2

Basic Immunocytochemistry for Light Microscopy

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1. Introduction

1.1. What is Immunocytochemistry?

Immunocytochemistry may be defined as the identification of a cell- or tissue-bound antigen *in situ*, by means of a specific antibody–antigen reaction, tagged microscopically by a visible label. Successful immunocytochemistry therefore requires (1) preservation of the antigen in a form that is recognizable by the antibody, (2) a suitable antibody, and (3) an appropriate label. The basic technique was first described by Coons and colleagues ***(1–3)***, who employed antibody directly labeled with a fluorescent tag to identify antigen in tissue sections. Since that time, the technique has been refined and expanded enormously. Some significant developments include the use of horseradish peroxidase ***(4)*** and alkaline phosphatase ***(5)*** as label molecules; the development of many, increasingly sensitive, multilayer detection methods; and exploitation of the strong binding between avidin and biotin in detection techniques ***(6,7)***.

1.2. Range of Applications

Immunocytochemistry is appropriate for a remarkably wide range of applications. Any cell- or tissue-bound immunogenic molecule can, theoretically, be detected *in situ* using the technique. It is a technique of particular interest in metastasis research, as it facilitates the detection of virtually any molecule of interest to the researcher in samples of tumor or normal tissues or cells. Of particular interest in this field is the heterogeneity in expression by cells within a morphologically homogeneous tumor mass, or between normal vs cancer cells. The gain or loss of expression of certain antigens by tumor cells at different stages in the natural history of the disease and in relation to metastatic potential is also of great relevance. For example, loss of expression of cell

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adhesion molecules by tumor cells may be instrumental in their breaking away from the primary tumor mass. Immunocytochemistry is the only technique that allows detection of such molecules *in situ*. Examples of immunocytochemistry as applied to metastasis research applications are explored in more depth in a number of chapters in this volume, including Chapter 5 by Braun and Pantel on immunocytochemical detection and characterization of individual micrometastatic tumor cells, Chapter 6 by John and Pignatelli on assessment of integrin expression, Chapter 8 by Turner and Harris on the measurement of microvessel density in primary tumor, Chapter 9 by Gillett on assessment of cellular proliferation, Chapter 13 by Kilic and Ergün in the companion volume on methods to evaluate the formation and stabilization of blood vessels and their role for tumor growth and metastasis, and Chapter 14 in the companion volume on galectin-3 binding and metastasis by Nangia-Makker et al.

1.3. Types of Cell and Tissue Preparations

As the first requirement for successful immunocytochemistry is preservation of the antigen, the primary consideration must be what type of cell or tissue preparation to employ. Immunocytochemistry can be performed on a range of different cell and tissue preparations of interest in metastasis research, including cell suspensions, cell smears, frozen (cryostat) sections and fixed, paraffin wax-embedded sections. Some of the advantages and disadvantages of these types of preparation are summarized in **Table 1**.

Of particular interest may be the application of immunocytochemistry to routinely formalin-fixed, paraffin wax-embedded archival tissues to facilitate mapping of expression of molecules of interest retrospectively with the benefit of long-term patient follow-up. Many antigens are well preserved for many years in such tissues, and retrospective analysis can be successfully carried out after years (possibly decades) of tissue storage, making this a powerful and informative approach. The only limitations are, first, that lipids are dissolved out and lost during processing to paraffin wax, and therefore their antigenic structures are not present. Second, an antibody is required that successfully recognizes antigen preserved in this manner. Some antigens may be damaged, sequestered, or altered by fixation and processing to paraffin wax, and many antibodies will therefore no longer recognize them and will give successful results only on fresh, frozen (cryostat) tissue sections or fresh cell preparations. Enzyme and heat-mediated antigen retrieval techniques are described later in this chapter and may often be very successful in partially, or fully, reversing the alterations caused by fixation and processing and facilitate successful detection of otherwise undetectable antigens. The relatively recent popularity of heat-mediated antigen retrieval (e.g., *see* **refs. *8–11***) has vastly expanded the repertoire of antibodies that can be used successfully on fixed

Table 1
Advantages and Disadvantages of Different Tissue Preparations

Preparation	Suitable for	Advantages	Disadvantages
Cell suspensions	Living cells, e.g., blood cells, cultured cells, cells released from solid tissue masses. Direct method using fluorescent-labeled antibodies most suitable.	Unaltered antigen expression in the living cell seen. Excellent for cell surface antigens.	Not suitable for demonstration of cytoplasmic antigens. Cells seen in isolation; no indication of tissue distribution of antigen.
Cell smears	Any living cells in suspension, e.g., blood, cultured cells. Any staining method is suitable.	Quick and easy. Good for cytoplasmic antigens.	Morphology sometimes indistinct.
Frozen sections	Any fresh solid animal or human tissue. Any staining method is suitable.	Relatively quick. Fairly good morphology; spatial relationships of cells within tissues seen. Good for cytoplasmic and cell surface antigens.	Technically more demanding than suspensions or smears.
Paraffin sections	Any solid animal or human tissue. Any staining method is suitable.	Tissue preserved indefinitely. Excellent morphology. Relationships between cells in tissues seen. Cell surface and cytoplasmic antigens seen.	More time consuming than other methods. Glycolipids lost. Fixation and processing may damage some antigens.

and processed tissues. Expression of molecules by preparations of cultured cell lines or of cell suspensions from body fluids such as ascites, blood, or pleural effusions may also be of particular interest in metastasis research, and immunocytochemistry on such preparations in the form of cytospins, cell smears, or cells cultured on coverslips is generally very successful.

For the simpler, quicker immunocytochemical methods, cell and tissue preparations will adhere well to clean, dry glass microscope slides. For longer, multistep techniques, use of an adhesive is recommended. Silane treatment, described in this chapter, is probably one of the most effective adhesives available and is cheap and simple to use. Its use is essential if heat-mediated antigen retrieval methods are going to be used subsequently.

1.4. Choice of Antibody

The second requirement for successful immunocytochemistry is the availability of a suitable antibody directed against the antigen of interest. Detailed description of the raising and production of antibodies lies beyond the scope of this volume, but, in brief, the choice lies between monoclonal and polyclonal antibodies. A huge range of both types of antibodies, directed against thousands of antigens of potential interest, are available commercially, and both types of antibodies can be produced ''in house'' if the appropriate facilities and expertise are available. Commercial companies exist that will produce ''tailor made'' antibodies directed against peptide sequences requested by the customer at (relatively) modest cost.

It is essential to choose an antibody appropriate for immunocytochemistry specifically, as antibodies developed for other applications, for example, enzyme-linked immunosorbent assay (ELISA), may simply not work well. It is also important to realize that all commercially available antibodies directed against the same molecule, or epitope on a particular molecule, may not be equally effective in immunocytochemistry and some ''shopping around'' may be helpful. Many commercial companies will provide small samples of antibodies free of charge for researchers to evaluate.

The choice of monoclonal or polyclonal antibody depends largely on what antibodies directed against the antigen of interest are available. Each type of antibody has specific advantages and disadvantages and it cannot be assumed that either polyclonal or monoclonal antibodies are invariably superior. In brief, polyclonal antibodies contain a cocktail of immunoglobulins directed against different epitopes of the antigen of interest, and other, irrelevant antigens also. They can therefore sometimes crossreact with molecules other than the one of interest and give spurious results or ''dirty'' background staining. The cocktail of immunoglobulins present may, however, react with multiple epitopes on the antigen molecule of interest, resulting in stronger and more effective labeling

than achieved with a comparable monoclonal antibody. They are usually raised in rabbit (or sometimes other large animals such as goat or sheep; chicken antibodies are also gaining in popularity), and tend to be cheaper to buy than monoclonal antibodies. Monoclonal antibodies are usually raised in mice or rats, and, as the name suggests, represent immunoglobulins produced by a single immortalized clone of cells, and therefore directed against a single epitope. They tend to be more expensive than polyclonal antisera, but can sometimes be used at extremely high working dilutions. The great advantage of monoclonal antibodies is their absolute specificity, which means that labeling results are often very clean. Many modern monoclonal antibodies are raised to synthetic peptide sequences, which has the advantage that the precise epitope they recognise is known. Crossreactivity can sometimes occur even with monoclonal antibodies if the epitope they are directed against is shared by other, irrelevant molecules.

