

Lectin Histochemistry to Detect Altered Glycosylation in Cells and Tissues

Susan A. Brooks and Debbie M.S. Hall

Abstract

Lectins are naturally occurring carbohydrate-binding molecules. A very wide range of purified lectins are commercially available which exhibit a diversity of carbohydrate-binding preferences. They can be used in the laboratory to detect carbohydrate structures on, or in, cells and tissues in much the same way that purified antibodies can be employed to detect cell- or tissue-bound antigens using immunocytochemistry. As lectins can distinguish subtle alterations in cellular glycosylation, they are helpful in exploring the glycosylation changes that attend both transformation to malignancy and tumour progression. In this chapter, methodologies are given for appropriate preparation of many types of cell and tissue preparations, including cells cultured on coverslips (which can be used for live-cell imaging), cell smears, and frozen (cryostat) and fixed, paraffin wax-embedded tissue sections. Heat- and enzyme-based carbohydrate retrieval methods are covered. Basic detection methods, which can be readily adapted to the researcher's needs, are given for direct (labelled lectin), simple indirect (labelled secondary antibody directed against the lectin), and avidin–biotin (biotinylated lectin) and avidin–biotin complex. The use of both the enzyme label, horseradish peroxidase, and fluorescent labels is considered.

Key words: Histochemistry, Lectin, Glycan, Frozen, FFPE tissue, Direct, Indirect

1. Introduction

1.1. *Lectins and Their Applications*

A lectin is a “protein or glycoprotein of non-immune origin, not an enzyme, that binds to carbohydrates and agglutinates cells” (1). Lectins are naturally occurring substances, which occur ubiquitously in nature. Purified preparations used in laboratory research are most commonly derived from plant or, sometimes, invertebrate sources. They can be exploited in the laboratory to detect and reveal carbohydrate structures in or on the surface of cells or, for example, as elements of glycoproteins separated and immobilised by SDS-PAGE and Western blotting, in very much the same way that antibodies

can be used to reveal specific antigens. Lectins can detect subtle alterations in cellular glycosylation. This is of interest in metastasis research as there is evidence that marked glycosylation changes can attend both transformation to malignancy and tumour progression.

1.2. Nomenclature of Lectins

Lectins are named after the source from which they are derived—sometimes the Latin name (for example, *Bandeirea simplicifolia* lectin or *Dolichos biflorus* lectin), sometimes the common name (for example, peanut lectin or wheatgerm lectin), or sometimes by a slightly obscure historical term (for example, Concanavalin A or Con A for the lectin from *Canavalia ensiformis*, the jack bean). The term lectin is used fairly interchangeably with the older term “agglutinin”, as in peanut agglutinin (PNA) or *Helix pomatia* agglutinin (HPA). Lectins are often referred to by an abbreviation for their names, for example PNA or *Dolichos biflorus* agglutinin (DBA); obscurely, PHA which stands for phytohaemagglutinin is, for historical reasons, the abbreviation usually employed for the lectin derived from *Phaseolus vulgaris*. Many sources yield more than one lectin, termed isolectins, which may have quite different carbohydrate binding specificities (for example, the gorse, *Ulex europaeus*, yields two major isolectins *Ulex europaeus* agglutinin I or UEA-I, which has a strong binding preference for fucose, and *Ulex europaeus* agglutinin II or UEA-II, which has a strong binding preference for *N*-acetylglucosamine).

1.3. Monosaccharide Binding Specificity and Natural Binding Partners

The sugar binding specificity of a lectin is usually quoted in terms of the monosaccharide (or sometimes the slightly more complex structure, perhaps a disaccharide) that most effectively inhibits its binding to its naturally occurring complex—often undefined—binding partners. For example, the lectin from the Roman snail, *Helix pomatia* (HPA), is said to have a monosaccharide binding specificity for α -D-*N*-acetylgalactosamine, as does the lectin from the horse gram *Dolichos biflorus* (DBA). It is very important, however, to understand that the combining site of the lectin commonly encompasses more than the terminal monosaccharide (in this example, α -D-*N*-acetylgalactosamine) in an oligosaccharide chain, and that lectins with nominally identical monosaccharide binding specificities will actually recognise subtly different complex binding partners and will give quite different results in lectin histochemistry. In the example given above, HPA and DBA will give quite distinct labelling patterns in lectin histochemistry in spite of their apparent identical nominal binding specificities.

1.4. The Range of Lectins Available Commercially

Many hundreds of lectins are readily available, at modest cost, from commercial sources. Very few have well-defined binding characteristics, and little or nothing is known about some. In most cases, limited information, such as monosaccharide-binding preference, will be available. It is, therefore, often the case that the researcher

may need to test several lectins before the tool most ideally suited to the task is selected.

Although the literature abounds with lectin histochemical studies, surprisingly little useful data have yet been revealed by their use. This may be in the most part owing to the conservative nature of many lectin histochemical studies, which often concentrate on a limited panel of much-used and well-characterised lectins, probably chosen for their nominal coverage of the major monosaccharides present in human tissues and often purchased as a convenient kit from one of the major suppliers. Progress seems more likely if researchers are more adventurous and use a broader spectrum of the lectins available.

