cell interactions are of importance both in cell structure and cell function. Cell–cell interactions provide physical barriers (skin), boundaries (organ capsules), and tissue cohesion. They provide a mechanism of communication between cells (nervous tissue) and are the means of reproduction (the cell–cell interaction of ova and sperm). When these interactions occur in mobile cells (e.g., in the blood), they are the means of switching on, spreading, or regulating fundamental processes such as inflammation and coagulation.

Many cell–cell interactions are mediated via cell adhesion molecules (CAMs). CAMs are surface membrane structures designed or adaptable to binding with similar structures, termed ligands, on other cells. Many CAMs have now been identified, and their molecular structures have been elucidated. Based on structural homology, to date, six families of CAMs have been defined, but there are a number of new adhesion molecules that are yet to be grouped.

The families, so far, are the immunoglobulin family, the cadherin family, the integrin family, the selectins, the surface proteoglycan family, the sialomucin family, and a number of other that do not fit into these families. CAMs play an important role in signal transduction as well as mediating adhesion with other cells and matrix components. As such, CAMs may defined as morphoregulatory molecules that affect cellular processes, based on data about inside-out and outside-in signaling and signal transduction pathways. Most CAMs may also be found as soluble circulating proteins, and levels of these soluble CAMs have been found to be increased in inflammatory conditions.

## 14. Analysis of Cell–Cell Interactions

Our ability to examine adhesion molecules involved in cell–cell interactions in cells is greatly enhanced by flow cytometry (**Table 3**).

It is not possible to comprehensively discuss every adhesion molecule on every cell in which cell–cell interactions matter. Platelet–platelet, platelet–leukocyte, leukocyte–leukocyte, and leukocyte–endothelial interactions will be discussed below.

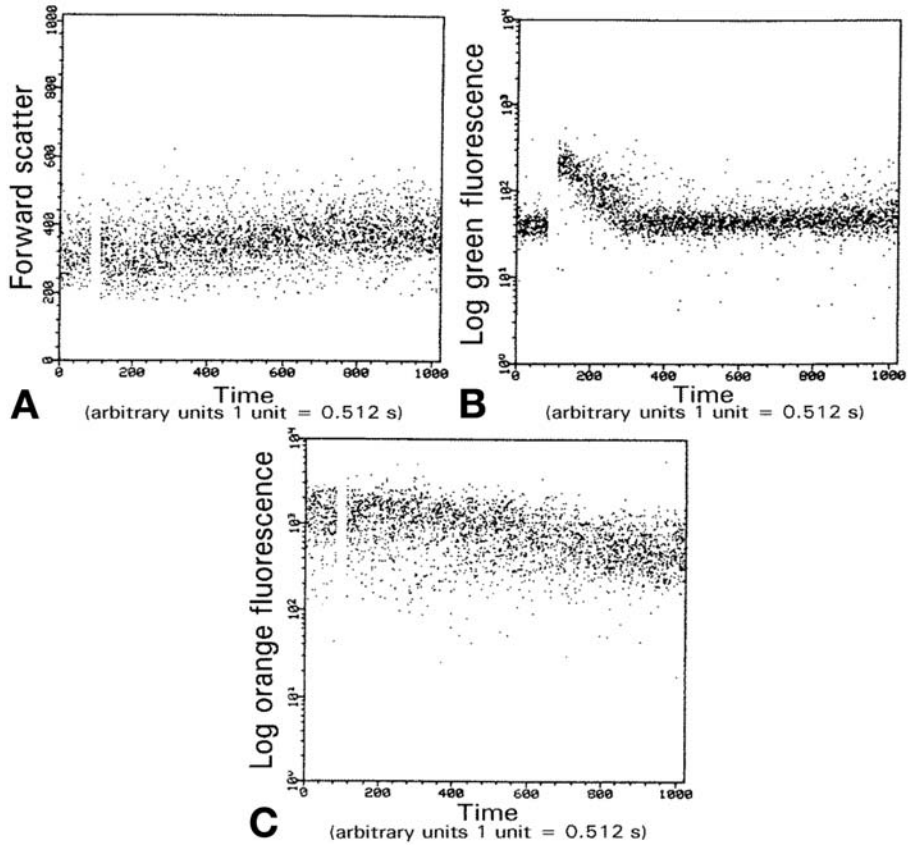


**Fig. 5.** The simultaneous continuous measurement of changes in size, calcium concentration, and L-selectin expression in neutrophils stimulated with the chemoattractant fMLP. Cells were labeled with Fluo-3, LDS-751, and PE-conjugated anti L-selectin (CD62L). Fluo-3 is a 488 nm-excitable $Ca2^{+}$ indicator that exhibits an increase in green fluorescence upon binding with free calcium. Neutrophils were identified as in histograms A and B of **Figure 5** above. Baseline levels of forward scatter **(histogram A)**, Fluo-3 **(histogram B)**, and phycoerythrin **(histogram C)** fluorescence were established in dual-parameter plots with time in seconds on the *x*-axis. At 60 s, the fMLP was added, thus producing a small gap in the histogram trace. **Histogram A** shows the small but gradual increase in size after addition of the fMLP. In **histogram B**, a marked increase in fluorescence is seen after addition of fMLP due to release of free calcium that binds to Fluo-3, resulting in a greater fluorescence emission. The green fluorescence rapidly falls back to the baseline level, producing a characteristic "calcium spike" associated with signal transduction. After activation, L-selectin is shed from the surface of the cell. This leads to a gradual loss of PE fluorescence as antibody bound to this adhesion molecule is also lost from the cell surface **(histogram C)**.

## 15. Platelet–Platelet Interactions

Increased platelet activation may lead to platelet aggregation and thrombosis. This can result in clinical syndromes such as myocardial infarction and stroke, both of which are major causes of morbidity and mortality. Reduced platelet–platelet interaction can result in bleeding problems, which although much rarer can also be serious.

Flow cytometry is increasingly used to accurately characterize the phenotypic alterations of platelets which are related to their cellular activation, hemostatic function, and maturation of precursor cells. More than 40 ***(64)*** molecules have been identified on the surface membrane of platelets, many of which change in relation to the activation state and to the function of the cell. Many of these are adhesion molecules, and the principal platelet adhesion molecules belong to the integrin and selectin families. The process of platelet activation is a receptor-mediated response of resting platelets to a variety of specific stimuli originating from activated proteins of the coagulation cascade (thrombin), subendothelial matrix proteins (collagen), or specific mediators such as ADP or PAF.

## 16. Flow Cytometric Analysis of Platelet Activation

### *16.1. Surface Membrane Changes*

Platelets express a variety of glycoproteins that are involved in adhesion and aggregation. Not all of these molecules are expressed on resting cells. It is functionally advantageous for some receptors to become expressed only on activated platelets (e.g., αIIb/ß3, which once expressed alters its molecular configuration to facilitate fibrinogen binding and platelet–platelet interaction that leads to thrombosis if sustained).

Platelet activation in vitro is associated with a decrease in the number of GPIb/IX complexes on the platelet surface, which are redistributed into the platelet canalicular system ***(65)***, and an increase in the number of αIIb/ß3 complexes on the platelet surface due to redistribution from intracellular pools such as storage organelle membranes ***(66)***. Exposure of the procoagulant surface is the result of a flip-flop in anionic phospholipids, predominantly phophatidylserine, from the inner to the outer leaflet of the membrane bilayer, which then forms a binding substrate for the prothrombinase complex. This change can be detected either by using antibodies to clotting factors Va or VIIIa ***(67)*** or by the binding of fluorochrome-labeled annexin V ***(68)***.

Upon activation, platelet surface αIIb/ß3 complexes undergo conformational changes generating neoepitopes detectable by using mAbs such as PAC-1 ***(69)***. The consequent changes in conformation can also be detected by monitoring changes in fluorescence energy resonance produced when two different mAbs directed against epitopes on the glycoprotein either are brought together or move

**Table 3**
**Cell Adhesion Molecules That May Be Measured by Flow Cytometry**

| CD number | Molecule | Family | Cellular expression |
|---|---|---|---|
| CD2 | Leukocyte function antigen-2 LFA-2 | Immunoglobulin | Leukocytes |
| CD9 | MRP-1 | Immunoglobulin | Platelet |
| CD11a | Leukocyte function antigen –1 (LFA-1) | Integrin α chain | Leukocytes |
| CD11b | Receptor for the C3bi complement component (C3biR) | Integrin α chain | Leukocytes |
| CD11c | Receptor for the C3 complement component (CR4) | Integrin α chain | Leukocytes |
| CD15 | Lewis X | Poly-N-acetyllactosamine | Leukocytes |
| CD15s | Sialyl-Lewis X | | Leukocytes |
| CD29 | GPIIa | Integrin β chain | Platelets, leukocytes, endothelial cells |
| CD31 | Platelet endothelial cell adhesion molecule – 1 (PECAM-1) | Immunoglobulin | Leukocytes, endothelial cells |
| CD34 | L-selectin ligand | Sialomucin | Leukocytes |
| CD35 | Receptor for the C1 complement component (CR1) | Regulator of complement activation | Leukocytes |
| CD41 | GPIIb | Integrin α chain | Platelets |
| CD42a | GPIX | Leucine-rich repeat | Platelets |
| CD42b | GPIb | Leucine-rich repeat | Endothelial cell |
| CD43 | Sialophorin | Sialomucin | Leukocytes |
| CD44 | Hyaladherin (H-CAM) | | Leukocytes |
| CD47 | Integrin-associated protein | Immunoglobulin | Platelets, leukocytes, endothelium |
| CD49 | Very late antigens (VLA) | Integrin α chain | Platelets, leukocytes, endothelium |
| CD50 | Intercellular adhesion molecule – 3 (ICAM-3) | Immunoglobulin | Leukocytes |

| | | | |
|---|---|---|---|
| CD51 | Integrin αV chain | Integrin α chain | Platelets, leukocytes, endothelium |
| CD54 | Intercellular adhesion molecule – 1 (ICAM-1) | Immunoglobulin | Leukocytes, endothelial cells |
| CD56 | Neuronal cell adhesion molecule (NCAM) | Immunoglobulin | Leukocytes |
| CD58 | Leukocyte function associated antigen – 3 (LFA-3) | Immunoglobulin | Leukocytes |
| CD61 | GPIIIa | Integrin β chain | Platelets |
| CD62E | E-selectin | Selectin | Endothelial cells |
| CD62L | L-selectin | Selectin | Leukocytes |
| CD62P | P-selectin | Selectin | Platelets |
| CD66 | Biliary glycoprotein | Immunoglobulin | Leukocytes |
| CD73 | Ecto-5′-nucleotidase | 5′-nucleotidase | Leukocytes |
| CD99 | MIC2 gene product. E2 antigen | Mucin | Leukocytes, endothelial cells |
| CD104 | Integrin β4 chain | Integrin β chain | Leukocytes, endothelial cells |
| CD106 | Vascular cell adhesion molecule – 1 (VCAM-1) | Immunoglobulin | Leukocytes, endothelial cells |
| CD144 | Vascular endothelial (VE)-cadherin | Cadherin | Endothelial cells |
| CD146 | Melanoma cell adhesion molecule (MCAM) | Immunoglobulin | Leukocytes, endothelial cells |
| CD147 | Intracellular matrix metalloproteinase inducer (EMMPRIN) | Immunoglobulin | Platelets, leukocytes, endothelial cells |
| CD151 | Platelet-endothelial cell tetra-span antigen (PETA-3) | Tetraspanin | Platelets, leukocytes, endothelium |
| CD162 | P-selectin glycoprotein ligand – 1 (PSGL-1) | Sialomucin | Leukocytes |
| CD164 | Mucin-like glycoprotein (MGC-24) | Mucin | Leukocytes |
| CD165 | Gp137 | Glycoprotein | Platelets, leukocytes |
| CD166 | Activated leukocyte adhesion molecule (ALCAM) | Immunoglobulin | Leukocytes |