There are no hard and fast rules as to choice of antibody —for some applications, a particular monoclonal antibody may be ideal; for others a polyclonal antiserum may give better results. It is important to note what class of antibody is being used. Most monoclonal antibodies used in immunocytochemistry are of the immunoglobulin G (IgG) class, but some may be immunoglobulin M (IgM). This is an important consideration in many immunocytochemical methods, as detection of antibody binding to antigen may be achieved by subsequent reaction with a secondary antibody directed against the first—for example, to detect a monoclonal mouse IgG binding, a labeled secondary antibody raised in, for example rabbit, against mouse IgG may be applied. A secondary antibody directed against mouse IgM would not be appropriate in this example.

For any application, the appropriate dilution of antibody must be determined. This can usually be ascertained only by performing a range a of dilutions and checking which gives optimum results in terms of strong specific labeling coupled with clean background. When using polyclonal antibodies, doubling dilutions are convenient, ranging from, perhaps, as a rough guide, 1:50–1:3200. Monoclonal antibodies may be tested in the range of, possibly, 1–50 μg/mL. In the more complex, multistep techniques, different dilutions of primary antibody and secondary labeling reagents may need to be titrated against each other in a ‘‘checkerboard of dilutions’’ to determine optimum working dilutions. An example of a typical ‘‘checkerboard of dilutions’’ is given in **Table 2**. One would expect the more concentrated solutions of primary and/or secondary antibody to give strong staining but unacceptably high background; too high a dilution of either primary or secondary antibody will yield low intensity, but probably very clean labeling. The optimum dilution of both in combination should yield deep, intense specific labeling allied to clean background and absence of nonspecific labeling. It is worth taking time and care over titration experiments to achieve optimal experimental results.

Table 2
Determination of Optimum Dilution of Primary and Secondary Antibody[a]

Dilution of secondary antibody, e.g., swine antisera raised against rabbit immunoglobulins	Dilution of primary antibody, e.g., polyclonal rabbit antisera raised against the molecule of interest						
	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
1:50							
1:100							
1:200							
1:400							
1:800							

[a]Sample "checkerboard of dilutions" used to determine the optimum dilution of primary and secondary antibody for use in, for example, an indirect detection method. The optimum combination of both will yield strong, specific labeling with clean background and no nonspecific labeling.

1.5. Choice of Labels for Immunocytochemistry

The third requirement for successful immunocytochemistry is the presence of a visible label. The choice of label usually lies between a fluorescent label or the colored product of an enzyme reaction, although other labels such as colloidal gold, silver, or ferritin can also be employed (usually in immunocytochemistry for electron microscopy, which is beyond the scope of this chapter).

Traditionally, the most commonly used fluorescent label is fluorescein isothiocyanate (FITC), which fluoresces a bright yellow-green ***(12)***. Alternatives include tetrarhodamine isothiocyanate (TRITC) and Texas red, which fluoresce red *(13)*. Many primary and secondary antibodies labeled with these compounds are available commercially. There is also an ever increasing range of other fluorescent labels that open up the possibility of multiple labeling, as described in Chapter 3 by Atherton and Clarke.

The most commonly used enzyme labels are horseradish peroxidase and alkaline phosphatase. Many primary and secondary antibodies and other immunocytochemical reagents labeled with these compounds are available. Other, less commonly used, enzyme labels include glucose oxidase and β-galactosidase. The principle of using any enzyme label is that its reaction with substrate plus a soluble chromagen yields a precipitated or insoluble colored product visible by light microscopy. For horseradish peroxidase reaction with hydrogen peroxide plus the chromogen diaminobenzidine (DAB) ***(14)*** yields a granular, brown, alcohol insoluble product or with 3-amino-9-ethylcarbazole (AEC) ***(15)*** yields a granular, red, alcohol-soluble product. Other chromagens are also available, but are less commonly employed. For alkaline phosphatase, reaction with naphthol phosphate as a substrate and a diazonium salt can yield a variety of colored—most typically red or blue—alcohol-soluble azo dyes as products ***(16)***.

Enzyme label detection kits, usually in the form of dropper bottles of concentrated reagents ready to be diluted in water or buffer, are commercially available as a convenient alternative to preparation of the necessary solutions ''in house.''

When enzyme labels are employed, the issue of endogenous, cell- or tissue-bound enzyme becomes an issue, and steps often need to be incorporated into the detection method to block endogenous enzyme prior to development of the final colored label product. It is worth noting that endogenous alkaline phosphatase is usually destroyed in processing to paraffin wax.

1.6. Range of Detection Methods Available

A number of fairly standard immunocytochemical techniques exist, which vary in terms of complexity and sensitivity. They range from the simple ''direct'' technique in which antigen is detected by the binding of a directly labeled antibody, through to much more complex, but highly sensitive multilayer techniques. Examples of a range of detection methods are outlined in this chapter and their relative advantages and disadvantages are summarized in **Table 3**. They are also represented diagramatically in **Figs. 1–5**. For any particular application, the choice of technique must usually be determined largely by trial and error. The simpler ''direct'' techniques are often employed for labeling of living cells—for example, cultured cells or cells from body fluids—as they are least likely to damage delicate cells. They are commonly used in conjunction with fluorescent labels, although enzyme labels can also be used. The more complex and sensitive, multilayer techniques are usually used in conjunction with more robust cell and tissue preparations and are particularly appropriate where antibody titres are low, or where antigen expression is scanty as the ''layering'' of reagent results in amplification of the final signal. Amplification is achieved because at every step, multiple reagent molecules have the opportunity to bind to the previous ''layer,'' resulting eventually in a much amplified ''cloud'' of label molecules marking the initial binding of antibody molecule to antigen. This important point is not shown in the figures illustrating the methods as, for the sake of clarity, the ''layers'' are represented in a simplified, linear manner.

In addition to the methods listed in this chapter, similar approaches are described in detail in Chapter 4 by Brooks and Hall on the related technique of lectin histochemistry. Lectin histochemistry facilitates the detection of carbohydrate structures, as part of, for example, glycoproteins, glycolipids, or glycosaminoglycans, *in situ*, by means of their recognition by a lectin.

1.7. Controls

The incorporation of appropriate positive and negative controls is, naturally, of paramount importance. The most appropriate positive control is a cell or tissue preparation that is known to express high levels of the antigen of inter-

Table 3
Advantages and Disadvantages of Different Detection Methods

	Advantages	Disadvantages
Direct method	Simplest method available. Quick. Limited number of reagents required. Works particularly well using fluorescent labelled antibody and cell suspensions.	Lacks sensitivity; therefore may not be appropriate for scantily expressed antigens. May suffer from high background.
Simple indirect method	Increased sensitivity over direct method (~20× more sensitive). Relatively quick and straightforward.	Requires more reagents than the direct method; therefore potentially more expensive. Extra step, therefore takes longer.
Simple avidin-biotin method	Relatively quick and simple, but highly sensitive and yields clean labeling.	Endogenous biotin may confuse interpretation in some cases. Glycosylated avidin may be recognized by tissue bound lectins or bind charged sites non-specifically.
ABC method	Highly sensitive (at least 100× more sensitive than the direct method). Clean results.	Large ABC complex sometimes causes steric hindrance. Tissue lectins may bind glycosylated avidin; avidin may attach nonspecifically by charge. Endogenous biotin may confuse interpretation. Cost implications of reagents.
PAP or APAAP methods	Highly sensitive (~50× more sensitive than direct method).	Very time consuming Cost implications of extra reagents. Largely superseded by ABC method.

