CHAPTER 15

Postembedding Immunocytochemical Techniques for Light and Electron Microscopy

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I. Introduction

Postembedding immunocytochemistry takes advantage of the fact that many proteins still react with antibodies directed against them in tissue that has been chemically fixed and embedded for microscopy. The tissue may by embedded in a resin (e.g., LR White, London Resin Co., London, England) that allows thin sections (1 μm thick) to be cut for light microscopy and ultrathin sections (70 nm thick) to be cut for electron microscopy. An antigen is localized to a specific tissue or a specific cellular or subcellular location by applying a specific antibody (the primary antibody) to the tissue section. The primary antibody is then detected by a secondary antibody conjugated to some molecule that allows its

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visualization by light or electron microscopy. The postembedding techniques differ from the preembedding techniques, in which the antibodies are applied to tissues (or cells in culture for another example) prior to embedment and sectioning. There are advantages and disadvantages to both techniques and these should be considered before choosing one. The postembedding techniques have a major advantage by virtue of being able to apply different antibodies to different (e.g., serial) sections. Within the limitations listed below one could, theoretically, use an infinite variety of antibodies on any given tissue as long as sections are available and the antibodies in question recognize antigens in fixed and embedded tissues. In this article we will demonstrate the embedding of tissue in one of the newer resins (LR White) that can be used for both light and electron microscopy. In this case, the resin remains with the tissue sections and only antigenic sites on the surface of the sections are available for reaction with the antibody. Resolution and tissue integrity are usually quite good but the number of sites available for antibody binding (hence labeling density or signal) are usually low. In other variants, the tissue is embedded in a medium that is removed after the sections are cut and prior to antibody labeling (e.g., paraffin, Paraplast Xtra; Sherwood Medical, St. Louis, MO). In this case labeling density is usually higher than is achieved by the first technique, because antibodies penetrate and bind to antigenic sites throughout the full thickness of the section, but resolution and structural preservation are usually less. Postembedding techniques can also be used with frozen tissue in which no fixative is used (other than the freezing itself) and the tissue remains unembedded or embedded in a water-soluble medium such as OCT compound (e.g., Tissue-Tek; Miles, Inc., Elkhart, IN). In general a technique that produces the highest signal has the lowest resolution.

For the postembedding techniques, tissue preservation must often be sacrificed in order to retain adequate antigenicity, that is, the ability of the primary antibody to recognize and bind to its specific antigenic site. Because this article considers both light and electron microscopic immunocytochemistry, the bias will be toward retaining maximum tissue preservation while still allowing the detection of a broad range of antigens. The inability to use osmium tetroxide as a secondary fixative, after primary fixation with aldehydes, as is commonly done in conventional tissue processing for electron microscopy, is a limitation of the technique. Osmium tetroxide provides better tissue preservation and contrast, and although it has been used for some immunolabeling experiments (Pelletier *et al.*, 1981; Schwendemann *et al.*, 1982; Smith and Keefer, 1982) its use as a fixative for immunocytochemistry is limited because it bonds covalently to proteins and alters their conformation (Lenard and Singer, 1968), usually to the ". . . extinction of antigenicity in the great majority of proteins" (Pearse, 1980).

The immersion of aldehyde-fixed tissue in a solution of uranyl acetate, either before or during dehydration, is also used to increase tissue contrast and is often referred to as *en bloc* staining. Uranyl acetate, used in this manner, also has

fixative properties (Silva *et al.*, 1968, 1971; Terzakis, 1968). However, the fixative action of uranyl acetate appears to be electrostatic (Hyatt, 1981), rather than covalent, thus reducing the problem of conformational changes. Thus we commonly use uranyl acetate as a secondary fixative after aldehyde fixation to obtain better tissue preservation and still allow the immunolabeling of a wide variety of proteins (Erickson *et al.*, 1987; Lewis *et al.*, 1989). This procedure has the additional advantage of quantitatively increasing the immunolabeling of a number of proteins when compared to immunolabeling after simple aldehyde fixation (Erickson *et al.*, 1987).

There are other important variables in tissue processing that can also affect the quality of fixation and immunolabeling, including the following.

1. The tissue preservation technique, which may involve freezing or the use of a coagulative (e.g., acetone or ethanol), or a chemical fixative
2. Temperature of solutions during fixation
3. Length of fixation
4. Type, pH, and osmolality of buffer(s)
5. Dehydrating agent(s)
6. Concentrations and temperatures of dehydrating agent(s) during dehydration
7. Choice of embedding medium

Because the conformation of proteins, and thus the ability of antibodies to recognize them, can be altered by virtually any of these variables the choice of tissue preparation procedures is largely empirical. However, we have included fixation protocols that we routinely use that work well for a broad range of proteins (cytoplasmic, cytoskeletal, membrane-bound and extracellular matrix) in mammalian tissues and at least some cells in culture. If these tissue-processing protocols give inadequate immunolabeling, consider decreasing the glutaraldehyde concentration or omitting it altogether (e.g., as is recommended in the paraffin-embedding protocol outlined below) as a first step before manipulating other parameters. Remember, however, that ultrastructural quality will be sacrificed.

The model system on which these protocols are based is the vertebrate retina (normal, degenerating, and regenerating), but the protocols are similar to those used on a variety of tissues and cells in culture. To replicate retinal degeneration reproducibly, we use an experimental retinal detachment procedure (Anderson *et al.*, 1983; Erickson *et al.*, 1983; Lewis *et al.*, 1989). The regenerative component is accomplished by surgical reattachment of the detached retina (Anderson *et al.*, 1986; Guerin *et al.*, 1989). Because of the expense of the experiments and the desire to use fewer animals, we have continued to experiment with fixation and immunolabeling regimens for postembedding analyses. By using postembedding techniques, it is possible to go back repeatedly to earlier experimental tissue (embedded in various substrates) and probe the tissue with

new antibodies or use newly developed labeling techniques. We have applied the postembedding techniques outlined below to a broad range of species, including squirrel, rabbit, cat, monkey, and human. These techniques may have to be significantly modified for use with plant tissue.