(*Continued*)

**Table 3 (*Continued*)**

| CD number | Molecule | Family | Cellular expression |
|---|---|---|---|
| CD168 | Receptor for hyaluronic acid mediated motility (RHAMM) | Hyaladherin | Leukocytes |
| CD169 | Sialic acid-binding immunoglobulin superfamily lectins (Siglec)-1 | Immunoglobulin | Tissue macrophage |
| CD170 | Sialic acid-binding immunoglobulin superfamily lectins (Siglec)-5 | Immunoglobulin | Macrophage, DC, neutrophils |
| CD171 | Neural cell adhesion molecule (N-CAM) L1 | Immunoglobulin | Leukocytes |
| CD172 | Signal regulatory protein (SIRP) | Type I transmembrane protein | Leukocytes, DC, stem cells |
| CD222 | Mannose 6-phosphate/insulin-like growth factor 2 receptor M6P-R | Type I transmembrane protein | Leukocytes |
| CD227 | Mucin-1 (MUC-1) | Mucin | Leukocytes, stem cells, epithelium |
| CD242 | Intercellular adhesion molecule (ICAM)-4 | Blood group glycoprotein | Erythrocytes |
| CD301 | Macrophage galactose-type C-type lectins (MGLs), MGL1 | Lectin | Macrophage, immature DC |
| CD324 | Epithelial-cadherin | Cadherin | Epithelium, platelets |
| CDw325 | Neuronal-cadherin | Cadherin | Epithelium, fibroblasts |
| CD326 | Epithelial cell adhesion molecule (Ep-CAM) | Type I transmembrane glycoprotein | Epithelium |

apart *(70)*. Binding of specific ligands such as fibrinogen to αIIb/ß3 after activation can also be detected using mAbs or fluorochrome-conjugated fibrinogen *(72,73)*.

### 16.2. Organizational Events

Three membrane glycoproteins have been characterized in platelet lysosomes: the lysosomal integral membrane protein CD63 antigen (also designated gp53, ME491 antigen, pltgp40, or granulophysin) *(73)*, lysosome-associated protein (LAMP)-1 *(74)*, and LAMP-2 *(75)*. So far, three dense body integral membrane proteins have been identified: the CD63 antigen, a LAMP-2 protein primarily associated with lysosomes, and P-selectin associated with the α-granules *(76)*. Three major α-granule membrane proteins (GMPs) have been identified: P-selectin (CD62P), GMP-33, and $\alpha_{IIb}/\beta_3$ *(77)*. After platelet activation, CD62P and GMP-33 are expressed on the plasma membrane by fusion of the granule with the plasma membrane. Several α-granule membrane receptors, CD9, CD31, CD36, and GPIIb/IX/V complex, may also be detected by flow cytometry *(78)*. Release of dense granules may be detected by a decrease in mepacrine staining *(79)*.

## 17. Methods for the Analysis of Platelet Adhesion and Activation Molecules

### 17.1. Preanalytical Variables: Anticoagulant

Accurate assessment of in vivo or in vitro cellular expression of molecules requires optimal preanalytical conditions to prevent in vitro artifactual activation. The choice of anticoagulant is one of the critical preanalytical conditions because anticoagulants exert different effects on the activation of cells ex vivo. Historically, sodium citrate has been the favored anticoagulant for use in the studies of platelet activation and function, including aggregation and adherence. However, recent studies have shown that the anticoagulant CTAD (a mixture of sodium citrate, theophylline, adenosine, and dipyridamole) is better for retaining the ex vivo status of platelets *(80)*. But it should be noted that this anticoagulant is light-sensitive and, when exposed, stable for only up to 4 h *(81)*. In addition, when CTAD is combined with EDTA (ethylenediaminetetraacetic acid) and the blood held at 4°C, platelet activation after venesection is inhibited for at least 6 h after venesection *(82,83)*. The use of fixatives is best avoided given that these have been shown to cause platelet activation *(84)*.

### 17.2. Venepuncture for Flow Cytometry Samples

1. The patient should be seated with the arm extended and comfortably supported. Only a light tourniquet positioned above the antecubital fossa for a brief period should be used.

2. A 19-gauge needle is inserted into the antecubital veins, the tourniquet released, and blood venesected into a standard plastic syringe. The first 2 mL of blood is discarded. Vacutainers may be used.
3. The blood is immediately transferred to a glass tube containing citrate by gently dripping it down the side of the tube. The formation of bubbles is avoided at all stages. Samples should be placed in an upright rack and transported to the laboratory within 5 min.

### 17.3. Analysis of Platelet Surface Activation Antigens ($\alpha_{IIb}/\beta_3$)

The first antibody that recognized the activated conformation of the molecule glycoprotein $\alpha_{IIb}/\beta_3$ was developed in 1994. This antibody, PAC-1, is an IgM antibody that recognizes a sequence on the $\alpha_{IIb}/\beta_3$ molecule that is exposed on activation. It may not be used with samples that have been fixed with formaldehyde ***(85)***. The normal conformation of $\alpha_{IIb}/\beta_3$ is dependent on the presence of divalent ions (predominantly $Ca2^+$) at physiological concentrations ***(86)***. Removal of divalent ions results in an altered conformation, and therefore blood samples in EDTA are unsuitable for study of the activated form of this molecule. EDTA may in itself cause dissociation of the $\alpha_{IIb}/\beta_3$ complex ***(69)***.

The mAbs against $\alpha_{IIb}/\beta_3$ may be conjugated to fluorescent molecules and analyzed by flow cytometry. Directly conjugated IgG antibodies such as RUU-SP2.41 (Beckman Coulter) are now available. These reduce the manipulation needed to measure antibody binding to activated $\alpha_{IIb}/\beta_3$ on platelets because a directly conjugated antibody obviates the need for a washing step.

### 17.4. Analysis of P Selectin in Whole Blood

1. Blood (4.5 mL in EDTA/CTAD) is drawn using the standardized techniques as above and cooled to 4°C.
2. 5 µL of antibody is added to 90 µL of Tyrode's solution at 4°C.
3. 5 µL of the blood is added to tubes already containing diluted antibody. The final concentration of platelets is approx $1–2 \times 10^8$ mL, and the final concentration of antibody 5 µg/mL.
4. The agonist (at a final volume of 3 µL) is reconstituted at varying concentrations and added to each tube. This serves to maintain a constant concentration of antibody.
5. Blood is incubated for 5–10 min and then diluted for analysis with 900 µL of cold Tyrode's solution.
6. Appropriate positive and negative controls are used in each experiment.
7. Analysis is then performed immediately using log FALS and log 90° LS **(Fig. 6)**.

Note: Where there may be problems with the analysis of platelets due to cellular fragments, dual-labeling with FITC-CD42a (which is specific for platelets) and PE-CD62P may be performed to ensure that platelet gating is accurate. In this case, platelets may be gated in a plot of FITC-CD42a logarithmic scale on the *x*-axis and SSC logarithmic scale on the *y*-axis.

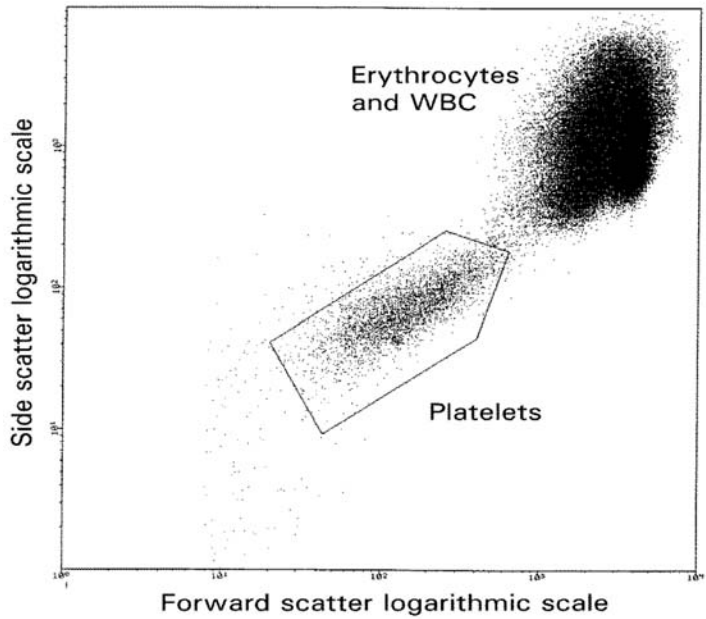


**Fig. 6.** The light scattering properties of platelets in diluted whole blood.

## 18. Platelet Microparticle Analysis

Activation by strong agonists such as collagen in combination with thrombin or complement (C5b-9) induces shedding of microvesicles from the platelet surface. This "budding" leads to vesicles that contain intracytoplasmic substances exclusively and are procoagulant and express CD40L and CD62P. Increases in platelet microparticles have been found in patients with sepsis or after cardiopulmonary bypass and are associated with thrombotic diseases ***(86)***. The procedure below is a modification of that first described by Bode et al. ***(87)*** and may also be found in Macey et al. ***(83)***. An alternative method is described in Amabile et al. ***(88)***, in which microparticle rich plasma is analyzed instead of whole blood.

### *18.1. Quantification and Characterization of Platelet Microparticles*

Blood (10 µL) preferably anticoagulated with EDTA/CTAD is incubated for 5 min at 4°C with FITC-isotype control (2l) and PE-isotype control (2l) or with FITC-CD42a (2l) and PE-CD62P (2l). Samples are diluted to 1 mL with HBSS-BSA containing LDS-751 (as above).

Unlabeled polystyrene spheres 1.09 m in diameter (at $1 \times 10^{-6}$ from the stock solution supplied Sigma-Aldrich) and EDTA/CTAD at 0.25 of the concentration used for preventing blood coagulation are added and analyzed immediately by flow cytometry.

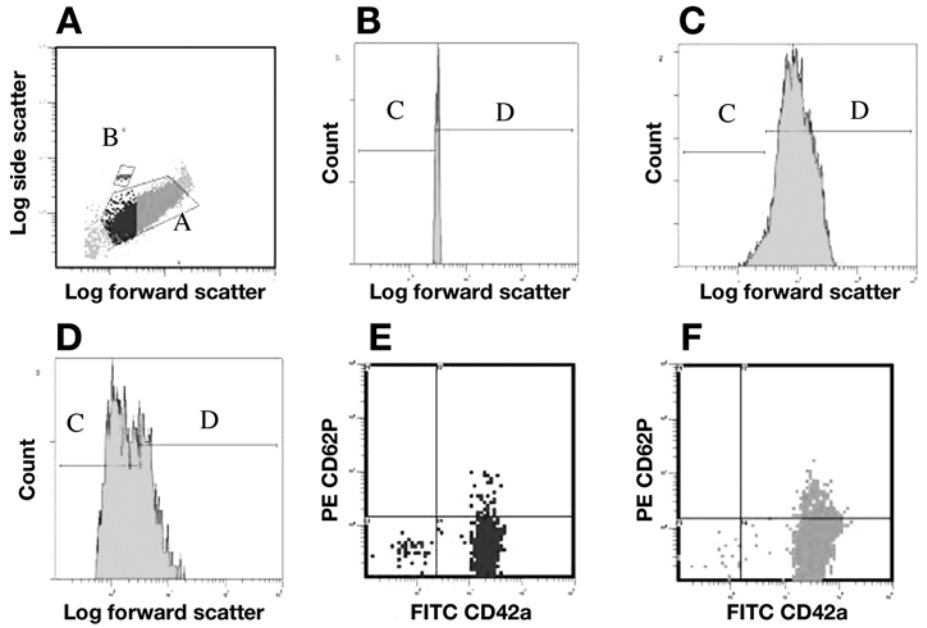


**Fig. 7.** Analysis of platelet microparticles and platelets. The blood sample was stained with fluorescein isothiocyanate (FITC)-conjugated CD42a and phycoerythrin (PE)-conjugated CD62P, and events were displayed in a plot of side light scatter (logarithmic scale, ordinate) and forward light scatter (logarithmic scale, abscissa; **dot plot A**). To assess CD62P expression and platelet and platelet microparticle numbers, one gate (A) was set to encompass platelets and putative platelet microparticles and another (B) was set to encompass just the 1.09-m-diameter polystyrene beads **(dot plot A)**. The events from A and B were counted and displayed in frequency histograms of forward light scatter (logarithmic scale); those from B are not illustrated in **plot B**, and those from A are shown in **plot C**. The value obtained for the mean forward light scatter signal of the 1.09-m-diameter beads (from B) was used to set the position of the cursor (in **plot B**) used to divide the events from A into putative microparticles (C) and platelets (D). **Plot B** shows the distribution of normal platelets, and **plot D** shows the distribution of aged blood (48 h), in which high numbers of microparticles have formed. The gated events in regions C and D are displayed in plots of PE-CD62P fluorescence (FL2; logarithmic scale, ordinate) versus FITC-CD42a fluorescence (FL1; logarithmic scale, abscissa; **plots E** and **F**, respectively). $CD42a^+$ events were considered microparticles **(plot E)** and platelets **(plot F)**, and the percentages of these that were also $CD62P^+$ and their mean fluorescence intensity were recorded.

