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Light Microscopy

Overview and Basic Methods

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1. Introduction

1.1. Why Use Lectins?

Lectins are proteins or glycoproteins of nonimmune origin derived from plants, animals, or microorganisms that have specificity for terminal or subterminal carbohydrate residues. They are sensitive, stable, and easy-to-use tools. Lectin histochemistry and cytochemistry can provide an extraordinarily sensitive detection system for changes in glycosylation and carbohydrate expression that may occur during embryogenesis, growth, and disease. Although carbohydrates occur in a vast range of permutations that may be present in and between cells, there is frequently a dominance and conservation of structures to give specific markers of cell types or differentiation. Lectin histochemistry or cytochemistry can reveal subtle alterations in glycosylation between otherwise indistinguishable cells.

Lectins will specifically recognize and bind to carbohydrate structures on the surface of cells, on cytoplasmic and nuclear structures, and in extracellular matrix in cells and tissues from throughout the animal and plant kingdoms, down to bacteria and viruses.

1.2. What Do Lectins Bind to?

Sugar combining specificity of lectins is usually quoted in terms of the monosaccharide that best inhibits its binding to cells; for example, *Helix pomatia* lectin is said to be specific for *N*-acetyl-galactosamine, concanavalin

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A for mannose, and so on However, this is a massive oversimplification, and the exact, complex structures recognized by most lectins remain unknown. Lectins bind approx 10 times more avidly to solid-phase or immobilized sugars as to the same sugars in solution, which probably reflects the association–dissociation equilibrium between the lectin binding sites and the sugar, and the multivalency of lectins A typical lectin binding site may consist of three monosaccharides, and includes their linkage and adjacent protein or lipid backbone. Furthermore, a lectin will bind increasingly in 10-fold steps between mono-, di-, and trisaccharides.

1.3. Cell and Tissue Samples

Lectin histochemistry to detect alterations in cellular glycosylation can be performed on living cells in suspension, on cell smears, tissue imprints, or fresh cryostat sections. Here, sugars in the extracellular matrix, glycolipids, and glycoproteins can be detected readily If archival tissues are of interest (e.g., in which cellular glycosylation is to be correlated with disease progression or prognosis in a retrospective study), then sections from formalin-fixed, paraffin-embedded tissues may be preferred Here, glycolipids will have been lost during tissue processing and glycans may be sequestered during fixation and processing. For this reason, glycans—although still present—may be hidden and unavailable for lectin binding. Such sequestration can usually be at least partially overcome by pretreatment with a digestive enzyme such as trypsin, which reveals hidden structures

1.4. The Range of Lectins Available

About 100 different lectins are readily available commercially, and hundreds of others have been detected and described, mostly by their agglutination of red cells. Many lectins have not been assessed for their histochemical staining of tissues and cells.

In the United Kingdom, two suppliers with a wide range of lectins are Sigma, Poole, Dorset, UK, and EY Laboratories (UK agents are Bradsure Biologicals, Market Harborough, Leicestershire).

There undoubtedly exist hundreds, thousands, or tens of thousands of lectins in nature that remain to be discovered and purified. It is very simple to produce a crude lectin preparation in the laboratory that will give good results in immunohistochemistry Excellent sources include virtually any beans or seeds, and most green plant tissue like stems and leaves. The easiest method of detecting lectin binding with such preparations, or with unlabeled commercially purchased lectins in which no antibody is available, is through biotinylation of the lectin

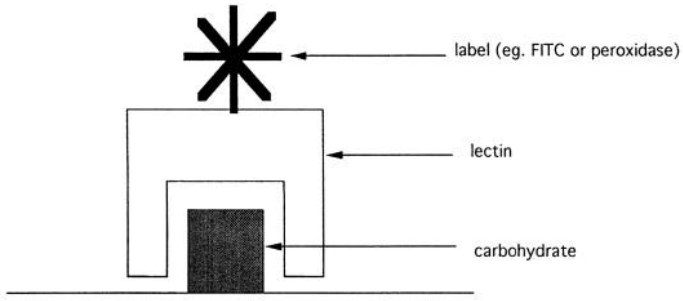

Fig. 1. The direct method.

1.5. Histochemical Methods

1.5.1. The Direct Method

The simplest methods for lectin histochemistry are the so-called direct methods, which rely on detection of lectin binding through the presence of a label conjugated directly onto the lectin molecule. The most commonly used labels are either fluorescent labels such as fluorescein isothiocyanate (FITC), which are visible when viewed under UV light, or enzyme labels like horseradish peroxidase, which yield a visible, colored reaction product (*see* Note 1). The principle of the direct method is illustrated in Fig. 1. The advantages of a direct method is that it is quick, easy, and cheap. The disadvantages are that it is generally less sensitive than the more complex methods (*see* Section 3.6.4.) and that the incorporation of a relatively large labeling molecule, like horseradish peroxidase, may interfere sterically with the combining site of the lectin and therefore slightly alter binding characteristics.

1.5.2. The Indirect Antibody Method

Here, the binding of native, unconjugated lectin is revealed by a second step in which a labeled (again, fluorescent or enzyme label) antibody (usually polyclonal, raised in rabbit) is added. The principle of this technique is illustrated in Fig. 2. The addition of a second layer has the advantage of increasing sensitivity and removes the potential problem of the label molecule interfering with binding specificity. Disadvantages are that the method takes longer, it is slightly more expensive because extra reagents need to be purchased, and there is slightly more potential for nonspecific background staining.

1.5.3. Indirect Sandwich Methods

Further layers can be added. For example, native, unconjugated lectin can be layered with first an unlabeled primary antibody (for example, a polyclonal

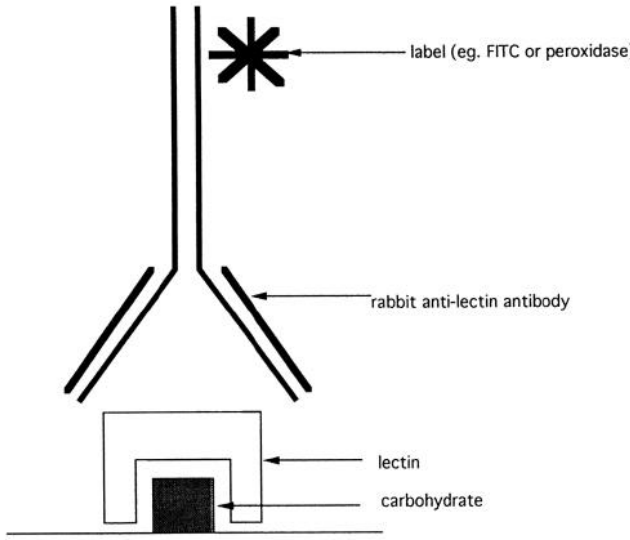

Fig. 2. The indirect antibody method.

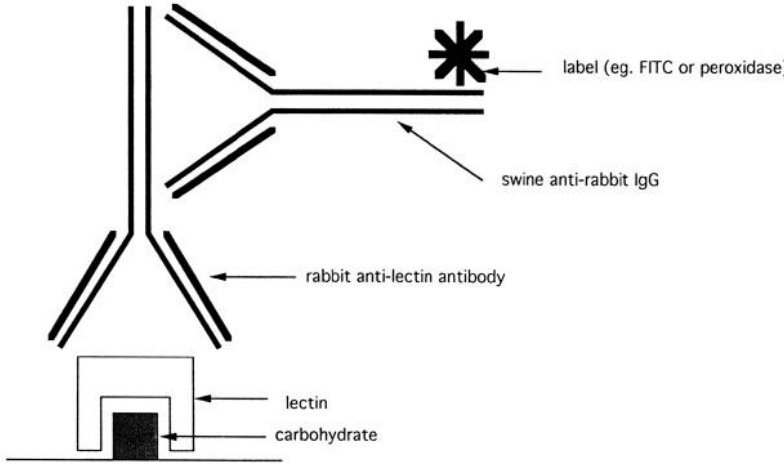

Fig. 3. An indirect sandwich method.

antibody raised in rabbit against the lectin), then a labeled second antibody (for example, a peroxidase labeled polyclonal antibody raised in swine against rabbit immunoglobulins). The principles of this method are illustrated in Fig. 3. Again, the advantage of adding extra layers is increased sensitivity. The disadvantages are, as before, that the method takes longer, is slightly more expensive, and there is slightly more potential for nonspecific background staining.

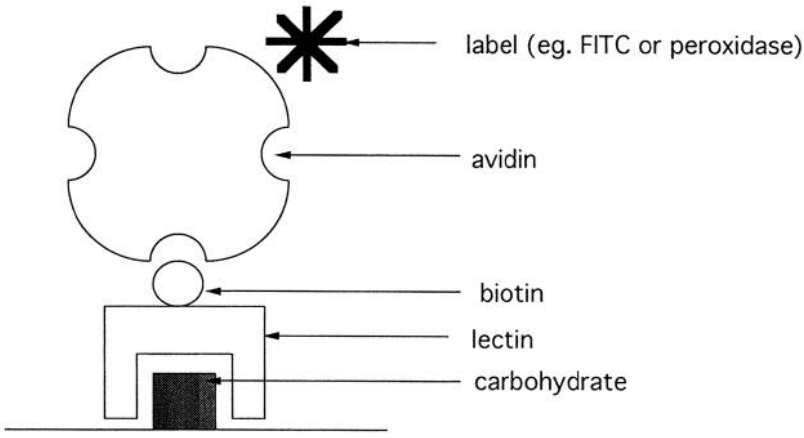

Fig. 4. The simple avidin–biotin method.

1.5.4. Simple Avidin–Biotin Method

Avidin–biotin techniques rely on the great avidity with which avidin (a protein originally derived form egg white) binds to biotin (one of the B group of vitamins). Lectins can be purchased ready labeled with biotin, or can be biotinylated in the laboratory using a simple method (*see* Sections 2.9. and 3.5.). Streptavidin, derived from *Streptomyces avidinii,* has largely replaced avidin, as it gives a cleaner staining result.

The simplest avidin–biotin technique relies on detection of the biotinylated lectin by streptavidin linked to a label such as peroxidase. The principle of this technique is illustrated in Fig. 4.

1.5.5. Multistep Avidin–Biotin Methods

The avidin–biotin interactions can be exploited in more complex multistep techniques. For example, unlabeled lectin is layered with a biotinylated antibody directed against it (e.g., polyclonal antisera raised in rabbit), followed by streptavidin peroxidase. Alternatively, lectin is layered first with unlabeled primary antibody (e.g., polyclonal antisera raised in rabbit) and then a second antibody (e.g., a polyclonal antibody raised in swine to rabbit immunoglobulins) labeled with biotin. This, again, would be followed by streptavidin peroxidase. The principle of these techniques is illustrated in Fig. 5.

1.5.6. Other Methods

Variations on these techniques can be developed to suit individual needs. It is probably better to start with a simple, quick, direct method before going on to more complex systems. Companies such as Dako (High Wycombe, United

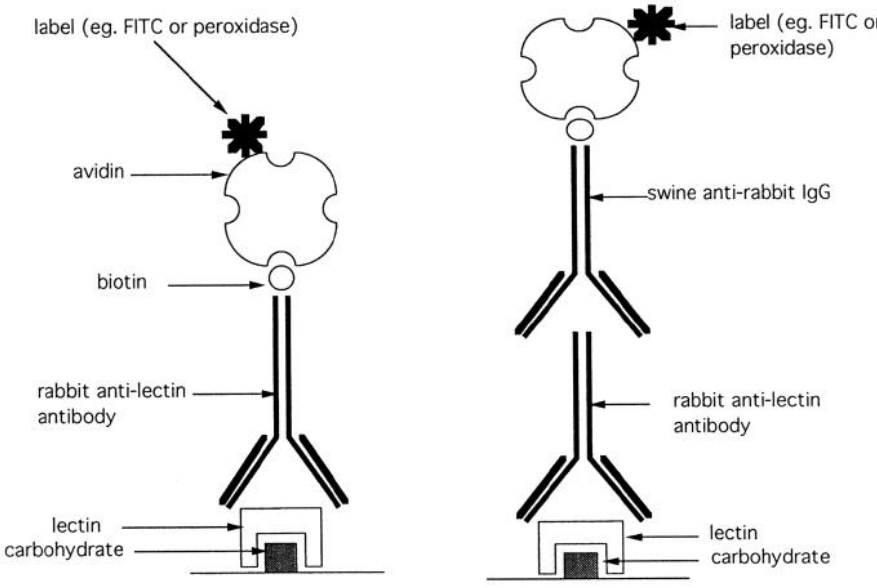

Fig. 5. Examples of multistep avidin–biotin methods.

Kingdom) supply easy-to-use kits of the main steps in some of the more complicated techniques, as well as a comprehensive range of antibodies.

1.6. A Philosophical Point Before Starting

Although numerous lectins have been commercially available for years, remarkably little progress has resulted. Histologists seem reluctant to try something new and the majority of lectin publications report on the same 10 or 12 lectins, frequently purchased as a lectin kit and chosen by the chemical company according to availability rather than a scientific basis. *(So be daring!)*

We recommend that interested researchers:

1. Start by using a well-characterized lectin that is almost certain to work.
2. Progress to try lesser known lectins.
3. Choose a biological phenomenon, a diagnostic problem, or some *a priori* hypothesis to test.
4. Or, select one lectin and explore the staining of a range of tissues; or select one tissue and explore a selection of lectins with a theme, such as galactose-binding or fucose-binding lectins (*see* Note 2).
5. Look for new or little used and uncharacterized lectins.

1.7. Which Lectins to Use and Which to Avoid

To get started, choose a cheap, well-documented lectin that binds to a wide variety of tissues. Concanavalin A is a splendid starter—it is cheap

and binds to most cells and tissues. *Ulex europeus* lectin UEAI is interesting in that it binds to blood vessel endothelium. Peanut lectin is also a good starter, as it binds very specifically to a variety of cell types, giving very beautiful staining patterns.

Some lectins are highly toxic, and should be avoided whenever possible. Lectins from *Ricinus communis,* the castor oil bean (also called Ricin), *Abrus precatorius,* the Jaquerty bean (also called Abrin), and *Viscum album* mistletoe, are all extremely toxic

Toxicity and hazard data are unavailable for most other lectins Generally, although dust and aerosols should obviously be avoided, no special precautions are needed.

1.8. Which Tissues to Use

To get started, you need a readily available, reproducible source of tissue that binds a wide variety of lectins. Kidney is very good for this—even fresh pig kidney from a supermarket. Epithelial rich tissue is generally successful Pituitary (for hormone precursors) and salivary glands and gut (different mucins) usually give interesting staining patterns.

1.9. The Way Forward

Lectin histochemistry is really a first step. Binding of lectins to tissue sections can demonstrate differences or changes in glycosylation, but their identification is more difficult By competitive inhibition of lectin binding by mono- and oligosaccharides and by enzyme digestion of tissue sections before lectin staining, it is possible to get some idea of the structures recognized; and this may be extremely sensitive and clinically useful But to identify the structure really needs extraction, separation, isolation of the carbohydrate, probably linked to a protein or lipid, then expensive and time-consuming procedures such as mass spectrometry, sequential enzyme digestion, and computer-assisted comparison with other known structures

1.10. Examples of the Useful Application of Lectin Histochemistry

1.10.1. Ulex europaeus *Lectin UEAI and Endothelial Cells*

Ulex europeus (gorse) has two main lectins—UEAI, which binds to fucose; and UEAII, which binds to *N*-acetyl glucosamine. UEAI is an excellent marker of human endothelial cells in tissue sections and in culture. It is probably a better marker than the more commonly used Von Willebrand factor. UEAI binding gives intense staining, particularly on fresh tissues or after trypsin digest in paraffin sections. Not all endothelial cells stain—for example, those

in gut may be negative—but some, like those in capillaries and muscle, do so intensely The binding is not absolutely specific for endothelial cells—for example, normal intestinal mucosal cells may sometimes stain. Some cancers, including prostate and pancreas, also stain strongly, presumably reflecting a change in fucosylation.

UEAI shows some species specificity, and different lectins may be more suitable for animal endothelium. *Bandeirea simplicifolia* isolectin 1 (BS-I), peanut lectin, and wheat germ lectin all stain endothelium in mouse, rat, pig, and sheep, and work well—though not as well as UEAI—in human tissues too

1 10 2. Helix pomatia *Lectin (HPA) and Cancer*

The lectin from *Helix pomatia* (the Roman or edible snail) recognizes terminal *N*-acetyl galactosamine, and to a lesser extent *N*-acetyl glucosamine and galactose sugars. It appears to be a very good prognostic marker in breast and other cancers. Approximately 80% of breast cancers will stain strongly for the binding of the lectin, whereas approx 20% are completely negative The positive cancers tend to be aggressive, to spread to other sites (metastasize), and have a very poor prognosis. The negative cases tend not to spread and have a much more favorable prognosis *(1,2)*. Studies have shown that HPA binding is also a marker of poor prognosis in other cancers, including colorectal *(3,4)*, gastric *(5)*, esophageal *(6)*, and prostatic cancers *(7)*. The HPA appears to recognize complex sugar(s) that are involved in aggressive biological behavior and spread of cancers.

2. Materials

2.1. Lectin Buffer (see Note 3)

1. 60 57 g Tris (Sigma), 87 g NaCl, 2.03 g $MgCl_2$, 1 11 g $CaCl_2$ dissolved in 1 L distilled H_2O.
2. Adjust pH to 7 6 using concentrated HCl
3. Make up to a total volume of 10 L with distilled water

2.2. Preparation of Tissue Imprints

1. Piece of fresh tissue approx 1 cm^3 in size.
2. Glass microscope slides
3. Acetone or methanol

2.3. Preparation of Cell Smears

1. Cells in suspension (e g., in tissue culture fluid, any suitable buffer, or in body fluids like blood)
2. Glass microscope slides
3. Acetone or methanol

2.4. Preparation of Frozen (Cryostat) Sections

1 Piece of tissue approx 0 5 cm^3 in size
2 Glass microscope slides
3 OCT embedding medium (Tissue-Tek, supplied by Raymond A. Lamb)
4 Isopentane or hexane
5. Liquid nitrogen
6 Acetone or methanol

2.5. Preparation of Formalin-Fixed, Paraffin-Embedded Sections

1 Formalin-fixed, paraffin-embedded blocks of tissue
2 Glass microscope slides.
3 20% ethanol in distilled water.
4. CNP30 (BDH) or xylene
5 99% v/v and 70% v/v ethanol in distilled water
6 Distilled water.
7 Lectin buffer

2.6. Trypsinization of Paraffin Sections

Trypsin solution should be made fresh immediately before use Glassware, buffers, and so on should be prewarmed to 37°C, and trypsinization should be carried out at 37°C either in a water bath or an incubator.