II. Protocols

All procedures in all protocols are performed at room temperature, and in glass containers, unless otherwise stated. Recipes for the solutions are found in Section III. Use a 10× volume (minimum) of solutions per volume of tissue. *Gently* swirl (do not "agitate") the tissue in the various solutions on an intermittent basis, unless otherwise stated.

A. Fixation and Embedding

1. Tissue Processing Steps: Standard Protocol for Tissue to Be Embedded in LR White Resin

1. Dissect the tissue into suitably sized pieces (e.g., 1 mm^3, but this can vary with tissue type) for traditional fixation for electron microscopy and transfer immediately to fresh primary fixative. Leave the tissue in the primary fixative for 1.0 hr. (*Note:* For retinal tissue, place the entire eye into primary fixative and excise the anterior third of the globe, near the limbus, with a razor blade. Remove any residual vitreous from the eye cup and transfer the eye cup into fresh primary fixative.)

2. Transfer the tissue into fixative wash buffer. Wash the tissue three times in fresh fixative wash buffer, 10 min per wash. (*Note:* For retinal tissue, cut the eye cup into quadrants at this time.)

3. Dehydrate the tissue:
   a. Methanol (15%): distilled H_2O (v/v) for 10 min
   b. Methanol (30%) for 10 min
   c. Methanol (50%) for 10 min

4. Immerse the tissue in uranyl acetate fixative for 1.0 hr.

5. Continue the dehydration:
   a. Methanol (85%) for 10 min
   b. Methanol (95%) for 10 min
   c. Methanol (100%) for 10 min

6. Infiltrate the tissue with a 1:1 ratio of 100% methanol:LR White resin (London Resin Co.; store LR White at 4°C); cap the vials containing the tissue and rotate them (at approximately 45° and about 2 rpm) overnight at 4.0°C.

7. The next day, place the tissue in 100% LR White resin, in uncapped vials, and rotate for 2.0 hr at 4.0°C.

8. Transfer the tissue to fresh LR White and rotate at room temperature (uncapped) for 2.0 hr.

9. While the tissue is rotating at room temperature, place fresh LR White resin (stored at 4.0°C) into 4- or 7-dram polyethylene vials (VWR, San Francisco, CA; use 4 or 8 ml of resin) and allow the resin to equilibrate to room temperature.

10. Place the tissue in the fresh, room-temperature LR White resin and cap the vials. There should be an air space between the top of the resin and the cap; once the resin polymerizes, the top of the resin will be clear and allow visualization of the tissue.

11. Polymerize by placing the vials at 52°C for 2 days or until hard.

2. Tissue Processing Steps: Alternative Protocol for Tissue to Be Embedded in LR White Resin

This protocol uses uranyl acetate as a fixative before dehydration and also during the entire dehydration series. The structural integrity of the tissue is improved compared to the previous protocol and preliminary evidence with numerous antibodies suggests the immunolabeling is also improved, but a rigorous quantitative study similar to that performed for the previous protocol (Erickson *et al.*, 1987) has yet to be undertaken.

1. Dissect the tissue into suitably sized pieces (e.g., 1 mm^3, but this can vary with tissue type) for traditional fixation for electron microscopy and transfer immediately to fresh primary fixative. Leave the tissue in the primary fixative for 1.0 hr. (*Note:* For retinal tissue, place the entire eye into primary fixative and excise the anterior third of the globe, near the limbus, with a razor blade. Remove any residual vitreous from the eye cup and transfer the eye cup into fresh primary fixative.)

2. Transfer the tissue into fixative wash buffer. Wash the tissue three times in fresh fixative wash buffer, 10 min per wash. (*Note:* For retinal tissue, cut the eye cup into quadrants at this time.)

3. Wash the tissue three times in maleate wash buffer (pH 5.2), 10 min per wash.

4. Immerse the tissue in 2.0% uranyl acetate in maleate buffer (pH 4.75) for 2.0 hr (secondary fixation).

5. Fix/dehydrate the tissue (*Note:* These steps are performed at 4°C):
   a. Uranyl acetate (2.0%) in 15% methanol/distilled H_2O (v/v) for 10 min
   b. Uranyl acetate (2.0%) in 30% methanol for 10 min
   c. Uranyl acetate (2.0%) in 50% methanol for 10 min
   d. Uranyl acetate (2.0%) in 70% methanol for 10 min
   e. Uranyl acetate (2.0%) in 85% methanol for 10 min

f. Uranyl acetate (2.0%) in 95% methanol for 10 min

g. Uranyl acetate (2.0%) in 100% methanol for 10 min

6. Wash out the uranyl acetate in 100% methanol (three times, 10 min each, at 4°C).

7. Infiltrate the tissue with a 1 : 1 ratio of 100% methanol:LR White resin (at 4°C); cap the vials containing the tissue and rotate them (at approximately 45° and about 2 rpm) overnight at 4°C.

8. The next day, place the tissue in 100% LR White resin (at 4°C), in uncapped vials, and rotate for 2.0 hr at room temperature.

9. Change to fresh, room-temperature LR White and continue to rotate at room temperature for 2.0 hr.

10. Repeat step 9.

11. Place the tissue in fresh LR white resin (room temperature) that is in 4- or 7-dram polyethylene vials (VWR; use 4 or 8 ml of resin).

12. Cap the vials. There is an air space between the top of the resin and the cap; once the resin polymerizes, the top of the resin will be clear and allow visualization of the tissue.