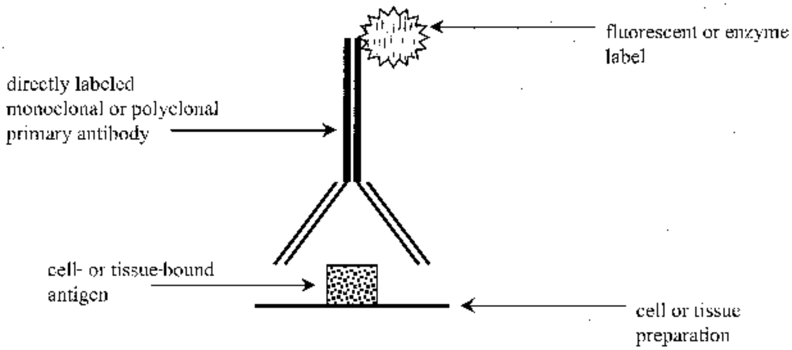

Fig. 1. Direct method. Cell- or tissue-bound antigen is detected by binding of drectly labeled primary antibody,

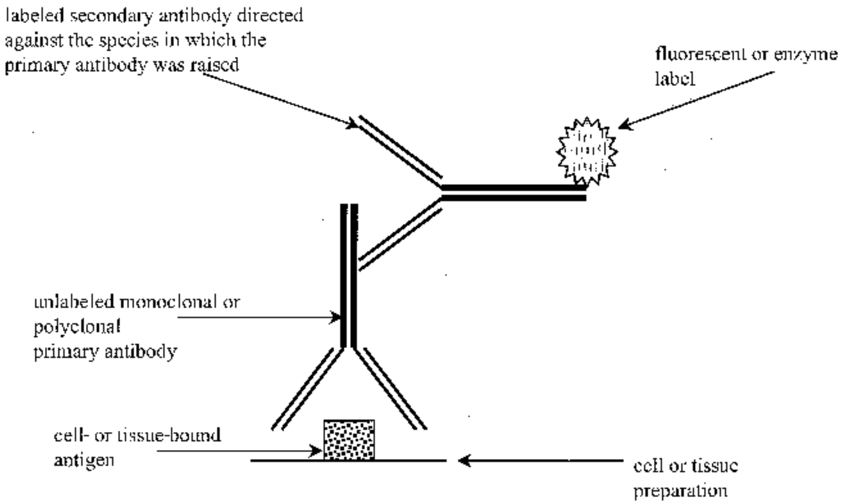

Fig. 2. Simple indirect method. Cell- or tissue-bound antigen is detected by binding of unlabeled primary antibody, then labeled secondary antibody directed against the species in which the primary antibody was raised.

est. The simplest negative control is to omit the primary antisera, and replace it with either buffer, preimmune serum at the same working dilution as the antibody, or an antibody directed against an irrelevant antigen. Specificity of binding can be confirmed by competitive inhibition in the presence of, or preabsorption of the antisera with, the antigen of interest.

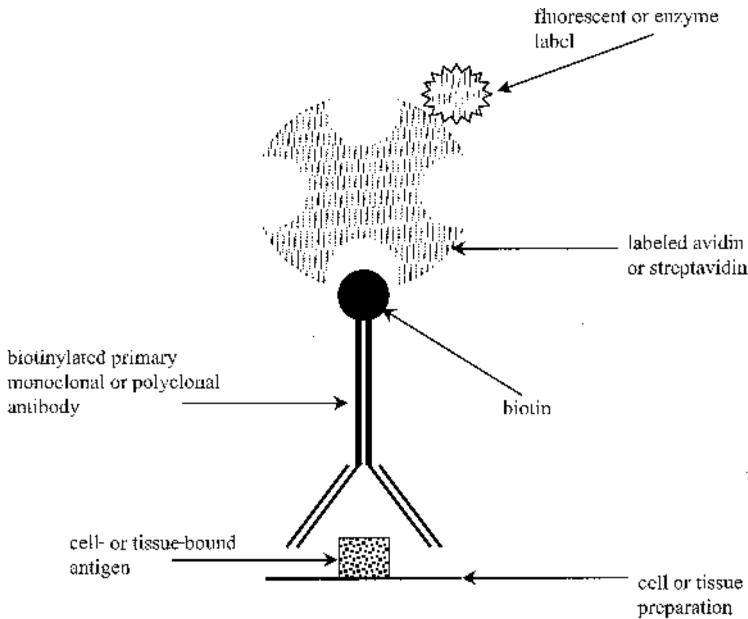

Fig. 3. Simple avidin–biotin method. Cell- or tissue-bound antigen is detected by binding of a biotinylated primary antibody, then labeled avidin or streptavidin.

2. Materials

*2.1. Silane Treatment of Microscope Slides (*see *Note 1)*

1. Acetone.
2. Acetone/silane solution: 2% v/v aminopropyltriethoxysilane or 3-(triethoxysilyl)-propylamine in acetone; make fresh on the day of use. Discard after treating a maximum of 1000 slides.
3. Distilled water: Discard and refresh after every five racks of slides have passed through.

2.2. Preparation of Cells Cultured on Coverslips

1. Cells cultured under standard conditions.
2. Fetal calf serum (FCS) cell free culture medium.
3. Alcohol- or autoclave-sterilized round glass coverslips (13 mm diameter, thickness 0).
4. Dental wax or Parafilm.
5. 0.1 *M* PIPES buffer, pH 6.9: Stir 12.1 g of PIPES (piperazine-*N*,*N*'-*bis*-[2-ethane-sulfonic acid]) into 50 mL of ultrapure water to give a cloudy solution. Add approx 40 mL of 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 400 mL.

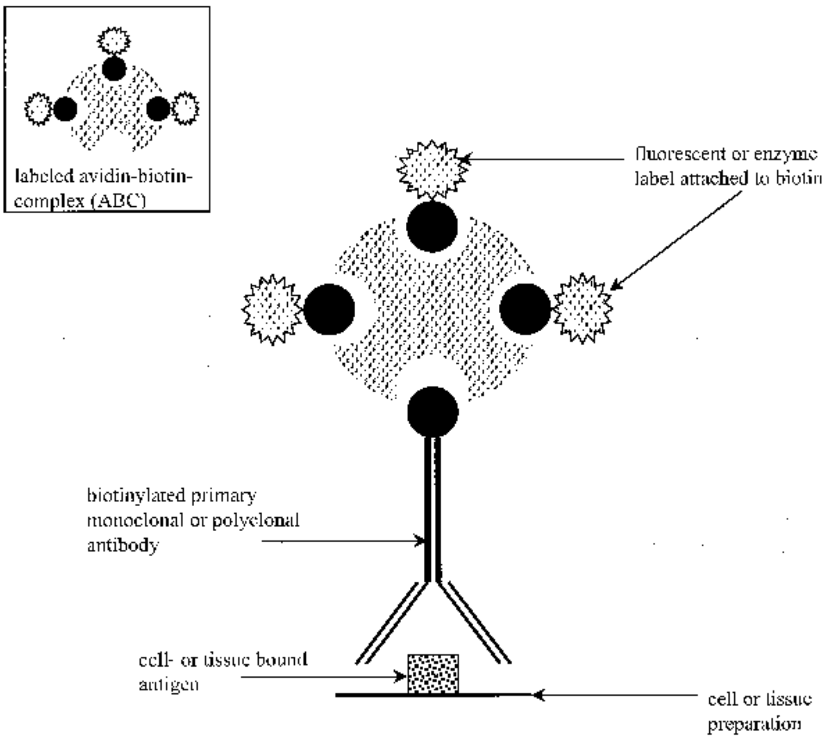

Fig. 4. Avidin–biotin complex (ABC) method. Cell- or tissue-bound antigen is detected by binding of a biotinylated primary antibody, then labeled ABC.