Labeled samples are first displayed in a plot of SSC (logarithmic scale) versus forward light scatter (logarithmic scale) **(Fig. 7)**. Events with the light scatter characteristics of platelets and putative platelet microparticles are displayed in a

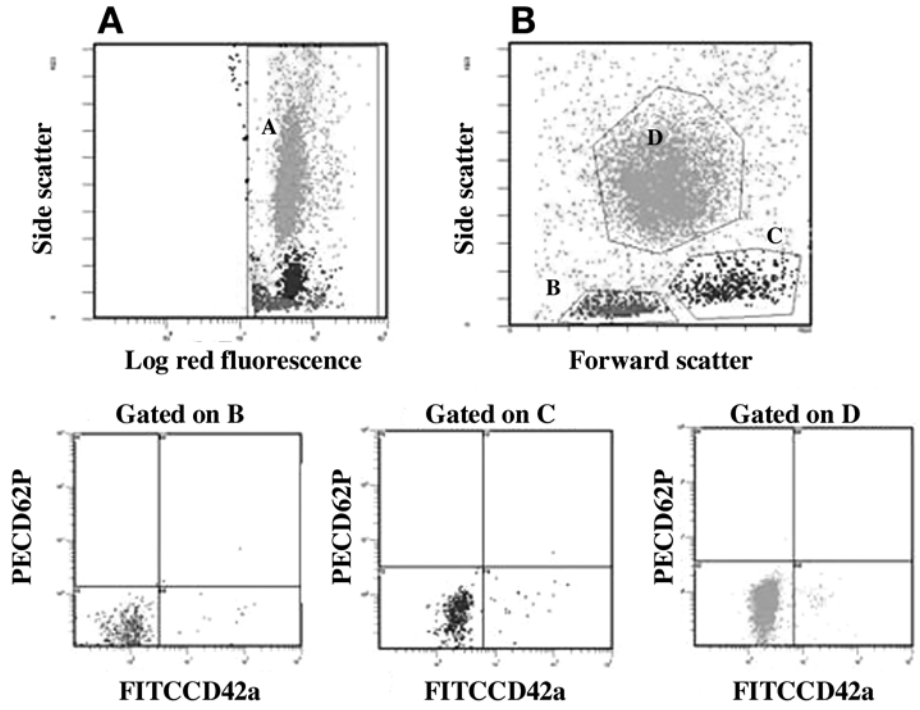


**Fig. 8.** The flow cytometric analysis of platelet leukocyte aggregates (PLAs) in whole blood. The cells in blood are analyzed for LDS-751 expression, and leukocytes positive for this are gated by A in **histogram A** to exclude red cells and platelets. These events are then backgated to a histogram of forward scatter and side scatter **(histogram B)**. Amorphous regions B, C, and D are set around the lymphocytes, monocytes, and neutrophils, respectively. These are then analyzed in a dual-fluorescence histogram of PE-CD62P and FITC-CD42a. Events in regions B-D that are positive for CD42a are considered to be platelet leukocyte conjugates. Single positive events are considered to be platelet-free leukocytes. The relative percentage of platelet leukocyte conjugates may then be determined. The use of CD62P allows the identification of activated platelets bound to the leukocytes. Quantum Red-conjugated CD3, CD14, and CD16 may also be used to identify lymphocytes, monocytes, and neutrophils, respectively.

frequency histogram of forward light scatter. Those with forward light scatter signals greater than that of 1.09-m-diameter polystyrene beads are considered platelets, whereas those with lesser signals are considered putative microparticles. The gated events (i.e., platelets and putative platelet microparticles) are displayed in plots of PE-CD62P fluorescence versus FITC-CD42a fluorescence; $CD42a^+$ events are considered platelets or platelet microparticles, and PE-CD62P fluorescence data are recorded.

## 19. Platelet–Leukocyte Interactions

Although most blood cells circulate as single cells, small percentages can also exist in the form of aggregates. These can be homotypic or heterotypic, such as platelet–platelet aggregates or platelet leukocyte aggregates (PLAs). PLAs may be important in thrombotic and inflammatory disease states ***(89–91)***. Li et al. ***(92)*** developed a rapid and reliable procedure for measuring PLAs in whole blood which is consistent with the recommendations of the European Working Group on clinical cell analysis ***(93)***.

### *19.1. Procedure for Measuring Platelet–Leukocyte Aggregates*

1. Blood is collected by venepuncture, without stasis, into siloconized Vacutainer tubes (BD, Oxford, UK) containing 1:10 volume of 3.8% trisodium citrate.
2. Within 3 min of collection, 5 µL of blood is added to 45 µL of HEPES-buffered saline (150 n*M* NaCl, 5 m*M* KCl, 1 m*M* MgSO4, and 10 m*M* HEPES, pH 7.4) containing appropriately diluted FITC-CD42a (Beb1; Becton Dickinson) and Rhodopycea phycoerythrin (RPE)-CD45 (T29/33; DakoCytomation, Ely, UK) mAbs (final concentrations 1.25 and 20 µg/mL, respectively).
3. The blood is incubated for 20 min at room temperature without agitation, then diluted by mild fixation with 0.5% formaldehyde in saline, and analyzed by flow cytometry within 3 h.

## 20. Leukocyte–Leukocyte Interactions

Methods for leukocyte–leukocyte interactions have been developed to investigate various processes in the immune response, such as antigen presentation and natural killer cell function ***(94)***. The following assay was developed to detect specific cell-mediated cytotoxicity ***(95)***. The target cells are labeled with an orange fluorescent membrane dye PKH-26, effector cells may be identified by the addition of fluorescein-conjugated mAbs, and cell death is detected by the differential uptake of the red fluorescent dye PI.

### *20.1. Method for Measuring Leukocyte–Leukocyte Interaction*

1. Target cells K562 are washed once in RPMI-1640 supplemented with L-glutamine (0.3 g/L), penicillin (100 U/mL), and streptomycin (100 µg/mL) and resuspended in PKH cell labeling buffer (Sigma-Aldrich).
2. The cells are mixed with an equal volume of 4 m*M* PKH-26 (Sigma-Aldrich) for 2 min at 21°C. The labeling reaction is stopped by the addition of an equal volume of FCS for 1 min, and the cells washed twice RPMI-1640 supplemented with 10% FCS and resuspended to $1 \times 10^5$/mL.
3. Peripheral blood mononuclear effector cells (100 µL) at a concentration of $0.6–10.0 \times 10^6$/mL are mixed with 100 µL of PKH-26-labeled target cells and 25 µL of PI solution (1 µg/mL) (Sigma-Aldrich) in tubes and gently mixed.

4. The tubes are centrifuged at 50*g* for 5 min and then incubated at 37°C in 5% $CO_2$ in air.
5. After 1 h, tubes are placed on ice until analyzed.
6. Spontaneous cell death is determined by incubating both targets or effectors alone. Samples are run in duplicate.
7. The targets and effector cells are identified on a two-parameter histogram of FSC and SSC, and the proportions of red and orange fluorescent cells are determined in a histogram of log-orange and log-red fluorescence. This allows the enumeration of four subpopulations: (1) live targets (orange fluorescent only), (2) killed targets (orange and red fluorescent), (3) live effector (nonfluorescent), and (4) dead effectors (red fluorescent only).
8. The effector/target ratio can then be calculated. At least 2000 target events should be enumerated.

The spectral overlap of the fluorochromes is electronically compensated using PKH-26-labeled targets alone and unstained targets the membrane of which has been permeabilized by treatment with 0.1% Tween 20 detergent (Sigma-Aldrich) in PBS (Sigma-Aldrich) for 10 min at 37°C. These Tween-treated targets are then washed twice and incubated with PI (0.1 μg/mL). This preparation contains both live and dead targets.

The cell-mediated cytotoxicity is determined by subtracting the background cell death percentage from the specific cell death percentage.

## 21. Leukocyte–Endothelial Cell Interactions

Epithelial cells and endothelial cells express high levels of adhesion molecules, and assays to quantify the adhesive interactions between leukocytes and activated epithelial and endothelial cells have been developed. The following assay was developed to identify different subpopulations of lymphocytes adhering to cultured parenchymal cells treated with proinflammatory cytokines ***(96)***.

### *21.1. Procedure for Measuring Leukocyte–Endotheial Cell Interactions*

1. Confluent endothelial or epithelial cells in 24-well plates are washed, and 500 μL of RPMI-1640 medium without phenol red is added to each well.
2. Then, $5 \times 10^5$ resting peripheral blood lymphocytes or mitogen-activated T cells are added in a further 500-μL volume of medium. The plates are incubated for 1 h at 37°C in a humidified chamber with 5% $CO_2$.
3. Nonadherent cells are resuspended by mechanical plate shaking for 3 min at 150 oscillations per minute, and the wells are washed gently three times with PBS containing 5% serum.
4. The remaining cells are detached from the plastic and completely dissociated by treatment with trypsin-EDTA. The cell suspensions are washed and labeled with an optimum concentration of FITC-conjugated CD45.

5. Cells are analyzed in a histogram of log-green fluorescence and log SSC. The results are expressed as a ratio of the number CD45-positive lymphocytes to the number of CD45-negative parenchymal cells.

The phenotype of the adherent cells may be further characterized by the addition of PE-conjugated CD4, CD8, or CD56. The proportion of each lymphocyte subset in the adherent population is then compared with the proportion in the original cell preparation, and the results are expressed as a ratio. Values greater than 1.0 indicate preferential adhesion.

## Conclusion

Many of the assays described here have been used primarily as research tools, but recently they have begun to be used in clinical settings. Their use for clinical applications has been limited by those factors, referred to at the beginning of this chapter, relating to the requirement of rapid transport and handling of the sample. However, if flow cytometry is to be used on a more routine basis in clinical laboratories, there is one further problem that needs to be addressed, that of standardization.

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# 11

# Cell Sorting by Flow Cytometry

**Derek Davies**

**Summary**

Flow sorters have become a widespread and vital resource in the biological sciences and beyond. Their main purpose is to retrieve populations of interest from a heterogeneous population for further study. If a cell or particle can be specifically identified by its physical or chemical characteristics, it can be separated using a flow sorter. This chapter discusses the ways in which this may be done, the principles behind particle sorting, and the practicalities of a successful sorting experiment.

**Key Words:** Cell sorting; flow cytometry; fluorescence.

## 1. Introduction

Flow cytometry is an extremely powerful technology that allows the individual measurement of physical and chemical characteristics of particles as they pass one by one through a light source. Flow sorting is a process that allows the physical separation of a cell or particle of interest from a heterogeneous population. If a population can be identified in an analytical cytometer, it may be retrieved using a flow sorter.