13. Polymerize by placing the vials at 52°C for 2 days or until hard.

3. Tissue Processing Steps: Protocol for Tissue to Be Embedded in Paraffin

1. Dissect the tissue into suitably sized pieces (e.g., 1- to 2-mm thick slices, but this can vary with tissue type) and transfer immediately to fresh paraffin primary fixative. Leave the tissue in the paraffin primary fixative for 1.0 hr. (*Note:* For retinal tissue, place the entire eye into paraffin primary fixative and excise the anterior third of the globe, near the limbus, with a razor blade. Remove any residual vitreous from the eye cup and transfer the eye cup into fresh paraffin primary fixative.)

2. Transfer the tissue into fixative wash buffer. Wash the tissue three times in fresh fixative wash buffer, 10 min per wash. (*Note:* For retinal tissue, cut the eye cup into quadrants at this time.)

3. Dehydrate the tissue (perform each dehydration step at 4°C):

a. Ethanol (15%)/distilled H_2O (v/v) for 10 min

b. Ethanol (30%) for 10 min

c. Ethanol (50%) for 10 min

d. Ethanol (70%) for 10 min

e. Ethanol (85%) for 10 min

f. Ethanol (95%) for 10 min

g. Ethanol (100%) for 10 min

4. Infiltrate the tissue with toluene and Paraplast Xtra (Sherwood Medical) at the indicated temperatures:
   a. Toluene (100%) for 15 min at room temperature
   b. Repeat step a
   c. Toluene (100%) for 30 min at 60°C
   d. Toluene (100%) 1 : 1 with 100% Paraplast Xtra for 30 min at 60°C
   e. Paraplast Xtra (100%) at 60°C overnight
   f. Change to fresh 100% Paraplast Xtra at 60°C for 30 min
5. Embed in fresh 100% Paraplast Xtra, placed in a plastic, aluminum, or stainless steel mold, and allow Paraplast Xtra to solidify (it turns from clear to white).
6. Once the Paraplast Xtra starts to solidify (becomes somewhat white and the tissue does not move), place the entire mold in ice water for a few minutes to speed the solidification. (*Note:* Paraplast Xtra and other paraffin blocks cut better on a microtome if they are chilled somewhat before sectioning.)

4. Other Options for Embedding

Just as there are options for fixation, there are options for embedding media. One embedding medium we have used successfully for light and electron microscopic immunocytochemistry is Lowicryl K4M (Erickson *et al.*, 1987). For light microscopic immunocytochemistry, acrylamide embedding (Johnson and Blanks, 1984) or straight immersion freezing plus cryosectioning without embedding media (Vaughan *et al.*, 1990) also work well for some applications.

B. Immunocytochemistry Controls

Because of the complexity of immunocytochemistry in general, and the limited nature of this article, we are assuming the following.

1. Characterization of the antibody and antigen: The antibodies that are being used have been well characterized, that is, they are specific for the protein/antigen of interest. These issues are covered in other articles in this volume.
2. Characterization of the antibody and tissue with which the antibody is to be used: Protein gels of whole-tissue homogenates and immunoblots of these gels (the so-called Western blots) have shown the antibodies are immunolabeling *only* the protein/antigen of interest and not some additional protein(s) within the tissue of interest (Fig. 1). (For descriptions of this technique see Smith, 1987; Winston *et al.*, 1987; see also article 6, this volume.)

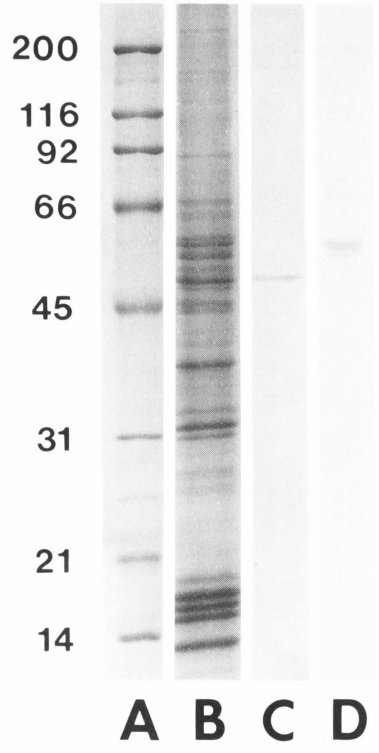

Fig. 1 A Western blot demonstrating antibody specificity. Lane A contains the molecular weight standards; lane B is a Coomassie-blue stained gel of retinal tissue homogenate; lane C is labeled with anti-GFAP (51 kDa); lane D is labeled with anti-vimentin (57 kDa). The antibodies were detected with an HRP color development reagent (Bio-Rad, Richmond, CA).

Immunolabeling Controls

It is essential that proper control experiments be performed in parallel with structural immunolabeling to ensure that tissue labeling as seen in the microscope is due to only one variable: the specific binding of the primary antibody. The following controls are appropriate (assuming the primary antibody is an IgG produced by a rabbit). Make the following substitutions for the primary antibody.

1. Preimmune serum or preimmune IgG: This serum or IgG fraction comes from the same rabbit that made the primary antibody; it is obtained *before* the rabbit makes the primary antibody.

2. Nonimmune serum or nonimmune IgG: This serum or IgG fraction comes from the same species (rabbit) that made the primary antibody, but not from the individual rabbit.

3. Phosphate-buffered saline–bovine serum albumin–sodium azide (PBS–BSA–NaN_3): This is the buffer into which the primary antibody is diluted, but without the antibody.

4. Nonspecific positive: This is an antibody that is specific to an antigen/protein in the tissue that is being labeled, but is *different* from the antigen/protein that is of interest. This should be an antibody known to give positive results with the tissue of interest. For example, if the antigen of interest is opsin and the primary antibody is an anti-opsin (which labels only the retinal photoreceptor cells), a reasonable nonspecific positive control would be an anti-vimentin to label the retinal Müller cells and capillary endothelial cells, but not the photoreceptor cells.

5. Nonspecific negative: This is an antibody that is not related to any antigen/protein in the tissue being labeled. This control is usually satisfied by the preimmune or nonimmune antibodies.