6. 3% v/v paraformaldehyde in 0.1 *M* PIPES buffer, pH 6.9: Place 3 g of 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, then add 50 mL of 0.2 *M* PIPES buffer, pH 6.9 (*see* **step 5**). Divide into 10-mL aliquots and store frozen. Defrost in a warm water bath for use.
7. 0.1% v/v Triton X-100 or Saponin in 0.1 *M* PIPES buffer, pH 6.9.

2.3. Smears Prepared from Cells in Suspension

1. Cells in suspension (*see* **Note 2**).
2. Silane-treated glass microscope slides (*see* **Subheading 2.1.** and **Note 1**).
3. Aluminum foil or "cling film."
4. Acetone.

2.4. Frozen (Cryostat) Sections

1. Chunk of fresh tissue >0.5 cm^3 in size.
2. Cryostat embedding medium, for example, OCT or similar.
3. Isopentane or hexane.

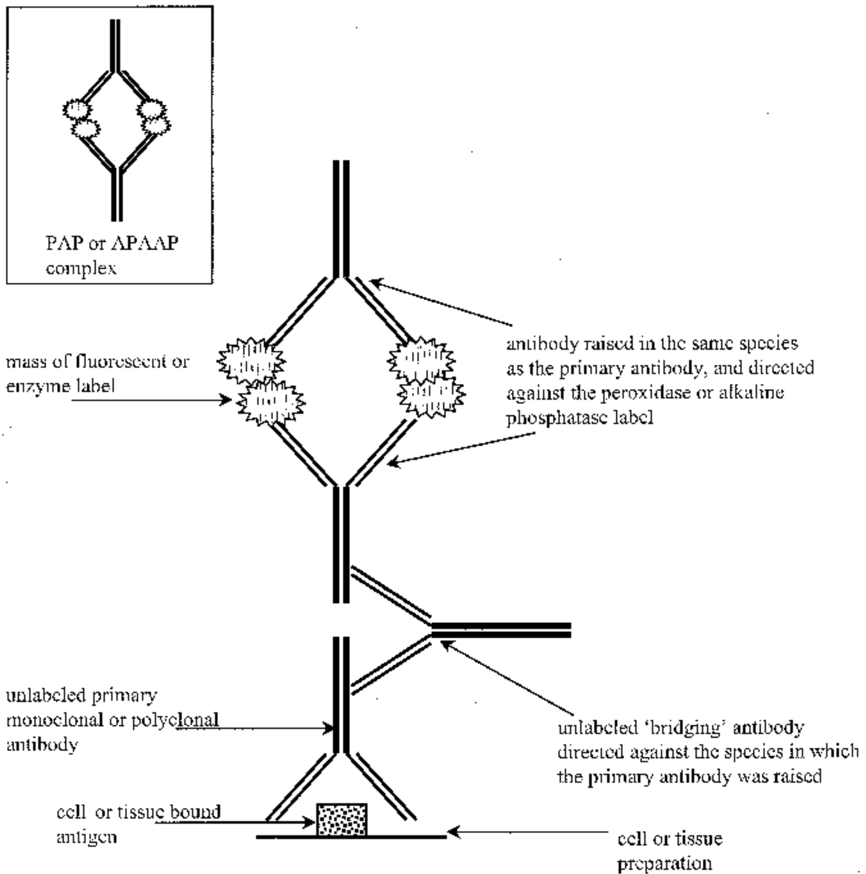

Fig. 5. Peroxidase–antiperoxidase (PAP) or alkaline phosphatase–antialkaline phosphatase (APAAP) method. Cell- or tissue-bound antigen is detected by, first, unlabeled primary monoclonal or polyclonal antibody, then a "bridging" antibody directed against the species in which the primary antibody was raised, and finally a labeled PAP or APAAP complex raised in the same species as the primary antibody. The labeled PAP or APAAP consists of a mass of enzyme label complexed with antibodies directed against it.

4. Liquid nitrogen.
5. Silane-coated clean glass microscope slides (*see* **Subheading 2.1.** and **Note 1**).
6. Acetone.
7. Aluminum foil or "cling film."

2.5. Fixed, Paraffin Wax-Embedded Sections

1. Paraffin wax-embedded tissue blocks.
2. 20% v/v ethanol or industrial methylated spirit in distilled water.

3. Silane-treated glass microscope slides (*see* **Subheading 2.1.** and **Note 1**).
4. Xylene (*see* **Note 3**).
5. Absolute ethanol or industrial methylated spirit.
6. 70% v/v ethanol or industrial methylated spirit in distilled water.
7. Distilled water.

2.6. Buffers for Blocking, Dilutions, and Washes

1. Washing buffer: Tris-buffered saline (TBS), pH 7.4–7.6: 60.57 g of Tris, 87.0 g of NaCl dissolved in 1 L of distilled water. Adjust pH to 7.4–7.6 using concentrated HCl. Make up to total vol 10 L using distilled water. This buffer is recommended for all washes, unless otherwise stated.
2. Blocking buffer: 5% v/v normal horse or goat serum in washing buffer.
   All immunocytochemical methods (*see* **Subheading 3.9.**) incorporate a step in which cell and tissue preparations are incubated with a blocking buffer to reduce nonspecific binding of antibodies (*see* **Note 4**).
3. Dilution buffer for antibodies: 3% v/v normal horse or goat serum in blocking buffer. Antibodies are diluted to their working concentration in buffer containing a low percentage of normal serum. This, again, reduces nonspecific binding of antibodies and minimizes ''dirty'' background staining (*see* **Note 4**).

*2.7. Enzyme-Based Antigen Retrieval Methods (*see* Note 5)*

1. Trypsin solution: 1 mg/mL of crude, type II trypsin, from porcine pancreas (*see* **Note 6**) and 1 mg/mL of calcium chloride in washing buffer (*see* **Subheading 2.6.**) warmed to 37°C.
2. Protease solution: Protease XXIV, bacterial, 7–14 U/mg in washing buffer (*see* **Subheading 2.6.**) prewarmed to 37°C.
3. Pepsin solution: pepsin from porcine stomach, 1:2500, 600–1000 U/mg in 0.01 *M* hydrochloric acid, prewarmed to 37°C.
4. Neuraminidase solution: neuraminidase (sialidase), type V from *Clostridium perfringens* at a concentration of 0.1 U/mg in 0.1 *M* sodium acetate pH to 5.5 with citric acid, containing 0.01% w/v calcium chloride, prewarmed to 37°C.

*2.8. Microwave Oven Heat-Mediated Antigen Retrieval Method (*see* Note 7)*

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

2.9. Quenching Endogenous Enzyme

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

2.10. Examples of Some Histochemical Staining Techniques

2.10.1. Direct Method

1. Blocking buffer (*see* **Subheading 2.6.**).
2. Monoclonal or polyclonal antibody labeled with a fluorescent or enzyme label made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.**).
3. Washing buffer (*see* **Subheading 2.6.**).