The history of flow cytometry can be traced back to the experiments of Andrew Moldavan ***(1)***, who in the 1930s designed a photoelectric cell apparatus to count individual cells flowing through a capillary tube mounted on a microscope stage. In 1953, Crosland-Taylor ***(2)*** used the principles of hydrodynamic focusing to center cells within a fluid stream and this still forms the basis of most modern flow cytometers. In the 1950s, Wallace Coulter ***(3)*** began the development of an instrument that could electronically calculate cell volume. These early cell analyzers were further developed by Fulwyler, Kamentsky, and their colleagues ***(4–6)*** and provided important biological information – principally cell size or volume, and DNA and protein content – in a wide range of cell types. The

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invention and refinement of ink-jet printers by Richard Sweet, at Stanford, *(7)* led to a way of retrieving specifically defined cells or particles for further study, and early sorters were developed from the close interactions at the time of biologists and engineers *(8,9)*.

The development and early history of cell sorters is dealt with in several fascinating reviews *(10–14)*. Early flow sorters were very much machines that were hand-made for a specific purpose, but the commercial potential of being able to sort cells was quickly realized, and mass-produced cell sorters became available in the early 1970s. Since then, they have become much more widely available and a valuable resource in the biomedical and other fields.

## 2. Applications

In many situations in which a complex population is under investigation, there is frequently a need to isolate unique populations for further studies. The power of flow cytometry is that it is able to use multiparametric analysis to identify highly specific populations. Moreover, it is not just phenotypic characteristics identified by a specific antibody-antigen interaction that can be measured; it is also possible to measure the DNA content of cells *(15)*, the RNA content *(16)*, or even assess functional characteristics such as ion flux or pH *(17)* or altered cell states such as apoptosis and cell death *(18)*.

Having identified a specific subpopulation using an analytical cytometer, why would it be important to sort or isolate particles? There is a large body of literature based on isolation of subpopulations by flow sorting that shows how useful the technology has become. It is possible to sort under aseptic conditions to isolate specific cell populations for expansion in culture (e.g., green fluorescent protein-tagged transfected cells) *(19)*. Cells may be sorted to include in functional assays *(20)* or for transplantation into laboratory animals *(21)* or human patients *(22)*. It is also possible to sort sperm for subsequent insemination to allow sex selection of offspring *(23)*. Although flow sorters are used in the main to sort mammalian cells, it is more accurate to refer to particle sorting given that flow sorters have also been used to sort yeast *(24,25)*, bacteria *(26,27)*, and phytoplankton *(28)*. Indeed, whole cells are not always needed as it is also possible to sort subcellular organelles such as Golgi complex *(29)* or chromosomes *(30)*. Flow sorting is the only practical way of isolating large numbers of specific chromosomes from humans, other primates *(31)*, or plant species *(32)*, and flow sorters proved invaluable during the human genome sequencing project *(31)* and more recently in the production of chromosome paints *(33)*. In addition, as the newer scientific fields of genomics and proteomics have evolved, flow sorting has also become important in, for example, sorting large numbers of specific subsets of cells for microarray analysis *(34)*. At the other end of the scale, single particles may also be sorted into individual wells of a plate for cloning *(35)* or

for polymerase chain reaction (PCR) analysis ***(36)***. Therefore, the applications of flow sorting are wide-ranging and a flow sorter, or access to one, is an invaluable resource.

## 3. Principles of Particle Sorting

### *3.1. Electrostatic Sorting*

Most analytical flow cytometers are enclosed in that cells are aspirated from a reservoir and hydrodynamically focused so that they pass one by one through a light source, generally from one or more lasers. At this point, scattered light and fluorescence signals are generated, detected, and measured. After this, cells are removed under vacuum to a waste reservoir. In general, flow sorters use a principle involving the electrostatic deflection of charged droplets similar to that used in ink-jet printers. To sort particles by this method, the process has to be performed in a more open system where cells are ejected into air in a stream of sheath fluid.

Any fluid stream ejected into air will break up into droplets but this is not a stable process; the distance from the orifice that the stream begins to break up will depend on many factors such as the orifice size, the pressure of the sheath fluid, the ambient temperature, and the viscosity of the fluid. However, if a stationary wave of vibration of known frequency and amplitude is applied to the fluid stream, it is possible to stabilize the break-off, and for a given set of conditions, the size of the droplets and the distance between drops will also stabilize. In a flow sorter, this vibration is produced by a transducer, which is generally a piezo-electric crystal acoustically coupled to the nozzle. As cells are ejected from a nozzle, they pass through one or more laser beams and at this point – the moment of analysis – information is gathered about the cell or particle (**Figure 1**). The distance, and therefore time, between the point of analysis and the point at which the cell breaks off from the solid stream in a droplet is constant and under given conditions can be calculated. This distance between the laser intercept and the break-off point is measured in drop equivalents and is often referred to as the drop delay. The calculation and monitoring of this is critical and is the factor that makes a sort successful or not. The drop break-off can be observed microscopically under stroboscopic illumination to allow the break-off point to be monitored. The drop delay is calculated by determining how many drops are in the distance between the analysis point and the break-off point; drops will start to form as soon as the stream emerges from the nozzle but will be coalesced until the break-off point. There are several ways of measuring the drop delay: by counting the number of drops formed in a known distance, by sorting beads onto slides at varying drop delays and checking microscopically, or by viewing fluorescent beads in sorted side streams. The precise way of calculating drop delay will vary with the type of sorter used.

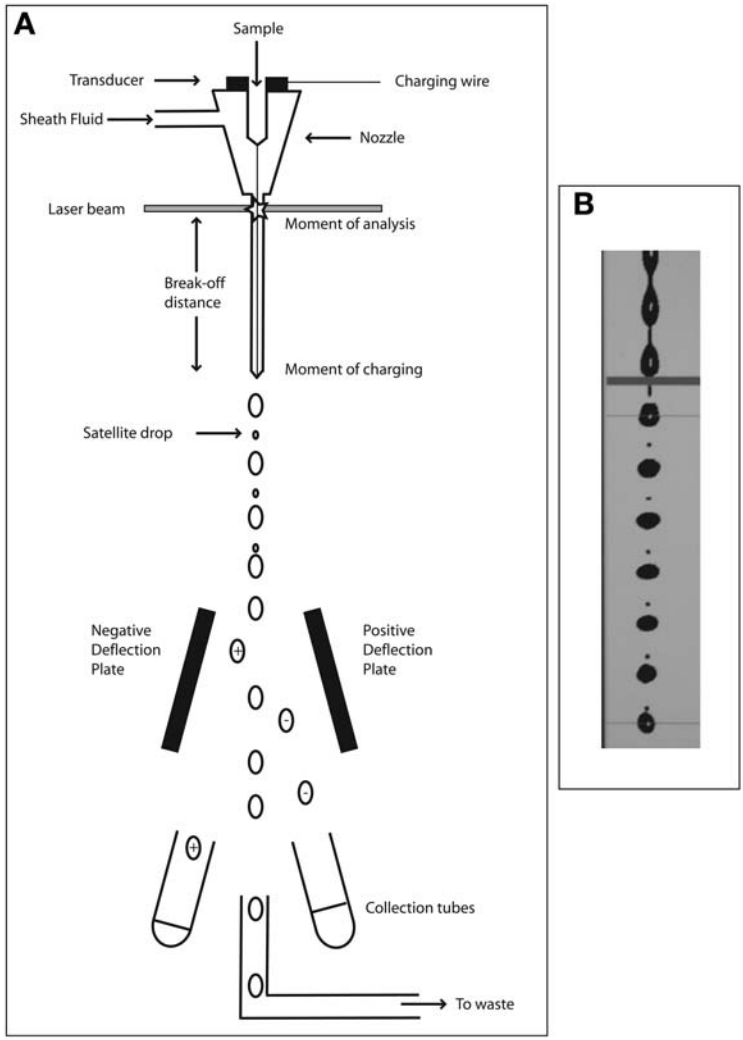


**Fig. 1.** A diagram of a generalized cell sorter **(A)**. Particles are introduced into a column of pressurized sheath fluid, and as they emerge from the nozzle, they pass through one or more laser beams. At this point, the moment of analysis, the cytometer gathers information about the fluorescence characteristics of the particle. After passing through the stream for the break-off distance, the stream is charged when the cell breaks off into a drop (moment of charging). There will be a variable number of satellite drops that are formed from the fluid connecting the drops as they form. These satellites should be "fast-merging" (i.e., quickly become coalesced with the preceding drops). Charged drops then pass through two high-voltage deflection plates and are deflected into collection vessels or aspirated to waste. The break-off point is seen in real time under stroboscopic illumination **(B)**.

Once the drop delay is calculated, it is possible to charge through the stream at the precise moment that the first drop is forming. Therefore, individual drops, as they break away from the solid stream, can be independently charged and will carry a positive charge, a negative charge, or will remain uncharged. The individual drops then pass through a static electrical field created by two charged plates. The voltage between the plates will be in the range of 2000 to 6000 V depending on the flow sorter used and the number of populations required from the sort. Charged drops are attracted to the plate of opposite polarity and will be deflected into collection vessels, which may be, for example, Eppendorf tubes, 6-mL centrifuge tubes, 15- or 50-mL conical tubes, or multiwell plates (anything up to 384-well plates). Initially, flow sorters were able to sort only two populations, one to the right and one to the left. However, the most recently introduced sorters (MoFlo [Dako, Fort Collins, Co.], FACS Aria [BD Biosciences, San Jose, CA], and Influx [Cytopeia Incorporated, Seattle, WA]) have the ability to sort four populations and they do this by using a variable charge so two populations may be sorted to the right and two to the left of the unsorted center stream.

The formation of drops and the determination of the drop delay are the factors that enable the flow sorter to be able to sort cells. However, to sort a pure population, it is necessary to ensure that the particles of interest be contained in a drop that does not also include an unwanted event. To ensure purity, normally only the drop containing – or likely to contain – the required event is charged. Although droplet formation in the absence of a sample is stable, stream dynamics and minor disturbances in the flow mean that the position of the event within the drop is not certain and that the charging pulse may not always be in phase with the drop production. This is especially true if an event is near the edge of a drop, and it may appear in the preceding or the following drop, and so to avoid cells loss more than one drop may be charged. Whether this is the preceding or following drop depends on the position of the event within the drop.

Sorting will never be a 100% efficient process, and some particles of interest will be missed and lost. Particles may arrive at the analysis point too close together to be analyzed separately and these are often termed hardware aborts; the flow sorter will ignore these events, as it cannot be sure whether they are wanted or unwanted. The number of hardware aborts will be influenced by the dead time of the electronics – that is, the time needed to finish processing one event before the next can be analyzed – and this is more important in analog systems than in more modern digital systems. Additional considerations are the sample pressure, the size of the cell, the cell concentration, and "stickiness" of the cells.

Another way that events of interest may be lost is when they can be analyzed separately but are too close together in the fluid stream to be included in

separate drops; these are often termed software or coincidence aborts. Rather than compromise purity, the drop would not normally be sorted if it contained an unwanted event and these events would go to waste. However, it is possible to override this if the investigator is interested in a particular population even at the expense of purity; this is often termed enrich mode.

In fact, all sorters give the user the ability to vary how the sort is performed depending on the user's needs. Each cytometer does this in a slightly different way, but broadly speaking there are three sort modes: one that is optimized for purity, which would be the general default mode; there will be some cell loss where there are high coincidence aborts. The second mode is the enrich mode, where all events of interest are sorted at the expense of some reduction of purity. The third mode is often termed counter or single-cell mode and this is used when high count accuracy is required (e.g., when sorting single cells into multiwell plates for cloning or for sorting known numbers of cells for functional assays or transplantation). Here, the cytometer electronics will sort a drop only when it contains only one event of interest, when that event is in the center of the drop, and there are no possible coincident events in adjacent drops.