6. Positive tissue and negative tissue controls: Additional controls involve using a tissue preparation known to contain the antigen of interest (positive tissue control) and one known to be devoid of the antigen of interest (negative tissue control). These controls can be addressed with a single tissue if (1) it contains numerous cell types; (2) some of the cell types contain the antigen of interest and some are devoid of the antigen; and (3) the positive and negative cell types are readily identifiable and easily distinguishable from each other.

C. Light Microscopic Immunocytochemistry

This section discusses coating of glass microscope slides to enhance tissue section adhesion and three protocols for light microscopic immunocytochemistry: (1) immunofluorescence for paraffin-embedded tissue, (2) immunoperoxidase for paraffin-embedded tissue, and (3) immunogold silver enhanced for LR White-embedded tissue.

1. Coating of Glass Microscope Slides to Enhance Tissue Section Adhesion

Tissue sections do not adhere well to glass microscope slides during lengthy incubations in aqueous solutions. We have tried numerous coatings to improve the adhesion of tissue sections to slides (including gelatin–chrome alum, Haupts, low molecular weight polylysine, high molecular weight polylysine, straight gelatin, and Formvar) and have found that 3-aminopropyltriethoxysilane [APTES; Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO); L. Angerer, personal communication] works most reliably.

1. Clean the slides: dip into distilled H_2O and wipe with a paper towel.
2. Add 2 ml of APTES to 98 ml of acetone (make fresh).

3. Dip the slides into the 2% APTES in acetone solution for 30 sec.
4. Dip the slides briefly in 100% acetone.
5. Dip the slides briefly in distilled H_2O (change after about 20 slides).
6. Air dry or oven dry the slides (the slides can be stored for future use).
7. The slides are now ready for paraffin or LR White sections:
   a. Place a drop of distilled H_2O on the slide.
   b. Float the tissue section on the drop of H_2O.
   c. Remove excess H_2O (e.g., with a needle/syringe).
   d. Air dry and dry on a warm plate (about 40–50°C) or in an oven (same temperature).

2. Immunofluorescence

This protocol is for immunofluorescence with paraffin-embedded tissue (Color Plate 3A and B and Fig. 2A and B).

1. Deparaffinize the tissue sections by dipping the slides in 100% xylene or a xylene:HemoD (3 : 1; HemoD is from Fisher Scientific, Pittsburgh, PA) solution (5 min, with gentle agitation about once a minute).
2. Repeat step 1 with fresh xylene or xylene:HemoD (3 : 1).
3. Repeat step 1 again, with fresh xylene or xylene:HemoD (3 : 1).
4. Rehydrate the tissue:
   a. Dip the slides into 100% ethanol for 1 min.
   b. Dip the slides into 50% ethanol for 1 min.
   c. Dip the slides into distilled H_2O for 1 min.
5. Dip the slides into PBS–BSA for 5 min.
6. Remove the excess PBS–BSA by blotting around the tissue sections with a paper towel (do not allow the tissue sections to dry out), and place sufficient blocking antibody (about 100 μl) on top of the sections so that they stay wet for 30 min. (*Note:* It is beneficial to keep the slides in a humidified chamber during the antibody incubations so that the solutions do not evaporate; an inexpensive solution is to invert a large casserole dish over the slides and have some petri dishes filled with water in the "chamber.")
7. Rinse away the blocking antibodies by gently dripping PBS–BSA onto the slide while holding the slide at a 45° angle.
8. Wipe around the tissue sections and add the primary antibody (about 150 μl) to the sections; place the sections in a humidified chamber for 2 hr.
9. Rinse dropwise with PBS–BSA, then put about 200 μl of PBS–BSA on top of the sections, letting them soak for about 5 min.
10. Repeat step 9.
11. Repeat step 9 again.