2.10.2. Simple Indirect Method

1. Blocking buffer (*see* **Subheading 2.6.**).
2. Unlabeled monoclonal or polyclonal antibody made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.**).
3. Washing buffer (*see* **Subheading 2.6.**).
4. Fluorescent- or enzyme-labeled secondary antibody directed against the immunoglobulins of the species in which the primary antibody was raised, made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.**).

2.10.3. Simple Avidin–Biotin Method

1. Blocking buffer (*see* **Subheading 2.6.**).
2. Biotin-labeled monoclonal or polyclonal primary antibody made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.**).
3. Washing buffer (*see* **Subheading 2.6.**).
4. Avidin or streptavidin labeled with a fluorescent or enzyme label made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.** and **Note 8**).

2.10.4. Avidin–Biotin-Complex Method

1. Blocking buffer (*see* **Subheading 2.6.**).
2. Biotin-labeled monoclonal or polyclonal primary antibody made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.**).
3. Washing buffer (*see* **Subheading 2.6.**).
4. Fluorescent- or enzyme-labeled avidin–biotin complex (ABC) made up according to the manufacturer's instructions (*see* **Note 9**).

2.10.5. Peroxidase–Anti-Peroxidase or Alkaline Phosphatase–Anti-Alkaline Phosphatase Methods (see **Note 10**)

1. Blocking buffer (*see* **Subheading 2.6.**).
2. Unlabeled monoclonal or polyclonal antibody made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.**).
3. Washing buffer (*see* **Subheading 2.6.**).
4. Unlabeled "bridging" antibody directed against the species in which the primary antibody was raised made up in excess concentration (*see* **Note 11**) in dilution buffer (*see* **Subheading 2.6.**).
5. Peroxidase–anti-peroxidase (PAP) or alkaline phosphatase–anti-alkaline phosphatase (APAAP) raised in the same species as the primary antibody (e.g.,

when using a mouse monoclonal primary, use *mouse* PAP or APAAP; when using rabbit polyclonal primary antibody, use *rabbit* PAP or APAAP), made up at optimum working dilution in dilution buffer (*see* **Subheading 2.6.**).

2.11. Enzyme Development Methods

2.11.1. DAB for Horseradish Peroxidase Label

1. Washing buffer (*see* **Subheading 2.6.**).
2. DAB–H_2O_2: 0.5 mg/mL of 3,3-diaminobenzidine tetrahydrochloride (DAB) in washing buffer (*see* **Subheading 2.6.**). Add H_2O_2 to give a concentration of 0.03% v/v immediately before use. This substance is potentially carcinogenic (*see* **Note 12**).

2.11.2. Fast Red for Alkaline Phosphatase Label

1. TBS, pH 8.2–9.0: 6.57 g of Tris, 8.7 g of NaCl dissolved in a total volume of 1 L of distilled water. Adjust pH to 8.2–9.0 using concentrated HCl.
2. Stock solution of naphthol phosphate: Dissolve 20 mg of naphthol AS-MX phosphate sodium salt in 500 μL of *N,N*-dimethylformamide in a small glass vessel (*see* **Note 13**).
3. Stock solution fast red salt: Dissolve 20 mg of fast red salt in 1 mL of TBS, pH 8.2–9.0.
4. Levamisole hydrochloride.

2.12. Counterstaining

1. Mayer's hematoxylin solution (*see* **Note 14**).
2. 1% v/v ammonia in tap water.

2.13. Mounting

1. For fluorescently labeled preparations: An antifade mounting medium, for example, Citifluor or similar.
2. For alkaline phosphatase/fast red labeled, or other preparations labeled with an alcohol-soluble chromogenic product: An aqueous mountant, for example, Aquamount or similar.
3. For horseradish peroxidase/DAB labeled, or other preparations labeled with an alcohol-insoluble chromogenic product: 70% v/v ethanol or industrial methylated spirit in distilled water; 95% v/v ethanol or industrial methylated spirit in distilled water; absolute alcohol; xylene (*see* **Note 3**); and an appropriate xylene-based mounting medium, for example, Depex or similar.

3. Methods

3.1. Silane Treatment of Microscope Slides (see Note 1)

1. Place slides in a slide carrier and immerse in acetone for 5 min.
2. Immerse in acetone/silane solution for 5 min.
3. Immerse in two consecutive baths of either acetone or distilled water for 5 min each.
4. Drain slides, dry either at room temperature or in a warm oven, and store in closed boxes at room temperature indefinitely.

3.2. Preparation of Cells Cultured on Coverslips

1. Wash cultured cells in fresh FCS free culture medium.
2. Aspirate and discard the medium.
3. Scrape cells from the flask using a rubber policeman and resuspend in fresh FCS cell free culture 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% CO_2.
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 of 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 or 0.1% v/v Saponin in 0.1 *M* PIPES buffer, pH 6.9, for 10 min.
10. Wash thoroughly in 0.1 *M* PIPES buffer, pH 6.9.

3.3. Smears Prepared from Cells in Suspension

1. Place a drop of cells in suspension approx 5 mm from one end of a silane-treated glass microscope slide.
2. Place a second microscope slide on top of the first, allowing approx 1 cm of glass to protrude at either end, and allowing the drop to spread between the two.
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 approx 5 min. They may then be used at once, or wrapped individually in foil or "cling film" and stored in the 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.

3.4. Frozen (Cryostat) Sections

1. Using a sharp, clean blade, cut a solid tissue block of fresh tissue of approx 0.5 cm^3 (*see* **Note 15**).
2. Place the tissue on cryostat chuck thickly coated in cryostat embedding medium (*see* **Note 16**).
3. Using long-handled tongs, pick up the chuck and immerse chuck and tissue in isopentane or hexane precooled in liquid nitrogen for approx 1–2 min (*see* **Note 17**).
4. Place the frozen chuck in the cabinet of the cryostat and leave to equilibrate for approx 30 min.
5. Using the cryostat, cut 5–10 μm thick sections and pick them up on clean, dry silane-treated microscope slides. Allow to air-dry for between 1 h and overnight.
6. Sections may then either be stored until required by wrapping individually or back-to-back in aluminum foil or "cling film," sealing in polythene bags or boxes containing desiccant, and storing in the freezer, or may be used at once. If stored frozen, allow to thaw and equilibrate to room temperature before opening.
7. Immediately before use, dip slides in acetone for 1–10 min and air-dry for approx 5 min.

3.5. Fixed, Paraffin Wax-Embedded Sections

1. Cool wax-embedded tissue blocks on ice for approx 15 min.
2. Cut 4–7 µm thick sections by microtome (*see* **Note 18**).
3. Float sections out on a pool of 20% v/v ethanol or industrial methylated spirit in distilled water on a clean glass plate supported by a suitable receptacle such as a glass beaker or jar (*see* **Note 19**).
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 20**).
5. Separate out individual sections very gently using the tips of fine, bent forceps.
6. Pick up individual sections on silane-treated microscope slides.
7. Allow slides to drain by up-ending them on a sheet of absorbent paper for 5–10 min.
8. Dry slides either in a 37°C incubator overnight or in a 60°C oven for 15–20 min (*see* **Note 21**). 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 approx 15 min to remove the paraffin wax.
10. Transfer through two changes of absolute ethanol or industrial methylated spirit, then through one change of 70% v/v ethanol or industrial methylated spirit in distilled water, then distilled water, agitating the slides vigorously for 1–2 min at each stage to equilibrate (*see* **Note 22**).

*3.6. Enzyme-Based Antigen Retrieval Methods (*see *Note 5)*

When using fixed, paraffin embedded tissues *only*, methods to retrieve antigens damaged or sequestered by harsh fixation and processing procedures may be necessary, or may significantly enhance results. These methods are *not* appropriate for other types of cell or tissue preparation (*see* **Note 23**).