The speed of sorting depends on the time taken to generate a droplet, which depends on the frequency of the transducer. The frequency of droplet production in a stream-in-air sorter is determined by the jet velocity and the jet diameter and is defined as f = v/4.5 d, where v is the velocity of the fluid and d the diameter of the orifice ***(37)***. A typical sorter of the mid-1980s, sorted using a sheath pressure of approx 12.5 psi, will generate a sheath velocity of approx 10 m/s. This allows about 27,000 drops per second to be produced. Typically, flow rates for cells would be approx 5000 so we would expect only one drop in five to contain an event. To sort more quickly at a given orifice size, flow sorters have had to be developed that run at higher pressure and therefore greater sheath velocity. Increasing the sheath pressure to 60 psi allows approx 100,000 drops to be produced every second, giving an approx fourfold increase in the speed of sorting. The optimal drop drive frequency will vary with the nozzle orifice diameter. **Table 1** shows typical sort pressures, drop drive frequencies, and sort times for a range of common nozzle sizes. The maximum speed of sorting is limited not by the number of drops that may be produced but by the sheath pressure needed to produce them; a drop frequency of 250,000 per second is possible but only if a pressure of 500 psi is used and this would be contraindicated for live cells although these pressures have been used in sorting chromosomes ***(38)***.

### *3.2. Mechanical and Other Forms of Sorting*

Although they are not as commonly encountered, mechanical or fluidic switching sorters are worth mentioning. Mechanical sorters do not use drops,

**Table 1**
**The Number of Drops That Can Be Produced Per Second for a Variety of Nozzle Sizes at Low and High Sheath Fluid Pressure**

| Nozzle size (μm) | Pressure (psi) | Frequency (drops/s) | Pressure (psi) | Frequency (drops/s) |
|---|---|---|---|---|
| 50 | 15 | 37,000 | 80 | 160,000 |
| 70 | 12 | 26,000 | 60 | 100,000 |
| 100 | 10 | 15,000 | 40 | 50,000 |

because they are enclosed, but instead use a motor-driven syringe to aspirate fluid containing the event of interest. The decisions as to which "drops" to sort are governed by the same sort logic procedures as on a stream-in-air sorter. However, they are comparatively slow (500 events/s) and are able to sort only one defined population at a time. However, because the system is enclosed, there is reduced contamination risk, they are alignment-free, and they are easier to set up so no dedicated operator is required.

As seen in **Table 1**, stream-in-air sorters are able to sort at speeds of up to 30,000/s but this is still slow in relation to bulk separation methods such as cell filtration or cell affinity methods ***(39,40)***, fractionation ***(41)***, or centrifugal elutriation ***(42)***. Another way to sort cells is to use magnetic beads ***(43)***; it is possible to positively select cells of interest by adding magnetically labeled antibodies to specifically select the population of interest or by negatively selecting a population by adding antibodies, coupled to magnetic beads, specific for cells other than those of interest. Cells are then passed through a column between a strong magnetic field to either elute or retard the population of interest. However, a flow sorter should give a higher purity as well as be able to separate populations on the basis of multiple parameters, the expression of fluorescent proteins, nucleic acid content, and the level of fluorescence expression.

Stream-in-air sorters are also more versatile, being able to be modified, especially in terms of the excitation light sources and the emission optics used. In addition, up to four specifically identified subpopulations may be isolated simultaneously. However, they are more expensive to purchase, maintain, and run and, for optimal efficiency, need a dedicated operator.

## 4. Practicalities of Cell Sorting

### *4.1. Sample Preparation*

Sample preparation prior to sorting is important; in fact, successful sorting depends almost entirely on the state of the input sample. It is a prerequisite for flow cytometry that cells or particles be in a monodispersed suspension. This is

relatively easy when the cells used are in a natural suspension (e.g., blood cells or suspension cultured cells) but more problematic when using cells from adherent cultures or cells from solid tissue. However, there are several well-established methods for preparing samples for flow sorting.

### 4.2. Preparation from Suspension Cells

1. Take cells directly from a culture flask into 50-mL conical tubes and centrifuge at 400*g* for 5 min.
2. Discard the supernatant and resuspend in medium (cell culture medium or phosphate-buffered saline [PBS] with 1% bovine serum albumin).
3. Centrifuge again at 400*g* and discard supernatant. Count cells and resuspend at an appropriate concentration, which will vary with sorter used but will be in the range of $1 \times 10^6$–$1 \times 10^7$ per mL. The final suspension medium will depend on the cell types to be sorted. In general, a low protein concentration is recommended because this will lead to less cell clumping although the addition of 5 m*M* EDTA (ethylenediaminetetraacetic acid) will also help this.

### 4.3. Preparation from Adherent Cells

1. Harvest cells by using trypsin (0.25% w/v) or versene (0.2% w/v). Transfer cells to 50-mL conical tubes and centrifuge at 400*g* for 5 min.
2. Discard the supernatant and resuspend in medium (cell culture medium or PBS with 1% bovine serum albumin).
3. Centrifuge again at 400*g* and discard supernatant. Resuspend the cells in a small volume of medium and aspirate up and down through a pipet several times to help disaggregate clumps. Count cells and resuspend at an appropriate concentration. In practice, adherent cells tend to be larger, and a lower concentration is recommended. It is always better to keep the concentration high prior to sorting and dilute to an appropriate concentration immediately prior to a sort.

### 4.4. Preparation from Solid Tissue

1. Place tissue in a sterile Petri dish. Tease tissue apart using a needle and scalpel or alternatively use an automated system such as a MediMachine (Consults, Italy) ***(44)***. In addition, enzymatic disaggregation (e.g., collagenase (220 U/mL) may also help free single cells.
2. Decant cells into a 50-mL conical tube and centrifuge at 400*g* for 5 min.
3. Discard the supernatant and resuspend in medium (cell culture medium or PBS with 1% bovine serum albumin).
4. Centrifuge again at 400*g* and discard supernatant. Resuspend the cells in a small volume of medium and count cells as above.

All preparations may be filtered through sterile nylon mesh prior to sorting; a range of pore sizes from 20 to 70 μm will be suitable for most cell types encountered.

### 4.5. Flow Sorter Setup

It is a truism that a clean sorter is a happy sorter. It is important to make sure that all fluidics lines be cleaned and/or replaced on a regular basis. If a sort is aseptic (i.e., one in which cells will be required to be recultured or transplanted), a sterilization procedure will be needed. In general, this involves running 70% ethanol through all fluidics lines for 30–60 min before flushing this with distilled water (30 min) and finally sterile sheath fluid (at least 30 min before commencing a sort). At all times, fluids pass through a 0.22-µm filter immediately after leaving the sheath tank. It is important to remember that the compressed air used to generate the pressures needed to run a sorter should also pass through an in-line filter and that all filters should be replaced periodically. All areas where the cells can potentially be in contact with the atmosphere (i.e., the sample line and sort chamber) should also be cleaned with ethanol prior to a sort. To check the sterility of a flow sorter, it is useful to periodically remove fluid from key locations (sheath tank, nozzle, and sample line) and put this into culture in an appropriate medium; cultures should remain sterile for at least 7 d.

The next consideration is nozzle size. The cell type will influence the size of orifice used. A general rule of thumb is that for blockage-free sorting and coherent side streams a cell should be no more than one fifth the diameter of the nozzle. In practice, this means that small round cells such as lymphocytes or thymocytes would require a 70-µm nozzle whereas many cultured adherent cell lines and primary cells such as keratinocytes would require a 100-µm nozzle. Other larger cells such as plant cells require larger nozzles, typically approx 150–200 µm ***(45)***. In practical terms, a larger nozzle will use more fluid and will need a longer time from the emergence of the stream into air to break up into drops, because it will run at a lower pressure.

In contrast to analytical benchtop cytometers, laser alignment should be checked on a daily basis on a stream-in-air system. It is important that the laser or lasers hit the stream in parallel and that the beams be focused correctly. Alignment may be checked using fluorescent latex microspheres (beads) that are excited by a particular wavelength of light and have a broad emission spectrum; it is also important to monitor the sensitivity of the sorter using multipeak beads with a variety of fluorescence intensities. The number of lasers used and the fluorochromes to be detected will also vary and need to be determined before the flow sorter is prepared.

Once aligned, the frequency and amplitude of the drop drive are altered to achieve a minimum stable break-off with coherent side streams using a test-mode sort. Although a given nozzle size has an approximate resonance frequency (**Table 1**), there will be a variability between individual nozzles of a given size and there will also be variability on a day-to-day basis depending on conditions.

A stable break-off point will be found at several harmonic resonance frequencies and only by experience will an operator be able to find the most stable of these. It is possible to alter the amplitude of the transducer during the sort to keep drop break-off stable and keep the charging pulse in phase. Many modern sorters have automated systems that will detect movement of the break-off point and change the amplitude accordingly. If the change in the break-off is greater than user-defined parameters, the sort will interrupt and alert the operator. It is always worth rechecking drop delay if there is a block and/or the break-off seems unstable.

### *4.6. Sort Setup*

The setup of a sort depends entirely on which cells or particles are needed. As already noted, it is possible to sort according to antigen expression, fluorescent protein expression, nucleic acid content, or functionality. In all cases, a preliminary experiment on an analytical cytometer is always worthwhile. In addition, in most cases, a dye that will specifically identify dead cells should be added in order to exclude them from the sort. Although these cells may not be problematic when cells are to be recultured, it would be important to exclude them if specific numbers of cells were needed for functional studies or if cells were to be used to analyze protein expression or mRNA content. Examples of viability dyes include propidium iodide (used at 5 µg/mL), 7-aminoactinomycin-D (2 µg/mL), TO-PRO-3 (200 n*M*), and DAPI (4′, 6-Diamidino-2-phenylindole; 200 ng/mL). The dye used will depend on which combination of fluorochromes will be used in the sort to identify the populations of interest.

Although hydrodynamic focusing will help align particles in the center of a stream and to some extent keep them separate, some particle doublets will always be present. This can have a deleterious effect on sorting purity. For example, if a positive event of interest were measured at the same time as a negative event, both would be sorted because to the sorter electronics it is a positive event, so there would be a concomitant reduction in sort purity. Therefore, a way of excluding these is useful on a flow sorter; many flow sorters give the user the capability of measuring pulse width or time of flight, which is a measure of how long a particle takes to traverse the laser beam. Doublets and clumps tend to become oriented lengthways due to the fluidics of the cytometer ***(46)***, and potential doublets or clumps may be excluded on the basis of their pulse width.