1.5. The Range of Lectin Histochemistry Methods

Lectin histochemistry can be carried out on a range of different cell and tissue preparations, including, as described in this chapter, frozen or cryostat sections of fresh tumour tissue, formalin-fixed, paraffin wax-embedded surgical specimens, cells in suspension (either cultured cell lines or clinical samples, such as blood samples, ascites, or pleural effusions), and cultured cancer cell lines either grown on coverslips or pelleted, fixed, and processed to paraffin wax blocks. If cells or tissues are fixed and processed to paraffin wax, antigen retrieval methods (trypsinisation or heat-mediated retrieval methods) may assist the recovery of sequestered carbohydrates and improve labelling results significantly.

A very large number of histochemical labelling techniques are available for detection of lectin binding to cells and tissues. Each has advantages and disadvantages, and the simple and basic techniques described here should all work well and give good results. Lectin binding may be visualised by either a fluorescent (especially suitable for fresh cell smears or cells cultured on coverslips) or an enzyme label which yields a coloured product (the enzyme label in the methods given here is horseradish peroxidase, which yields a deep chocolate brown product with the chromogenic substrate diaminobenzidine (DAB)/H₂O₂, but alternative enzyme labels, such as alkaline phosphatase are also commonly used). Detection methods range from the very straightforward, but relatively insensitive, “direct method” to the more complex, multi-step, and more sensitive “avidin–biotin complex” (ABC) method. Other, even more sensitive, but further complex methods exist.

1.6. Some Examples of Glycosylation Changes, Detectable by Lectin Histochemistry, of Interest in Metastasis Research

There are a number of major alterations in cellular glycosylation, detectable by lectin histochemistry, that appear to be of interest in metastasis research. This field is reviewed elsewhere (2–4). Some examples, to be briefly discussed here, include altered sialylation, increased synthesis of complex β 1-6-branched *N*-acetylglucosamine oligosaccharides (detected by the lectin PHA), and incomplete synthesis and/or synthesis of truncated structures. It seems likely that in the rapidly advancing field of glycobiology, as the functions of

complex carbohydrates and their interactions with their receptors become better understood, their relevance and interactions in the complex processes of metastasis will increasingly be revealed.

1.6.1. Alterations in Sialylation

Sialic acids (neuraminic acids) are commonly found at the non-reducing termini of oligosaccharide chains, and disturbances in sialylation associated with malignancy and tumour progression have been reported. Increased synthesis of sialylated structures, for example the sialylated form of the Tn epitope (sialyl Tn, sTn), sialyl Lewis A (sLe^a), and sialyl Lewis X (sLe^x), have been associated with malignancy and metastatic competence, as has over-expression of sialyltransferase enzymes involved in synthesis of sialylated structures (5–8). Conversely, other studies have correlated failure in sialylation mechanisms with experimental metastatic capacity (9).

1.6.2. PHA Binding to Detect Complex β 1-6-Branched Oligosaccharides

Increased synthesis of complex β 1-6-branched oligosaccharides, detected by the lectin PHA, has been associated to increased metastatic potential of tumour cells in a number of studies (10–12).

1.6.3. Incomplete Synthesis/Synthesis of Truncated Structures

Incompletely synthesised or truncated carbohydrate structures, resulting from a failure in normal chain extension, are a feature of cancer cells. Attachment of a single *N*-acetylgalactosamine residue to the polypeptide backbone yields a simple structure, known as the Tn antigen. This is extended in normal cells, either by addition of sialic acid to yield sialyl Tn (sTn) or addition of other monosaccharides to build more complex structures. However, it is frequently unelaborated in cancer where its presence has been described as a marker of poor prognosis and an interesting potential target for immunotherapy (see Subheading 2 for review). *Helix pomatia* lectin (HPA) recognises Tn and other oligosaccharides terminating in α -D-*N*-acetylgalactosamine. Positive labelling of cancers by HPA using lectin histochemistry is associated with metastatic competence and poor patient survival in a range of human adenocarcinomas, including breast, colon, gastric, prostate, and oesophageal (13–16).

2. Materials

2.1. Silane/APES Treatment of Microscope Slides (See Note 1)

1. Acetone.
2. Acetone/silane: 7 ml 3-(triethoxysilyl)-propylamine (APES) in 400 ml acetone; make fresh on the day of use. Discard after treating a maximum of 1,000 slides.
3. Distilled water.

2.2. Cell and Tissue Preparations

2.2.1. Frozen or Cryostat Sections

1. Chunk of fresh tissue approximately 0.5 cm³ in size.
2. OCT embedding medium.
3. Isopentane.
4. Liquid nitrogen.
5. APES-coated clean glass microscope slides (see Note 1).
6. Acetone.
7. Aluminium foil.

2.2.2. Fixed, Paraffin Wax-Embedded Sections

1. Paraffin wax-embedded tissue blocks (see Note 2).
2. 20% v/v ethanol (or industrial methylated spirits, IMS) in distilled water.
3. APES-coated glass microscope slides (see Note 1).
4. Xylene.
5. Absolute ethanol or IMS.
6. 70% v/v ethanol or IMS in distilled water.
7. Distilled water.

2.2.3. Carbohydrate Retrieval from Fixed, Paraffin Wax-Embedded Sections; Trypsinisation

1. Tris-buffered saline (TBS), pH 7.6: 60.57 g Tris, 87.0 g NaCl dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water.
2. Trypsin solution: 1 mg/ml crude, type II trypsin, from porcine pancreas (see Note 3) in TBS, pH 7.6.