1. Trypsinization: Immerse slides in a bath of trypsin solution at 37°C, in an incubator or water bath, for 5–30 min, then wash in running tap water for 5 min.
2. Protease treatment: Place slides face up in a suitable chamber (*see* **Note 24**) and apply a few drops of protease solution to cover the tissue preparation. Incubate in an incubator at 37°C, for 5–30 min, then wash in running tap water for 5 min.
3. Pepsin treatment: Place slides face up in a suitable chamber (*see* **Note 24**) and apply a few drops of pepsin solution to cover the tissue preparation, or immerse slides in a bath of pepsin solution. Allow to digest in an incubator at 37°C for 5–30 min, then wash in running tap water for 5 min.
4. Neuraminidase treatment: Place slides face up in a suitable chamber (*see* **Note 24**) and apply a few drops of neuraminidase solution to cover the tissue preparation. Incubate in an incubator at 37°C for 5–30 min, then wash in running tap water for 5 min.

3.7. Microwave Oven Heat-Mediated Antigen Retrieval Method

An alternative to enzyme mediated antigen retrieval is antigen retrieval mediated by heat. The method given in the following is for heat-mediated treatment using a microwave oven, but other methods exist using, for example, pressure cooking or autoclaving (*see* **Note 7**).

1. Immerse slides in citrate buffer, pH 6.0, in any suitable microwave-safe container, such as a plastic sandwich box (*see* **Note 25**).
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 boils 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 another 5 min.
4. Allow slides to cool at room temperature for 30 min (*see* **Note 26**).
5. Wash under running tap water for 5 min.

3.8. Quenching Endogenous Enzyme

If horseradish peroxidase is to be employed as the label molecule, then endogenous peroxidase must be quenched as follows. This step is most conventionally performed immediately prior to the addition of the primary antisera.

1. Immerse slides in methanol–hydrogen peroxide solution for 20 min.
2. Wash under running tap water for approx 5 min.

If alkaline phosphatase is to be employed as the label molecule it may be necessary to quench endogenous alkaline phosphatase, although it is usually destroyed by processing to paraffin wax rendering this procedure unnecessary. If required, 1 m*M* levamisole is added to the final enzyme development medium (*see* **Subheadings 2.11.2.** and **3.11.2.**).

3.9. Examples of Some Histochemical Staining Techniques

As described in the Introduction, a number of basic immunocytochemical techniques are available that vary in their relative complexity and sensitivity. Illustrative examples are listed here that should give good results, but the researcher is urged to experiment and adapt these basic technique to give optimum results in his or her experimental system. Other techniques also exist. The methods outlined here are also illustrated diagramatically in **Figs. 1–5**.

3.9.1. Direct Method (see *Fig. 1*)

1. Incubate slides with blocking buffer for 30 min.
2. Drain slides and wipe around cell or tissue preparation using a clean, dry tissue.
3. Incubate slides with directly (fluorescent or enzyme) labeled monoclonal or polyclonal antibody in a humid chamber (*see* **Note 24**), for 30 min to 2 h at room temperature, or at 4°C overnight.
4. Wash in three changes of washing buffer (*see* **Note 27**).
5. If fluorescently-labeled antibody is employed, mount and view directly using a fluorescent microscope. If enzyme-labeled antibody is used, proceed to enzyme development as described in **Subheading 3.10.** onwards.

3.9.2. Simple Indirect Method (see *Fig. 2*)

1. Incubate slides with blocking buffer for 30 min.
2. Drain slides and wipe around cell or tissue preparation using a clean, dry tissue.

3. Incubate slides with unlabeled monoclonal or polyclonal antibody in a humid chamber (*see* **Note 24**), for 30 min to 2 h at room temperature, or at 4°C overnight.
4. Wash in three changes of washing buffer (*see* **Note 27**).
5. Incubate slides with either fluorescent- or enzyme-labeled secondary antibody directed against the immunoglobulins of the species in which the primary antibody was raised (*see* **Note 28**) in a humid chamber (*see* **Note 24**) for 1 h.
6. Wash in three changes of washing buffer (*see* **Note 27**).
7. If fluorescently-labeled secondary antibody is employed, mount and view directly using a fluorescent microscope. If enzyme-labeled secondary antibody is used, proceed to enzyme development as described in **Subheading 3.10.** onwards.

*3.9.3. Simple Avidin–Biotin Method (*see ***Fig. 3****)*

1. Incubate slides with blocking buffer for 30 min.
2. Drain slides and wipe around cell or tissue preparation using a clean, dry tissue.
3. Incubate slides with biotin-labeled monoclonal or polyclonal primary antibody in a humid chamber (*see* **Note 24**), for 30 min to 2 h at room temperature, or at 4°C overnight.
4. Wash in three changes of washing buffer (*see* **Note 27**).
5. Incubate with avidin or streptavidin labeled with fluorescent or enzyme label, in a humid chamber (*see* **Note 24**), for 30 min.
6. Wash in three changes of washing buffer (*see* **Note 27**).
7. If fluorescently-labeled avidin or streptavidin is employed, mount and view directly using a fluorescent microscope. If enzyme-labeled avidin or streptavidin is used, proceed to enzyme development as described in **Subheading 3.10.** onwards.

*3.9.4. ABC Method (*see ***Fig. 4****)*

1. Incubate slides with blocking buffer for 30 min.
2. Drain slides and wipe around cell or tissue preparation using a clean, dry tissue.
3. Incubate slides with biotin-labeled monoclonal or polyclonal primary antibody in a humid chamber (*see* **Note 24**), for 30 min to 2 h at room temperature, or at 4°C overnight.
4. Wash in three changes of washing buffer (*see* **Note 27**).
5. Incubate with fluorescent- or enzyme-labeled ABC in a humid chamber (*see* **Note 24**) for 30 min.
6. Wash in three changes of washing buffer (*see* **Note 27**).
7. If fluorescently-labeled ABC is employed, mount and view directly using a fluorescent microscope. If enzyme-labeled ABC is used, proceed to enzyme development as described in **Subheading 3.10.** onwards.

*3.9.5. PAP or APAAP Methods (*see ***Fig. 5****)*

1. Incubate slides with blocking buffer for 30 min.
2. Drain slides and wipe around cell or tissue preparation using a clean, dry tissue.
3. Incubate slides with unlabeled monoclonal or polyclonal primary antibody in a humid chamber (*see* **Note 24**), for 30 min to 2 h at room temperature, or at 4°C overnight.

4. Wash in three changes of washing buffer (*see* **Note 27**).
5. Incubate with an unlabeled "bridging" antibody (*see* **Note 11**) for 1 h in a humid chamber (*see* **Note 24**).
6. Wash in three changes of washing buffer (*see* **Note 27**).
7. Incubate with PAP or APAAP for 1 h in a humid chamber (*see* **Note 24**).
8. Wash in three changes of buffer (*see* **Note 27**).
9. Proceed to enzyme development as described in **Subheading 3.10.** onwards.

3.10. Enzyme Development Methods

3.10.1. DAB for Horseradish Peroxidase Label

1. Wash in three changes of washing buffer (*see* **Note 27**).
2. Incubate with DAB–H_2O_2 for 10 min (*see* **Note 12**).
3. Wash under running tap water for 5 min.
4. Proceed to counterstaining (*see* **Subheading 3.11.**) and mounting (*see* **Subheading 3.12.**).