In terms of practical setup for a sort, **Figure 2** illustrates the procedure used. The population or populations to be sorted are identified initially on the basis of their fluorescence characteristics. Dead cells are excluded on their positivity for the viability dye, the doublets are excluded as far as possible according to their pulse width, and finally debris and events, which are clearly not intact cells,

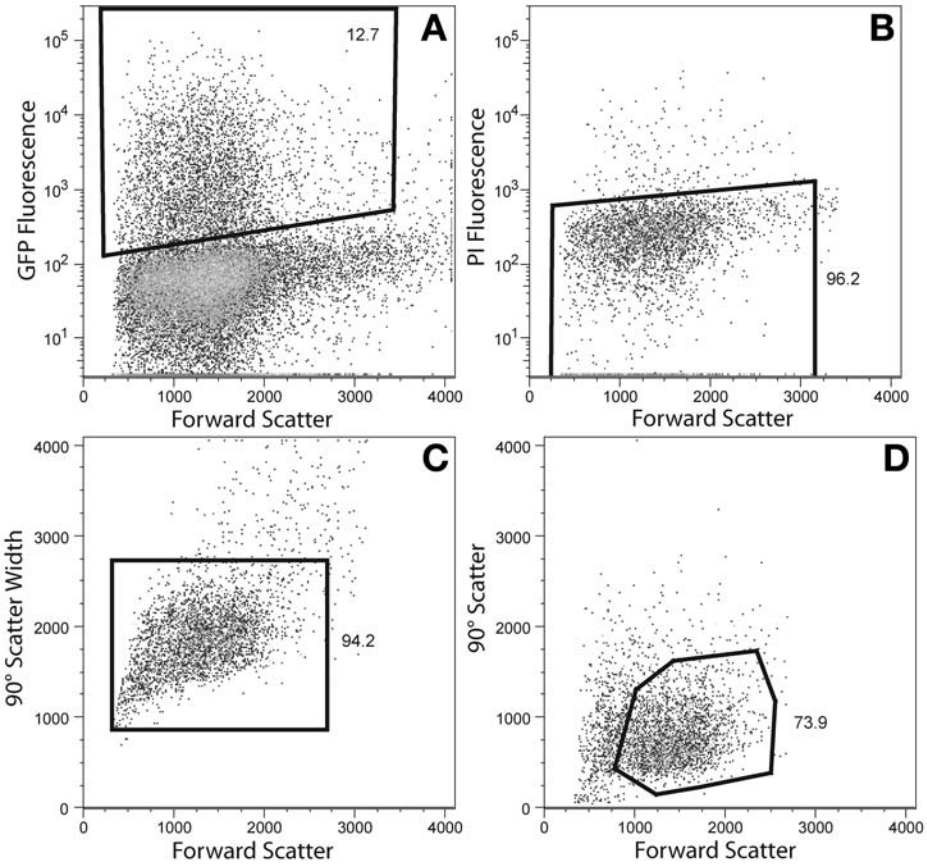


**Fig. 2.** Setting sort gates. Cells are initially selected on the basis of fluorescence characteristics **(A)**, in this case GFP fluorescence. Nonviable cells that fall in this gate are then excluded **(B)** on the basis of propidium iodide positivity. Next, doublets are excluded on the basis of the width of their 90° scatter signal **(C)**. Finally, cells that fulfil all three criteria are selected on the basis of scatter characteristics **(D)**.

are excluded on the basis of their forward and 90° scatter signals. As a rule of thumb, dot plots should be used to set up sort parameters.

Once the population to be sorted has been identified, the sort mode to be used has to be decided. In practice, there is a balance between purity, recovery, and yield of the sort. In the majority of sorts, only one drop per event will be sorted; this will give the greatest purity because drops containing unwanted cells would not be sorted, which is the usual requirement in a sorting experiment. However, if the event is toward the edge of a drop, it is possible that it may not be in the charged drop when that cell reaches the break-off point; this will lead to

**Table 2**
**Time Taken to Sort a Given Number of Cells in an Ideal Situation Assuming a High Pressure Sorter Running at 10,000 Cells/s ($3.6 \times 10^7$/h)**

| Number of cells requested | Percentage of population | | | | |
|---|---|---|---|---|---|
| | 0.1% | 1% | 5% | 10% | 40% |
| 1000 | 1.7 min | 10 s | 5 s | 1 s | 0.25 s |
| 10,000 | 17 min | 1.7 min | 50 s | 10 s | 2.5 s |
| 100,000 | 2.8 h | 17 min | 8.3 min | 1.7 min | 25 s |
| 1,000,000 | 28 h | 2.8 h | 1.4 h | 17 min | 4.2 min |

With the time taken to set up the sorter and cell losses due to aborts, it is reasonable to assume a 50% increase in the actual time needed.

reduced recovery. Increasing the number of drops sorted will increase recovery but may reduce yield because of increased coincidences. As seen above, most modern cell sorters give the user the capability of varying the sort mode depending on the wishes of the user. The default mode is as pure as possible, but sometimes a precious population is needed, and in these cases we can override the coincidence abort circuits so all the desired cells are sorted. Experience will allow the sorter operator to strike a balance between the sort efficiency and the requirements of the experiment.

Electronic thresholding is a more important consideration on a flow sorter than it is in analytical cytometry. Most cytometers use an electronic threshold, a "bar" above which an event must pass before it is analyzed. Events that do not reach this are, in effect, invisible to the electronics of the machine. However, because these events are still in the stream of fluid, if they are not seen, they may end up in the sorted tube. This can be a problem, particularly if cells are being used, for example, for PCR amplification of RNA. So it is advisable to set the minimum threshold possible before commencing a sort.

Two practical considerations are the number of particles that can be sorted and the number that can be recovered in a given period of time. **Table 2** shows the relationship between the number of cells required and the time taken to sort them at a range of initial percentages. There are clearly times when the number needed is high and the initial percentage low, and in these cases either a pre-enrichment, possibly using magnetic beads, or an enrich-mode sort followed by a second, purity, sort would be indicated.

At the end of the sort, if possible, it is advisable to re-analyze a portion of the sorted cells to assess the purity and recovery of the sort and how efficient the sort has been in terms of cell yield. The purity of the sort is judged by analyzing the cells in the sorted tube and by assessing the percentage of cells that fulfil the sort criteria (**Figs. 3–5**). When using the sorter to check purity, it is

important to make sure that the sample line has been adequately cleaned to ensure no sample carryover. Typically, a purity of greater than 98% would be expected. At this stage also, if live cells are sorted, the viability should be determined to ensure that it is similar to that in the unsorted cells. It is worth mentioning that a cell sorter will always sort what the user has asked it to sort; problems arise when the sort criteria may be fulfilled by cells in which the investigator is not interested (e.g., doublets, dead cells, and debris).

The sort recovery is defined as the percentage of events that the sort counters have indicated which actually ended up in the sorted tube. This should typically be more than 80%. The best way to count cells is to remove a small aliquot directly from the sort tube; do not centrifuge the tube at all. If possible, use a cell counter rather than a hemocytometer to assess cell numbers. Recovery will never be 100%, because not all particles will find their way into the collection tube. This can be due to cells repelling each other (because all events sorted into the same collection tube will have the same charge); some particles may miss the tube, which will be true if there has been fanning of the side streams during a sort; and some particles may hit the side of the tube rather than the medium. If sorting a small percentage population, it is possible to sort into tubes that are almost filled with collection medium to try to prevent loss. Collection tubes may also be coated with fetal calf serum to help prevent cell loss and improve cell viability.

The yield of a sort is defined as the number of wanted cells recovered divided by the number that were in the original sample (i.e., the sample to be sorted has $1 \times 10^7$ cells and the population of interest forms 10%, the maximum theoretical yield is $1 \times 10^6$). The yield is defined as the recovery divided by the theoretical maximum expressed as a percentage. At the end of a sort, all fluidics lines and machine parts that have come into contact with the specimen should be decontaminated. Again, all exposed parts should be treated with dilute bleach (10%) and detergent, and these fluids should also be run through the sample line, ending with a tube of distilled water. If consecutive sorts are to be performed, sufficient time between sorts should be allowed for this procedure.

### *4.7. Tips and Troubleshooting*

Although the theory of flow sorting is relatively straightforward, as with many technologies there are several "tricks of the trade" that are learned only with experience.

Poor purity may be seen when too many doublets are present (i.e., when a target event is associated with a nontarget event); for this reason, negative sorting is always slightly purer than positive sorting. Here, cell preparation is important because dead cells can increase the incident of doublets and clumps due to release of DNA which causes stickiness; adding DNAse I (100 mg/mL with

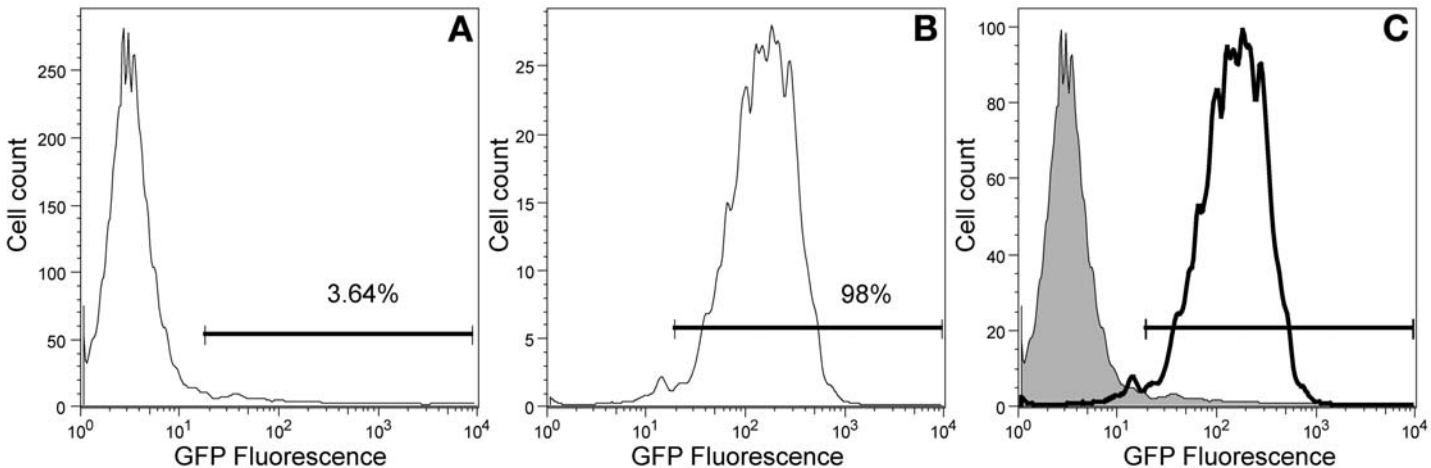


**Fig. 3.** Sorting according to GFP fluorescence. Cells were transfected with a GFP-tagged protein and selected as in **Figure 2**. **(A)** The initial GFP positive population. **(B)** The sorted sample re-analysed after the sort. **(C)** An overlay histogram of the pre- and post-sort samples.

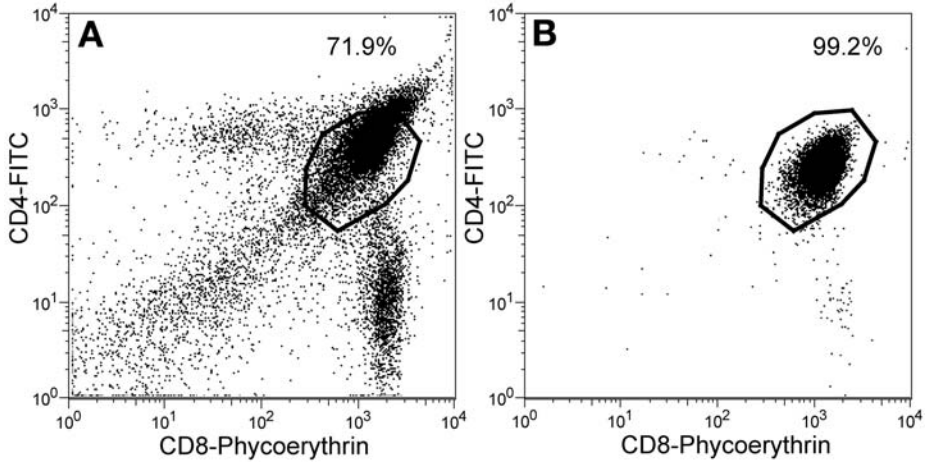


**Fig. 4.** Two-color sorting. In this case, thymocytes labeled with antibodies to CD4 and CD8 were sorted and the double-positive population was selected **(A)**. Re-running the sample after the sort shows the sort purity **(B)**.

5 m*M* $MgCl_2$) will help reduce aggregates. In addition, keeping the sample cool can help maintain a monodispersed suspension. Purity in four-way sorts may also be influenced by the quality of the side streams, and in addition, the larger populations should be sorted to the inner left and right streams.