2.2.4. Carbohydrate Retrieval from Sections Using Heat

1. Citrate buffer, pH 6.0: 2.1 g citric acid dissolved in 1 l distilled water. pH to 6.0 using concentrated NaCl.
2. Distilled water.

2.2.5. Cell Smears

1. Cells in suspension (see Note 4).
2. APES-coated glass microscope slides (see Note 1).
3. Aluminium foil.
4. Acetone.

2.3. Preparing Cultured Cells on Coverslips

1. Cells should be cultured under standard conditions to 70% confluence or as appropriate.
2. Cell culture medium, without foetal calf serum (FCS).
3. Alcohol- or autoclave-sterilised round glass coverslips (13 mm diameter, thickness 0).
4. Piperazine-*N,N'*-bis-[2-ethanesulfonic acid] (PIPES) buffer 0.2 M, pH 6.9: Stir 12.1 g PIPES into 50 ml ultrapure water to give a cloudy solution. Add approximately 40 ml 1 M NaOH and the solution should clear. Check pH and adjust to 6.9, if

necessary, using 1 M NaOH. Add ultrapure water to give a final volume of 200 ml.

5. PIPES buffer 0.1 M, pH 6.9: Prepare 0.2 M PIPES buffer, as above, but add ultrapure water to give a total volume of 400 ml, instead of 200 ml, or take a small volume, e.g. 50 ml of 0.2 M PIPES buffer, and dilute 1:1 with ultrapure water.
6. 3% v/v paraformaldehyde in 0.1 M PIPES buffer, pH 6.9: Place 3 g paraformaldehyde in a 250-ml conical flask, add 30 ml ultrapure water, loosely stopper, and heat on a 60°C hotplate, in a fume cupboard, for about 30 min to give a cloudy solution. Add 1 M NaOH (the purest grade), with continual stirring, until the solution clears. Add ultrapure water to give a total volume of 50 ml, and then add 50 ml 0.2 M PIPES buffer, pH 6.9 (see above). Divide into 10-ml aliquots and store frozen. Defrost in warm water bath for use.
7. 0.1% v/v Triton X-100 in 0.1 M PIPES buffer, pH 6.9.

2.4. Preparing Cultured Cells in Cell Pellets or Appropriate, for Paraffin Wax Embedding

1. Cells cultured to 70% confluence under standard conditions.
2. Cell medium, without FCS.
3. 4% v/v formol saline in distilled water, at 4°C: Dissolve 4.25 g NaCl in 500 ml of a 4% v/v formaldehyde solution in distilled water.
4. Lectin buffer: 60.57 g Tris, 87.0 g NaCl, 2.03 g MgCl₂, 1.11 g CaCl₂ dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water (see Note 5).
5. 4% w/v agarose in lectin buffer: Suspend 4 g low-gelling-temperature agarose in 100 ml lectin buffer and heat gently (in a water bath or microwave oven, with regular stirring) until the agarose dissolves (approximately 60°C). Cool to approximately 37–40°C (the agarose should thicken slightly as it begins to gel) for use.

2.5. Quenching Endogenous Peroxidase

1. Methanol/hydrogen peroxide solution: 3% v/v hydrogen peroxide in methanol. Make up fresh every 2–3 days.

2.6. Labelling Methods

2.6.1. Direct Fluorescent Label Method

1. Fluorescently labelled lectin, for example fluorescein isothiocyanate (FITC)- or tetramethylrhodamine (TRITC)-labelled lectin.
2. Lectin buffer: 60.57 g Tris, 87.0 g NaCl, 2.03 g MgCl₂, 1.11 g CaCl₂ dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water (see Note 5).

*2.6.2. Direct Horseradish
Peroxidase Label Method*

1. Horseradish peroxidase-labelled lectin.
2. Lectin buffer: 60.57 g Tris, 87.0 g NaCl, 2.03 g MgCl_2 , 1.11 g CaCl_2 dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water (see Note 5).
3. DAB- H_2O_2 : 3,3-diaminobenzidine tetrahydrochloride, 0.5 mg/ml, in lectin buffer. Add H_2O_2 to give a concentration of 5% v/v immediately before use (see Notes 6–8).
4. Mayer's haematoxylin (see Note 9).

*2.6.3. Biotin-Labelled
Lectin: Simple
Avidin-Biotin Method*

1. Biotin-labelled lectin.
2. Lectin buffer: 60.57 g Tris, 87.0 g NaCl, 2.03 g MgCl_2 , 1.11 g CaCl_2 dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water (see Note 5).
3. Streptavidin labelled with horseradish peroxidase.
4. DAB- H_2O_2 : 3,3-diaminobenzidine tetrahydrochloride, 0.5 mg/ml, in lectin buffer. Add H_2O_2 to give a concentration of 5% v/v immediately before use (see Notes 6–8).
5. Mayer's haematoxylin (see Note 9).

*2.6.4. Indirect Antibody
Method*

1. Native, unlabelled lectin.
2. Lectin buffer: 60.57 g Tris, 87.0 g NaCl, 2.03 g MgCl_2 , 1.11 g CaCl_2 dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water (see Note 5).
3. Peroxidase-labelled rabbit polyclonal antibody directed against the lectin (see Note 10).
4. DAB- H_2O_2 : 3,3-diaminobenzidine tetrahydrochloride, 0.5 mg/ml, in lectin buffer. Add H_2O_2 to give a concentration of 5% v/v immediately before use (see Notes 6–8).
5. Mayer's haematoxylin (see Note 9).