Dissolve 400 mg trypsin (crude type II from porcine pancreas, Sigma) and 400 mg $CaCl_2$ in 400 mL lectin buffer warmed to 37°C. Filter. Immerse slides in the solution at once and incubate at 37°C in an incubator or water bath (*see* Note 4).

2.7. Blocking Endogenous Peroxidase (see Note 5)

3% v/v solution of hydrogen peroxide in methanol. Make fresh as required.

2.8. Preparation of Crude Mushroom Lectin Extract

1. Mushrooms (the white, closed cup variety stocked by most supermarkets work well).
2. Laboratory tissue homogenizer, or domestic food processor or juicer
3 Lectin buffer or distilled water.

2.9. Labeling with Biotin

1. 0.1*M* $NaHCO_3$ in distilled water
2 *N*-hydroxysuccinimidobiotin (NHS-biotin, Sigma) (*see* Note 6).
3 Dimethylformamide or dimethyl sulfoxide
4 Lectin buffer

2.10. Biotinylated Mushroom Lectin Binding to Gut Sections

1 Paraffin or frozen sections of human or animal gut (materials listed in Section 2 4 or 2 5. and in 2.7 required)

2 Biotinylated crude mushroom extract (materials listed in Sections 2 8 and 2 9 required)
3 Lectin buffer
4 Streptavidin conjugated with horseradish peroxidase (Calbiochem, La Jolla, CA) diluted 1/400 in lectin buffer
5 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma). 0 5 mg/mL in lectin buffer Hydrogen peroxide added to give a concentration of 5% v/v immediately before use (*see* Note 7)
6 Mayers hematoxylin solution (Sigma)
7 70% v/v, 95% v/v, and 99% v/v ethanol in distilled water
8 CNP30 (BDH) or xylene
9 Depex resinous mounting medium (BDH)

2.11. Direct Method to Detect Peroxidase-Labeled Peanut Lectin Binding to Kidney Sections

1 Paraffin or frozen sections of human or animal kidney (materials listed in Section 2 4 or 2 5 required, materials listed in Sections 2 6 and 2 7 required)
2 Peroxidase-labeled peanut lectin (PNA, Sigma) at a concentration of 10 µg/mL in lectin buffer
3 Lectin buffer
4 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma) 0 5 mg/mL in lectin buffer Hydrogen peroxide added to give a concentration of 5% v/v immediately before use (*see* Note 7)
5 Mayers hematoxylin solution (Sigma).
6 70% v/v, 95% v/v, and 99% v/v ethanol in distilled water
7 CNP 30 (BDH) or xylene
8 Depex resinous mounting medium (BDH).

2.12. Direct Method to Detect FITC-Conjugated UEAI Binding to Endothelial Cells

1 Paraffin sections, frozen sections, or imprints of human striated muscle, or smears of cultured human endothelial cells (materials listed in Sections 2 2.–2 4 , or 2 5. required)
2 Fluorescein isothiocyanate (FITC)-labeled *Ulex europeus* isolectin I (UEAI) (Sigma) at a concentration of 10 µg/mL in lectin buffer
3 Lectin buffer

2.13. Indirect Antibody Method to Detect UEAI Binding to Endothelial Cells

1 Paraffin sections, frozen sections, or imprints of human striated muscle, or smears of cultured human endothelial cells (materials listed in Sections 2 2 –2.4 , or 2 5 required; materials listed in Sections 2 6 and 2.7 required)
2 *Ulex europeus* isolectin I (UEAI; Sigma) 10 µg/mL in lectin buffer

3 Lectin buffer
4 Horseradish peroxidase-labeled polyclonal rabbit anti-UEAI (Dako, Santa Barbara, CA) diluted 1/200 in lectin buffer (*see* Section 2 1.)
5 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma) 0 5 mg/mL in lectin buffer Hydrogen peroxide added to give a concentration of 5% v/v immediately before use (*see* Note 7)
6. Mayers hematoxylin solution (Sigma)
7 70% v/v, 95% v/v, and 99% v/v ethanol in distilled water
8 CNP30 (BDH) or xylene
9 Depex resinous mounting medium (BDH)

2.14. Helix pomatia *Lectin Binding to Breast Cancers*

1. Paraffin sections of breast cancers (materials listed in Sections 2.5.–2 7 required)
2 *Helix pomatia* lectin (HPA, Sigma) at a concentration of 10 µg/mL in lectin buffer
3 Lectin buffer (*see* Section 3 1)
4. Polyclonal rabbit anti-HPA (Serotec, Oxford, UK) diluted 1/200 in lectin buffer
5 Biotinylated polyclonal swine antibody against rabbit immunoglobulins (Dako) diluted 1/400 in lectin buffer.
6 Streptavidin-labeled horseradish peroxidase (Calbiochem) diluted 1/400 in lectin buffer
7 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) 0 5 mg/mL in lectin buffer. Hydrogen peroxide added to give a concentration of 5% v/v immediately before use (*see* Note 7)
8. Mayers hematoxylin solution (Sigma)
9 70% v/v, 95% v/v, and 99% v/v ethanol in distilled water
10. CNP30 (BDH) or xylene
11 Depex resinous mounting medium (BDH).

3. Methods

3.1. Preparation of Samples

3.1.1. Tissue Imprints

1 Slice through a piece of fresh tissue using a clean, sharp scalpel blade
2 Touch the cut surface briefly, gently, and cleanly onto a clean microscope slide Do not allow the tissue to drag on the surface of the slide and do not press hard—a light touch is enough
3. Air-dry for 5 min (slides may then be stored wrapped in foil in the freezer until required)
4. Fix by dipping in acetone or methanol for 1 min and then air-dry

3.1.2. Cell Smears

1 Smear cells onto two clean glass microscope slides by placing a drop of cell suspension near one end of the first slide, placing the second slide on top, and dragging one slide against the other in a swift, smooth movement

2 Air-dry for 5 min (slides may then be stored wrapped in foil in the freezer until required)
3 Fix by dipping in acetone or methanol for 1 min

3 1.3. Frozen (Cryostat) Sections

1 Snap freeze a 0 5-cm^3 piece of fresh tissue on an OCT-coated cryostat chuck by immersion in isopentane or hexane, precooled in liquid nitrogen Allow to equilibriate to the temperature of the cryostat before cutting
2 Cut 5-μm thick sections using a cryostat
3 Air-dry for 5 min (slides may then be stored wrapped in foil in the freezer until required)
4 Fix by dipping in acetone or methanol for 1 min

3.1.4. Formalin-Fixed, Paraffin-Embedded Sections

1 Cool tissue blocks on ice for approx 15 min
2 Cut 5-μm thick sections by microtome
3 Float sections on a pool of 20% ethanol in distilled water on a clean glass plate
4 Carefully float sections from the ethanol solution to the surface of a water bath heated to 40°C
5 Pick the sections up on clean glass slides Allow to drain for 5–10 min (*see* Note 8)
6 Dry in an incubator at 37°C overnight, then at 60°C for 20 min Slides may then be cooled, stacked, or boxed, and stored at room temperature until required
7 When required, soak in CNP30 or xylene for 20 min to dissolve the wax (*see* Note 9)
8 Transfer through two changes of 99% ethanol, through one change of 70% ethanol and finally to distilled water, agitating the slides for 1–2 min at each stage to equilibriate
9 Allow to stand for 5 min in lectin buffer

3.2. Trypsinization of Paraffin Sections (see Note 4)

1 Place sections in a bath of trypsin solution and incubate at 37°C for 5–30 min, depending on the tissues and/or lectins used.
2 Wash in running tap water for 5 min
3 Allow to stand for 5 min in lectin buffer

3.3. Blocking Endogenous Peroxidase (see Note 5)

1 Immerse sections in methanol/hydrogen peroxide solution for 20 min
2 Wash in running tap water for 5 min
3 Allow to stand for 5 min in lectin buffer

3.4. Preparation of a Crude Mushroom Lectin Extract (see Note 10)

1 Chop mushrooms roughly with a scalpel blade
2. Homogenize in a laboratory tissue homogenizer or domestic food processor or juicing machine A small volume of distilled water or lectin buffer may be added if the extract is too thick or viscous

3 Filter.
4. The extract may be stored frozen until required

3.5. Labeling Lectins with Biotin (see Note 6) (8–10)

1 Dissolve the lectin at a concentration of 1 mg/mL in 0 1*M* $NaHCO_3$, or dialyze crude extract or lectin solution against 0 1*M* $NaHCO_3$ overnight.
2. Remove NHS-biotin from the freezer and allow to thaw for approx 30 min (*see* Notes 6 and 11).
3 Dissolve NHS-biotin in dimethylformamide or dimethylsulfoxide at a concentration of 1 mg/mL
4 As rapidly as possible after dissolving the NHS biotin, add the biotin solution to the lectin solution in a ratio of approx 120 µL biotin solution·1 mL lectin solution. Mix immediately and well. React at room temperature, preferably with constant gentle mixing, e g , on an end-over-end mixer, for 2 h or overnight
5 Dialyze overnight against lectin buffer, and store at 4°C

3.6. Examples of Staining Methods (see Note 12)

3.6.1. Direct Method to Detect Peroxidase-Labeled Peanut Lectin Binding to Kidney Sections

1 Paraffin sections of human or animal kidney should be used, treated as described in Sections 3 1 4 and 3 3 , or fresh, frozen sections treated as described in Sections 3 1 3. and 3 3 Paraffin sections should be trypsinized for 20 min as described in Section 3 2
2 Incubate sections with peroxidase-labeled PNA for 1 h
3. Wash in lectin buffer (*see* Note 13)
4 Incubate with DAB for 10 min (*see* Note 7)
5 Wash in running tap water
6 Counterstain by immersing in Mayers hematoxylin for 2 min
7 Wash in running tap water for 5–10 min
8 Pass through 70% ethanol, 95% ethanol, and two changes of 99% ethanol Agitate the slides at each stage until equilibriated.
9 Agitate for 1–2 min in CNP30 or xylene.
10 Mount in a resinous mountant such as Depex.

3 6.2. Biotinylated Mushroom Lectin Binding to Gut Sections

1. Paraffin sections of human or animal gut should be used, treated as described in Sections 3.1.4. and 3 3., or fresh, frozen sections treated as described in Sections 3 1.3 and 3 3
2. Incubate with biotinylated mushroom lectin extract, prepared as described in Sections 3 4 and 3 5
3. Wash in lectin buffer (*see* Note 13).
4 Incubate with strepatavidin peroxidase for 30 min.
5 Wash in lectin buffer.
6. Treat as described in steps 4–10 in Section 3.6.1

3 6.3. Direct Method to Detect FITC-Conjugated UEAI Binding to Endothelial Cells

1 Paraffin sections of human striated muscle should be used, treated as described in Sections 3 1 4 , and trypsinized for 10 min as described in Section 3 2 , or fresh, frozen sections treated as described in Sections 3 1 3 , or imprints treated as described in Section 3.1 1., or smears of cultured human endothelial cells treated as described in Section 3 1 2
2. Incubate with FITC-UEAI for 1 h
3 Wash in lectin buffer (*see* Note 13)
4 View wet under UV light using a fluorescent microscope

3.6.4 Indirect Antibody Method to Detect UEAI Binding to Endothelial Cells

1 Paraffin sections of human striated muscle should be used, treated as described in Sections 3 1 4 and 3 3 , and trypsinized for 10 min as described in Section 3 2 , or fresh, frozen sections treated as described in Sections 3 1 3 and 3 3, or smears of cultured human endothelial cells treated as described in Sections 3 1 2. and 3 3
2 Incubate with UEAI for 1 h
3 Wash in lectin buffer (*see* Note 13).
4 Incubate with peroxidase conjugated rabbit anti-UEAI for 1 h
5 Wash in lectin buffer
6 Treat as described in steps 4–10 in Section 3 6 1

3.6.5. Helix pomatia *Lectin Binding to Breast Cancers*

1 Paraffin sections of human breast cancer should be used, treated as described in Sections 3 1 4 and 3 3 , and trypsinized for 20 min as described in Section 3 2
2 Incubate with HPA for 1 h
3 Wash in lectin buffer (*see* Note 13).
4 Incubate with polyclonal rabbit anti-HPA for 1 h
5. Wash in lectin buffer
6 Incubate with biotinylated swine antirabbit for 1 h
7 Wash in lectin buffer.
8 Incubate with streptavidin peroxidase for 30 min
9. Wash in lectin buffer
10 Treat as described in steps 4–10 in Section 3 6 1

4. Notes

1 Other enzyme labels are available—for example, alkaline phosphatase, alcohol dehydrogenase, and glucose oxidase. Other fluorescent labels are available—for example, Texas red and tetramethylrhodamine isothiocyanate (TRITC). Electron-dense labels such as ferritin and colloidal gold can also be used
2 Lectins with the same nominal sugar binding specificity—for example, "fucose binding lectins" or "galactose binding lectins"—will actually have quite differ-

ent specificities for complex sugar structures (as described in Section 1.2) and will thus give very different histochemical staining patterns on the same tissues.

3. The binding of some lectins is dependent on the presence of calcium and other metals. Buffers containing phosphate ions, like phosphate-buffered saline (PBS), are therefore generally unsuitable for lectin histochemistry, as the phosphate ions bind with and sequester the metals. We therefore recommend the use of a general lectin buffer for all dilutions and washes. This is basically a Tris-HCl buffer, pH 7.6, but with added calcium chloride and magnesium chloride.
4. Digestion with an enzyme such as trypsin can often be helpful in revealing carbohydrate structures hidden or damaged during fixation and processing of paraffin-embedded tissues. Different tissues, fixed and processed in different ways, and different lectins, vary enormously in their requirement for trypsinization. We would recommend that when a new method is being tested, the effect of a range of trypsinization times is assessed (e.g., no trypsin, 5 min trypsinization, 10 min trypsinization, 20 min trypsinization, and 40 min trypsinization). Trypsinization can sometimes dramatically enhance staining. It is better to use a fairly crude preparation of trypsin—we recommend the type II crude trypsin from porcine pancreas stocked by Sigma—as sometimes the impurities (like chymotrypsin) actually enhance its effect! Trypsin solutions should be made up fresh when required, as they lose their activity over time. For this reason, it is rarely worth continuing trypsin digestion for more than 30–40 min. Over-trypsinization will result in overdigestion of tissue and loss of morphology. Trypsinization should be carried out at 37°C—all glasswear, buffers, and so on should be prewarmed before use. Trypsinization is only appropriate for formalin-fixed, paraffin-embedded tissues, it is *not* appropriate for cell imprints, smears, or frozen sections.
5. If horseradish peroxidase is to be used as a label, it is necessary to quench any endogenous peroxidase in the tissue sections. Failure to carry out this step will result in high levels of background staining. It is, obviously, not necessary to carry out this step if a fluorescent tag, such as fluorescein isothiocyanate, is used, or if other enzyme labels such as alkaline phosphatase are used. If other enzyme labels are used, it may be helpful to carry out a different blocking step appropriate to that enzyme—for example, if alkaline phosphatase is used, endogenous alkaline phosphatase can be blocked using levamisole.
6. We purchase NHS-biotin in 5-mg aliquots—the smallest supplied by Sigma. For each biotinylation experiment, one 5-mg aliquot is allowed to thaw, then dissolved in 5 mL dimethylformamide. Any unused NHS-biotin is discarded at the end of the procedure. We find this gives consistently successful biotinylation, and is a more satisfactory approach than buying a larger quantity of NHS-biotin, and thawing and weighing out for each experiment.

Biotinylation is a very quick, cheap, and easy way of labeling lectins. For lectins purchased in an unlabeled form in which no antibody is commercially available, or for homemade or obscure lectins, it is often the easiest—sometimes the only—practical way of detecting their histochemical binding.

7 DAB is potentially carcinogenic and should therefore be handled with care Wear gloves Avoid spillages and aerosols Work in a fume cupboard. After use, all equipment, glasswear, and so on should be soaked in diluted bleach overnight before washing; worktops should be swabbed down with dilute bleach and rinsed with plenty of water

To limit the risks associated with weighing out, we purchase DAB in 1-g batches An entire 1-g batch is dissolved in 200 mL distilled water and 1-mL aliquots transferred to 10-mL capped plastic tubes and frozen until required. When required, a tube is thawed, and 9 mL lectin buffer and the appropriate volume of hydrogen peroxide are added

8 If sections are damaged in any way—for example, if they have tiny scores or tears—or if a very long staining method is to be undertaken, it may be a good idea to precoat the clean glass slides with an adhesive such as poly-L-lysine to help the tissue section stick firmly to the glass. A tiny drop of a 0.1% w/v aqueous solution of poly-L-lysine (Sigma) is applied near one end of a clean glass microscope slide A second slide is placed against it, and one is dragged against the other in a fast, smooth movement to leave a very thin film on both slides The slides should be allowed to air dry for about 5–10 min, then sections applied to them as usual

9 Once paraffin sections have been dewaxed, it it imperative that they not be allowed to dry out at any point, as this will result in high levels of dirty, nonspecific background staining

10 An extremely crude method for extraction of lectin from mushrooms is given In spite of its obvious limitations, it works very well and should give excellent histochemical results. This type of approach is equally applicable to many other lectins—for example, a very adequate preparation of peanut lectin can be obtained by soaking peanuts in distilled water or lectin buffer overnight. Experiment!

11. It is critical to exclude water from the NHS-biotin before it mixes with the lectin solution For this reason, the vial should be thoroughly thawed before the cap is removed in order to discourage condensation of water vapor from the air.

12 The methods are given to act as examples The scientist is encouraged to try other lectins, other tissues, other staining protocols—the applications are limitless!

13. Thorough washing is critical for good histochemistry results. We recommend washing the sections in at least three changes of lectin buffer Each wash should last at least 1–2 min Agitation helps a great deal. We would recommend placing the slides in a metal slide rack and jiggling them up and down in the buffer quite vigorously! If problems with background staining are encountered, one of the major culprits is likely to be insufficiently vigorous washing

14. If a high level of nonspecific background staining is observed, and this cannot be corrected by vigorous washing (*see* Note 13), a blocking solution may be required In traditional immunohistochemistry, the approach would be to incorporate 1–5% normal rabbit or swine serum into antibody dilutions and washes This is *not* appropriate for lectin histochemistry as many lectins bind to glycoproteins in serum We recommend incorporating 1–5% bovine serum albumin

into the lectin and antibody solutions. This can sometimes dramatically improve staining results

15 The optimum temperature for lectin binding may be lower than the usual room temperature and, although this should not normally cause any problems, some experimentation is worthwhile Remarkably, some mammalian lectins appear to bind best at 7 or 8°C, and minimally at 37°C

16. The optimum binding of lectins may be pH dependent Again, this should not cause problems, and most lectin histochemistry will work well at pH 7.6 If problems are encountered with unusual or little-used lectins, some experimentation may be helpful.