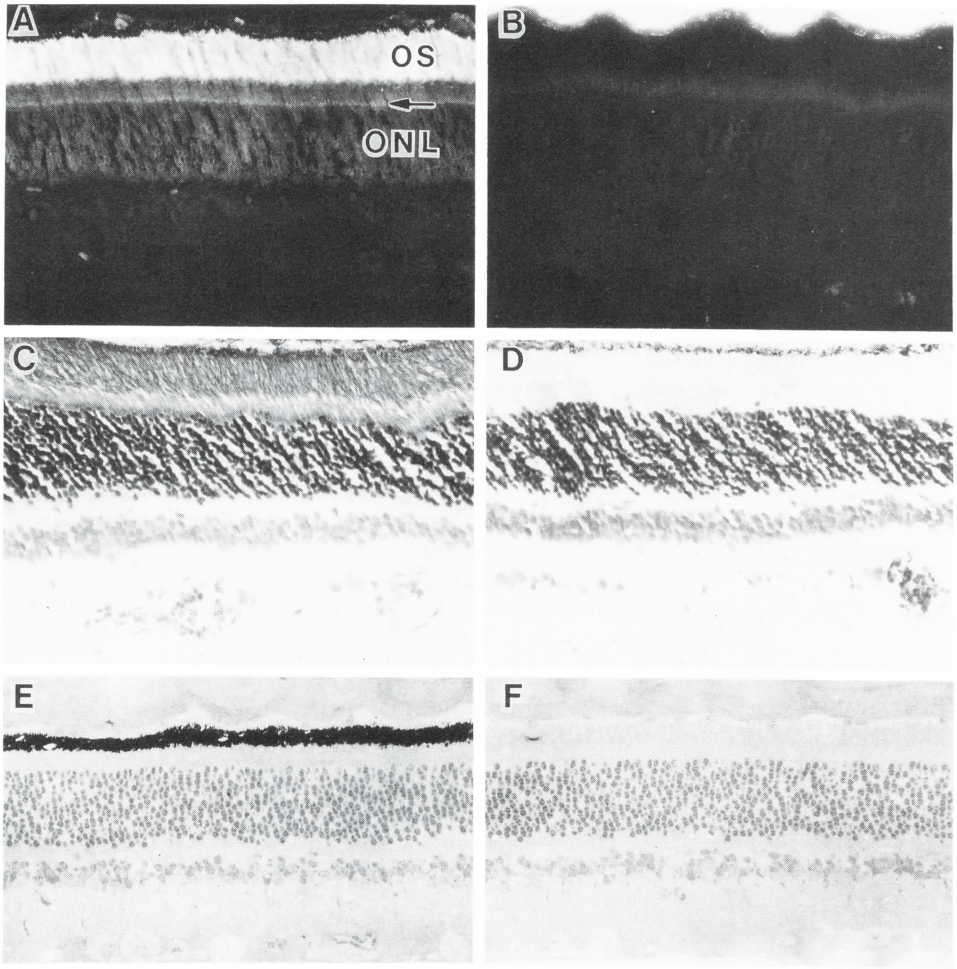

Fig. 2 Three different methods are shown for detecting an antibody against the photoreceptor-specific protein opsin (A, C, and E) along with their controls (B, D, and F). (A and B) Immunofluorescence technique on paraffin-embedded tissue. The antibody labels the photoreceptor outer segment layer (OS), photoreceptor cell bodies in the outer nuclear layer (ONL), and the Golgi apparatus found in the "inner segment" region of the photoreceptor cells (arrow). The faint labeling in the control (B) is the background level whereas the white at the top of the figure is the autofluorescence from the reflective tapetal region. (C and D) Immunoperoxidase technique on paraffin-embedded tissue. The outer segment layer labeling is observed as in (A); however, the nuclei are counterstained with hematoxylin, making them appear black (C). The control (D) shows no labeling of the outer segment layer. (E and F) Immunogold silver-enhanced technique on LR White-embedded tissue. Tissue preservation is improved but, because only the surface of the section is labeled, antigen binding is reduced; the black reaction product appears only over the outer segments and the faint labeling of the Golgi and cell bodies seen in (A) is not evident. These sections are counterstained with basic fuchsin to add tissue contrast. (Magnification: × 162.)

12. Rinse dropwise with PBS–BSA, wipe the excess from around the tissue sections, and add the secondary fluorescent antibody (about 150 μl) for 1 hr [place in humidified chamber; to reduce fading (bleaching/quenching) of the fluorescently tagged secondary antibody, cover the chamber with aluminum foil].

13. Rinse dropwise with distilled H_2O and then place about 200 μl of distilled H_2O on top of the sections and let soak for about 5 min (cover the slides with aluminum foil).

14. Repeat step 13.

15. Repeat step 13 again.

16. Mount in 5% *n*-propyl gallate (Sigma, St. Louis, MO) in glycerol (NPGG) by blotting excess H_2O from around the tissue sections, adding the NPGG to the sections, and coverslipping.

17. Seal the edges of the coverslips with clear nail polish.

18. Store the slides in the dark at 4°C.

Notes

During the antibody incubations, a smaller volume of antibody solution can be used by placing a coverslip on top of the antibody solution (for overnight incubations, seal the edges of the coverslip with rubber cement; on the next day simply peel off the rubber cement). To remove the coverslip, dip the slide vertically in PBS–BSA and agitate gently (use different containers for different antibodies to protect against antibody cross-contamination of the tissue sections) or rinse gently by applying PBS–BSA from a squirt bottle until the coverslip slides off. Rinse gently (do not "jet wash"). There are mounting media other than NPGG that may reduce fading (bleaching/quenching) more effectively, depending on the particular fluorochrome (see, e.g., Bock *et al.*, 1985), but good results can be obtained with NPGG.

3. Avidin–Biotin Conjugate Immunoperoxidase

This protocol is for paraffin-embedded tissue (Color Plate 3C and D and Fig. 2C and D). We have tried many variations of this protocol and because of cost, quality, consistency, and ease of use we have settled on using the elite ABC kit (Vector Laboratories, Burlingame, CA) for the biotinylated antibody and avidin–biotin conjugate (ABC) reagents and follow their protocols for dilutions. We also use the Vector Laboratories diaminobenzidine (DAB) substrate kit for horseradish peroxidase (HRP) and follow their protocol.

1. Deparaffinize the tissue sections by dipping the slides in 100% xylene or xylene:HemoD (3 : 1; obtainable from Fisher Scientific) solution (5 min, with gentle agitation about once a minute). The HemoD acts as a clearing agent.

2. Repeat step 1 with fresh xylene or xylene:HemoD (3 : 1).

3. Repeat step 1 again, with fresh xylene or xylene:HemoD (3 : 1).

4. Rehydrate:
   a. Dip the slides into 100% ethanol for 1 min.
   b. Dip the slides into 50% ethanol for 1 min.
   c. Dip the slides into distilled H_2O for 1 min.

5. Block endogenous peroxidase, for 15 min, by adding 0.03% H_2O_2 in methanol to the tissue sections. This can be done by adding the H_2O_2 dropwise to the sections or by dipping the slides into a container holding the H_2O_2.

6. Dip the slides into PBS–BSA for 5 min.

7. Remove the excess PBS–BSA by blotting around the tissue sections with a paper towel (do not allow the tissue sections to dry out), and place sufficient dilute blocking serum (about 100 μl) on top of the sections so that they stay wet for 30 min. (*Note:* It is beneficial to keep the slides in a humidified chamber during the antibody incubations so that the solutions do not evaporate; an inexpensive solution is to invert a large casserole dish over the slides and have some petri dishes filled with water in the "chamber.")

8. Rinse away the dilute blocking serum by gently dripping PBS–BSA onto the slide while holding the slide at a 45° angle.

9. Wipe around the tissue sections and add the primary antibody (about 150 μl) to the sections and place them in a humidified chamber for 2 hr.

10. Rinse dropwise with PBS–BSA, then put about 200 μl of PBS–BSA on top of the sections and let them soak for about 5 min.