*2.6.5. Avidin-Biotin
Complex Method*

1. Biotin-labelled lectin.
2. Lectin buffer: 60.57 g Tris, 87.0 g NaCl, 2.03 g MgCl_2 , 1.11 g CaCl_2 dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water (see Note 5).
3. Horseradish peroxidase ABC (see Note 11).
4. DAB- H_2O_2 : 3,3-diaminobenzidine tetrahydrochloride, 0.5 mg/ml, in lectin buffer. Add H_2O_2 to give a concentration of 5% v/v immediately before use (see Notes 6–8).
5. Mayer's haematoxylin (see Note 9).

**2.7. Dehydration,
Clearing, and
Mounting Slides
for Viewing by
Microscopy**

1. 70% v/v ethanol or IMS in distilled water.
2. 100% ethanol or IMS.
3. Xylene.
4. Xylene-based mounting medium, for example “Depex”.
5. Coverslips.

3. Methods

**3.1. APES Treatment
of Microscope Slides
(See Note 1)**

1. Place up to a maximum of 1,000 clean glass microscope slides in slide carriers.
2. Place the first slide carrier in a trough containing enough acetone to cover the slides, and leave for 5 min.
3. Transfer, smoothly and without agitation, to a trough containing enough acetone/APES to cover the slides, and leave for 5 min.
4. Transfer, smoothly and without agitation, to a trough containing enough distilled water to cover the slides, and leave for 5 min. Discard the water and replace with fresh after five carriers of slides have passed through.
5. Transfer, smoothly and without agitation, to a second trough of distilled water, and leave for 5 min. Discard the water and replace with fresh after five carriers of slides have passed through.
6. Transfer to absorbent paper and allow to drain.
7. Allow to dry thoroughly—either at room temperature or more quickly in a suitable incubator, glass drying cabinet, or oven.
8. When completely dry, store in labelled boxes until required.

**3.2. Different Types
of Cellular and Tissue
Preparations**

Cells and tissues can be prepared in a number of different ways for lectin histochemistry. Some of the most common and more useful preparations are listed below.

**3.2.1. Frozen or Cryostat
Sections**

1. Using a sharp, clean blade, cut a solid tissue block of approximately 0.5 cm³ (see Note 12).
2. Place the tissue on an OCT-coated cryostat chuck (see Note 13).
3. Using long-handled tongs, pick up the chuck and immerse chuck and tissue in isopentane pre-cooled in liquid nitrogen for approximately 1–2 min (see Note 14).
4. Place the frozen chuck in the cabinet of the cryostat and leave to equilibrate for approximately 30 min.

5. Using the cryostat, cut 5- μ m-thick sections and pick them up on APES-coated (see Note 1) clean glass microscope slides. Allow to air-dry for approximately 5 min.
6. Sections may then either be stored until required by wrapping individually in aluminium foil and storing in the freezer or may be used at once. If stored frozen, allow to thaw and equilibrate to room temperature before use.
7. Immediately before use, dip slides in acetone for 1 min and air-dry for approximately 5 min.

3.2.2. Fixed, Paraffin Wax-Embedded Sections

1. Cool wax-embedded tissue blocks on ice for approximately 15 min.
2. Cut 5- μ m-thick sections by microtome (see Note 15).
3. Float sections out on a pool of 20% v/v ethanol in distilled water on a clean glass plate supported by a suitable receptacle, such as a glass beaker or a jar (see Note 16).
4. Carefully transfer the sections, floating on the alcohol, onto the surface of a water bath heated to 40°C—they should puff out and become flat (see Note 17).
5. Separate out individual sections very gently using the tips of fine, bent forceps.
6. Pick up individual sections on clean, APES-coated glass microscope slides (see Note 1).
7. Allow slides to drain by upending them on a sheet of absorbent paper for 5–10 min.
8. Dry slides either in a 37°C incubator overnight or on a hot-plate at 60°C for 20 min. Slides may then be cooled, stacked, or boxed, and stored at room temperature in a dust-tight container until required.
9. When required, soak slides in xylene for approximately 15 min to remove the paraffin wax.
10. Transfer through two changes of absolute ethanol or IMS, then through one change of 70% v/v ethanol or IMS in distilled water, and then distilled water, agitating the slides vigorously for 1–2 min at each stage to equilibrate (see Note 18).

3.2.3. Trypsinisation for Carbohydrate Retrieval (See Note 19)

1. Immerse slides in trypsin solution at 37°C, in an incubator or water bath, for 5–30 min (see Notes 3 and 20).
2. Wash in running tap water for 5 min.

3.2.4. Heat-Mediated Carbohydrate Retrieval

1. Immerse slides in citrate buffer, pH 6.0, in a suitable microwave-safe container, such as a plastic sandwich box (see Note 21).
2. Place in a conventional microwave oven and heat on full power until the buffer boils.

3. Reduce the power to “simmer” or “defrost” for 5 min so that the buffer simmers gently. After 5 min, check the level of the buffer, and top up with hot distilled water if necessary. Heat on “simmer” or “defrost” for a further 5 min.
4. Allow slides to cool at room temperature for 30 min (see Note 22).
5. Wash in running tap water for 5 min.