Poor recovery will be seen if events miss the collection receptacle. Changing to polypropylene or glass tubes coated with fetal calf serum can help prevent the electrostatic charge build-up that leads to poor recovery. It may also be due to poor side streams. In general, these are caused either by too much protein in the sample fluid or by the particles being sorted being relatively large compared with the size of a drop; changing the medium and increasing the size of the nozzle may help. It may also be the result of poor deflections; it is important to keep the sort chamber scrupulously dry. In addition, poor recovery may be due to changes in sheath flow and drop generation during a sort which are caused by changes in fluid viscosity due to temperature changes. It is important that temperature be kept constant to avoid this as far as possible.

Reduced yield will be influenced by the number of coincident events during a sort. This should be monitored during a sort; all flow sorters will allow the user to monitor the hardware aborts and these should be kept to approx 5% of the total flow rate. If it is too high, reducing the flow rate and/or increasing the nozzle size may again help.

Poor viability or cell functionality indicates that the sort conditions are too harsh for the cell type being sorted. Sorting cells into complete medium with high protein concentration (20% serum) may help, as may cooling the sample.

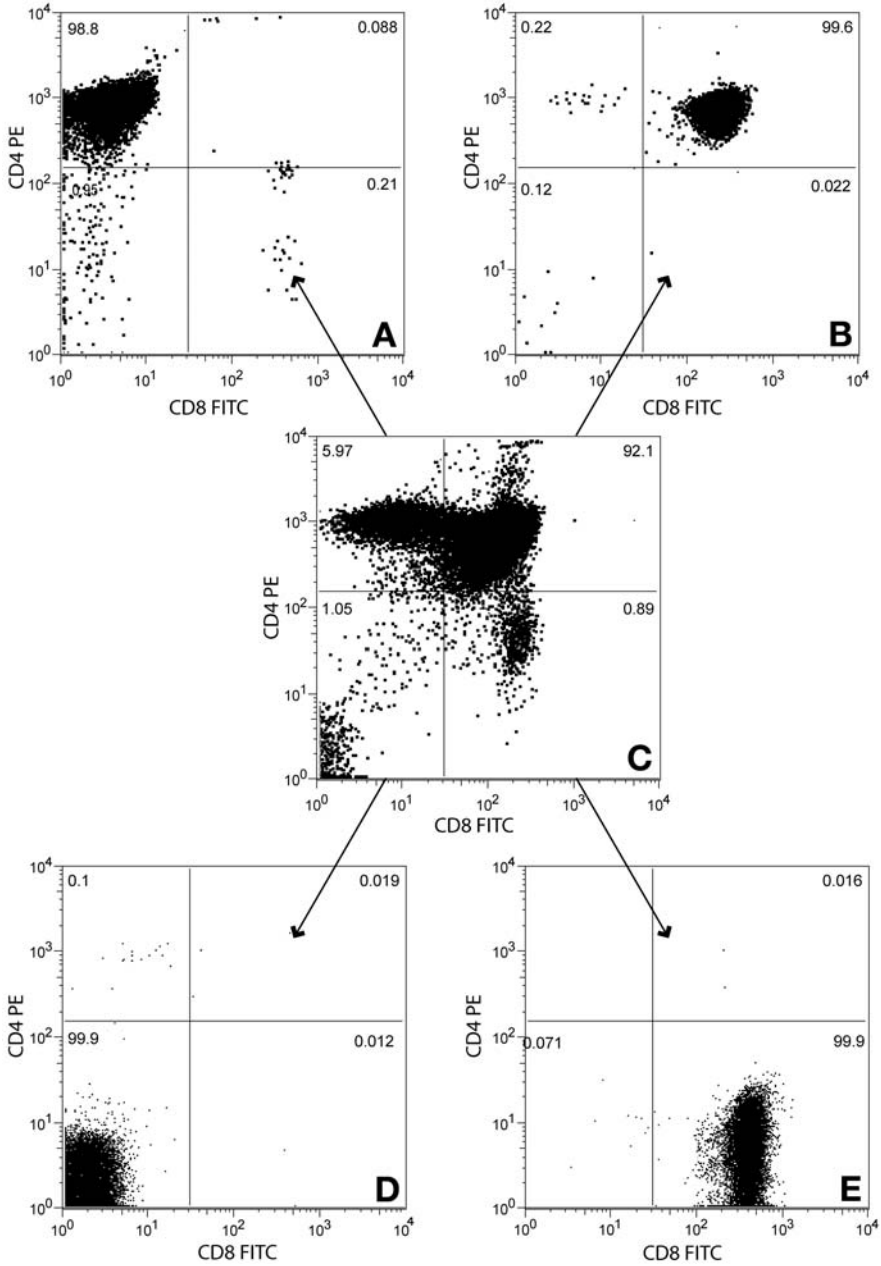


**Fig. 5.** Four-way sorting. In this case, the populations to be sorted are defined by their expression of CD4 and CD8, and the two single-positive, the double-positive, and the double-negative populations were sorted **(C)**. Re-analysis **(A, B, D,** and **E)** shows sort purity.

However, more likely is that the cells are adversely affected by the sheath pressure or nozzle size, so decreasing the pressure and/or increasing nozzle diameter is indicated. In extreme cases, altering the sheath fluid to the medium the cells grow in may be worthwhile. Maintaining an optimal pH for the cells being sorted is important and addition of a nonphosphate buffering system (e.g., 25 m*M* HEPES) to the medium in which the cells are sorted may help viability.

Blockages are the bane of the sorter operator's life. A particle will clearly not pass through a blocked nozzle, but partial blockages may also cause perturbations in the stream and cause instability in drop formation and hence alter drop delay. Blockages can be avoided by cooling the sorting sample, using sample agitation, filtering immediately prior to sorting, and sorting serial aliquots of the sample. Nozzles may be cleaned by sonication sequentially in a detergent solution and distilled water.

Because stream-in-air systems have detection optics physically further away from the excitation point, the light-collection efficiency is lower, and although it is possible to compensate for this by using higher excitation powers, it may still be that the sensitivity of stream-in-air systems is less than an enclosed system. This is important to bear in mind when designing experiments, particularly when looking for weakly expressed antigens or where the signal-to-noise ratio is low or where using fluorochromes such as PerCP (peridinin chlorophyll protein), which may be photobleached by high laser powers. It is also possible to use a combination of an enclosed system for fluorescence measurement before the production of drops and sorting (e.g., the recently introduced FACS Aria). However, despite the advent of new flow sorters, old advice still holds true today ***(47)***.

## 5. Health and Safety Considerations

All stream-in-air sorters will by their nature produce aerosols, so operators and users of these machines need to be aware of the potential biosafety implications. There are published guidelines for containment procedures ***(48,49)***, but guidance from local health and safety officers is important and should be sought. In all cases, operators should be aware that all unfixed particles are potentially infectious and appropriate safety measures should be taken, including the use of gloves, appropriate laboratory coats and face masks, as well as fitting an aerosol management system to the flow sorter.

As well as considering biological hazards, operators need to be aware of chemical hazards from the dyes and fluorochromes used in flow cytometry, especially the DNA-binding dyes (which are potentially mutagenic), and of laser hazards. Appropriate barrier goggles should be worn during laser alignment, and there should be controlled access to rooms containing Class IV lasers.

The cost involved in the purchase of a state-of-the-art flow sorter is not trivial, and there is frequently a need for a trained and experienced operator to ensure that initial investment is cost-effective. The principles of sorting are relatively straightforward, but successful sorting is very much experience-based, and a basic grounding and understanding of lasers, fluidics, and computing as well as biology are essential if a flow sorter is to be used to its full potential.

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# Appendix

## Useful Internet Sites

### Sites of General Interest for Cytometry

http://www.isac-net.org
Home page of the International Society for Analytical Cytology. Contains a Compendium of Internet

### *Sites With Direct Links to an Exhaustive Collection of Sites That Address issues in Cytometry*

http://www-ls.lanl.gov/NFCR
Los Alamos National Laboratory National Flow Cytometry Resource.

http://www.bio.umass.edu/mcbfacs/flowcat.html
Catalogue of free flow cytometry software (last updated 24/10/1997).

http://nucleus.immunol.washington.edul/ISAC.html
Compendium of cytometry internet sites.

http://www.biochem.mpg.de/research-groups/vale/cytorel.htl
G Valet's cytorelay site providing information on recent developments in cytometry and useful links to other sites.

http://www.cytometry.org
The USA Clinical Cytometry Society home page; a directory of suppliers and useful links.

http://www.cyto.purdue.edu
Purdue University cytometry pages; a major site for cytometry information.

http://www.cyto.purdue.edu/hmarchive/Cytometry
Purdue University mailing list archives. Questions and answers in cytometry.

http://pingu.salk.edu/fcm/sitelink.html

http://carmen.salk.edu/fcm/sites.html
Flow cytometry on the Web. Comprehensive links list for cytometry maintained by the Salk Institute, La Jolla, CA

From: *Flow Cytometry: Principles and Applications*
Edited by: M. G. Macey © Humana Press Inc., Totowa, NJ

**Table 1**
**Directories of Suppliers**

| Name | Internet address |
|---|---|
| Abcam (dynamic search for antibodies) | http://www.abcam.com |
| Anderson's List of Scientific companies | http://www.actg.com |
| BioSupplyNet | http://www.biosupplynet.com |
| Matt's Scientific Suppliers List | http://cmgm.stanford.edu/~footer/companies.html |
| NetSci's Biotech & Pharmaceutical YellowPages | http://www.netsci.org/Resources/Biotech/Yellowpages/xyz.html |
| SciQuest™ | http://www.sciquest.com |
| The Antibody Resource Page | http://www.antibody resource.com |

http://www.meds.com/leukemia/leukemia.html
Medicine Online Leukemia Information Library.

http://www.meds.com/leukemia/flow/flow0.html
Medicine Online Leukemia Library: Atlas with flow cytometry in AML.

http://www.hss.edu/research/flow/
The Fannie E. Rippel Foundation Flow Cytometry Core Facility at the Hospital for Special Surgery contains very useful links to many cytometry related sites and resources.

http://www.ncbi.nlm.nih.gov/prow
A useful CD guide

http://www.bork.emblheidelberg.de/Modules
Useful information on the structure of modules and domains found in leucocyte surface antigens.

http://www.cf.ac.uk/uwcm/hg/hoy/index.html
Royal Microscopical Society (Cytonet UK)

http://www.immune-source.com/html/favorite_links.html
Immune Source link site for flow cytometry

http://bioinformatics.weizmann.ac.il/hotmolecbase/
HotMolecBase: information resource and search engine for 'Hot' research molecules.

http://www.dti.gov.uk/bioguide/hsafety.html
United Kingdom Health and Safety Executive page providing access to biosafety information.

http://www.antibody resource.com
The Antibody Resource Page: A multidisciplinary page on antibodies with links to suppliers, databases, immunology and biotechnology sites.

http://www.wiley.com/products/subject/life/cytometry/flo_823710246.html
Wiley: Flow cytometry web site

## Suppliers of Instruments, Reagents, Antibodies, and Other Materials

**Table 1** gives the internet addresses of some directories of suppliers which may be searched alphabetically by name or by product, whereas **Table 2** gives the internet addresses of suppliers.