17. Ten micrograms per milliliter seems to be a good, optimal concentration for most lectins in histochemistry and, if a new lectin is to be tried, it is a sensible concentration to start with We recommend trying a range of concentrations (e g , 2 5, 5, 10, 20, and 40 μg/mL) to assess which gives the best results Some lectins, such as pokeweed, work best at very low concentrations (~1 μg/mL), others, such as *Limulus polyphemus*, require much higher concentrations (~100 μg/mL)

18 Lectins are usually purchased as a dry powder They can be conveniently stored as a stock solution of 1 mg/mL in lectin buffer at 4°C, and will be stable for months or years Most native lectins are also reasonably stable, stored at 4°C at their working dilution (e g., 10 μg/mL in lectin buffer) However, some lectins (such as *Limulus polyphemus*) are remarkably labile

19 Specificity of lectin binding may be confirmed by competitive inhibition using the appropriate monosaccharide Lectin is incubated on the tissue section in the presence of 0 1*M* monosaccharide—for example, UEAI in the presence of 0.1*M* fucose Alternatively, bound lectin may be displaced by subsequent incubation with the sugar solution With some lectins, competitive inhibition is very strong, giving complete abolition of staining; with other lectins, a significant reduction in staining intensity, but not complete abolition, may be expected

Suggestion for Further Reading

A publication that is out of print but still full of interesting detail (beautiful electron micrographs and 50 pages of references on lectin cytochemistry and histochemistry) is: Schrevel, J., Gros, D., and Monsigny, M (1981) Cytochemistry of Cell Glycoconjugates. 250 page monograph in *Progress in Histochemistry and Cytochemistry* **14(2),** Gustav Fischer Verlag, Stuttgart, New York.

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a α-mannosidase in 0 1*M* citrate buffer, pH 4 5, at 26°C *(32)*
b α-L-fucosidase in 0 1*M* phosphate buffer, pH 6 5, at 26°C *(32)*.
c ß-galactosidase (Grade VIII from *Escherichia coli*, Sigma) in 0 1*M* phosphate buffer, pH 7 3, with 1 1 × 10^{-6}*M* magnesium chloride and a trace of mercaptoethanol at 37°C *(26,32)*
d. ß-*N*-acetylglucosaminidase in 0 1*M* citrate buffer, pH 5 0, at 26°C *(32)*

During an incubation period of 1 h, four separate aliquots of enzyme are applied to the sections, each one for 15 min, so that the enzyme is not exhausted Each aliquot may subsequently be tested by incubation with the appropriate *p*- or *o*-nitrophenyl glycoside, which turns yellow on the addition of a few drops of 10*N* sodium hydroxide if the enzyme is active Negative controls, with sections being incubated in the buffer alone, should always be carried out

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1 Dewax, block endogenous peroxidase, and trypsinize if necessary
2 Rinse in TBS 3 × 5 min
3 Rinse briefly in distilled water
4 Make up the following mixture and allow to warm up to 45°C
   a 0 954 g potassium hydroxide, and
   b 40 mL distilled water
   Allow to dissolve and add
   a 50 mL dimethyl sulfoxide, and
   b 10 mL Analar ethanol (Potassium hydroxide solution· 0 17*M* final concentration)
5 Incubate sections for 40–60 min at 45°C
6 Carefully wash twice in 10 m*M* HCl to neutralize Sections tend to detach at this stage!
7 Wash in distilled water followed by TBS for 3 × 5 min
8 Continue staining with lectins as normal

4. Notes

1 Tris-buffered saline—0 5*M* stock solution
   a Dissolve 60 57 g of Tris (hydroxymethyl) methylamine and 81 g of sodium chloride in 800 mL of distilled water
   b Adjust pH to 7 6 using concentrated hydrochloric acid (using correct safety precautions)
   c Make up to 1 L with distilled water (to give 0 5*M* stock solution)
      This may be stored in a refrigerator in a stoppered bottle until required, when it should be diluted with 9 L of distilled water to make the working concentration of 0 05*M* TBS
      Warning: Hydrochloric acid is corrosive, very toxic, and an irritant!! Always wear acid resistant gloves and eye protection Store in acid cupboard and measure out in fume cupboard.
2 The use of a high molarity buffer eliminates nonspecific binding of avidin–peroxidase to tissue sections *(44)*. This is particularly useful when staining highly anionic structures such as mast cells and matrix components *(4,27,33)*
3 There are other techniques utilizing colloidal gold the direct staining method using a lectin conjugated directly to gold particles, as well as the method using biotinylated lectins followed by an avidin–gold or streptavidin–gold solution
4 The lectins chosen for any particular study will depend on the purpose of the study and the amount of information, if any, already available as to the glycans present in the tissues of interest. Tables of lectin-binding characteristics are given in the Appendix and in refs. *26–28*, *30*, and *31*, together with citations of studies defining their specificities
5 Apart from neuraminidase, other glycosidases (e g , from Sigma) at a variety of concentrations (normally between 0.1 and 10 U/mL but always in excess) may also be used with appropriate buffers, at various temperatures depending on the source of the enzyme·

7 Place grids in antibiotin gold (10 nm) diluted 1 30 with TBS + 1% (w/v) BSA for 1 h at room temperature
8 Rinse in drops of distilled water, wash in 5 vials of distilled water and dry
9 Contrast with 2% (w/v) uranyl acetate and Reynold's lead citrate prior to examination in the electron microscope

3.9. Pretreatment of Ultrathin Epoxy Resin Sections for Lectin Histochemistry

1 Cut pale gold/gold sections and mount on nickel or gold (but not copper) grids
2 Etch sections in 1% (v/v) saturated sodium ethoxide in ethanol for 1 min or longer (up to 5 min)
3 Rinse (10 dips) in absolute alcohol, 90% (v/v) alcohol, 70% (v/v) alcohol
4 Rinse in distilled water
5 Remove osmium, if necessary, with 0 1 g/mL sodium periodate for 40 min on orbital shaker This may also etch the resin and may be included with nonosmicated tissue
6 Distill water washes 3 × 5 min
7. Proceed with staining protocol

3.10. Glycosidase Treatment of Sections (42) (see Note 5)

The presence of sialic acid may be confirmed by incubating sections in the enzyme neuraminidase, which cleaves off terminal sialic acid residues. Neuraminidase may be derived from many different sources; the one used in our laboratory is obtained from *Clostridium perfringens* (Type VI, Sigma). Different neuraminidases will vary in their substrate specificity, some being specific for 2–3 or 2–6 linkages.

1. Remove resin, and block endogenous peroxidase activity as in Section 3 4.
2 Treat with trypsin if necessary. Wash in TBS and then in distilled water
3 Apply neuraminidase solution. 0.1 U/mL in 0 2*M* acetate buffer, pH 5.5, with 1% (w/v) $CaCl_2$ for 1 h at 37°C in a humidity chamber. Gently remove excess enzyme and replace with fresh solution. Leave for another hour as before As neuraminidase may be contaminated with proteases, too long of an incubation may result in digestion of the tissue by these enzymes.
4. Rinse off with distilled water, then TBS, and continue staining with the lectins, and so on. Sialic acid may also be removed by mild hydrolysis with 1*N* sulfuric acid at 60–70°C for 1 h

3.11. ß-Elimination (35)

Unlike their *N*-linked counterparts, *O*-linked glycans are susceptible to the action of mild alkali, which removes them from the protein backbone (ß-elimination). This technique can be applied to tissue sections to distinguish between *N*- and *O*-linked glycan *in situ*. It is not recommended for plastic sections, or for sections not fixed to the slide by means of an adhesive.

3.6. Lectin Staining of Semithin, Acrylic Resin Sections (Fig. 2)

The method is used as in Section 3.5., but the DAB (step 12) is increased to 10 min and then an intensification method may be used, such as the gold–sulfide–silver technique *(36)*

1 After treating with DAB for 10 min, wash in distilled water for 6 min
2 Treat with 0 02% (w/v) gold chloride for 3 min
3 Wash in distilled water for 6 min
4 Apply neutralized 0 3% (w/v) sodium sulfide for 3 min
5 Wash in distilled water for 6 min
6 Develop in Gallyas' silver solution for about 4 min or until sections appear fully stained; monitor this with a microscope.
7 Fix in 1% (v/v) acetic acid, 3 × 10 min
8 Dehydrate, clear and mount

3.7. Lectin Staining of Ultrathin Sections, Acrylic Resin (L.R. White), Using a Peroxidase-Based Revealing System (33)

1 Mount pale gold acrylic sections onto nickel or gold (but not copper) grids Air-dry
2 Float sections face down on 10% (v/v) hydrogen peroxide for 10 min to etch the sections and also block endogenous peroxidase activity
3 Wash in distilled water, then TBS
4 Incubate in 10 µg/mL biotinylated lectin in TBS with 1 m*M* $CaCl_2$ for 15 min at room temperature
5 Jet wash in TBS + $CaCl_2$, then place in drops of 5 µg/mL avidin peroxidase in 0 125*M* TBS containing 0 374*M* NaCl for 30 min at room temperature
6 Jet wash in TBS followed by the DAB-H_2O_2 procedure described in Section 3 5. for 8 min
7 Intensify the reaction with 0 1% (w/v) gold chloride for 1 min followed by thorough washing (This step is optional)
8 Air-dry sections and examine without further staining Over time, the gold particles may aggregate to form a precipitate over the sections

3.8. Lectin Staining of Ultrathin Sections Using Colloidal Gold (43) (see Note 3)

1. After appropriate etching pretreatments, immerse grid in drops of TBS + 1% (w/v) BSA for 5 min Blot edge of grid
2 TBS + 1% (w/v) gelatin for 10 min Blot
3 TBS + 0 2% (w/v) glycine for 3 min Blot
4 Place grids in drops of TBS + 1 m*M* $CaCl_2$ + 0 5% Tween-20, wash in three vials of the same, and place in fresh drops again
5 Blot and transfer to drops of biotinylated lectin, 10 µg/mL in TBS + 1 m*M* $CaCl_2$ for 1 h at room temperature
6 Wash as in step 4 Blot

8. After wiping around the section to remove excess TBS, pipet on the biotinylated lectin (10 µg/mL in TBS + 1 m*M* $CaCl_2$) Incubate for 30 min at room temperature in a humidity chamber
9 Wash off with TBS + $CaCl_2$ and give 3 × 5 min washes in TBS + $CaCl_2$
10 Wipe off excess buffer and incubate in avidin peroxidase 5 µg/mL in 0 125*M* TBS, pH 7.6, containing 0 374*M* NaCl (made by diluting 1 part 0 5*M* stock TBS with 3 parts distilled water, *see* Note 2) for 1 h at room temperature in a humidity chamber
11 Wash off with TBS and then wash 3 × 5 min in TBS
12 Reveal sites of lectin binding in 0 05% (w/v) diaminobenzidine tetrahydrochloride (Aldrich) in TBS with 0.015% (v/v) hydrogen peroxide (diluted from 100 vol) for 5 min at room temperature
13. Counterstain sections in hematoxylin or methyl green before washing, dehydration, and mounting in XAM (Gurr, BDH)

3.5. Lectin Staining of Semithin, Epoxy Resin Sections (38) (Fig. 1)

1 Cut sections 0 75 µm thick and mount on APES-coated slides
2 Dry on a hotplate and then at 50°C for 2 d to ensure adhesion.
3 Remove resin in 50% (v/v) saturated sodium ethoxide in ethanol for 15 min at room temperature
4 Wash in absolute ethanol and gradually rehydrate to distilled water Wash in TBS 3 × 2 min
5 Treat with trypsin if necessary warm two baths containing 300 mL TBS to 37°C in a water bath To one, add 300 mg calcium chloride Place slides in the other bath and add either 100 or 300 mg trypsin (type II porcine, crude, Sigma) to the bath containing the calcium chloride After 5 min, transfer the slides to the trypsin/$CaCl_2$ mixture and incubate for 4 min at 37°C
6. Wash in running cold water to stop the reaction
7 Wash in TBS 3 × 5 min
8 After wiping around the section to remove excess TBS, pipet on the biotinylated lectin (10 µg/mL in TBS + 1 m*M* $CaCl_2$) Incubate for 45 min at 37°C in a humidity chamber.
9 Wash off with TBS + $CaCl_2$ and give 3 × 5 min washes in TBS + $CaCl_2$
10. Wipe off excess buffer and incubate in avidin peroxidase 5 µg/mL in 0 125*M* TBS, pH 7.6, containing 0.374*M* NaCl (made by diluting 1 part 0 5*M* stock TBS with 3 parts distilled water; *see* Note 2) for 1 hour at 37°C in a humidity chamber
11. Wash off with TBS and then wash 3 × 5 min in TBS
12 Reveal sites of lectin binding in 0.05% (w/v) diaminobenzidine tetrahydrochloride (Aldrich) in TBS with 0.015% (v/v) hydrogen peroxide (diluted from 100 vol) for 5 min at room temperature; then wash in water.
13 Counterstain with hematoxylin if required, wash, then air-dry sections and mount in XAM (Gurr, BDH)

2 Immerse in a container of xylene or similar solvent and soak at 60°C for several hours until the wax has melted Change the xylene two or three times
3 When there are no more signs of wax around the tissue and the tissue appears translucent, soak in xylene for a further 30 min to ensure there is no residual wax in solution
4 Transfer the tissue to absolute ethanol for 15–30 min (depending on the size of the tissue) Change and repeat once more
5. Soak in propylene oxide, two changes of 15 min each Infiltrate in propylene oxide epoxy resin (1.1) for 1 h at room temperature
6 Infiltrate in propylene oxide.resin (1 3) at 4°C overnight.
7 Next day, infiltrate with undiluted resin at 45°C for 1 h, and repeat twice before embedding the tissue either in capsules or planchettes/foil molds and polymerizing the resin as recommended by the manufacturer (24–72 h)

3.3. 3-Aminopropyltriethoxysilane (APES) Coating of Microscope Slides

This is an excellent adhesive for both paraffin and plastic sections, and does not react with the reagents used in lectin histochemistry

1 Clean slides with acid alcohol or 95% (v/v) ethanol for at least 2 min
2 Rinse in tap water for 1 min
3 Rinse in 99% (v/v) ethanol
4. Air-dry in slide racks
5 Place in fresh 3% (v/v) 3-aminopropyltriethoxysilane in acetone for 30 s
6 Rinse in acetone then distilled water
7 Dry slides and protect from dust

3.4. Lectin Staining of Paraffin Wax Sections Using the Avidin–Peroxidase Method (42)

1 Cut sections 4 μm thick and mount on APES-coated slides Dry in oven at 70°C
2 Dewax in xylene and wash in absolute ethanol
3. Block endogenous peroxidase activity for 30 min in:
   a 400 mL analar methanol,
   b 1.6 mL 1*N* hydrochloric acid, and
   c 2 0 mL 30% (v/v) hydrogen peroxide
   The hydrogen peroxide must be added just before use.
4 Rinse briefly in tap water and then wash in 0 05*M* TBS, pH 7 6
5 Trypsinize if necessary Warm two baths containing 300 mL TBS to 37°C in a water bath To one, add 300 mg calcium chloride Place slides in the other bath and add 300 mg trypsin (type II porcine, crude, Sigma) to the bath containing the calcium chloride. After 5 min, transfer the slides to the trypsin/$CaCl_2$ mixture and incubate for 5–30 min (normally 15 min).
6 Wash in running cold water to stop the reaction.
7 Wash in TBS for 3 × 5 min

6 Diaminobenzidine tetrahydrochloride (Aldrich, Gillingham, UK), 0.05% (w/v) in TBS with 0 015% (v/v) hydrogen peroxide (diluted from 30%, 100 vol)
7 Neuraminidase (Type VI from *Clostridium perfringens*, Sigma), 0.1 U/mL in 0 2*M* acetate buffer, pH 5 5, with 1% (w/v) $CaCl_2$
8 Various glycosidases (Boehringer-Mannheim, UK) (*see* Note 5)
9 For ß-elimination Potassium hydroxide, dimethyl sulfoxide, analytical grade ethanol, 10 m*M* hydrochloric acid
10 For the gold–sulfide–silver intensification method of semithin acrylic sections. 0 02% (w/v) gold chloride, neutralized 0 3% (w/v) sodium sulfide, 1% (v/v) acetic acid, Gallyas' solution (1982) made as follows
   a. 5 g sodium carbonate (anhydrous) in 100 mL distilled water
   b 0 2 g ammonium nitrate
      0 2 g silver nitrate
      1 0 g tungstosilicic acid
      0 5 mL 37% (v/v) formaldehyde
      100 mL distilled water

   Add reagents in the above order Glassware must be acid cleaned For use, add equal parts of a to b, quickly Apply immediately If the solution becomes chalky, the glassware has not been adequately cleaned A faint opalescence is normal All reagents from BDH (Merck, Poole, Dorset, UK)
11. For gold intensification of peroxidase-stained ultrathin sections: 0 1% (w/v) gold chloride (BDH)
12. Antibiotin gold (10 nm) (Biocell Research, Cardiff, UK) diluted 1·30 in TBS + 1% (w/v) BSA for colloidal gold staining of ultrathin sections
13 For contrasting ultrathin sections after gold labeling 2% (w/v) uranyl acetate, and Reynold's lead citrate (1 33 g lead nitrate and 1 76 g trisodium citrate in 30 mL distilled water, shaken for 1 min then left 30 min with intermittent shaking Add 8 mL 1*N* NaOH and make up to 50 mL with distilled water) Reagents from BDH (Merck)

3. Methods

3.1. Fixation of Tissues

1. Excise tissue, dice into small pieces with a sharp razor blade, and fix immediately in either 2 5% (v/v) glutaraldehyde in 0 1*M* sodium cacodylate buffer, pH 7 2, or in 4% (w/v) paraformaldehyde in 0.1*M* sodium cacodylate buffer or 0.1*M* phosphate buffer, pH 7 2, with 1% (v/v) glutaraldehyde, at room temperature for 4 h
2. Wash in 0.1*M* sodium cacodylate buffer, pH 7.2, with 3 m*M* $CaCl_2$, three times over 24 h, using a large volume of liquid relative to the size of the tissue Store in buffer at 4°C until required for further processing

3.2. Method for Re-Embedding Paraffin Block in Plastic

1 Locate the area of interest and carefully cut it out of the paraffin wax block Trim away as much excess wax as possible

1.5. Hazardous Lectins

A small number of lectins are liable to be contaminated with plant toxins or to be inherently toxic. Those of *Ricinus communis*, *Abrus precatorius*, and *Viscum album* are the most likely to be encountered because of their commercial availability. If possible, their use should be avoided, but if they *are* used, care should be taken to handle them according to the supplier's recommended instructions and to dispose of wastes safely All lectins are potentially allergenic and their handling as dry solids should be minimized. Toxic lectins should never be handled in a dry form, but only in solution. They are usually supplied in vials with septum caps to enable them to be made up into solution *in situ*

2. Materials

2.1. Tissue Fixation

1 2 5% (v/v) glutaraldehyde in 0 1*M* sodium cacodylate buffer, pH 7 2, prepared by mixing 1 part 25% glutaraldehyde (v/v) (E M grade, Taab Laboratories, Aldermaston, UK) with 9 parts buffer immediately before use
2 4% (w/v) paraformaldehyde in 0 1*M* phosphate buffer, pH 7.2, or 0 1*M* sodium cacodylate buffer, pH 7 2, dissolved by heat and cleared with a few drops of 1*N* NaOH, to which glutaraldehyde is added to a final concentration of 1% (v/v), freshly prepared
3 Buffer wash 0 1*M* sodium cacodylate buffer, pH 7 2, with 3 m*M* $CaCl_2$

2.2. Plastic Embedding

1 Analar ethanol, propylene oxide, Taab embedding resin, or epoxy resin equivalent (Taab Laboratories)
2 For coating slides 3-aminopropyltriethoxysilane (APES) (Sigma, Poole, UK), 3% (v/v) in acetone
3 To remove resin· 50% (v/v) saturated sodium ethoxide (excess sodium hydroxide pellets in absolute ethanol, left for 3 d minimum, then diluted with an equal volume of ethanol)
4 L R White acrylic resin (Taab or London Resin, Reading, UK)

2.3. Lectin Histochemistry

1. Hydrogen peroxide, 30% or 100 vol.
2 0.05*M* Tris-buffered saline (TBS) (*see* Note 1), pH 7 6, with and without 1 m*M* $CaCl_2$
3 Trypsin (Sigma, type II porcine, crude)
4 Biotinylated lectins (Sigma, and EY Laboratories, San Mateo, CA, supplied by Bradsure Biologicals, Loughborough, UK), 10 µg/mL in 0 05*M* TBS with 1 m*M* $CaCl_2$ (*see* Note 4)
5. Avidin-peroxidase (Sigma), 5 µg/mL in 0 125*M* TBS containing 0.374*M* NaCl, pH 7 6 (made from diluting 1 part 0 5*M* stock TBS with 3 parts distilled water).

ing. It should be noted that some lectins show slower, largely irreversible reactions with their ligands, leading to an increase in apparent affinity constant and increasing difficulty in reversing their reaction: to minimize this, the lectin and its competitor should be premixed or placed on the specimen simultaneously.