3.2.5. Cell Smears

1. Place a drop of cells in suspension approximately 5 mm from one end of a clean APES-coated glass microscope slide (see Note 1).
2. Place a second clean APES-coated glass microscope slide on top of the first, allowing approximately 1 cm of glass to protrude at either end and allowing the drop to spread between the two slides.
3. Drag one slide over the other in a rapid, smooth movement, spreading the cells in a thin smear over the surface of both slides.
4. Air-dry the slides for approximately 5 min. They may then be used at once or wrapped individually in foil and stored in a freezer until required. If stored frozen, allow to thaw to room temperature before use.
5. When ready for use, fix by dipping in acetone for 1 min and air-dry.

3.2.6. Methods for Preparing Cultured Cells

Grown on Coverslips

1. Wash cultured cells, grown to 70% confluence, in fresh medium.
2. Aspirate and discard the medium.
3. Scrape cells from the flask using a rubber scraper and re-suspend in fresh medium.
4. Count cells, and subculture 1×10^5 cells into Petri dishes.
5. Place sterile coverslips in Petri dishes, and allow cells to proliferate for 24 h at 37°C under 5% CO₂.
6. Carefully remove coverslips using fine forceps or the edge of a scalpel blade.
7. Place coverslips, cell side up, onto a piece of dental wax or Parafilm for support, and cover each with 100 µl cold 3% v/v paraformaldehyde in 0.1 M PIPES buffer, pH 6.9, for 15 min.
8. Wash thoroughly in 0.1 M PIPES buffer, pH 6.9.
9. Permeabilize in 0.1% (v/v) Triton X-100 (diluted in 0.1 M PIPES buffer, pH 6.9) for 10 min (see Note 23).
10. Wash thoroughly in 0.1 M PIPES buffer, pH 6.9.

Fixed and Processed for
Paraffin Wax Embedding

1. Wash cultured cells, grown to 70% confluence, in several changes of fresh medium without FCS.
2. Scrape cells from the flask using a rubber scraper.
3. Transfer to a 50-ml centrifuge tube and centrifuge at $1,100 \times g$ for 5 min to pellet the cells.
4. Gently re-suspend the pellet (see Note 24) in fresh medium without FCS, and centrifuge at $1,100 \times g$ for 5 min. Repeat.
5. Aspirate the supernatant and discard.
6. Gently re-suspend the cell pellet (see Note 24) in 4% v/v formal saline for 2 h at 4°C to fix the cells.
7. Centrifuge, as previously, aspirate, and discard the supernatant.
8. Wash the cell pellet in three changes of lectin buffer—centrifuge, aspirate, and discard the supernatant, add fresh buffer, and re-suspend the cells, as previously.
9. Gently re-suspend the cell pellet in a drop of warm (37°C) liquid agarose and allow to set at room temperature (see Note 25).
10. Process the cell/agarose pellets for paraffin wax embedding and then cut sections as detailed in Subheading 3.2.2. Carbohydrate retrieval methods (Subheading 3.2.3 and 3.2.4) may be appropriate.

**3.3. Quenching
Endogenous Peroxidase
(See Note 26)**

1. Immerse slides in methanol/hydrogen peroxide solution for 20 min.
2. Wash in running tap water for approximately 5 min.
3. Proceed to Subheading 3.4.6.

**3.4. Examples of Some
Histochemical
Labelling Techniques
(See Note 27)**

*3.4.1. Direct Fluorescent
Label Method (See Note 28)*

1. Incubate slides with fluorescently labelled lectin at a concentration of 10 µg/ml in lectin buffer, in a humid chamber, for 1 h (see Note 29).
2. Wash in three changes of lectin buffer (see Note 30).
3. Proceed to Subheading 3.4.6.

*3.4.2. Direct Horseradish
Peroxidase Label Method
(See Note 31)*

1. Incubate slides with horseradish peroxidase-labelled lectin at a concentration of 10 µg/ml in lectin buffer, in a humid chamber (e.g. a sandwich box lined with damp filter paper), for 1 h (see Note 29).
2. Wash in three changes of lectin buffer (see Note 30).
3. Incubate with DAB-H₂O₂ for 10 min (see Notes 6–8).
4. Wash in running tap water for 5 min.
5. Counterstain in Mayer's haematoxylin for 2 min (see Note 9).
6. “Blue” in running tap water for approximately 5 min (see Note 32).
7. Proceed to Subheading 3.4.6.

*3.4.3. Biotin-Labelled
Lectin: Simple
Avidin-Biotin Method
(See Note 33)*

1. Incubate slides with biotin-labelled lectin at a concentration of 10 µg/ml in lectin buffer, in a humid chamber, for 1 h (see Note 29).
2. Wash in three changes of lectin buffer (see Note 30).
3. Incubate with streptavidin labelled with peroxidase at a concentration of 5 µg/ml in lectin buffer, in a humid chamber, for 30 min.
4. Wash in three changes of lectin buffer (see Note 30).
5. Incubate with DAB-H₂O₂ for 10 min (see Notes 6–8).
6. Wash in running tap water for 5 min.
7. Counterstain in Mayer's haematoxylin for 2 min (see Note 9).
8. "Blue" in running tap water for approximately 5 min (see Note 32).
9. Proceed to Subheading [3.4.6](#).