3.10.2. Fast Red for Alkaline Phosphatase Label

1. Wash slides briefly in TBS, pH 8.2–9.0.
2. Take 18.5 mL of TBS pH 8.2–9.0, add 500 μL of stock solution of naphthol phosphate and mix, then add levamisole to give a 1 m*M* solution and mix, then add 1 mL of fast red solution and mix. Filter and apply to slides immediately.
3. Immerse the slides in fast red solution for 5–30 min (*see* **Note 29**).
4. Wash under running tap water for 5 min.
5. Proceed to counterstaining (*see* **Subheading 3.11.**) and mounting (*see* **Subheading 3.12.**).

3.11. Counterstaining

1. Immerse in Mayer's hematoxylin solution for 3–5 min.
2. "Blue" by immersing in running tap water for 5 min, or dip briefly in 1% v/v ammonia in tap water, then wash in tap water (*see* **Note 30**).
3. Proceed to mounting (*see* **Subheading 3.12.**).

3.12. Mounting

1. Preparations labeled using fluorescent tags should be mounted directly in an antifade fluorescent mountant such as Citifluor or similar.
2. Preparations labeled using alcohol-soluble chromagens such as fast red for alkaline phosphatase should be mounted directly in an aqueous mountant such as Aquamount or similar.
3. Preparations labeled using alcohol-insoluble chromagens such as DAB for horseradish peroxidase should be dehydrated by immersing, with agitation, for 1 min each in 70% v/v, 95% v/v ethanol or industrial methylated spirit, then two changes of absolute ethanol, cleared by immersion, with a agitation, in two changes of xylene, then mounted in a xylene-based mountant such as Depex or similar (*see* **Note 31**).

3.13. Viewing, Interpretation, and Quantification of Labeling Results

Slides should be viewed by light or fluorescence microscope, as appropriate. Fluorescently labeled preparations should be should be stored in the dark and in the refrigerator until viewing. They should be viewed as soon as possible after labeling, as fluorescence will fade over time, and photographic images should be made for permanent record. Enzyme labels should be permanent, and slides may therefore be stored for longer. Some aqueous mountants deteriorate over time. Good labeling is indicated by a strong specific label and low, or preferably nonexistent, background and nonspecific labeling.

It is often helpful to score labeling on an arbitrary scale where the observer estimates the percentage of cells, for example cancer cells, labeled (10%, 50%, 95%, etc.) and the intensity of labeling on a scale of – (no labeling at all), + – very weak labeling, + (weak but definite labeling) to ++++ (extremely intense labeling) to give results ranging from completely negative to 100% ++++. Preferably, this should be carried out by at least two independent observers and results compared.

Many attempts have been made to quantify immunocytochemistry results using automated, computer-based approaches, but the author is unaware of any truly satisfactory and reproducible system.

3.14. Some Common Problems and the Most Likely Suggested Solutions to Them

3.14.1. High Nonspecific Background Staining

Possibly the most common problem, this usually can be caused by a number of different factors. The most usual is probably insufficient washing between steps (*see* **Note 27**). The second most likely cause is employment of too high a concentration of one or more of the reagents. All reagents (primary antibody, secondary antibody, avidin and biotin products, PAP or APAAP, etc.) should be titrated carefully to give optimum results (*see* **Subheading 1.4.** and **Table 2**). When using horseradish peroxidase as a label, residual endogenous peroxidase may sometimes be a problem — check by incubating a slide that has been treated simply with the standard methanol–hydrogen peroxide solution (*see* **Subheading 2.9.**) with the chromogenic substrate DAB–H_2O_2 (*see* **Subheading 2.11.1.**) — there should be no brown staining present; if there is, this indicates the presence of unquenched endogenous peroxidase. If this is the case, try freshly made methanol–hydrogen peroxide, increase the concentration of hydrogen peroxide or the incubation time in this step, or try an alternative label, for example, alkaline phosphatase.

If the problematic high background staining is absent in a negative control where primary antibody is omitted, this would indicate a crossreaction between the primary antibody and some cell or tissue component. This may be remedied

by one or more of the following: increasing the concentration of blocking serum or protein in blocking buffer (*see* **Subheading 2.6.** and **Note 4**), incorporating more sodium chloride (up to 0.1 *M*) into blocking, dilution, and washing buffers, or adding a small amount of detergent (e.g., 0.05% v/v Tween-20) to washing buffers.

In avidin–biotin based methods, endogenous biotin can sometimes cause confusing results. It can be blocked by applying unconjugated avidin (which binds to tissue-bound biotin), then saturating with further free, excess, unlabeled biotin. Avidin may also sometimes attach to charged cell/tissue sites: This may be most easily remedied by increasing the pH of washing, dilution, and blocking buffer to 9.0, or may be avoided by using the more costly streptavidin products instead of avidin.

3.14.2. Weak or Absent Labeling

Obviously, the positive control—a preparation known to express the antigen of interest—should be checked. If satisfactory labeling is achieved here, it would suggest that the antigen is present in only low levels, or absent, in the test slides. If low levels only are present, perhaps indicated by weak labeling, a more sensitive detection technique should be employed. If fixed, paraffin-embedded material is being examined, antigen retrieval methods should be tried. If positive controls show inadequate labeling, all reagents should be systematically checked for reactivity.

4. Notes

1. We use silane-treated slides for *all* cell and tissue preparations in immunocytochemistry. Alternative, commercial, brand-named preparations are also available, but tend to be more expensive. Silane, or equivalent, treatment is *essential* if a heat-mediated antigen retrieval method is to be employed subsequently. Slides should not be agitated in the baths of reagents, as air bubbles will prevent the silane solution reaching the glass surface and will result in patchy and inadequate treatment.
2. Any cells in suspension are suitable—for example, blood, cancer cells in ascites or in pleural effusions taken from patients or from animal models, cells derived from solid tissue tumors and released into suspension, or cultured cells in suspension.
3. Xylene is potentially hazardous and should be handled with care in a fume cupboard. Modern, safer chemical alternatives are commercially available, but it is our experience that they give slightly less satisfactory results.
4. Blocking buffer and buffer for dilution of antibodies: The use of normal (nonimmune) animal serum and its incorporation into working solutions of antibodies effectively reduces non-specific background staining. Goat or horse serum is recommended here for simplicity as it is unlikely that the detection methods employed will involve specific recognition of antibodies raised in these species.

It is also appropriate to employ normal (nonimmune) serum from the final antibody-producing species in any detection method — for example, in an indirect method in which a labeled rabbit secondary antibody is employed to detect binding of an unlabeled mouse monoclonal antibody, nonimmune rabbit serum would be entirely appropriate. Solutions of an inert protein or protein mixture such as bovine serum albumin, casein, or commercially available dried skimmed milk powder are also routinely used, and are cheaper than nonimmune serum. As a guide, a solution of 1–5% w/v solution in buffer is appropriate.