11. Repeat step 10.

12. Repeat step 10 again.

13. Rinse dropwise with PBS–BSA, wipe the excess from around the tissue sections, and add secondary biotinylated antibody (about 150 μl; diluted 1:200 in PBS-BSA; Vector Laboratories) for 1 hr (in a humidified chamber).

14. Repeat step 10 three times with PBS (no BSA, because most BSA is contaminated with minor amounts of biotin that might interfere with the next step).

15. Rinse dropwise with PBS, wipe the excess from around the tissue sections, and add ABC reagent (about 150 μl; Vector Laboratories) for 1 hr (in a humidified chamber). *Note:* The ABC reagent must be prepared at least 30 min before using at this step.

16. Repeat step 10 three times with PBS (no BSA).

17. Rinse dropwise with PBS (no BSA), wipe the excess from around the tissue sections, and add DAB reagent (about 100 μl; Vector Laboratories) for 5 min.

18. Repeat step 10 with distilled H_2O.

19. Dip the slides briefly in 50% ethanol, then in 100% ethanol, and then soak in xylene until ready to coverslip. Do not let them air dry. Coverslip with Permount (Fisher Scientific).

Notes

During the antibody and ABC incubations, a smaller volume of solution can be used if a coverslip is placed on top of the solution (for overnight incubations, seal the edges of the coverslip with rubber cement; on the next day simply peel off the rubber cement). To remove the coverslip, dip the slide vertically in PBS–BSA and agitate gently (use different containers for different antibodies to protect against antibody cross-contamination of the tissue sections) or rinse gently with PBS–BSA from a squirt bottle until the coverslip slides off. Rinse gently (do not "jet wash"). If counterstaining is desired, do so after step 17, and use aqueous hematoxylin (2 min; Biomeda Corp., Foster City, CA) for cytoplasmic antigens (because hematoxylin is a nuclear stain) and eosin (1 min; Lerner Laboratories, Pittsburgh, PA) for nuclear antigens (because eosin is a cytoplasmic stain). After counterstaining, repeat step 10 three times, using PBS only, then move on to step 18.

4. Immunogold with Silver Enhancement

This protocol is for tissue embedded in LR White resin (Figs. 2E and F and 3E and F).

1. Dip the slides into PBS–BSA for 5 min.
2. Remove the excess PBS–BSA by blotting around the tissue sections with a paper towel (do not allow the tissue sections to dry out), and place sufficient blocking antibody (about 100 μl) on top of the sections so that they stay wet for 30 min. (*Note:* It is beneficial to keep the slides in a humidified chamber during the antibody incubations so that the solutions do not evaporate; an inexpensive solution is to invert a large casserole dish over the slides and have some petri dishes filled with water in the "chamber.")
3. Rinse off the blocking antibodies by gently dripping PBS–BSA onto the slide while holding the slide at a 45° angle.
4. Wipe around the tissue sections, add the primary antibody (about 150 μl) to the sections, and place them in a humidified chamber for 2 hr.
5. Rinse dropwise with PBS–BSA, then put about 200 μl of PBS–BSA on top of the secretions and let them soak for about 5 min.
6. Repeat step 5.
7. Repeat step 5 again.
8. Rinse dropwise with PBS–BSA, wipe the excess from around the tissue sections, and add the secondary immunogold antibody (about 150 μl) for 1 hr (in a humidified chamber).
9. Repeat step 5 three times, using PBS only (no BSA).
10. Fix the antibodies to each other and the tissue by wiping the excess PBS from around the tissue sections and adding about 150 μl of glutaraldehyde fixative for 30 min.

11. Repeat step 5 three times, using PBS only (no BSA).

12. Repeat step 5 three times, using distilled H_2O. [*Note:* At this point distilled H_2O can remain on the tissue sections while other slides are being silver enhanced a few at a time (which is the next step in this protocol).]

13. Just before use, combine equal amounts of silver enhancing solutions [e.g., IntenSE II or IntenSE M (Amersham, Arlington Heights, IL), light microscopy kit (BioCell U.S. Distributor is Goldmark Biologicals, Phillipsburg, NJ), or LI silver (Nanoprobe, New York, NY); follow the instructions of the manufacturer]. Use this silver enhancing solution immediately and time each slide so that they each have the enhancing solution on the tissue sections for identical times (it helps to stage the slides about 30 sec apart; do not attempt too many slides at the same time, and make up a fresh batch of silver enhancing solutions for each new set of slides). These two solutions (initiator and enhancer, 1 : 1) are then placed on the tissue sections after wiping the excess distilled H_2O from around the tissue sections. Incubation times with the silver enhancing solution will be determined empirically, but try to stay within the 5- to 25-min range to help control the immunolabeling results (if too fast, it is difficult to coordinate slide timing; if too long, self-nucleation of the silver occurs and the background is "noisy"). What really determines the timing is how intense the antibody labeling is prior to silver enhancing (abundant signal requires less enhancing).

14. Repeat step 5 three times, using distilled H_2O.

15. It is possible to silver enhance again if the signal is too low (the signal can be observed with a light microscope during the enhancing); if repeating step 13 is desired, do so now. Once silver enhancing is completed, rinse the tissue sections one more time with distilled H_2O and let air dry; once dry, counterstain (e.g., with basic fuchsin) for a few seconds if desired, rinse with distilled water, and air dry again; coverslip with Permount (Fisher Scientific).

Notes

During the antibody incubations, a smaller volume of solution can be used if a coverslip is placed on top of the solution (for overnight incubations, seal the edges of the coverslip with rubber cement; on the next day simply peel off the rubber cement). To remove the coverslip, dip the slide vertically in PBS–BSA and agitate gently (use different containers for different antibodies to protect against antibody cross-contamination of the tissue sections) or rinse gently with PBS–BSA from a squirt bottle until the coverslip slides off. Rinse gently (do not "jet wash").