In cases in which a lectin has two or more subsites within its overall binding site, paradoxical patterns of competitive inhibition may arise. For example, the lectin of *Maclura pomifera* has subsites for Galß1- and GalNAcα1-, and binds with high affinity to Galß1,3GalNAcα1- However, this binding is inhibited by ß-anomeric glycosides of galactose and α-anomeric glycosides of 2-deoxy,2-acetamido galactose *(40)*, which led, for some time, to confusion as to the real specificity of the lectin In such cases, controls need to be carefully designed. Even where clear-cut competitive inhibition is shown, it proves only that the lectin is binding via its binding site. for example, concanavalin A binds to α-mannosyl or α-glucosyl termini and α-methyl mannopyranoside will abolish *both* bindings—it does *not* prove that terminal α-mannose is present in the glycan

For these reasons, the use of competitive inhibition should be treated with care. It is necessary and can be helpful, but can also mislead, confuse, or cause overinterpretation. Hence, it is often desirable to use exoglycosidases, either singly or in panels, to supplement or replace competitive inhibition; they often provide additional structural information as well *(26,32,33)*. Some care must be exercised to show that the enzymes are pure glycosidases, free of proteolytic activity, and remain active during their usage. Also, some glycosidases (especially ß-galactosidase) are very sensitive to the positions of linkages or other structural features of glycans, as well as to anomeric configuration, and may prove unexpectedly unreactive, though this also can be structurally informative *(26)*.

Used in panels, lectins can act as controls on each other, though this demands that their binding requirements have been well-defined by chemical methods.

Discrimination between *O*- and *N*-glycans is possible only with the limited set of glycans that have a requirement for α-mannose in the structures to which they bind, in mammals this sugar is restricted to *N*-glycans and to glycosylated phosphoinositides *(1,15,23,41)*. Whereas some saccharides tend to be more common in *O*- or *N*-glycans, it is dangerous to use the lectins that bind to them as markers of *O*- or *N*-linkage, unless there is additional chemical information to validate this. If, for example, the lectin of *Dolichos biflorus* shows staining that is prevented by pretreatment of the specimen with alkali *(35)* (ß-elimination; *see* Section 3 11), there are good grounds for attributing its ligand to an *O*-glycan. If staining is unaffected, an *N*-glycan is suggested, but not proven. This requires further chemical analysis

both the light and electron optical levels, whereas the large size of the peroxidase complex generated in the latter method makes it difficult to penetrate resin sections If higher resolution is required, the disclosing system can then be changed from a peroxidase to a gold-based one, allowing the two systems to be compared at lower power, to check their equivalence, before moving to higher magnification It is this flexibility that makes lectin histochemistry on semithin sections so attractive and powerful a tool

1.4. Control Procedures

As with other techniques of lectin histochemistry, controls must be applied to its use on semithin sections. These are of two kinds, namely controls on the action of the disclosing systems and controls on the specificity of the lectins used

Disclosing systems are normally controlled by the omission of reagents at each step, which should abolish all reactions thereafter Residual staining in the direct avidin–peroxidase system, for example, can arise from the action of endogenous peroxidase, nonspecific adsorption of avidin–peroxidase, the presence of endogenous biotin, or the nonspecific adsorption and oxidation of diaminobenzidine, and these can be separated by appropriate simple controls. More complex disclosing systems require control at each step and are, therefore, more costly of material. Since a major advantage of semithin sections is their economy of valuable material, there is some logic in using the most direct disclosing system possible and using a single disclosing system for a panel of lectins, so that only one set of controls on disclosure is required

The control of lectin specificity creates more subtle problems. Most studies have used competitive inhibition with free sugars, glycosides, or oligosaccharides to show that the binding of lectin can be selectively abolished. In practice, some lectins such as erythro- and leuko-phytohemagglutinins (e-PHA and l-PHA) have no simple sugar competitors and the oligosaccharides that are their ligands are not sufficiently available to be usable at appropriate competitive concentrations Similarly, competition with lectins that bind to sialyl glycans, using sialic acids or small sialyl saccharides, cannot be achieved without a concomitant variation in acidity or ionic strength, which will render the controls questionable. In both of these situations, enzymatic controls are to be preferred (*see* Section 3 10 and Note 5).

Complete abolition of lectin binding cannot always be achieved and, since the competition will approximate to pseudo-first-order kinetics, this is to be expected where the affinity constant of the lectin for its target glycan is high relative to that for the free sugar or glycoside. Hence, somewhat paradoxically, it is difficult to prove the specificity of highly specific bind-

Examination or photography of the specimen through a blue or cyan filter is also a useful way of improving contrast. Disclosing systems based on peroxidase yield a highly insoluble colored product that does not diffuse perceptibly at the light microscopic level, whereas those based on alkaline phosphatase are subject to some diffusion. Although this is usually acceptable on paraffin sections, it can be a limitation at the semithin level.

It is important to recognize that the intensity of stain given by the disclosing system will be proportional to the amount of lectin bound, or glycan sequence present, only over a limited range. Hence, very sensitive disclosing systems may show all of the sites at which a particular ligand occurs, but fail to reveal variations in its density that may be better shown by less sensitive systems Changes in image may occur in switching from paraffin sections to semithin sections, because of this type of effect. Titration of the staining pattern against various concentrations of lectin will often reveal its basis

1.3. The Adaptability and Applicability of Lectin-Stained Semithin Sections

Lectin histochemistry of paraffin sections often shows features that, although interesting, cannot be resolved sufficiently to show exactly where the stain is located If all the available tissue has already been processed for paraffin sectioning, as is often the case with pathological material, a solution to the difficulty may be to dewax a portion of the paraffin block and re-embed the tissue in an epoxy resin. This can also be a useful approach in cases in which sampling of an infrequent feature is required and the size of block needed for direct semithin sectioning would be too small to be efficient A feature found by paraffin sectioning can be excised, re-embedded, and examined at higher resolution and at the electron microscope (EM) level if required. Very often, semithin optical sections will reveal quite sufficient detail to make electron microscopy unnecessary *(38)*.

In going from paraffin sections to successively higher levels of resolution with lectin staining, it is important to ensure, wherever possible, that only one variable is changed at each step. In going to semithin sections from paraffin-embedded sections, the embedding medium is altered, but the staining and disclosing steps can remain essentially unaltered if they are peroxidase-based, so that the images obtained remain comparable. Similarly, the step to low power EM may involve only a change in the microscope system, although sometimes it may be more convenient to use an acrylic resin embedding medium, and the staining procedure again remains unchanged since the peroxidase product is electron-dense In our laboratory, we routinely use an avidin–biotin–peroxidase system *(37,38)* rather than the avidin–biotin–peroxidase complex (ABC) method of Hsu and Raine *(39)* because the former gives excellent results at

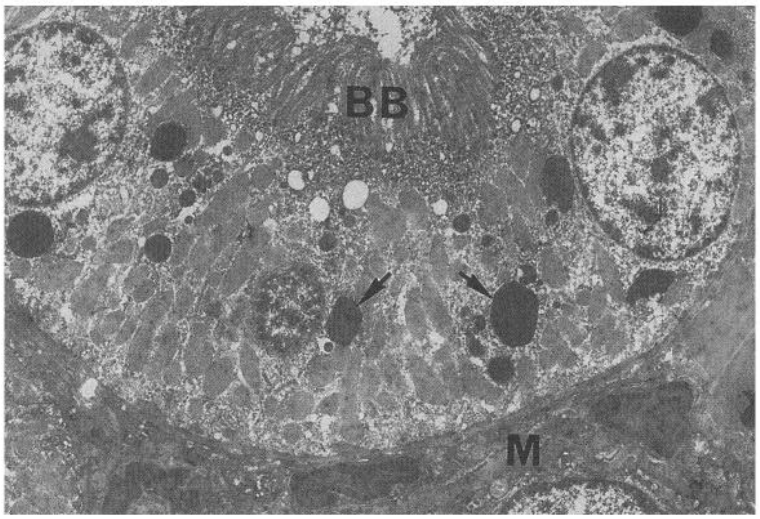

Fig. 2. Ultrathin acrylic (L.R. White) resin section of a rat kidney proximal tubule stained *(37)* with WGA (*Triticum vulgaris*, wheatgerm) lectin showing the strong binding to intracellular granules (arrows), as well to the brush border (BB) and matrix components (M) (×5600).

tem may be limited because of the size of the avidin molecule and its peroxidase conjugates: alternative disclosing systems tend to have similar problems. Acrylic resin will, however, allow even these large molecules to penetrate to a limited extent, unlike epoxy resin, and is the ideal medium for staining at the ultrastructural level. In our laboratory, we routinely embed tissue in epoxy resin (Taab embedding resin) for light microscopic staining with lectins, and in acrylic resin (L.R. White) for ultrastructural examination of the lectin binding properties of tissues. If it is essential to utilize the same block for both light and electron microscopy, the epoxy resin can be gently etched prior to staining grids bearing ultrathin sections, but it is advisable to cut the sections slightly thicker than usual.

It is desirable to control section thickness and the etching conditions carefully, in order to achieve reproducible results and to facilitate comparisons between sections. Because sections may be exposed to a succession of treatments and washings, it is essential to use cleaned and coated glass slides to avoid their detachment.

Because of the thinness of the sections used, the final image must be of relatively greater density than would be required for a paraffin section. Peroxidase-based disclosing systems will produce dense brown images, which can be intensified by treatment with salts of metals such as silver or cobalt *(36,37)*.

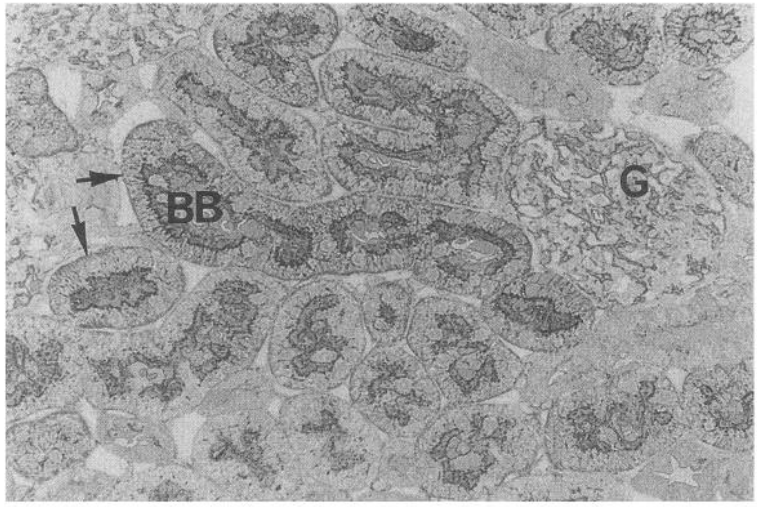

Fig. 1. Semithin epoxy (Taab) resin section of rat kidney stained *(37)* with e-PHA (*Phaseolus vulgaris*, erythroagglutinin, kidney bean lectin) showing binding to the brush border (BB), basal plasma membrane (arrows) and intracellular organelles of proximal tubules, and to components of glomeruli (G) (×350).

Araldite, Epon, Taab embedding resin, Agar 100) has been used for many years for routine electron microscopy and gives excellent results as an embedding medium for use in lectin histochemistry at the light microscopic level (Fig. 1). This is because the resin can be easily removed by a simple etching procedure using alcoholic sodium ethoxide, which, surprisingly, does not degrade glycans nor remove *O*-linked residues by ß-elimination *(35)*, but leaves a thin layer of tissue to be stained as required with the lectins. Alternatively, acrylic resin (e.g., L.R. White, Lowicryl) can be used (Fig. 2), but this resin is not easily removed. In order to visualize the stain at the light microscopic level, when it binds only to the surface molecules of the section and is barely visible even when viewed in the light microscope, stain intensification methods utilizing silver and gold enhancement have to be used *(36)*, some of which we have found to be rather capricious. Acrylic resin has the property, however, of being much more tolerant to water than epoxy resins: L.R. White resin can be infiltrated in a 3:1 mix with 70% (v/v) alcohol, thus avoiding the use of absolute alcohol and the subsequent destruction of sensitive epitopes, which is important when using immunocytochemical techniques to demonstrate protein antigens; glycans are, however, very hardy structures and will tolerate full dehydration during the processing program. Penetration by the disclosing sys-

selectivity as markers in studies of the type indicated above. These general surveys of glycan distribution make heavy demands on material for sectioning, but may not require very high resolution, at least in the first instance They are often made on paraffin-embedded material, which can have the advantage of a larger sample size than is easily possible with plastic-embedded specimens. However, problems of resolution can occur and re-embedding in plastic may be desirable (*see* Section 1.3).

The extension of the use of lectin specificity in conjunction with that of enzymes to the partial sequencing of larger glycans is a relatively new development *(32,33)*, and is not yet very widely applied Like the general surveys above, it is very demanding of material in that it requires relatively large numbers of sections, but is often also demanding in terms of resolution, making semithin sections a useful option

1.2. The Advantages and Limitations of Resin Embedding

In order to cut semithin (0.5–1 0 μm) sections of good quality, it is necessary to embed suitable well-fixed tissues in plastics that are more rigid and supportive than paraffin wax This creates five sets of problems, relating to fixation, the choice of embedding agent, the sectioning of the tissue and adherence of the sections to the slide, the achievement of penetration by the lectin and the disclosing system, and the production of a suitable image

Fixation should be of the best possible quality and, ideally, of a standard that would be acceptable for electron microscopy, since the optical microscopy of semithin sections should yield a resolution comparable with that of low-power electron microscopy No general method exists, however, because optimal fixation of human soft tissues, for example, requires conditions different from those needed for the fixation of marine invertebrates. The general rule is that fixatives that are satisfactory at the ultrastructural level can be used with confidence, with the proviso that metals, such as osmium, lead, or mercury should be avoided if possible, as they may alter the appearance of the final image by generating artifacts with the disclosing system Care should also be taken to avoid powerful oxidizing agents, such as chromates, which may degrade glycans, and strong acids, which can hydrolyze sialyl glycosides on prolonged exposure *(34)*. Aldehyde fixatives based on glutaraldehyde, formaldehyde, or a mixture of the two generally give the best results, but excess aldehyde must be washed away and any free aldehyde residues should be blocked (for example, with saturated glycine in 70% [v/v] alcohol) before exposure to lectin in order to minimize the risk of nonspecific attachment of lectin by crosslinking

There are two main types of plastic embedding material, and each has its advantages and disadvantages for use in microscopy. Epoxy resin (e.g.,

sites than this, with few interacting with linear glycan sequences of more than 3–4 monosaccharide units. MAbs show similar restrictions and tend to have slightly smaller binding sites than lectins

Most glycans are too big for their entire structure to be accommodated by a lectin or antibody and so, in general, only limited parts of their structures can be defined by the application of a single lectin or antibody probe However, the use of a set of probes with overlapping binding requirements, particularly in conjunction with a set of exoglycosidases, can enable the analysis of glycans larger than the sequences complementary to single binding sites This can be done by classical biochemistry, by blotting techniques, and, *in situ*, by histochemical methods

Histologically, lectins display both specificity and selectivity; it is important to distinguish between these. Specificity defines the minimal chemical structure that can interact with the binding site of the lectin to give a binding of more than a certain, chosen affinity constant In practice, specificity is often defined operationally in terms of inhibition of binding or red-cell agglutination by a range of saccharides, and the lower cutoff of affinity is in the range 10^3–10^4 $moles^{-1}$. Occasionally there are X-ray crystallographic or NMR data available to delineate more precisely the chemistry of sugar binding to lectins. Selectivity is a histological term referring to the ability of a lectin to bind to one or more structures in a section of a chosen tissue. It is not directly related to specificity, in that a highly specific lectin may bind to a common sequence present either in many different glycans or on numerous histological features and so may display poor selectivity. However, most highly selective lectins also show fairly high specificity in that they absolutely require specific chemical features in the ligand. These typically include oxygen atoms in various of the hydroxyl groups, in the pyranose rings of the sugars, or in glycosidic linkages, and nitrogen atoms in acetamido- groups of the 2-deoxy, 2-acetamido-sugars Hence there is great conformational and configurational specificity.