*3.4.4. Indirect Antibody
Method (See Note 10)*

1. Incubate slides with unlabelled lectin at a concentration of 10 µg/ml in lectin buffer, in a humid chamber, for 1 h (see Note 29).
2. Wash in three changes of lectin buffer (see Note 30).
3. Incubate with horseradish peroxidase-labelled rabbit polyclonal antibody directed against the lectin, at a dilution of 1/100 in lectin buffer, in a humid chamber, for 1 h.
4. Wash in three changes of lectin buffer (see Note 30).
5. Incubate with DAB-H₂O₂ for 10 min (see Notes 6–8).
6. Wash in running tap water for 5 min.
7. Counterstain in Mayer's haematoxylin for 2 min (see Note 9).
8. "Blue" in running tap water for approximately 5 min (see Note 32).
9. Proceed to Subheading [3.4.6](#).

*3.4.5. Avidin-Biotin
Complex Method
(See Note 34)*

1. Incubate slides with biotin-labelled lectin at a concentration of 10 µg/ml in lectin buffer, in a humid chamber, for 1 h (see Note 29).
2. Wash in three changes of lectin buffer (see Note 30).
3. Incubate with horseradish peroxidase ABC, prepared according to the manufacturer's instructions (see Note 11) in a humid chamber, for 30 min.
4. Wash in three changes of lectin buffer (see Note 30).
5. Incubate with DAB-H₂O₂ for 10 min (see Notes 6–8).
6. Wash in running tap water for 5 min.
7. Counterstain in Mayer's haematoxylin for 2 min (see Note 9).
8. "Blue" in running tap water for approximately 5 min (see Note 32).
9. Proceed to Subheading [3.4.6](#).

**3.4.6. Dehydration,
Clearing, and Mounting
Slides for Viewing by
Microscopy (See Note 35)**

1. Dehydrate by passing through 70% v/v ethanol or IMS spirit in distilled water, then two changes of 100% ethanol or IMS, and then clear in xylene. Agitate slides for 1–2 min at each stage to equilibrate.
2. Horseradish peroxidase-labelled slides should be mounted in a xylene-based mounting medium, for example Depex. Fluorescently labelled slides should be mounted in a commercially available fade-resistant mounting medium.

**3.5. Positive and
Negative Controls
and Confirmation of
Specificity of Labelling**

1. Positive control: A cell or tissue preparation that is known to give positive labelling from a previous experiment makes the ideal positive control. If this is not possible, kidney—either human or animal—makes an excellent positive control for labelling with many lectins owing to its diverse glycosylation patterns.
2. Negative control: Omit the lectin. Labelling should be completely abolished.
3. Confirmation of specificity: Add 0.1–0.5 M monosaccharide for which the lectin shows the greatest affinity (for example, for *Ulex europaeus* isolectin I, add fucose; for *Helix pomatia* lectin, add *N*-acetylgalactosamine) to the lectin solution, at its working dilution, about 30 min prior to incubation with the cell or tissue preparation (see Note 36).

**3.6. Interpretation
of Labelling Results**

Slides should be viewed using a light or fluorescence microscope. Good labelling is indicated by a strong specific label (either deep brown for horseradish peroxidase/DAB or fluorescent), low, or preferably non-existent, background, and non-specific labelling (see Note 37). Omission of the lectin should abolish labelling completely. The competitive inhibition experiment with an appropriate monosaccharide should abolish or at least dramatically diminish labelling.

It is often helpful to score labelling on an arbitrary scale, where the observer estimates the percentage of cells, for example cancer cells, labelled (10%, 50%, 95%, etc.) and the intensity of labelling on a scale of – (no labelling at all), +- (very weak labelling), and + (weak but definite labelling) to ++++ (extremely intense labelling) to give results ranging from completely negative to 100% ++++.

4. Notes

1. APES alters the electrical charge on the glass of the microscope slide so that cell and tissue preparations adhere much more firmly. We routinely APES treat all our microscope slides before use. It is absolutely essential if slides are to be subjected to

heat-mediated retrieval methods as the aggressiveness of the treatment will otherwise dislodge even the best cell/tissue preparation. It is desirable, but not essential, for other applications. Alternative, commercial, brand-named preparations are also available, but are more expensive. Other adhesives, for example glycerol gelatin or poly-L-lysine, will also improve adherence of cells/tissues to slides but are not as effective and are inadequate for preparations that are to be subjected to heat-mediated retrieval methods. Once acetone/APES solution is prepared, it should be used the same day and then discarded. 400 ml acetone/7 ml APES is sufficient to treat up to about 1,000 microscope slides; it is, therefore, a good idea to treat a batch of 1,000 slides, and then store them for use as required.

2. Paraffin wax-embedded tissue blocks are often the most convenient source of material for clinical studies as tumour specimens are routinely fixed in formalin and processed to paraffin wax for sectioning, labelling, and histopathological diagnosis in most clinical centres. Such blocks are often stored in hospital archives for years or decades, providing a hugely valuable resource for retrospective studies on glycosylation as related to the metastatic potential of tumours.
3. Crude, type II trypsin from porcine pancreas works best as the presence of impurities (e.g. chymotrypsin) assists its effect. Do not use purer (and more expensive!) products.
4. Any cells in suspension are suitable—for example, blood, cancer cells in ascites or in pleural effusions taken from patients or animal models, cells derived from solid tissue tumours and released into suspension, or cultured cells in suspension.
5. Lectin buffer is TBS with CaCl_2 and MgCl_2 added, and is good for all lectin histochemistry applications. We recommend it for all dilutions and washes. Many lectins are known to require Ca^{2+} and Mg^{2+} to stabilise their carbohydrate-binding site(s). The requirements of other lectins remain unknown. As a starting point, it is most inadvisable to use phosphate-buffered saline (PBS) for lectin histochemistry as the phosphate ions may bind with and sequester metal ions from the lectin-binding sites.
6. DAB is potentially carcinogenic and should be handled with care. Wear gloves. Avoid spillages and aerosols. Work in a fume cupboard. After use, soak all glassware, etc. in a dilute solution of bleach overnight before washing. Wash down worktops with dilute bleach after use. Clean up spillages with excess water, and then wash with dilute bleach.
7. DAB is available in dropper bottle kit form from major suppliers. This is more expensive than preparing solutions from powdered reagent, but is convenient and safer.