D. Electron Microscopic Immunocytochemistry

The following sections present techniques routinely used in our laboratory. There are many variations on these techniques, for example, with the proper equipment frozen sections can be used for electron microscopy, ferritin can be

used as an electron-dense label, enzymes and chromagens [such as peroxidase and DAB (see Section II,C)] can be used, and so on. Immunogold (the electron-dense label described in the following section) is an excellent label for electron microscopy due to its spherical structure, its ease of detection, and the fact that different-sized gold spheres can be used for labeling more than one antigen (Fig. 3).

1. Single-Label Immunoelectron Microscopy

For the sake of convenience the following protocol is separated into three 1-day segments. All of the immunocytochemistry procedures are done on a laboratory bench at room temperature unless otherwise stated.

Day 1

1. Clean a few glass slides, dip them into 0.5% Formvar (e.g., LADD Research Industries), and let them air dry. Float the Formvar onto distilled water by scraping the surface of the glass slide around the edges, thus allowing the Formvar film on top of the glass slide to float off the top once the slide is gently submerged into the H_2O at about a 30° angle; place 200-hex nickel grids (e.g., SPI Supplies, Westchester, PA; gold grids can also be used, but do not use copper grids because of the formation of electron-dense precipitates during the protocol) onto the Formvar. Lift the grids and Formvar off the distilled H_2O by touching a piece of filter paper (that is larger than the Formvar) onto the top of the Formvar and grids, and let them dry in a covered petri dish.
2. Cut thin sections (silver to pale gold, approximately 70 nm thick) and place on the Formvar-coated nickel grids.

Note

It is also possible simply to put the thin sections directly onto the grids; if these grids are submerged at each step during the following protocol, the antibodies will label both sides of the section and the labeling density will be higher; careful rinsing of the grids between steps is essential if this variation is used because of the thin sections not being supported by a Formvar film and because of antibody binding between the grid bars on the underside of the tissue sections.

Day 2

1. Make the following solutions (see Section III):

Phosphate-buffered saline (PBS)
Phosphate-buffered saline plus bovine serum albumin plus sodium azide (PBS–BSA–NaN_3)
Dilute blocking serum
Dilute primary antibody
Dilute control antibodies

2. Float the grids onto dilute blocking serum (e.g., normal goat serum) for 15–30 min. We usually use 12-well ceramic plates; each well easily holds 200 μl of solution and numerous grids (for grids without Formvar, submerge the grids).

3. Use filter paper to blot the dilute blocking serum from the grids (hold the grids with jeweler's forceps and also blot between the forcep blades; do not rinse or completely dry the grid) and float or submerge the grids onto (or into) the primary antibody [e.g., rabbit antiglial fibrillary acidic protein (GFAP) or non-immune IgG or other control antibody]. Let the sections incubate on (or in) the primary antibody overnight; cover the grids and primary antibody to ensure that the primary antibody does not evaporate (e.g., invert a casserole dish); include petri dishes of water in the enclosed environment to increase the humidity.

Note

It is important to do a dilution series with the primary antibody (see Section III). Although it is possible to incubate the tissue with the primary antibody for a shorter period of time, this requires a more concentrated antibody solution. Overnight incubation, at a reduced antibody concentration, frequently results in a higher signal-to-noise ratio (specific labeling to nonspecific labeling).

Day 3

1. Make the following solutions (see Section III):
   Dilute secondary antibody
   Aqueous uranyl acetate (1% uranyl acetate in distilled H_2O)
   Reynold's lead citrate;
   Osmium tetroxide in sodium phosphate buffer

2. Rinse the grids, gently, with several drops of PBS–BSA–NaN_3, immerse in PBS–BSA–NaN_3 for at least 10 min and then rinse dropwise again with PBS–BSA–NaN_3.

3. Incubate the grids in secondary antibody, [e.g., goat–anti-rabbit IgG complexed to 5-nm gold spheres (Gar-G5; Amersham)] for 1.0 hr (because the grids are wet from rinsing, they probably will not float; simply submerge them, tissue side up, in the secondary antibody solution).

Note

A dilution series is appropriate here also; however, it is possible to obtain an excellent signal-to-noise ratio with a 1.0-hr incubation.

4. Rinse dropwise with, and then immerse in, PBS–BSA–NaN_3.

5. Rinse dropwise with distilled H_2O, blot the grids with filter paper, and set aside to air dry.

6. Stain the sections with aqueous uranyl acetate for 10 min.

7. Stain with Reynold's lead citrate for 10 min (do this in an enclosed petri dish; do not breathe on the lead citrate or lead precipitate may form on the tissue sections).

8. Stain with the vapors of OsO_4–$NaPO_4$ in an enclosed environment, in a fume hood, for 1.0 hr. (*Caution:* OsO_4 vapors are very toxic and *will* fix corneas, skin, lungs, etc., so be careful to keep the vapors in the fume hood. *Never* pipette OsO_4, by mouth.) If the tissue contrast is sufficient to omit this step, do so (before processing the grids through this step, look at a grid in the electron microscope and determine if extra contrast is needed; if needed, perform this step).
9. Carbon coat the sections.
10. View in the transmission electron microscope.

2. Double-Label Immunoelectron Microscopy

Several methods may be used to immunolabel two antigens simultaneously, on the same tissue section, with two different primary antibodies. The method covered here works according to the following theory: If the primary antibodies are made in different species (e.g., species "R" is rabbit and species "M" is mouse), the different primary antibodies can be distinguished with a species-specific secondary antibody (e.g., goat–anti-R will recognize R but not M, whereas goat–anti-M will recognize M but not R). If goat–anti-R has a 5-nm gold sphere attached to it (GAR-G5) and goat–anti-M has a 15-nm gold sphere attached to it (GAM-G15), they can be distinguished based on the size of the gold shperes. Thus antigen "A" will be labeled with primary antibody "A" made in rabbit (R), which will be labeled by GAR-G5, and antigen "B" will be labeled with primary antibody "B" made by a mouse monoclonal antibody (M), which will be labeled by GAM-G15. In the electron microscope two sizes of gold spheres will appear overlying the tissue: 5-nm spheres locating antigen A and 15-nm spheres locating antigen B.