Lectins are used histologically in two general ways. Selectivity may be exploited by using them as markers of particular structures, such as the nuclear cap and acrosomes in developing rat spermatozoa *(26)*. In this type of study, the emphasis is on obtaining maximal resolution, so that the position of a single type of glycan sequence can be defined as closely as possible. This places severe restrictions on section thickness, so that paraffin sections may prove inadequate, but it is not heavily demanding of material.

Specificity is exploited in making general surveys of glycan distribution in tissues. A carefully selected panel of lectins of well-defined specificities is used to stain successive sections and, from their known binding requirements, a series of inferences can be drawn as to where particular sorts of glycan occur *(27–31)*. Such investigations should precede the use of selected lectins of high

of accomplishing this, since their first application as fluorochrome-labeled probes to paraffin sections in the early 1970s *(2,24)*. More recently, fluorescence has been largely supplanted by nonfluorescent disclosing systems, such as biotin–avidin–peroxidase and, though paraffin sections are still widely used, resin-embedding of specimens has proved advantageous in terms of resolution and economy of material at the light microscopic level.

1.1. The General Application of Lectins in Glycobiology and Histochemistry

Lectins are carbohydrate-binding proteins that, by definition, have at least two binding sites, are not immunoglobulins, and do not show glycosidase activity *(25)*. Most of those in histochemical use are obtained from plants and the largest group derive from the *Leguminosae* These show considerable sequence homology, despite a remarkable diversity in the glycans to which they bind In other orders of plants, related lectins seem less diverse in their ligands

The majority of lectins in common use have molecular weights in the range 60–130 kDa, are themselves glycoproteins (though they do not bind to their own glycans and seldom do so to glycans of each other), usually contain subunits, are often globular proteins, and are all freely soluble in aqueous buffers in the pH range 6–8. Most are stable to freeze drying, either before or after labeling with biotin, and many require small amounts of calcium for binding. Several also contain transition metals, which may be lost in the presence of the chelating agent EDTA. They are usually slightly smaller proteins than immunoglobulins of class G and mostly show comparable stability and ease of labeling. Both native and labeled forms of many lectins are now commercially available, often at much lower prices than those of monoclonal antibodies (MAbs). In general, lectins can be used in all ways analogous to antibodies, so that histochemical analysis can be supplemented by adsorption studies, the use of blotting, rocket electrophoresis, lectin adsorption assays (analogous to ELISA), and similar techniques.

Hence, though lectins and saccharide-directed MAbs are essentially complementary reagents to each other, there are occasions when a lectin may be the reagent of choice on grounds of cost alone.

With a few exceptions (such as *Helix pomatia* lectin), lectins have extended binding sites and in many plant lectins these are cleft-like. If the cleft is open-ended, the lectin may be able to interact with both terminal and internal saccharide sequences of glycans, but if it is closed at one end, the lectin will only interact with nonreducing terminal sequences *(25)*.

The size and shape of lectin molecules impose restrictions on the largest size of glycan that could be accommodated in a binding site: for a linear glycan this is about 6–7 sugar residues. In practice, most lectins have smaller binding

2

Lectin Histochemistry and Cytochemistry—Light Microscopy

Avidin–Biotin Amplification on Resin-Embedded Sections

Robert W. Stoddart and Carolyn J. P. Jones

1. Introduction

Within the last 25 yr, the study of glycans, their structures, and their distribution in tissues has emerged from relative obscurity to become a major theme of molecular and cellular biology: "glycobiology" *(1)*. Glycans are major components of cellular surfaces *(2,3)*, extracellular matrices *(4,5)*, and secretions *(6)*, and play important roles in cell–cell and cell–matrix recognition and adhesion *(7,8)*. They regulate the surface environment of cells by influencing the structure of water *(6,9)*, by modulating diffusion, by sequestering metabolites such as metal ions, by presenting various growth factors to their receptors *(10,11)*, by acting as ligands in recognition-adhesion systems *(11–13)*, and by making major contributions to cell surface charge *(2,14)*. In secretions, they are variously determinants of molecular folding *(15)*, hydration, interaction, and targeting, and they serve in the mechanisms that monitor the aging of glycoconjugates in circulation *(6,9,16,17)*. They are now implicated in mechanisms of molecular targeting and segregation within the endomembrane systems of cells *(18–20)*, in calcium transport in mitochondria *(21)*, the handling of mRNA and its export from the nucleus, the regulation of transcription *(19)*, and in their classical role as energy stores. In all of this, the association of anatomical or ultrastructural localization with biological function is of paramount importance. specific glycans occur in specific places *(22,23)* The challenge for the histochemist is to reconcile the achievement of sufficiently precise anatomical localization of glycans with the maximum of chemical information about their nature. The lectins have been the main means

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4. Notes

1 Formaldehyde is freshly prepared from paraformaldehyde by heating to 70°C Solution is cooled to room temperature, filtered with filter paper, and pH is adjusted to 7 0–7 4 if only formaldehyde is used for fixation

The fixative is usually prepared using Hanks balanced salt solution or PBS, and used either freshly prepared or stored frozen at –20°C We perform fixation either at 35–37°C (tissues and cell cultures) or at ambient temperature

2 The PVP is added to prevent osmotically induced changes of the extracellular spaces and $NaNO_2$ to paralyze blood vessel's musculature

3 Due care should be exercised to use quartz-distilled water or equivalent to prepare the solutions required for signal amplification. Furthermore, the glassware used for the silver reaction needs to be scrupulously cleaned and rinsed with double-distilled water It is generally recommended that freshly prepared solutions A and B be used However, we noticed that silver acetate and hydroquinone solution stored in a refrigerator for up to 4 wk produced the same staining results as freshly prepared ones In this case, due care is needed to be taken to warm the solutions to room temperature before use since the photochemical process is temperature dependent

4 This step is performed prior to the lectin incubation to prevent or minimize nonspecific interactions between the lectin and constituents contained in the paraffin sections. The blocking step may not be required if sections of Lowicryl K4M-embedded tissues are used

5 Fixation is required to prevent low pH-induced loss of labeling during silver intensification Alternatively, sections may be quickly rinsed in water to remove salts and air-dried before silver intensification.

6 Mix solution A and B a few seconds before use Place sections vertically to prevent fallout of autocatalytically formed silver precipitates Such precipitated silver cannot be removed by rinsing We found it useful to siliconize the glassware and mandatory to avoid metal slide holders and metal forceps The silver intensification is performed under daylight and can be monitored microscopically, the slides being observed upside down In our hands, a developing time of 18 min between 20 and 22°C results in optimal signal amplification. The deposition of silver proceeds exponentially and reaches optimal levels during the last 3–4 min of the developing time Toward the end of the developing time, the developer starts to turn dark

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Table 2
Various Sialidases and Their Specificities

Source	Specificity	Relative cleavage rate
Vibrio cholerae	(O-acyl) NeuAcα2-X	α2,3 > α2,6 > α2,8
Arthrobacter ureafaciens	NeuAcα2-X or NeuGlcα2-X	α2,6 > α2,3 > α2,8
Clostridium perfringens	NeuAcα2-X or NeuGlcα2-X	α2,3 > α2,8 ≈ α2,6
Newcastle disease virus	(O-acyl) NeuAcα2-3 X	α2,3 > α2,8, but
(Hitchner B1 strain)	(O-acyl) NeuAcα2-8 NeuAc	not α2,6

7 Incubate with gold-labeled anti-HRP antibody (diluted with TTBPBS to $A_{525\ nm}$ = 0 05) for 1 h at ambient temperature in a moist chamber
8 Rinse sections with PBS (2 × 5 min)
9 Immerse slides with sections in 1% glutaraldehyde in PBS for 20 min (*see* Note 5)
10. Rinse sections quickly with PBS and thoroughly with double-distilled water followed by air drying The air-dried sections can be stored up to several months or can be immediately processed by silver amplification

In this protocol, steps 6–8 can be replaced by a single incubation step with streptavidin–HRP/anti-HRP antibody gold complex (*see* item 4 in Section 2 2.2.).

3.2.3 Silver Intensification

1 Silver intensification· Place slides with the sections in solution B to which an equal volume of distilled water has been added for 2–5 min at ambient temperature. Transfer the slides in the developer consisting of equal volumes of solution A and B and incubate for 18–25 min at 20–22°C (*see* Note 6)
2. Rinse briefly with double-distilled water
3. Place slides with the sections in photographic fixative for 2–3 min
4. Rinse thoroughly with tap water
5. Counterstain as required, and mount.

3.2.4. Specificity Controls

1. Incubate lectin solution with the inhibitory and noninhibitory mono- and oligosaccharides, neoglycoproteins, or purified glycoproteins for 30 min at ambient temperature and use in parallel with standard lectin solution in step 3 of the protocols listed under Sections 3 2 1 and 3 2 2. Correct pH when acidic reagents (sialic acids) are used. *See* Table 1 for working concentrations of inhibitory substances.
2 Replace the lectin–DIG or lectin–biotin conjugate with the diluting buffer
3. Incubate tissue sections after conditioning with the required exo- and endoglycosidase (*see* Table 2) Incubation conditions according to suppliers' instructions Run control with enzyme-free buffer under identical conditions
4 ß-elimination reaction Float Lowicryl sections on large droplets of 0 1–0 2*N* NaOH for 12–36 h at 37°C in a moist chamber Afterward, rinse four times, 5 min each with double-distilled water, mount sections on poly-L-lysine-activated slides, air-dry at 40°C overnight, and proceed with lectin labeling

Table 1
A List of Selected Lectins with Indications of Their Use

Lectin source	Name	Specificity	Lectin–DIG concentrations	Inhibitors	Inhibitor concentrations
Amaranthus caudatus	ACA	Gal β1,3 GalNAc, Neu5Ac α2,3 Gal β1,3 GalNAc[a]	25 μg/mL	Synthetic T antigen T antigen neoglycoprotein	10 m*M* 10 m*M*
Arachis hypogaea	PNA	Gal β1,3 Gal NAc	25 μg/mL	D-Gal	100 m*M*
Canavalia ensiformis	Con A	Branched hexoses	5 μg/mL	Methyl-α-mannopyranoside	5 m*M*
Datura stramonium	DSA	GlcNAc (Gal) β1-4 GlcNAc and oligomers	10 μg/mL	Lactosamin Asialofetuin Asialoovomucoid *N-N*-chitobiose	200 m*M* 2 μg/mL 500 μg/mL 500 μg/mL
Glycine max	SBA	GalNAc α1,3 Gal	25 μg/mL	GalNAc	10 m*M*
Helix pomatia	HPA	GalNAc	1 μg/mL	GalNAc	≤10 m*M*
Limax flavus	LFL	Sialic acids[b]	0 5 μg/mL	Neu5Ac	10 m*M*
Maackia amurensis	MAA	Neu5Ac α2,3 Gal β1,4 GlcNAc	25 μg/mL	α2,3 sialyllactose	10 m*M*
Phaseolus vulgaris	PHA-L	GlcNAc β1,6 Man α1	10 μg/mL	Thyroglobulin	≈9 μ*M*
Sambucus nigra	SNA	Neu5Ac α2,6 Gal (GalNAc)	25 μg/mL	α2,6 sialyllactose	1 m*M*
Anguilla anguilla	AAA	αFuc			
Dolichos biflorus	DBA	GalNAc α1,3 GalNAc			
Erythrina cristagalli	ECA	Gal β1,4 GlcNAc			
Galanthus nivalis	GNA	Man α1,3 Man			
Lens culinaris	LcH	Branched mannoses with α fucose			
Lotus tetragonolobus	Lotus	Fuc α1,3 Gal β1,4 [Fuc α1-3] GlcNAc			
Phaseolus vulgaris	PHA-E	Gal β1,4 GlcNAc β1,2 Man α1,6→			
Triticum vulgare	WGA	$(GlcNAc\ \beta1\text{-}4\ GlcNAc)_n$			
Ulex europaeus	UEA-1	Fuc α1,2 Gal β1,4 GlcNAc			

[a]To determine whether binding is owing to the presence of sialylated or nonsialylated Gal β1,3 GalNAc, sections were pretreated with galactose oxidase (50 U/mL at 37°C for 4 h or 5 U/mL at 37°C overnight) followed by Schiff reagent (15 min) *See* refs *37* and *40*

[b]*See* ref *41*

2.2.4. Specificity Controls

1. Various mono- and oligosaccharides, neoglycoproteins, and purified glycoproteins can be obtained from different commercial sources (Sigma; Glycosystems; Chembiomed, Alberta, Canada).
2. Various exo- and endoglycosidases can be obtained from different commercial sources (Sigma; Boehringer Mannheim; Calbiochem, La Jolla, CA) For preparation of working solutions and conditions of use and storage, consult product information sheets of the respective suppliers.
3. ß-elimination solution 0 1*N* NaOH
4. Solution for acidic methanolysis: 0 1*N* HCl in methanol

3. Methods

3.1. Fixation, Embedding, and Tissue Sections

1. Deparaffinize and rehydrate paraffin sections according to standard protocol
2. Rehydrate Lowicryl K4M sections by covering them with PBS Resin removal is not required (nor possible owing to the nature of the chemical bonds) for subsequent lectin labeling (or immunostaining)

3.2. Lectin Histochemistry

3 2 1. Lectin–Digoxigenin Technique

1. Condition sections with TBPBS or TTBPBS for 5 min at ambient temperature (*see* Note 4).
2. Drain conditioning solution Avoid drying of sections
3. Incubate with diluted lectin–DIG conjugate (*see* Table 1) for 45 min at ambient temperature in a moist chamber.
4. Wash sections in PBS (2 × 5 min)
5. Incubate with 8 nm gold-labeled anti-DIG antibody diluted in TTBPBS ($A_{525\ nm}$ = 0.05) for 1 h at ambient temperature in a moist chamber
6. Wash sections in PBS (2 × 5 min).
7. Immerse slides with sections in 1% glutaraldehyde in PBS for 20 min (*see* Note 5)
8. Rinse sections quickly with PBS and thoroughly with double-distilled water followed by air drying. The air-dried sections can be stored up to several months or can be immediately processed by silver amplification (*see* Section 3 2.3)

3.2.2. Lectin–Biotin Technique

1. Condition sections with TBPBS or TTBPBS for 5 min at ambient temperature (*see* Note 4)
2. Drain conditioning solution Avoid drying of sections
3. Incubate with diluted lectin–biotin conjugate for 45 min at ambient temperature in a moist chamber
4. Rinse sections with PBS or TBS (2 × 5 min)
5. Incubate with streptavidin–HRP conjugate. Working dilution (1 25 µg/mL) was prepared with TTBPBS
6. Rinse sections with PBS (2 × 5 min)

2.2. Lectin Histochemistry

1 TBPBS· PBS containing 1% bovine serum albumin (BSA) and 0 05% Tween-20 plus 0 02% NaN_3
2 TTBPBS· PBS containing 1% BSA, 0 05% Tween-20, and 0 01% Triton X-100 plus 0 02% NaN_3

2.2.1. Lectin–Digoxigenin Technique

1 The various lectin–DIG conjugates were obtained from Boehringer Mannheim (Mannheim, Germany) Working dilutions of lectins were made up with TBPBS According to the manufacturer's instructions, stock solutions are stored at 4°C, and working dilutions are always freshly prepared before use Since most lectins are mitogenic and some are quite toxic, due care should be exercised
2 Anti-DIG antibody–gold complex prepared with 8-nm gold particles *(32)* or purchased from Boehringer Mannheim Working dilutions were prepared with TTBPBS and stored at 4°C

2.2.2. Lectin–Biotin Technique

1 The various lectin–biotin conjugates were purchased from different commercial sources (Sigma, St Louis, MO, Boehringer Mannheim; Vector Laboratories, Burlingame, CA) Working dilutions of lectins were made up with TBPBS According to the manufacturer's instructions, stock solutions are stored at 4°C, and working dilutions are always freshly prepared before use
2. Streptavidin–HRP conjugate was obtained from Jackson ImmunoResearch Laboratories (West Chester, PA) and working dilutions were prepared with TBPBS
3 Affinity-purified goat anti-HRP antibody–gold complex (4-nm gold particles) was obtained from Jackson ImmunoResearch Laboratories
4 Streptavidin–HRP/anti-HRP antibody–gold complex Mix equal volumes of streptavidin–HRP conjugate (1 25 µg/mL) and anti-HRP antibody gold ($A_{525\ nm}$ = 0 1) 30–60 min prior to use Complexes can be stored for up to 2 wk at 4°C

2.2.3 Silver Intensification

1 Solution A: 0.2% w/v silver acetate (from Fluka, Buchs, Switzerland) in double-distilled water (e g., 100 mg of silver acetate dissolved in 50 mL double-distilled water). Dissolve with a magnetic stirrer in an Erlenmeyer covered with a sheet of aluminum or sonicate for 5 s in a water bath Silver acetate dissolves slowly; prepare at least 30 min in advance (*see* Note 3)
2. Solution B: 0.5% w/v hydroquinone in citrate buffer (0 05*M*, pH 3.8) (e.g , 250 mg hydroquinone in 50 mL citrate buffer) Generally make twice as much solution B as solution A
3 Photographic fixative (i.e., Superfix from Agfa).
4. 1% glutaraldehyde in PBS
5 Commercially available silver intensification kits

tion, respectively Replacement of the lectin-conjugates with the diluting buffer in the incubation sequences serves as a further control.

2. Materials

2.1. Fixation, Embedding, and Preparation of Tissue Sections

1 Paraformaldehyde (*see* Note 1)
2 Glutaraldehyde, 25% aqueous, vacuum distilled, stored under protective gas atmosphere
3 HBSS· Hanks balanced salt solution, pH 7 4
4 PBS: 10 m*M* sodium-phosphate buffer, 150 m*M* NaCl, pH 7 4
5 PVP polyvinyl pyrrolidone, 30,000 mol wt.
6 Sodium nitrite, $NaNO_2$
7 Fixative for tissue: 3% paraformaldehyde—0.1% glutaraldehyde in HBSS or PBS containing 4% PVP and 70 m*M* $NaNO_2$ (*see* Note 2) Use freshly prepared fixative or store frozen at –20°C Alternatively, 10% formaldehyde in 100 m*M* sodium-phosphate buffer (pH 7 4) may be used Check pH of the fixative!
8 Fixative for cells· 2% paraformaldehyde—0 1% glutaraldehyde in HBSS or PBS (*see* Note 1) Use freshly prepared fixative or store frozen at –20°C Check pH of the fixative!
9 Quenching solution 50 m*M* NH_4Cl in PBS Store at ambient temperature and prepare freshly every 4 wk
10. Reagents and equipment for standard paraffin embedding.
11. Lowicryl K4M kit Lowicryl K4M monomers may cause severe allergic reactions and are toxic During embedding, it is strongly recommended that Lowicryl K4M-resistant gloves be used and that inhalation of the resin vapor be avoided by performing all steps in a well-ventilated fume hood
12. Equipment for low temperature dehydration, resin infiltration, and polymerization from Balzers or equivalent.
13. Paraffin microtome and auxiliary equipment for preparation of paraffin sections
14. Ultramicrotome and auxiliary equipment for preparation of Lowicryl K4M sections (0.1–1 μm).
15 Protein-coated glass slides for mounting of paraffin sections
16. Poly-L-lysine, 300,000–500,000 mol wt
17 Poly-L-lysine-activated glass slides for Lowicryl K4M sections Clean glass slides are marked on one side with a diamond; this region on the other side is covered with aqueous poly-L-lysine solution (1 mg/mL) for 5 min at ambient temperature After a quick rinse with distilled water, glass slides are air-dried and stored at ambient temperature until use
18 Mounting of Lowicryl K4M sections· Place sections with a wire loop onto a water drop in the marked region of poly-L-lysine-activated slides and allow to dry overnight at 40°C. Sections can be stored in an ordinary slide box at ambient temperature for prolonged periods of times.