5. It is not possible to predict if enzyme-based antigen retrieval methods will be effective, or which to choose; try them out and select conditions which work best for your application. Test a range of treatment times, for example, 0, 5,10, 20, and 30 min. Trypsinisation is probably most widely used, probably because it is relatively cheap, and can be extremely effective, if, sometimes a little brutal! The other enzymes tend to be more expensive (especially neuraminidase), but are more selective. Try heat-mediated antigen retrieval (*see* **Subheadings 2.8.** and **3.8.**) also. These methods should be used only in conjunction with fixed, paraffin-embedded tissue preparations mounted on silane (or equivalent; *see* **Note 1**)-treated glass slides.
6. Use crude, type II trypsin, from porcine pancreas. Impurities (e.g., chymotrypsin) enhance its effect. Do not use purer (and more expensive!) products.
7. It is not possible to predict if heat-mediated antigen retrieval methods will be effective in any particular case. This can be determined only by trial and error. Other heat-mediated antigen retrieval methods, such as autoclaving and pressure cooking also exist. Try enzyme-based antigen retrieval methods (*see* **Subheadings 2.7.** and **3.7.**) also. These methods should be used only in conjunction with fixed, paraffin-embedded tissue preparations mounted on silane (or equivalent, *see* **Note 1**)-treated glass slides.
8. Avidin is a large glycoprotein extracted from egg white that has four binding sites for the vitamin biotin. Streptavidin is a protein, similar in structure to avidin, and is derived from the bacterium *Streptomyces avidinii.* Avidin products are generally significantly cheaper than streptavidin products. Streptavidin is said to give a cleaner result, but avidin may be perfectly acceptable for most applications.
9. ABC is available commercially as convenient dropper bottle kits. Follow kit instructions, which usually require that avidin and labeled biotin are combined 30 min before use. Avidin and labeled biotin are mixed together in such a ratio that three of the four possible biotin-binding sites are saturated, leaving one free to combine with the biotin label attached to the primary antibody.
10. The PAP and APAAP techniques were extremely popular some years ago owing to their sensitivity, resulting from the multilayering. They are less commonly used today and have been superseded to some extent by the avidin–biotin and ABC methods.
11. The “bridging” antibody forms a “bridge” by linking the primary antibody with the PAP or APAAP complex. So, for example, if a monoclonal mouse primary antibody is used, the “bridging” antibody will be antisera raised against mouse

immunoglobulins; if a rabbit polyclonal primary antibody is used, the "bridging" antibody will be antisera directed against rabbit immunoglobulins. This antisera should be applied in excess. The "bridging" antibody needs to be present in excess so that only one of the two possible antigen binding sites of each "bridging" antibody molecule are occupied by primary antibody, leaving the second binding site free to bind to the PAP or APAAP complex. As a rough guide, in our experience, most commercially available secondary antibodies will work best in this context when diluted at approx 1:50.

12. DAB is potentially carcinogenic. It should be handled with care, using 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. Swab down working surfaces with dilute bleach after use. Clean up spillages with excess water, then swab with dilute bleach. We usually make a concentrated DAB stock solution at 5 mg/mL in distilled water and freeze in 1-mL aliquots in 10-mL plastic screw top tubes until required. This minimizes the risk of aerosols from weighing out powder when required. It is also available in convenient tablet or dropper bottle kit form, which minimizes hazards, but is more expensive.
13. Use glass, as plastic will dissolve in the dimethylformamide.
14. A number of different hematoxylin solutions are commercially available. Mayer's, a progressive stain, is particularly convenient, but other types are equally effective.
15. When cutting tissue, use a very sharp blade and use single, firm, swift, downward stokes. Avoid hacking and crushing, which will result in poor morphology. Cut the tissue into a straight edged, geometrical shape, most conveniently a cube, as this will make sectioning easier. Clearly, when handling potentially infective tissue, appropriate health and safety guidelines should be adhered to.
16. The cryostat embedding medium acts as a support for the tissue. Apply it generously to the chuck and immerse the tissue block into a pool of it. Align the tissue block with a straight edge parallel with the cutting edge of the chuck.
17. To obtain optimum morphological integrity, the tissue should be frozen as rapidly as possible, avoiding the formation of morphology-destroying ice crystals. The most effective method is to immerse the tissue in isopentane or hexane precooled in liquid nitrogen. These solvents conduct heat away from the tissue more rapidly that liquid nitrogen alone. Other methods of freezing the block—for example, by using a commercially available freezing spray, by blasting with CO_2 gas, or even simply placing it in the chamber of the cryostat until frozen are less effective.
18. Most tissues cut most best when chilled. An ice cube or handful of crushed ice should be applied to the surface of the tissue block every few minutes during cutting. This is particularly important when working in a warm room.
19. Small creases or wrinkles in the section should begin to flatten out; the effect may be enhanced by gentle manipulation using, for example, a soft paintbrush and/or forceps.
20. If the wax begins to melt, it is too hot. If sections remain wrinkled, it may indicate that the water is slightly too cold.
21. Do not heat directly on a hotplate, as this may damage some antigens.

22. Take careful note of the appearance of the slides during this process. When slides are transferred from one solvent to the next, they initially appear smeary as the two solvents begin to mix. Vigorous agitation, that is "sloshing them up and down" ensures that the slides equilibrate effectively with the new solvent. When they are equilibrated, the surface smearing will disappear. During the rehydration process, 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 immunocytochemistry results is inadequate removal of paraffin wax.
23. Make enzyme solutions fresh immediately before use. Use glassware, solutions, etc. that have been prewarmed to 37°C before use. Initially try a range of digestion times, for example, 0, 5, 10, 20, 30 min. Digestion times of >30 min are not recommended, as visible damage to tissue morphology becomes apparent. This is especially true when using trypsin.
24. The idea is to have a flat platform on which to place the slides, in a lidded, humid chamber. Humidity is important so that small volumes of solution placed onto the surface of the slides do not evaporate and therefore either dry out completely or become more concentrated. Drying of cell/tissue preparations will result in high nonspecific background staining. For small numbers of slides, it may be convenient to place them, face up, in a lidded Petri dish lined with a disc of dampened filter paper. For larger numbers of slides, specially designed incubation chambers—usually fashioned in Perspex, and containing raised ridges to support slides over troughs that may be partially filled with water to maintain a humid atmosphere—are commercially available. These tend to be expensive to buy. They can be made "in house" if appropriate facilities exist. It is possible to make a perfectly functional incubation chamber very simply using a large sandwich box with supports for slides formed from, for example, glass or wooden rods supported in "plasticine" or "blu-tack." Again, a small amount of water or dampened filter paper may be added to the base of the chamber to maintain humidity.
25. Slides can conveniently be placed in commercially available slide carriers, which typically hold up to about 12 or 25 slides and may be housed in, for example, appropriately sized plastic sandwich boxes, or, alternatively, upright in plastic Coplin jars. Space slides out evenly in the buffer. Do not overcrowd slides, as this results in "hot spots" and uneven antigen retrieval.
26. This cooling down period is part of the retrieval method and should not be skipped.
27. We recommend vigorous washing in three changes of washing buffer. Each wash should consist of vigorous "sloshing up and down" of slides in buffer for about 30 s to 1 min, 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 of dirty background staining.
28. For example, if a monoclonal mouse primary antibody was used, incubate with labeled secondary antisera raised against *mouse* immunoglobulins; if a rabbit polyclonal primary antibody was used, incubate with a labeled secondary antibody directed against *rabbit* immunoglobulins.

29. Monitor the progress of color development by periodic examination using a microscope. Stop development when specifically labeled structures show deep red and before nonspecific background staining begins to occur.
30. Cell/tissue preparations will initially stain deep purple-red after immersion in Mayer's hematoxylin. The stain changes to navy blue when exposed to mildly alkaline conditions (known as "bluing"). This is most commonly achieved by washing in the slightly alkaline tap water that is available in most areas. If "bluing" is unsuccessful owing to unusually acidic tap water, dip slides briefly in 1% v/v ammonia in tap water, then wash in tap water.
31. Take careful note of the appearance of the slides during this process. When slides are transferred from one solvent to the next, they initially appear smeary as the two solvents begin to mix. Vigorous agitation, that is, "sloshing them up and down," ensures that the slides equilibrate effectively with the new solvent. When they are equilibrated, the surface smearing will disappear. White clouding of the xylene (it appears "milky") indicates contamination with water. Slides should be passed back through graded alcohols (absolute ethanol, 95% ethanol, 70% ethanol) to tap water, solvents should be discarded and replaced, and the process repeated.

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Suggested Further Reading

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