8. We usually make a concentrated DAB stock solution at 5 mg/ml in lectin buffer and freeze in a 1-ml aliquot in 10-ml plastic screw-top tubes until required. This minimises the risk associated with weighing out powder.
9. Mayer's haematoxylin: A number of different haematoxylin solutions are commercially available. We find Mayer's, which is a progressive stain, convenient, although other types are equally effective.
10. Peroxidase-labelled rabbit polyclonal antibodies against lectins: Only a very limited range of polyclonal antisera against lectins are available commercially, so lack of availability may limit the application of this method.
11. ABC is available from major suppliers, often as convenient dropper bottle kits. Avidin has four binding sites for biotin. Avidin and labelled biotin are mixed together in such a ratio that three of the possible four binding sites on avidin are saturated with labelled biotin, leaving one free to combine with the biotin label attached to the lectin. To use these, follow the manufacturer's instructions; these usually require that the avidin and labelled biotin solutions are mixed together in the appropriate ratio at least 30 min prior to use.
12. When cutting tissue, use a very sharp blade, for example a new razor blade or scalpel, and use single, firm, swift, downwards strokes. Do not hack at the tissue and avoid crushing it as this will result in poor morphology. Cut the tissue into a geometrical shape with straight edges, preferably a square, as this will make sectioning easier.
13. OCT acts as a support for the tissue block during sectioning. Embed the tissue block in a generous pool of it. Align the tissue block, in the OCT, with one of its straight edges parallel with the cutting edge of the chuck, as this will make sectioning easier.
14. It is possible to freeze the tissue block in other ways—for example, by immersing in liquid nitrogen, using a commercially available freezing spray, blasting with CO₂ gas, or even simply placing it in the chamber of the cryostat until frozen. However, to obtain optimum morphological integrity, the tissue should be frozen as rapidly as possible, avoiding the formation of ice crystals, and the best way is to immerse in isopentane pre-cooled in liquid nitrogen. Hexane pre-cooled in liquid nitrogen is also very good. Isopentane/hexane are far better conductors of heat than liquid nitrogen alone and, therefore, facilitate extremely rapid and effective freezing. Safety note: Take appropriate precautions when using liquid nitrogen and solvents.

15. Most tissues can be cut effectively when very cold. Ice (for example, an ice cube) should be applied to the surface of the tissue block every few minutes during cutting.
16. During this step, if small creases or wrinkles are observed in the sections, these will flatten out. Gentle manipulation using, for example, a soft paintbrush and/or forceps can aid this.
17. If sections remain wrinkled, it may indicate that the water is slightly too cold. If the wax begins to melt, it is too hot.
18. When slides are transferred from one solvent to the next, they initially appear “smeary” as the two solvents begin to mix. Vigorous agitation, i.e. “sloshing them up and down”, ensures that the slides equilibrate effectively with the new solvent. When they are equilibrated, the surface smearing will disappear. If white flecks or patches become visible around the sections, this indicates that the wax has not been adequately removed—return the sections to xylene for a further 10–15 min. A common cause of poor labelling results is inadequate removal of paraffin wax.
19. Carbohydrate retrieval methods: In some cases, if the lectin histochemistry results on the fixed, paraffin wax-embedded tissues are disappointing, it may be because the carbohydrate moieties have been sequestered during tissue fixation and processing to paraffin wax. Results can often be improved by carbohydrate retrieval techniques, including trypsinisation or heat treatment. It is not possible to predict if carbohydrate retrieval methods will be effective or which to choose; try both and select which conditions work best for your application (other heat-mediated retrieval methods, such as heating using an autoclave or pressure cooking, may also be appropriate). N.B.: These methods are not appropriate for fresh cell/tissue preparations, such as frozen sections or cell smears.
20. Pre-warm all glassware, solutions, etc. to 37°C before use. Make up trypsin solution fresh immediately before use; trypsin will autolyse (self-digest) and lose activity over time. Initially, try a range of trypsinisation times, e.g. 0, 5, 10, 20, and 30 min. Incubation times of >30 min are not recommended as visible damage to tissue morphology becomes apparent.
21. Slides can conveniently be placed in commercially available slide carriers, which typically hold up to about 25 slides, or upright in Coplin jars. Do not overcrowd slides as this results in “hot spots” and uneven carbohydrate retrieval. When using microwave heat retrieval methods, do not use metal slide carriers!
22. The cooling down period is part of the retrieval method and should not be skipped.