1.2.3. Silver Intensification of Lectin–Gold Labeling

Trace amounts of particles of colloidal gold (and of any other metallic colloid) forming an invisible, latent labeling can be rendered visible for light microscopy by signal amplification using silver intensification The general process consists of the reduction of ionic silver on particles of colloidal gold, which act as catalysts, in the presence of hydroquinone as reducing agent under acidic conditions This creates a growing shell of metallic silver around the gold particles and, since metallic silver also catalyzes this reduction, the reaction becomes autocatalytic. Various silver compounds have been used as source for silver ions. The use of the light-sensitive silver nitrate and silver lactate proved not to be very practical the entire procedure had to be performed in the dark A breakthrough was achieved through the use of silver acetate *(34)*, which permitted the entire silver amplification to be performed under daylight *(32,35–39)*.

Following the silver intensification, the lectin labeling is indicated by the presence of a black staining, which is easily distinguishable from anthracotic and other pigments The silver-amplified gold particle labeling results in a nondiffusible, permanent staining. Sections can be counterstained with nuclear fast red, with hematoxylin alone, or in combination with eosin and then dehydrated and mounted in xylene-based mountants

1 2 4 Controls of Specificity

The classical protocol followed to establish the specificity of lectin staining consists in the incubation of the lectin–DIG conjugate with its appropriate binding substrate prior to its use for staining Most often mono- and oligosaccharides are used for this purpose, although they may not be identical to the lectin-binding sites of cellular glycoconjugates The closer the inhibitory substrate is to the natural structure recognized by the lectin, the lower the required concentrations are for complete inhibition of lectin binding to tissue sections. Thus, concentrations of 0.1–10 m*M* can be sufficient in inhibition studies when the respective lectins are used in the range of 1–20 µg/mL As also mentioned in the chapter by Zuber et al., this type of control also excludes any possible nonsugar-related lectin–protein interactions that sometimes occur. However, abolition of lectin binding by removal of the carbohydrate structure using the appropriate exo- and endoglycosidase provides direct evidence for the specificity of lectin labeling. Such experiments demonstrate not only specificity of lectin-carbohydrate interaction but, at the same time, provide structural information. Furthermore, lectin binding to *N*-glycosidically-linked oligosaccharides or to *O*-glycosidically-linked oligosaccharides can be differentiated by the use of the endoglycosidase PNGase F (endo-F) and the ß-elimination reac-

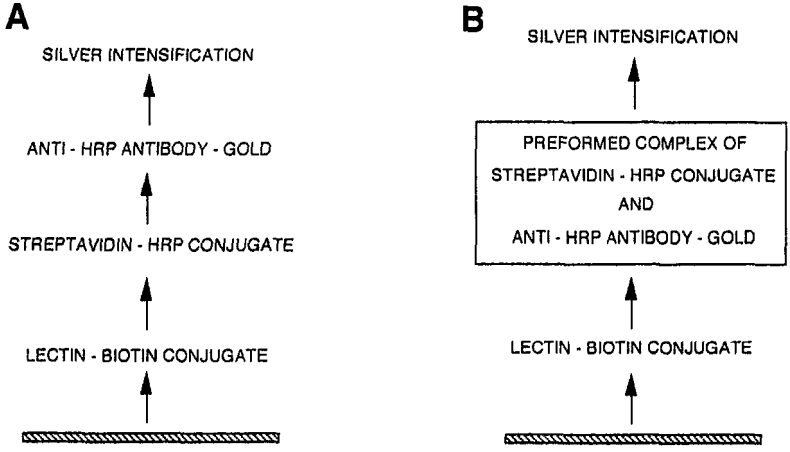

Fig. 1. Schematic presentation of lectin–biotin techniques involving the sequential use of streptavidin–HRP and anti-HRP antibody–gold complex **(A)** or of the preformed complex of streptavidin–HRP and anti-HRP antibody–gold **(B)**

cation of the gold particles (*see* Section 1.2.3.) As compared to the use of alkaline phosphatase- or horseradish peroxidase-labeled anti-DIG antibodies, the silver-intensified gold labeling provides improved resolution and higher contrast in paraffin sections *(32)*. Furthermore, the enzyme-labeled anti-DIG antibodies cannot be used on Lowicryl K4M sections since the enzyme reaction product does not stick to the sections *(32)*.

1.2.2. Histochemical Staining with Lectin–Biotin Conjugates

The indirect labeling technique using biotin-conjugated lectins described in this chapter is a modification of the well-known (strept)avidin–biotin principle and schematically shown in Fig. 1. The sections are first incubated with a lectin–biotin conjugate followed by incubation with streptavidin–peroxidase conjugate. Then, the histochemical visualization of catalytic activity of horseradish peroxidase (HRP) by the diamino benzidine reaction was replaced by the immunological detection of HRP with a gold-labeled anti-HRP antibody. Consequently, blocking of endogenous peroxidase activity was no longer required. Furthermore, the gold-labeled anti-HRP antibody shows no cross-reactivity with peroxidase molecules present in mammalian tissues. As mentioned in Section 1.2.2., the silver-intensified gold labeling provides better resolution and higher contrast in paraffin sections as compared to the DAB reaction product. Furthermore, the DAB reaction product does not stick to Lowicryl K4M sections *(32)*.

mitted the use of lectins for light microscopy in concentrations as applied for electron microscopy *(25,26)*.

A variety of direct and indirect lectin labeling techniques have been worked out during the last two decades *(19,27)* In this chapter, the use of biotin and digoxigenin-conjugated lectins in conjunction with colloidal gold detection systems and silver intensification for light microscopy will be detailed

1.1. Fixation, Embedding, and Tissue Section Preparation

Tissues, cells grown in suspension or in monolayer culture, and isolated cells can be fixed by buffered aldehydes. In contrast to immunohistochemistry, which often requires specific fixation protocols depending on a particular protein antigen to be detected, aldehyde-fixation-induced alteration of carbohydrate structures recognized by the various lectins is negligible. Formaldehyde and glutaraldehyde either alone or in combination are very suitable fixatives In our experience, tissues fixed in 10% buffered formalin, embedded in paraffin for diagnostic purposes, and stored for several years are very suitable for retrospective lectin histochemical studies. If at all possible, organs such as liver, kidney, lung, and brain should be fixed by vascular perfusion, whereas others such as stomach, intestine, and bladder can be conveniently fixed by immersion, particularly for studies on the mucosa. Aldehyde fixation should be terminated in a controlled manner by immersion of the cells and tissues in a buffer containing molecules with free amino groups to amidinate reactive aldehyde groups. This is followed by paraffin embedding according to a standard protocol or low temperature dehydration in graded ethanol followed by low temperature Lowicryl K4M embedding as described *(19)* (*see also* instructions for use provided by the manufacturer *[28,29]*). Paraffin or Lowicryl K4M sections mounted on glass slides can be stored at ambient temperature for prolonged periods of time.

1.2. Lectin Histochemistry

1 2 1. Histochemical Staining with Lectin–Digoxigenin Conjugates

The steroid hapten digoxigenin (DIG) has been introduced as a hapten marker for lectins. Digoxigenin provides a major advantage over biotin since it occurs naturally only in plants of the digitalis family and no crossreacting material has been reported to exist in mammalian tissues *(30–32)* This is in contrast to the natural occurrence of biotin in various tissues and cells, often necessitating section pretreatment to block the endogenous biotin *(33)*.

The lectin–DIG conjugate bound to tissue sections can be visualized using a single anti-DIG antibody gold complex, which is followed by silver intensifi-

3

Lectin–Gold Histochemistry on Paraffin and Lowicryl K4M Sections Using Biotin and Digoxigenin-Conjugated Lectins

Jürgen Roth, Christian Zuber, Tetsutaro Sata, and Wei-Ping Li

1. Introduction

A variety of staining reactions for the visualization of cellular and extracellular glycoconjugates at the light microscopic level are available that are based on the detection of carboxyl and sulfate groups or periodic acid reactive configurations *(1,2)*. Starting in the late 1960s, lectins have replaced many of these chemical staining reactions because of their high specificity for defined mono- and oligosaccharidic sequences in both *N*- and *O*-glycosidic-linked oligosaccharide side-chains of glycoproteins and glycolipids. In order to be useful as histochemical reagents, lectins must be tagged with appropriate markers and those employed in immunocytochemistry have been used successfully *(3–9)* Horisberger and coworkers were the first to prepare lectins labeled with particles of colloidal gold and used them in scanning electron microscopy *(10)* Subsequently, gold-labeled lectins were applied in transmission electron microscopy to study various aspects of cell surface expression and internalization of lectin-binding sites *(5,8,11,12)*, as well as in postembedding labeling of Lowicryl K4M thin sections *(13)*. Later, it was shown that gold-labeled lectins can be used to stain sections of paraffin-embedded tissues *(14–16)*, as well as semithin sections of Epon *(17)* and Lowicryl K4M-embedded tissues *(18,19)*. However, in order to achieve a visible pink staining, which is the natural color of particles of colloidal gold in transmitted visible light *(20)*, highly concentrated lectin–gold complexes had to be used, thereby allowing the possibility of nonspecific staining. A major improvement resulted through the application of a photochemical silver reaction for signal amplification *(21–24)*, which per-

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4. Notes

1 Using the Shandon Sequenza immunostaining center, it is possible to ensure that a uniform amount of lectin is delivered to the section in a known volume that is equally applied to all sections Washing of sections is far more efficient than with manual methods It is possible to incubate sections with lectins without this apparatus, but its use does give consistent, "clean" results, which are superior to sections incubated on a staining rack. The use of 100 µg lectin/mL is rather high, but it ensures that a reaction takes place if carbohydrate residues specific for that lectin are present. It is recommended that dilutions 50, 25, and 12.5 µg/mL be used to assess background to target ratios. Use of appropriate dilutions will result in considerable savings

2. In paraffin wax sections, the intensity of fluorescence may be graded semi-quantitatively if required It is also possible to undertake double labeling experiments wherein two or more different lectin-binding sites are identified using lectins with different markers attached, e g , FITC and TRITC (rhodamine) In double-labeling studies, it is imperative to ensure that no lectin–lectin interactions occur since this would result in a false positive reaction

3 Whereas the ability of sections of different tissues varies with respect to their abilities to bind labeled lectins, the latter may be detected in varying intensities Generally, fluorescence can be detected that is membrane-associated, and also that is located in intracellular organelles, often in endocytotic vesicles, which generally fuse with lysosomes

4. IL-2-containing supernatant as used in the cytophotometric studies to measure WGA uptake was prepared using 24-h culture medium (RPMI-1640/10% fetal calf serum) from cultures of Con A 2 5 µg/mL stimulated normal DBA/2 mouse spleen cells

5. The uptake of labeled lectins demonstrates that carbohydrate-containing binding sites are present, and that there are mechanisms to internalize the bound lectin glycoconjugate complexes Differently labeled lectins could be used to examine couptake and cointernalization The precise methodology used can result in differences in uptake and binding, particularly in intensity of fluorescence

6. The estimates resulting from cytophotometric studies allow a quantitative measure to be obtained that may be used in comparative studies. It also allows the monitoring of changes in lectin binding over a period of time, which indicates the changes in expression of cell surface receptors, as in Fig 3

7. The cytophotometric approach for quantitative studies of lectin uptake has the advantage over FACS analyses in that in the latter, large cells, including epithelial and tumor cells, sometimes stick to the tubing in the machine and block the flow If cells are grown on cover slips, no enzymatic pretreatment (trypsinization) for loosening of the cells has to be used. Since enzyme pretreatment can digest away lectin-binding sites under these conditions, it is not recommended (in contrast to fixed material)

8. Adherent cells grown on cover slips can be assessed by both the cytophotometric and FACS methods.

9 Large cell aggregates can be removed by passing the cells through a double layer of lens tissue or forcing the cells through a 25-gage syringe needle.

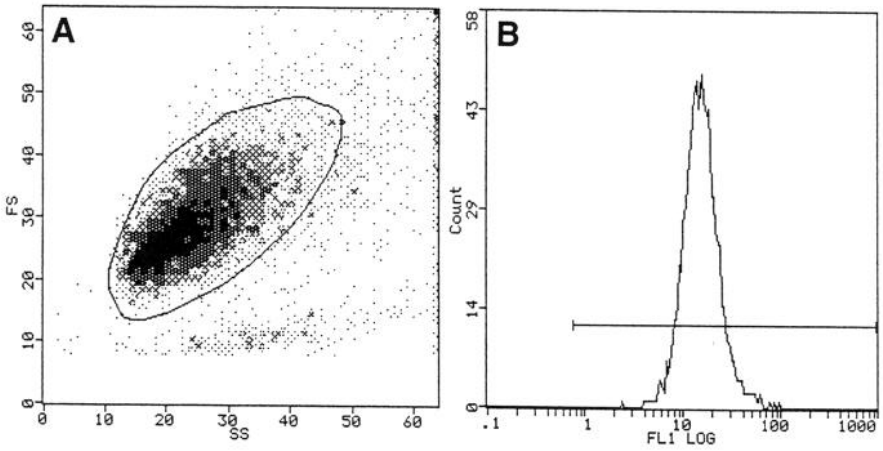


Fig. 8. The FACS analysis of UEA-1-labeled U937 cells. *See* Fig. 5 for details of axes.

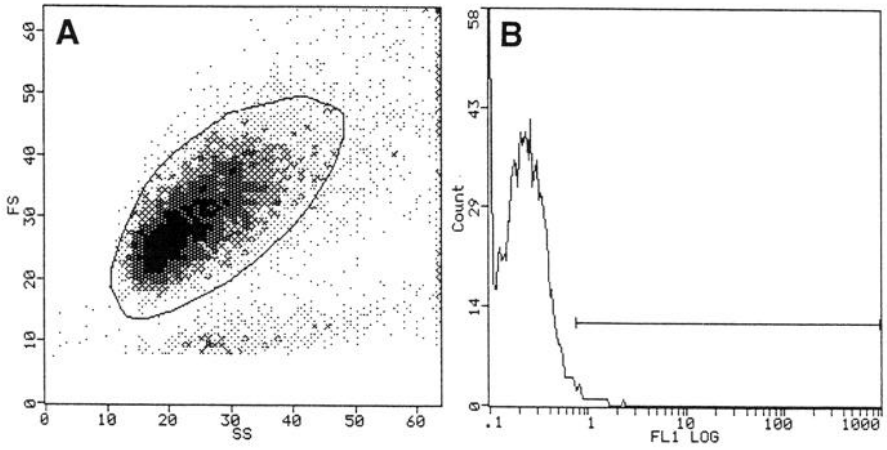
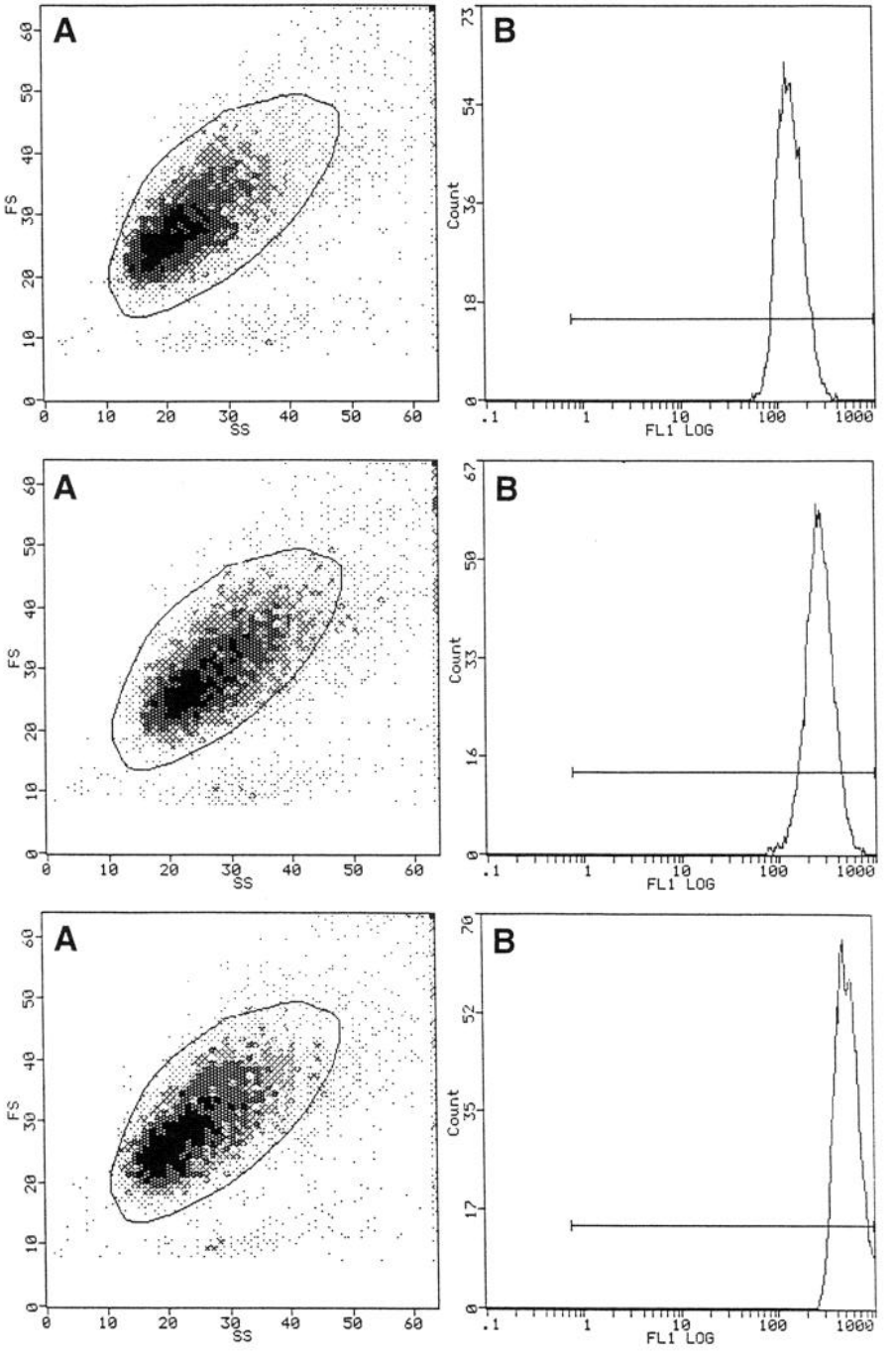


Fig. 9. The FACS analysis of U937 cells without lectin incubation. *See* Fig. 5 for details of axes.

ing to the lectin used; therefore, there is a possibility of sorting the cells into different lectin-positive and lectin-negative populations. Individual representations of FACS analyses for each of the lectins are shown in Figs. 5–9. In Fig. 5A, the field selected for measurement, set arbitrarily, is shown. Figure 5B indicates the fluorescence of cells labeled with WGA. This is the most intensive lectin labeling for U937 cells. In comparison, Fig. 6 shows the results for Con-A, Fig. 7 for PHA-L, and Fig. 8 for UEA-1 of successively decreasing lectin reactivity. The results of the negative control can be seen in Fig. 9, wherein relatively very low levels of fluorescence were detected (*see also* Notes 7–9).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Figs. 5–7.