**Table 2**
**Addresses of Suppliers**

| Name | Internet address | Products and/or services |
|---|---|---|
| Aber Instruments Ltd | http://www.aber-instruments.co.uk | Microcyte portable cytometer |
| Accurate | http://www.accurate-chemical.com | Antibodies, cell separation |
| Actigen | http://www.actigen.com | Affinity proteins, e.g. protein A |
| Alexis | http://www.alexis.com | Server down |
| Amersham Pharmacia Biotech | http://www.apbiotech.com | Molecular biology/ biotechnology |
| Amgen Ltd | http://www.amgen.com | Recombinant cytokines |
| Amrad (incorporates Silenus Laboratories) | http://www.amrad.com | Antibody-based diagnostic technology |
| Ancell | http://www.ancell.com | Antibodies, recombinant proteins |
| Applied Cytometry Systems | http://www.applied-cytometry.com | Flow cytometry software |
| Bangs Laboratories Inc. | http://www.bagslab.com | Latex microspheres |
| Beckman Coulter | http://www.beckman-coulter.com | Flow cytometers, antibodies, assay kits, antigens |
| Becton Dickinson (UK) Ltd | http://www.bdfacs.com | Flow cytometers, antibodies, assay kits, antigens |
| Bibby-Sterilin | http://www.bibby-sterilin.com | Cell culture plasticware, glassware, and equipment |
| BioCytex | http://www.biocytex.com http://www.alexis.com/ | Antibodies, apoptosis, and cell enumeration kits |
| Biodesign International | http://www.biodesign.com | Antibodies, antigens, and proteins |
| BioErgonomics | http://www.bioe.com | Preparation and standardization reagents, assay kits, for flow cytometry |
| Biogenex | http://www.biogenex.com | Molecular and cellular pathology diagnostics |
| Biomeda | http://www.biomeda.com | Immunochemicals |

(*Continued*)

**Table 2 (*Continued*)**

| Name | Internet address | Products and/or services |
|---|---|---|
| Bio-Rad Laboratories Ltd | http://www.biorad.com | Flow cytometers, confocal microscopes, clinical diagnostics, life sciences research products |
| Biosource | http://www.biosource.com | Antibodies, media, peptides |
| Bio-Synthesis Inc. | http://www.biosyn.com | DNA, peptides, antibodies |
| Boehringer-Mannheim (now the Diagnostics Division of Roche) | http://www.roche.com/diagnostics | Molecular biology chemicals, apoptosis, and cell proliferation, clinical chemistry |
| British Biotech plc | http://www.britishbiotech.co.uk | Metalloenzyme inhibitors |
| Calbiochem | http://www.calbiochem.com | Biochemicals, antibodies, assay kits, immunochemIcals, and reagents |
| Caltag Laboratories | www.caltag.com | Immunological reagents, particularly multicolor flow cytometry assays |
| Cambridge Bioscience | http://www.bioscience.co.uk | Molecular and cell biology and immunology research products |
| Cedarlane | http://www.cedarlane.com | Immunologicals, cell separation media |
| Chemicon | http://www.chemicon.com | Immunological reagents and kits, antibodies, and detection systems |
| Chroma Technology Corporation | http://www.techexpo.com/firms/chromatc.html | Optical filters, specializing in cytometry and microscopy |
| Chromaprobe | http://www.chromaprobe.com | Antibodies and fluorochromes for flow cytometry |
| CLB Reagents | http://www.clb.nl | Blood grouping reagents, antibodies, human cytokines |
| Coherent Auburn Group | http://www.cohr.com | Lasers and laser-based systems |
| Compucyte | http://www.compucyte.com | Laser scanning cytometer and OnCyte™ system |
| CP Pharmaceuticals | http:cppharma.com | Heparin, therapeutics |
| Cymbus Bioscience | http://www.cymbus.com | Monoclonal antibodies and reagents |
| Cytek Corporation | http://www.cytek.com | Support for electronic product design, software development |

(*Continued*)

**Table 2** (*Continued*)

| Name | Internet address | Products and/or services |
|---|---|---|
| Cytimmune Sciences Inc. | http://www.cytimmune.com | Cytokine and angiogenic immunoassay kits |
| Cytomation, Inc. | http://www.cytomation.com/contactus.htm | Flow cytometers, accessories, and upgrades |
| DAKO Diagnostics Ltd | http://www.dako.com | Immunocytochemistry, flow cytometry, immunological, immunocytochemistry, and microbiology reagents |
| DiaSorin | http://www.diasorin.com | Medical diagnostics, immunohistochemistry |
| Diatek | http://www.diatec.com | Monoclonal antibodies |
| Dojindo Molecular Technologies, Inc. | http://www.dojindo.com | Antibodies and biochemicals |
| Dynal | http://www.dynal.no | Dynabeads: magnetic bead separations for cells, DNA, proteins, etc. |
| Enzyme System Products | http://www.enzymesys.com | Synthetic substrates, inhibitors, and enzyme assay kits |
| Exalpha | http://www.exalpha.com | Antibodies, flow cytometry reagents, immunophenotyping kits |
| Exciton Inc. | http://www.exciton.com | Dyes for use with laser excitation |
| Flow Cytometry Standards Corporation (FCSC) | http://www.fcstd.com | Standardization, quality control, and quantitation reagents for flow cytometry |
| Pall Gelman Laboratory | http://www.pall.com/gelman | Filtration and separation devices |
| Harlan | http://www.harlan.com | Immunochemicals, laboratory animal models, isolators |
| Hoechst (now merged with Rhone Poulenc and Aventis | http://hoechst.com | Chemicals and reagents for the life sciences |
| ICN Biomedicals Ltd | http://www.icnbiomed.com | Biomedical, biochemical, immunological, molecular, and cell biology reagents |
| Immune Source Corporation | http://www.immune-source.com | Antibodies, kits, and reagents for flow cytometry |
| Immunicon Corporation | http://www.immunicon.com | Magnetic nanoparticles for cell selection/depletion |
| Immuno Quality Products | http://www.iqproducts.nl | Antibodies and assay kits for flow cytometry |

(*Continued*)

**Table 2** (*Continued*)

| Name | Internet address | Products and/or services |
|---|---|---|
| Inova Diagnostics Inc. | http://www.inovadx.com | Autoantibody assays/detection |
| Integrated Genetics | | Molecular and cell biology reagents, immunochemicals |
| Intergen | http://www.intergenco.com | Reagents for biomedical research |
| Jackson ImmunoResearch Laboratories Inc | http://www.jacksonimmuno.com | Secondary antibodies for flow cytometry and other immunodetection systems |
| KPL, Kirkegaard & Perry Laboratories, Inc. | http://www.kpl.com/open2.html | Antibodies, substrates, DNA, RNA, proteins, *in situ* detection, and analysis |
| Lab Vision NeoMarkers Corporation | http://www.labvision.com | Antibodies for molecular biology/medicine, apoptosis, cell cycle, and so forth |
| Lampire Biological Laboratories | http://www.lampire.com | Antibodies, antibody production, animal sera, and plasm |
| LEINCO TECHNOLOGIES | http://www.leinco.com | Antibodies, recombinant proteins |
| Life Technologies | http://www.lifetech.com | Cell culture media, sera, primers |
| Linscott's Directory | http://ourworld.compuserve.com/homepages/LINSCOTTS-DIRECTORY | Directory of immunological and biological reagents |
| LOT-Oriel | http://lot-oriel.com | Lasers, optics, filters |
| Luminex | http://www.luminexcorp.com | Luminex analysers and microspheres |
| Medarex Inc | http://www.medarex.com | Monoclonal antibodies, including bispecific, humanised and immunotoxins |
| Medis-El | http://www.medisel.com | Diagnostic and reseach CellScan instruments |
| Melles Griot | http://www.mellesgriot.com/OVRVIEW2.HTM | Lasers and optics |
| Merck | http://www.merck.com | Biomedical and pharmaceutical products. Also online access to the Merck Manual |
| Molecular Probes | http://www.probes.com | Fluorescent reagents and techniques for biomedical research |
| National Collections of Type Cultures (NCTC) | http://www.dti.gov.uk/bioguide/culture.html | Cell and microbial culture collections |

(*Continued*)

**Table 2** (*Continued*)

| Name | Internet address | Products and/or services |
|---|---|---|
| Novocastra Laboratories Ltd | http://www.novocastra.co.uk | Proteins, antibodies, detection systems |
| Novus Biologicals, Inc. | http://www.novus-biologicals.com | Antibodies, recombinant proteins, cDNAs |
| O.E.M. Concepts, Inc. | http://www.oemconcepts.com | URL wrong |
| Omega Optical, Inc. | http://www.omegafilters.com | Optical filters |
| Oncogene Research Products | http://www.apoptosis.com | Molecular and cell biology rsearch products |
| Orpegen Pharma | http://www.orpegen.com | Clinical diagnostics, assay kits |
| Ortho | Have no web address | Flow cytometers, diagnostic reagents |
| PanVera Corporation | http://www.panvera.com | Fluorescence polarization, recombinant proteins, molecular and cell biology reagents |
| Partec | http://www.partec.de | Laser and arc lamp flow cytometers, reagents and standards |
| PeproTech Inc. | http://www.peprotech.com | Cytokines |
| PerSeptive Biosystems, Inc. | http://www.pbio.com | Purification, analysis and synthesis fore biomedical research, and drug development |
| BD Pharmingen | http://www.pharmingen.com | Molecular and cell biology reagents, assay kits, antibodies |
| Phoenix Flow Systems Inc. | http://www.phnxflow.com | Flow cytometry software and assay kits |
| Pierce | http://www.piercenet.com | Antibodies, recombinant proteins, derivitization, and cross-linking reagents |
| Polysciences | http://www.polysciences.com | Microspheres, biochemicals, immunological reagents |
| Primm Labs, Inc. | http://www.primmlabs.com | DNA reagents |
| R & D Systems | http://www.rndsystems.com | Cytokines, antibodies, cell separation, immunoassay kits, flow cytometry kits |

(*Continued*)

**Table 2 (*Continued*)**

| Name | Internet address | Products and/or services |
|---|---|---|
| Research Diagnostics Inc | http://www.researchd.com | Distributor for immunochemicals |
| Riese Enterprises | http://www.riese.com | BioSure® standards and controls for flow cytometry, stains and reagents |
| Rochester MicroSystems Inc. | http://www.rochestermicro.com | Software for flow cytometry and sorting and for automatic color compensation |
| Rockland Immunochemicals, Inc. | http://www.rockland-inc.com | Monoclonal and polyclonal antibody, recombinant protein production |
| Santa Cruz Biotechnology | http://www.scbt.com | Antibodies and proteins for molecular and cell biology research |
| Serotec | http://www.serotec.co.uk | Immunological and cell culture reagents, recombinant cytokines |
| Sigma Chemical Company Ltd | http://www.sigma.sial.com | Chemicals, biochemicals, immunological reagents |
| Signet Laboratories, Inc. | http://www.signetlabs.com | Antibodies, detection kits, and associated reagents |
| Southern Biotechnology Associates | http://www.Southern-Biotech.com | Antibodies, enzyme substrates, apoptosis reagents |
| Spectra Physics | http://www.spectraphysics.com | Solid state lasers |
| Spherotech, Inc. | http://www.spherotech.com | Fluorescent, magnetic, colored microparticles |
| Stratagene | http://www.stratagene.com | Molecular biology products |
| Sysmex Uk Ltd | | |
| TCS Microbiology | http://www.tcsmicro.co.uk | Animal blood, plasma, and serum, diagnostic kits |
| The Binding Site | http://www.bindingsite.co.uk | Antisera, antibodies, and conjugates, immunodiagnostic kits and reagents |
| Trevigen, Inc. | http://www.trevigen.com | Molecular biology, DNA, apoptosis, flow cytometry reagents |
| Universal Biologicals Ltd | No web address | Distributor for immunological reagents |

(*Continued*)

**Table 2** (*Continued*)

| Name | Internet address | Products and/or services |
|---|---|---|
| Upstate Biotechnology | http://www.upstatebiotech.com | Recombinant enzymes, assay systems, and antibodies for modification states of proteins |
| Vector Laboratories Ltd | http://www.vectorlabs.com | Antibodies, immmunostaining, flow cytometry reagents |
| VMRD Inc. | http://www.vmrd.com | Monoclonal antibodies, immunoquantitation and reagents |
| Worthington Biochemical Corporation | http://www.worthington-biochem.com | Enzymes, e.g. for tissue dissociation |
| Zymed Laboratories Inc. | http://www.zymed.com | Cell biology, *in situ* hybridization and immunoassay reagents |

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