23. If cell surface carbohydrates only are of interest, this and subsequent steps may be omitted.
24. To re-suspend cells, either vortex in short sharp bursts or tap the tube sharply. Do not suck the cells up and down in a pipette as this will damage them.
25. To suspend cells in liquid agarose at 37°C, pre-warm a glass pipette to 37°C and transfer cells to a small drop (approximately 0.5 ml) of agarose in a small plastic tube, such as a 1.5-ml Eppendorf tube. Tap the tube several times sharply to mix. Leave agarose to set at room temperature for approximately 30 min. The agarose/cell pellet can be removed by either cutting the plastic tube away or dislodging the pellet from the tube using a syringe needle or pin.
26. If horseradish peroxidase is used to visualise lectin binding, endogenous peroxidase enzyme within the cell or tissue preparation may contribute to non-specific and confusing labelling. This may be overcome by quenching endogenous enzyme. Endogenous peroxidase is usually quenched immediately prior to incubation with the lectin. This step is not required if fluorescent labels are used. Other enzyme labels, for example alkaline phosphatase, are also commonly used and, although these will not require this step, they may require their own blocking procedure.
27. A large number of different histochemical techniques can be used to detect lectin binding to cell and tissue preparations. The choice of technique is dependent on the type of label preferred by the experimenter—fluorescent or enzyme (coloured)—and the balance between quick, simple techniques which generally lack sensitivity and the more sensitive techniques which tend to be more time consuming, contain more steps, and require more reagents (and are, therefore, slightly more expensive). All methods given here should give good results. Other methods also exist. The methods described in chapter 1 by Brooks in this volume can be readily adapted for use with lectins instead of primary antibodies using the general guidelines given in this chapter.
28. Fluorescent labels can give exceptionally beautiful results, but the fluorescence is ephemeral and preparations should be viewed (and if a permanent record is required, photographed) as soon as possible after labelling. Slides can be stored in a refrigerator, in the dark, for up to about 6 months, but some preparations will deteriorate significantly during that time. The fluorescence-based method described is especially good for cells in suspension and cell smears. It may also be used for tissue sections, and use of a commercially available fluorescent nuclear counterstain is then recommended to aid interpretation of tissue morphology.

29. Lectins are usually purchased as a purified powdered product. We routinely solubilise the powder in lectin buffer to give a stock solution of 1 mg/ml, which is stable in a refrigerator for several months. Lectin stock solution can then be diluted to the optimum working dilution as required. It is best practice to prepare working solutions immediately prior to use and discard unused solution at the end of the experiment. A working concentration of 10 µg/ml is often the optimal concentration for most lectin histochemistry. It may be a good idea to try a range of concentrations, e.g. 2.5, 5, 10, 20, and 40 µg/ml, to assess which gives the best results.
30. We recommend vigorous washing in three changes of lectin buffer. Each wash should consist of vigorous “sloshing up and down” of slides in buffer for about 30 s–1 min, and then allowing slides to stand in the buffer for about 4 min. Insufficient washing, in particular omitting one or more changes of buffer, can result in unacceptably high levels and dirty background labelling.
31. This method has the advantage of being quick, simple, and straightforward. It lacks the sensitivity of some of the longer, multi-step, and avidin–biotin methods. We have found that horseradish peroxidase-labelled lectins can sometimes give subtly different binding results to native (unlabelled) or biotin-labelled lectins, possibly owing to steric hindrance of lectin binding site by the relatively large peroxidase molecule (17).
32. Mayer’s haematoxylin is a port-wine red colour, and cell/tissue preparations will stain purple/red after immersion in it. The haematoxylin changes to a deep blue when exposed to mildly alkaline conditions (known as “bluing”). The mild alkalinity of tap water is usually sufficient. If bluing is unsuccessful owing to unusually acidic tap water, soak slides instead in tap or distilled water to which a few drops of ammonia or sodium hydroxide have been added.
33. This is a really useful method and is highly recommended for most applications; it is also quick, straightforward, and sensitive, generally giving good results. The biotin label appears to be small enough not to interfere with the combining site of the lectin and therefore does not appear, in our hands, to alter its binding characteristics (17) (see Note 31).
34. This is a very sensitive method that gives good, clean results. It should be employed when the simpler methods, for example the simple avidin–biotin method, give weak labelling.
35. After labelling, slides are in an aqueous medium. Before they can be mounted in a xylene-based mounting medium, they must therefore be dehydrated through graded alcohols and cleared in xylene. Aqueous mounting media are also commercially

available which, historically, tended to give inferior results to xylene-based mounting media. Some of the modern ones are, however, very good and may be used instead without the need for dehydration and clearing.

36. For a few lectins, this may not be possible as they are only inhibited by complex sugars, not monosaccharides (consult manufacturer's literature). If this is the case, inhibition with a heavily glycosylated glycoprotein, e.g. fetuin or asialofetuin, should give effective inhibition, as should incubation of the lectin in the presence of 5% v/v normal (human or animal) serum.
37. If dirty, non-specific background labelling is observed, this may indicate inadequate washing between stages in the labelling protocol (see Note 30). If careful attention to washing does not satisfactorily limit background labelling, incorporation of a blocking agent into lectin, antibody, and streptavidin-horseradish peroxidase solutions may be indicated. The simplest and most effective blocking agent is to include 3% w/v bovine serum albumin. Normal serum, often used as a blocking agent in immunocytochemistry, is not appropriate as the many heavily glycosylated molecules present will effectively inhibit specific labelling.

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Further Reading

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