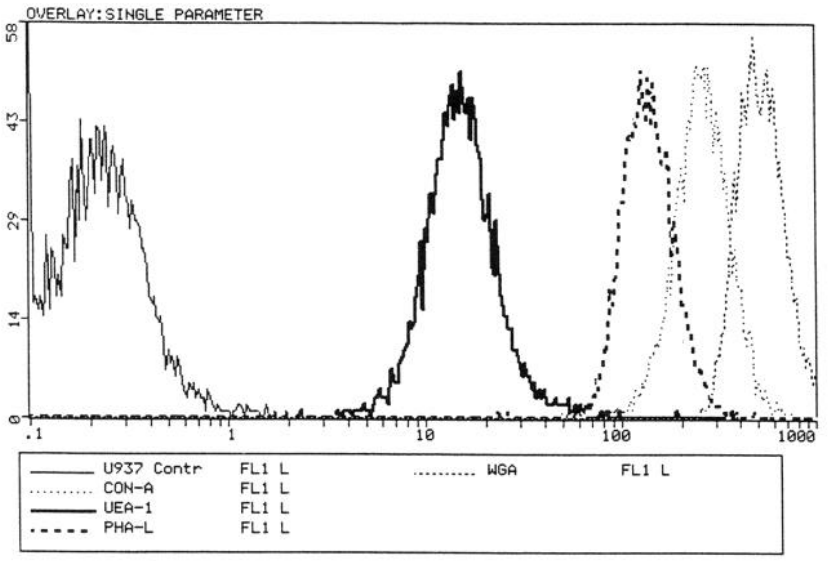

Fig. 4. An overlay of fluorescence intensity measured on a FACS machine for U937 cells incubated with Con A, UEA-1, PHA-L, or WGA lectins. Arbitrary units of relative cell numbers are shown on the *y*-axis, and of relative fluorescence intensity on the *x*-axis.

4. The cells pass through the sample nozzle (which has to be 50–100 μm in diameter depending on the diameter of the cells to be analyzed) in the FACS machine to produce a stream of single cells whose size/fluorescence intensity may be measured.
5. Determine the level of autofluorescence by passage of unlabeled cells through the FACS machine prior to labeled cells. For FITC, a 525-nm exciter filter is used.
6. Pass the cells that have been reacted with the lectin-FITC are then passed through the machine at a rate of 5000 cells/min.
7. Results are represented by Figs. 4–9. In Fig. 4, an overlay of the results is shown. It can be seen that the labeling of this particular population of cells varied accord-

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Fig. 5. *(opposite page; top)* The FACS analysis of PHA-L-labeled U937 cells. The black line encircling the scatter distribution in **(A)** represents the cell population that was assessed. On the *y*-axis is FS (forward scatter), which corresponds to cell granularity, and on the *x*-axis is SS (side scatter), which gives a relative measure of cell number. In **(B)** the relative intensity of fluorescence (log scale on the *x*-axis) has been plotted against relative cell number on the *y*-axis.

Fig. 6. *(opposite page; middle)* The FACS analysis of WGA-labeled U937 cells. *See* Fig. 5 for details of axes.

Fig. 7. *(opposite page; bottom)* The FACS analysis of Con A-labeled U937 cells. *See* Fig. 5 for details of axes.

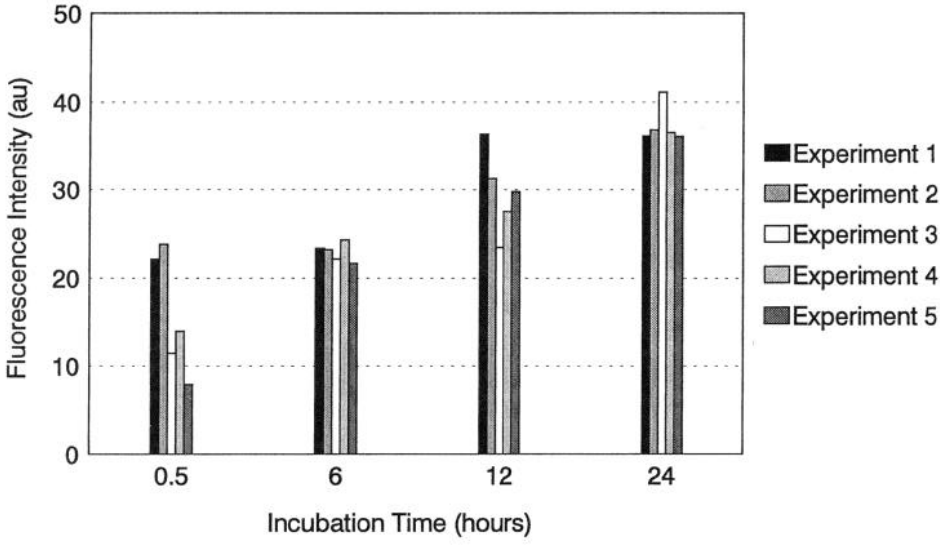

Fig. 3. A graphical representation of the results of an incubation experiment with WGA lectin. The graphs show the effect of extending incubation time on intensity of fluorescence. Experiments 1–5 are replicates.

2% heat inactivated fetal calf serum, 300 μg/mL glutamine, 16 μmol/mL sodium bicarbonate, 25 μmol/mL HEPES buffer, 50 U/mL penicillin and streptomycin, and 10% IL-2 containing supernatant (*see* Note 4).

2. Incubate stimulated cells (500,000/mL) with 4 μg/mL WGA.
3. Place the cells in suspension onto a glass microscope and measure the intensity of fluorescence with a microphotometer (MPV compact, Leitz, Wetzlar, Germany).
4. Examine cells with a ×70 oil immersion lens using a BG 12 excitation filter, and a green analysis filter (515–530 nm) and a 100-W mercury vapor lamp.
5. Measure the background fluorescence in areas free of labeled cells and subtract from the cell-associated fluorescence.
6. Examine 100–120 cells for each experiment to give measures of fluorescence intensity in arbitrary units, which allows a comparison between samples.
7. The results show an increase in WGA uptake, which varied with the length of incubation (*see* Notes 5 and 6, and Fig. 3).

3.4. Method 4: Use of FITC-Labeled Lectins in FACS Analysis

1. Incubate U937 (a myelomonocytic cell line) tumor cells at 4°C for 1 h with one of four lectins (25 μL/10^7): Con-A, UEA-1, PHA-L, and WGA all labeled with FITC.
2. Wash the cells twice with PBS.
3. Pass the cell suspensions (in PBS) through an EPICS(R) Coulter FACS analysis machine.

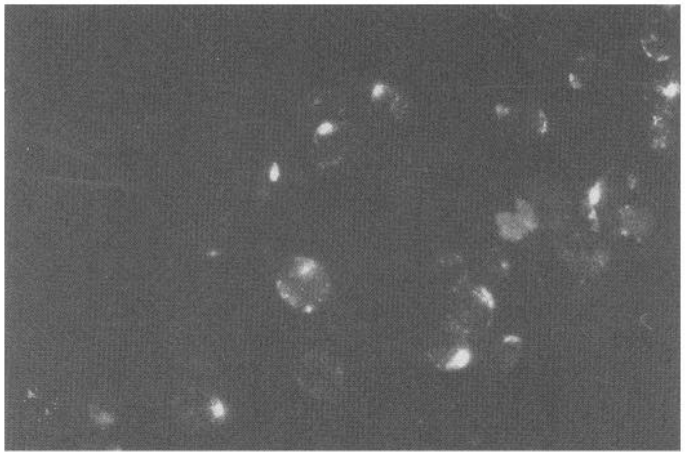

Fig. 2. Cells of the myelomonocytic cell line U937 after incubation with RCA-I lectin. Reactivity can be seen associated with the cell membrane and intracellular granules.

3.2. Method 2: Use of FITC-Labeled Lectins in Cytophotometric Analyses

FITC-lectins have been used in studies of uptake mechanisms in cells. Internalization occurs by endocytosis via both clatharin- and nonclatharin-mediated mechanisms.

1. Culture myelomonocytic cell lines (U 937 obtained from Sundström *[9]*, and HL 60 obtained from the Department of Experimental Haematology, University of Munich) in a tissue culture incubator with 5% carbon dioxide and 95% humidified air maintained at 37°C, using RPMI-1640 medium, supplemented with 10% fetal calf serum and 1% penicillin:streptomycin.
2. Incubate the cell lines (50 µL of 1×10^6 cells) for up to 24 h with a panel of FITC-labeled lectins (50 µg lectin/mL dissolved in 0.15*M* phosphate-buffered saline, pH 7.4).
3. Wash the cells twice after incubation with the lectins in PBS and then fix in 4% neutral-buffered formalin and photograph (*see* Note 7 and Fig. 2).

3.3. Method 3: Use of FITC-Labeled Lectins in Quantitative Internalization Studies

A modification to the methodology described in Section 3.2. is the quantitation of internalization using cytophotometric analysis. A cytophotometric method was used to measure wheatgerm agglutinin (WGA) uptake in activated lymphocytes.

1. Maintain lymphocytes from the cell line CTLL-1 (cytotoxic lymphocytes *[10,11]*) in Clicks/RPMI-1640 (50/50) tissue culture medium supplemented with

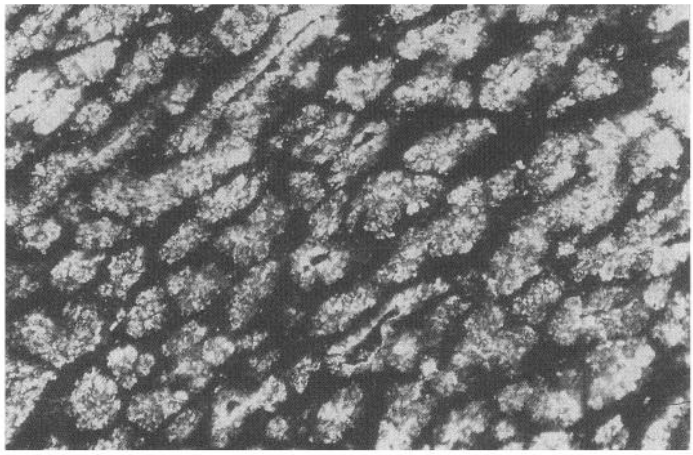

Fig. 1. A paraffin wax section of duodenum, showing Brunner's glands reacted for WGA lectin.

9. 16 μmol/mL Sodium bicarbonate.
10. HEPES buffer.
11. 50 U/mL penicillin and streptomycin.
12. 10% IL-2-containing tissue culture medium supernatant.
13. Shandon Sequenza immunostaining center, Life Sciences International (Basingstoke, Hampshire, UK).
14. Clicks tissue culture medium (Altick, Hudson, WI).
15. Citifluor (University of Kent at Canterbury, UK).

3. Methods

3.1. Method 1: Use of FITC-Labeled Lectins on Sectioned Material

1. Bring paraffin wax sections of duodenum containing Brunner's glands to 0.1*M* Tris-buffered saline (TBS), pH 7.4, containing 2% $CaCl_2$ and incubated for 30 min in a Shandon Sequenza immunostaining center with 100 μL of FITC-labeled lectin (100 μg/mL lectin; *see* Appendix) at room temperature (*see* Note 1).
2. Wash sections several times in TBS and mount in Citifluor.
3. Examine the sections on a fluorescence microscope, using exciter and barrier filters appropriate for FITC.
4. Check the specificity of each reaction by using a negative control in which the lectin is incubated with its appropriate binding sugar (*see* Appendix).
5. Examine and photograph sections (*see* Note 2 and Fig. 1) on a fluorescence microscope fitted with filters appropriate for FITC. Light microscopic localization of FITC-lectin binding sites is sufficient to visualize labeled membranes as well as intracellular organelles such as the Golgi apparatus (*see* Note 3).

variations in the binding of *Helix pomatia* agglutinin (HPA) were observed depending on whether living cells, fixed cells, or fixed and paraffin wax embedded cells were used In addition, the results were influenced by the technique used (direct with fluorescein isothiocyanate [FITC] versus peroxidase antiperoxidase technique) It was observed that the influence of fixation and processing on lectin binding is complex *(6)*. Although it was generally the case in this latter study that fixation and embedding reduced the amount of lectin binding, there was an increase in lectin binding with some lectins, probably owing to exposure of intracellular lectin-binding sites after sectioning. These results serve to emphasize the need to assess each lectin on an individual basis in order to obtain comparable data

The methods available for demonstration of lectin-binding sites at light level are mostly similar to those used in immunohistochemistry Those utilizing FITC, either directly or indirectly, are impermanent. The advantages of using FITC, however, are that it is a small molecule and it is less likely to influence the carbohydrate binding site of a lectin as could larger marker molecules like horseradish peroxidase

The FITC-labeled lectins may also be used in conjunction with the light microscope to investigate receptor-mediated endocytosis mechanisms *(7)* Studies in which single cells may be distinguished in flow cytometry are another way in which FITC-labeled lectins can be used to sort cells into those that express certain sugar groups and those that do not. Such methods can be of considerable use in quantitative population studies and cell function assays *(6,8)*. Fluorochrome lectins may also be used in double labeling (e.g., FITC and TRITC, rhodamine), and/or with antibodies in studies that facilitate cell population identification.

The methods described in this paper give short accounts of FITC-lectin labeling in paraffin wax sections, use of such lectins in cytophotometric analyses to quantify lectin uptake, and use of lectins in FACS analysis that enables studies of carbohydrate composition of glycoconjugates in studying cell populations.

2. Materials

1 RPMI-1640 tissue culture medium
2. Fetal calf serum
3 Penicillin (5000 U/mL) and streptomycin (5000 µg/mL)
4 FITC or otherwise (TRITC, rhodamine)-labeled lectins (*see* Appendix), obtained from Sigma (Poole, Dorset, UK)
5 0 1*M* phosphate-buffered saline, pH 7 4
6. 4% neutral-buffered formalin.
7 Heat-inactivated fetal calf serum
8 Glutamine

4

Use of Fluorochrome-Labeled Lectins in Light Microscopy

Udo Schumacher and Barry S. Mitchell

1. Introduction

The use of lectins with different carbohydrate specificities has demonstrated that there is a great diversity of carbohydrate residues, particularly in glycoconjugates of the cell membranes, but also in (sub)terminal carbohydrate residues of mucins and their precursors stored in the mucin granules of goblet and other mucin-producing cells *(1,2)*. The methods for the detection of these carbohydrate residues using fluorescence microscopy have mainly been carried out on cryostat or paraffin wax sections, although the use of fluorescent-labeled lectins on semithin sections is in principle possible, but not advisable owing to the lack of amplification signal. The particular advantages of using fluorochrome-labeled lectins are: first, the methodology is quick and easy to perform; second, quantification of the lectin binding intensity is possible by using fluorescence-activated cell sorters (FACS) or analyzers and digital imaging processing

Standard schedules for tissue processing may be used, since sugars to which lectins bind are usually fixed by most fixatives and not rendered too labile by standard processing (aside from glycolipids, which are soluble in the organic solvents used to process tissues to paraffin wax). In these cases, fresh or fixed cryostat sections may be the method of choice. Optimal lectin binding has been reported after use of ethanol or Bouin's or Carnoy's fixatives *(3)*. After formalin fixation, however, proteolytic enzymes will often be required to improve lectin binding *(4)*, though use of the latter must be optimized as in immunohistochemistry *(5)*. The precise localization of lectin-binding sites depends, therefore, to some extent on the fixation and tissue-processing regimen utilized *(6)* In a study of lectin binding in human breast cancer cell lines, considerable

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4 Fix the tissue slices in buffered formaldehyde solution *(see* Note 8)
5 See preceding protocol for lectin binding histochemistry

3.3. Treatment of Tissue Sections with Chondroitinase ABC

1 Treat tissue sections for 90 min at 37°C with chondroitinase ABC at 0 0125 U/50 µL per section in Tris-acetate buffer, pH 7 6, according to Hascall and Heinegård, Poole et al , and Hoedt-Schmidt *(2,16,17)*
2 Perform lectin-binding histochemistry (*see* Section 3 1)

3.4. Treatment of Tissue Sections with Neuraminidase

1 Incubate tissue sections for 60 min at room temperature with neuraminidase at 1.25 U/mL in acetate buffer, pH 5 5, to expose any sialic acid-covered binding sites *(see* Note 7)
2 *See* Section 3 1 for lectin-binding histochemistry

4. Notes

1 Fluorochromes other than fluorescein isothiocyanate are also available and can be used in lectin histochemistry
2 Many lectins (e.g , wheatgerm agglutinin) bind similarly to formaldehyde-fixed and frozen tissue, but there are some exceptions, particularly in terms of sensitivity *(18)* Therefore, it is advisable to perform lectin-binding experiments on frozen and formaldehyde-fixed tissue to compare the staining characteristics
3 The incubation time may vary between different lectins and should be tested
4 Some lectins are easier to inhibit than others *(18)* The concentration of inhibitory sugar is usually in the range of 0 2–2*M*
5. The equilibration time should be chosen depending on the thickness of the tissue
6 A mixture of proteinase inhibitors may be included, e g., ethylenediamine tetra-acetic acid (EDTA), *N*-ethylmaleimide, phenylmethanesulfonyl fluoride, pepstatin
7 The duration of the enzymatic digestion and the concentration of the enzyme applied has to be tested and is depending on the tissue of interest.
8 The time of fixation should be tested. Overfixation may influence the detection of lectin-binding sites

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6 Proteinase inhibitors Ethylenediamine tetraacetic acid (EDTA), *N*-ethylmaleimide, phenylmethanesulfonyl fluoride (PMSF), pepstatin
7 Incubation buffer for digestion with chondroitinase ABC 40 m*M* Tris-HCl, 40 m*M* sodium acetate, bovine serum albumin (BSA) 0 01% (w/v), pH 7 3
8 Incubation buffer for digestion with keratanase· 100 m*M* Tris-HCl, 30 m*M* sodium acetate, BSA 0 01% (w/v), pH 8 0, including a mixture of proteinase inhibitors consisting of 10 m*M* EDTA, 10 m*M* *N*-ethylmaleimide, 5 m*M* PMSF, 0 36 m*M* pepstatin
9 Incubation buffer for digestion with neuraminidase 100 m*M* acetate buffer, 40 m*M* $CaCl_2$, pH 5 5

3. Methods

3.1. Lectin Histochemistry of Formaldehyde-Fixed Tissue (see Note 2)

The lectin-binding studies can be performed on 5–8-μm deparaffinated tissue sections. In order to determine the affinity of lectins for tissue components, fluorescein isothiocyanate (FITC)-labeled lectins are reacted with sections at various concentrations The lectin conjugates should be diluted in 0.01*M* PBS (pH 7.4) and should be used in concentrations ranging from 3 to 100 μg/mL The sections are incubated in a 50-μL drop of lectin solution (concentration in stock solution is 1 mg/mL) in a moist chamber at room temperature

1. Mount 5–8-μm tissue sections on cleaned glass slides
2. Deparaffinate the sections in xylene and pass them through a decreasing alcohol series into PBS
3. Incubate with FITC-labeled lectin conjugate for 30–60 min at room temperature in concentrations ranging from 0.003 to 0 1 mg/mL *(see* Note 3)
4. Test the specificity of the obtained staining reaction by preincubating the lectin conjugates in a solution of the appropriate inhibitory sugar *(see* Note 4)
5 Wash off unbound lectin conjugate by 3 × 5-min washes in PBS
6. Estimate the autofluorescence of the tissue by incubations with PBS buffer alone.
7. Mount the sections using Mowiol *(15)* at pH 8 5
8 Examine sections using a microscope equipped for epifluorescence with a 450–490-nm excitation filter and a 520–560-nm barrier filter

3.2. Lectin Histochemistry After Pretreatment with Degrading Enzymes

1 Equilibrate the tissue slices in Hanks balanced salt solution containing glucose for 30–60 min *(see* Note 5).
2 Incubate with protease-free chondroitinase ABC, 0.2 U/mL at pH 7 3 and 37°C for 90 min *(see* Notes 6 and 7)
3 Incubate with keratanase, 0 2 U/mL at pH 8.0 for 90 min at 37°C with proteinase inhibitors *(see* Note 7)

Table 1
Properties of Various Lectins Useful in Cartilage Research and Their Corresponding Carbohydrate Specificity

Lectin	Carbohydrate specificity	Pretreatment
Wheatgerm agglutinin (WGA)	β-D-GlcNAc-(1-4)-β-D-GlcNAc-(1-4)-β-D-GlcNAc > β-D-GlcNAc > Neu5Ac	Not required
Peanut agglutinin (PNA)	β-D-Gal(1-3)-D-GalNAc > D-GalNH$_2$ = α-D-Gal	Neuraminidase, chondroitinase ABC, keratanase
Soybean agglutinin (SBA)	β-D-GalNAc, α-D-GalNAc	Chondroitinase ABC, keratanase
Ulex europaeus agglutinin I (UEA 1)	α-L-Fuc	Chondroitinase ABC, keratanase
Concanavalin A (ConA)	α-D-Man > α-D-Glc > α-D-GlcNAc	Not required

cartilage *(2)*. It may serve as a reproducible protocol to unmask lectin-binding sites in glycosaminoglycan-containing macromolecules

Table 1 demonstrates the properties of various lectins useful in cartilage research, their different sugar-binding specificities, and the required pretreatment for the detection of lectin-binding sites

2. Materials

2.1. Lectin Histochemistry

1 0.01*M* Phosphate-buffered saline (PBS), pH 7.4.
2. Fluorescein isothiocyanate-labeled lectins (various commercial suppliers) are dissolved in PBS buffer at pH 7.4 *(see* Note 1). The binding activities of some lectins (e.g., concanavalin A) depend on cations *(13)* The PBS buffer can be supplemented with 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 0 1 m*M* $MnCl_2$ *(14)* Some of the lectins possess mitogenic properties and others are associated with potent toxins, so they should be handled with care

2.2. Use of Fluorescein-Labeled Lectins in Combination with Enzymatic Digestion

1 Hanks balanced salt solution containing glucose
2. Protease-free chondroitinase ABC (Boehringer Mannheim, Mannheim, Germany)
3. Keratanase (Sigma, St Louis, MO)
4 Neuraminidase (Sigma)
5. 100 m*M* Tris-acetate buffer, pH 7 6

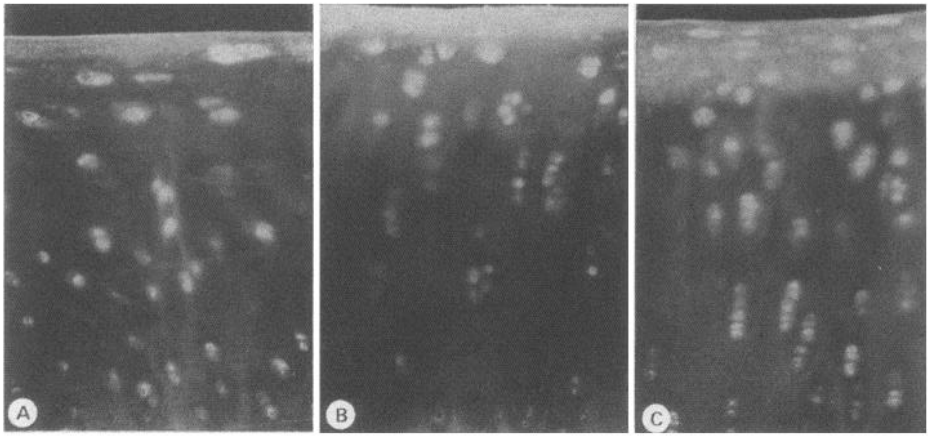

Fig. 1. Lectin-binding histochemistry with FITC-labeled wheatgerm agglutinin (WGA). Reprinted with permission from ref. *2*. **(A)** Normal bovine articular cartilage (control). **(B)** Cartilage degradation with chondroitinase ABC prior to fixation. **(C)** Digestion of cartilage tissue with chondroitinase ABC and keratanase.

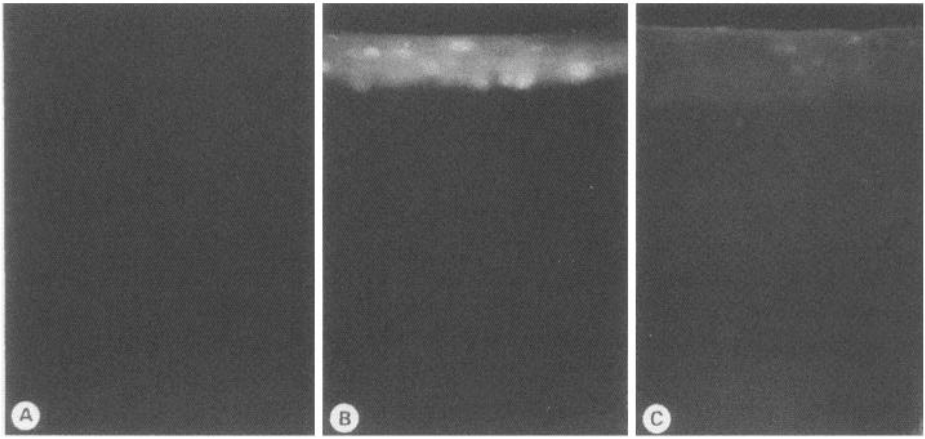

Fig. 2. Lectin-binding histochemistry with FITC-labeled peanut agglutinin (PNA). Reprinted with permission from ref. *2*. **(A)** Normal bovine articular cartilage (control). **(B)** Cartilage degradation with chondroitinase ABC prior to fixation. **(C)** Digestion of cartilage tissue with chondroitinase ABC and keratanase.

fate *(12)*. The use of these enzymes results in a defined degradation of cartilage matrix components *(see* Figs. 1 and 2). The changes obtained resemble, at least in part, the alterations in glycosaminoglycan content observed in osteoarthritic

1.1. Lectin Histochemistry

The application of lectins for the detection of carbohydrate structures in normal, healthy cartilage tissue results in negative staining patterns for *Ulex europaeus* agglutinin I (UEA I), soybean agglutinin (SBA), and peanut agglutinin (PNA), whereas wheatgerm agglutinin (WGA) and Concanavalin A (Con A) show a distinct positive staining reaction *(3)*. Some lectins detect internal sugar moieties of oligosaccharides or glycosaminoglycans; others bind only to terminal sugar residues It is thus likely that glycosaminoglycan chain breakdown as a result of osteoarthritic degradation may expose those sugar residues to lectin binding that are not accessible to the lectin in normal healthy tissue. Therefore, positive labeling of tissue with UEA I in osteoarthritic cartilage indicates matrix proteoglycan degradation. It has been shown that UEA I has a high affinity to extracellular matrix in fibrillated human patellar cartilage *(9)*.

In normal healthy cartilage, binding sites for a variety of lectins are masked by steric hindrance, owing to the densely packed proteoglycan aggregates in the matrix. Loss of proteoglycans and bondage breakdown, as can be seen in osteoarthritic cartilage, facilitates the binding of these lectins to specific carbohydrate structures resulting in distinct and defined binding patterns *(3)*. Thus, negative staining in lectin-binding histochemistry does not necessarily imply the absence of lectin-binding sites

The results of lectin-binding experiments have to be considered with care *(2,6)* Lectin affinities as established by in vitro analysis may not be consistent with lectin binding *in situ*, in which binding may be affected by charge density, hydrophobic interactions, and accessibility of the potential lectin-binding site *(10)*.

1.2. Use of Fluorescein-Labeled Lectins in Combination with Enzymatic Digestion

To visualize lectin-binding sites in tissue sections, lectins may be conjugated with various labeling substances The most widely used label is fluorescein isothiocyanate (FITC), a fluorochrome that can be covalently bound to proteins. Other labels that have been applied with lectins include various fluorochromes, ferritin, and horseradish peroxidase. Lectins may also be labeled with ^{3}H-acetyl groups for subsequent detection by autoradiography. Simultaneous double labeling of sugar moieties can be achieved using two lectins with different binding specificity and different labels *(11)*.

Glycosaminoglycan degrading enzymes such as chondroitin ABC lyase (chondroitinase ABC) and endo-β-D-galactosidase (keratanase) can effectively be used to induce a standardized matrix degradation increasing the accessibility of sugar moieties for lectin binding as well as epitopes recognized by MAbs Chondroitinase ABC removes chondroitin sulfate disaccharides from the proteoglycan monomer, whereas keratanase specifically degrades keratan sul-

5

The Use of Lectins in Combination with Enzymatic Digestion for the Study of Glycoconjugates in Cartilage

Sibylle Hoedt-Schmidt

1. Introduction

Cartilage is a highly specialized connective tissue that consists of cells embedded within an abundant extracellular matrix. The extracellular matrix of articular cartilage is composed of several types of collagens, proteoglycans, glycoproteins, noncollagenous proteins, polysaccharides, and water (for review, *see* refs. *1* and *2*). Besides type II collagen as a major constituent, the predominant proteoglycan of cartilage tissue is the large aggregating proteoglycan (aggrecan) consisting of a core protein to which glycosaminoglycans and *N*- as well as *O*-linked oligosaccharides are attached. This proteoglycan is able to form specific aggregates with hyaluronic acid and link protein

The integrity of cartilage matrix depends on the equilibrium between anabolic synthetic and catabolic degradative processes. Any perturbation of either the rate of synthesis or rate of catabolism must lead to pathological changes in the structural and functional constitution of the cartilage tissue. Using lectin-binding techniques, alterations in the composition of matrix components (e.g., proteoglycans) in osteoarthritic cartilage can be visualized *(3,4)*. The target structures of lectin-binding patterns in articular cartilage tissue have been evaluated using monoclonal antibodies (MAbs) against a variety of epitopes on proteoglycan molecules *(2)*.

Interactions between carbohydrates and lectins have also been studied for the identification of subcellular compartments containing oligosaccharide moieties *(5–7)*. Furthermore, carbohydrates as components of glycoproteins or glycolipids are important cell-surface molecules involved in cell recognition and growth *(8)*.

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tions in meningiomas, and PNA binding may represent a differentiation marker, with more intense binding seen in anaplastic and papillary meningiomas *(107)*.

Lectin histochemistry of routinely processed nerves shows that HPA and MPA bind selectively to pathologically altered nerves but are not specific for any type of injury *(108)*.

13. Microbiology

Most fungi will bind to lectins in routinely processed tissues, and PWM and Succ-ConA may be the most useful lectins as they show little binding to normal tissues *(109,110)*. It has been suggested that species-specific lectin binding may occur *(111)*, but this has not been confirmed Lectin histochemistry is unlikely to supercede established tinctorial methods to detect fungi but may provide biochemical data on changes in the fungal surface in disease.

14. Summary

The widespread diagnostic use of lectin histochemistry is still restricted to the identification of abnormal storage products in cells, of normal and neoplastic endothelial cells, and of fungi. There is increasing evidence that lectins may provide diagnostic information in cytological preparations of effusions, and that more prognostic information can be obtained on the behavior of a wide range of neoplasms. It is difficult to determine the clinical significance of changes in lectin binding owing to the heterogeneity of binding within a group of tumors, between groups of tumors and between laboratories. Most studies have been small and require validation using standardized methodology and multivariate analysis to test whether lectin-binding patterns are independent prognostic variables. Therapeutic decisions for individual patients require a high degree of certainty that the use or withholding of treatment is appropriate, and this is rarely available from lectin histochemical data.

In research, lectins are invaluable for the study of cell-surface interactions and may provide a more reproducible method for the grading of malignancy for many tumors than simple morphological examination. Nevertheless, it is essential that lectin-binding patterns are correlated with careful morphological evaluation of the cells to ensure that, for example, neoplastic rather than reactive cells are being studied

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10.4. Joint Pathology

Lectin histochemistry may reveal changes in the function of chondrocytes in normal and degenerating cartilage, with the breakdown of bonds in fibrillated cartilage making sugars accessible, particularly to SBA and PNA.

11. Hemopoietic and Lymphoid Cells

11.1. Bone Marrow

Several lectins bind to normal and neoplastic myeloid cells in routinely processed marrow biopsies, but there is no discrimination between benign and malignant cells *(97)*

11.2. Macrophages and Histiocytes

PNA binds strongly to the cytoplasm of tissue macrophages and histiocytes *(98)*. The phenotypic variation of macrophages in disease states makes PNA binding a useful adjunct to immunohistochemistry for their identification *(99)*. Interdigitating reticulum cells, and Langerhans cells in histiocytosis-X show strong paranuclear and surface membrane binding with weak cytoplasmic PNA binding *(100)*. Careful morphological interpretation is required since PNA also binds to the Golgi region of plasma cells, and shows membrane binding to neoplastic follicle center cells *(101)*.

11.3. Reed-Sternberg Cells

Reed-Sternberg cells in Hodgkin's lymphoma show a paranuclear dot, with light cytoplasmic staining and strong surface binding by PNA and BPA. Lectin binding identifies Reed-Sternberg cells in more cases and is less affected by formalin fixation than immunohistochemical labeling using Leu-M1 and Ber-H2 *(101,102)*.

11.4. Mast Cells

Human mast cells contain abundant *N*-linked sequences with few or no *O*-linked residues *(103)*. The preferential binding of DBA to mast cells has been used to identify these cells in skeletal muscle diseases *(104)*.

12. Central Nervous System

Lectins may be used to identify different types of cell in the central nervous system, and RCA1 has been used to identify microglia, since other glia and neurons are negative *(105,106)*. Lectins may be useful as differentiation markers in gliomas, since well-differentiated astrocytomas and oligodendrogliomas show more intense binding by ConA, RCA, and PNA than more poorly differentiated neoplasms *(106)* Lectins can be used to identify different cell popula-

carcinomas, there is more intense granular and reticular binding of WGA and ConA, but lectin binding is not of use in the diagnosis of malignancy *(87)*.

9.2. Thyroid

In normal thyroid, UEA1 positivity discriminates parafollicular from follicular cells. In hyperplastic thyroid follicles, adenomas, and carcinomas there is variable, focal, and inconsistent positivity for PNA and UEA1 of no diagnostic value *(88)*. In medullary carcinoma, lectin binding is not diagnostic, although most cells in most cases show binding for UEA1, ConA, RCA1, Succ-WGA, and SBA; amyloid in the stroma shows weak binding by RCA1 and ConA. Calcitonin (a precursor of which has affinity for ConA) and carcinoembryonic antigen in the neoplastic cells may be important contributors to lectin affinity *(89)*.

10. Musculoskeletal System

10.1. Fibrous Tissue

Many lectins show strong binding to glucose and galactose residues linked to hydroxyproline in collagen, and this may be used to demonstrate tissue architecture and changes in collagen *(90)*.

In a small study of malignant fibrous histiocytoma, RCA1 bound both fibroblastic and histiocytic cells, whereas few cells in fibrosarcomas labeled, and cases of malignant fibrous histiocytoma with foci of benign-appearing histiocytes tended to have a poor prognosis *(91)*.

10.2. Skeletal Muscle

Lectin histochemistry has been studied in muscular dystrophies, and increased binding of many lectins demonstrated in necrotic and regenerating muscle fibers *(92)* Loss of binding by RCA-1 to the muscle fiber surface in Duchenne muscular dystrophy *(93)* is probably related to the loss of integral membrane glycoproteins accompanying the defects of dystrophin *(94)*. The loss of UEA1 binding to capillaries, in addition to the deposition of complement, can be used to make a diagnosis of dermatomyositis in the absence of degenerative changes in the muscle fibers *(15)*.

10.3. Bone

Little is known about lectin binding to bone and its neoplasms. In nonneoplastic odontogenic cysts of the jaws, most epithelial layers are positive for UEA1 and BS1; a feature that may help to distinguish them from ameloblastoma in small biopsies in which characteristic morphological features may be absent *(95)*. Lectin binding has been studied in chordomas, uncommon neoplasms of the spine that arise from notochordal remnants, but there is no relationship between glycosylation and clinical course *(96)*.