This method works well (Fig. 3), but requires the following:

1. One primary antibody has been made in one species [e.g., a rabbit polyclonal antibody: (1 ° Ab-R)].
2. The other primary antibody has been made in a different species [e.g., a mouse monoclonal antibody: (1 ° AB-M)].
3. Single-label immunoelectron microscopy for *each* of the primary antibodies has been performed:
   a. Antibody–antigen specificity is well characterized.
   b. Antibody–tissue specificity is well characterized.
   c. Control experiments give the expected results.
   d. The optimum concentration of each of the primary antibodies is known (from the dilution series when the single-label immunoelectron microscopy was performed).
   e. The optimum concentration for each secondary antibody is known (from the dilution series when the single-label immunoelectrom microscopy was performed).

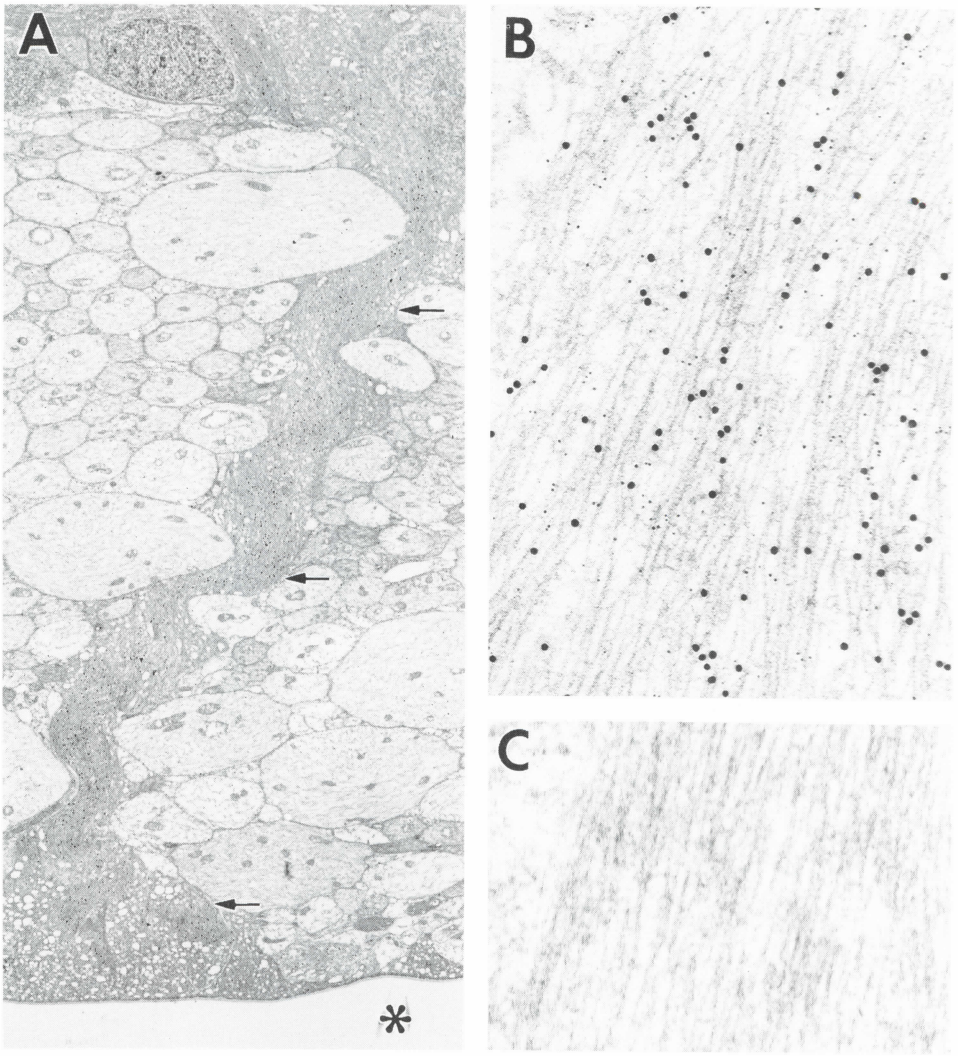

Fig. 3 Examples of double-label immunoelectron microscopy are shown, using antibodies against GFAP and vimentin on normal cat retina embedded in LR White. Both of these intermediate filament proteins are normally present in the cytoplasm of the specialized retinal astrocytes known as Müller cells. A portion of a Müller cell process (arrows) is shown, at low magnification, traversing through bundles of ganglion cell axons (A). The gold spheres are barely visible at this magnification. At higher magnification, anti-GFAP (small, 15-nm gold spheres) and anti-vimentin (large, 30-nm gold spheres) can be seen over the intermediate filaments (B). No gold spheres are present over the intermediate filaments in control sections (C). *, Vitreous cavity of the eye. [Magnification: (A) × 3300; (B) × 58,500; (C) × 78,000.]

Protocol: Day 1

Repeat the single-label immunocytochemistry protocol.

Protocol: Day 2

1. Make the mixture of primary antibodies solution (see Section III).
2. Repeat the day 2 single-label immunocytochemistry protocol, but substitute the mixture of primary antibodies for the single primary antibody.

Protocol: Day 3

1. Make the mixture of secondary antibodies solution (Section III).
2. Repeat the day 3 single-label immunocytochemistry protocol, but substitute the mixture of secondary antibodies for the single secondary antibody

III. Recipes

Stock sodium phosphate buffer (0.172 *M* $NaPO_4$, pH 7.2)

To make the listed volume of buffer, use the given amounts of monobasic and dibasic sodium phosphate. Add the phosphates sequentially to the desired (final) volume of distilled H_2O; adjust to pH 7.2. (*Note:* Save at least 100 ml of this 0.172 *M* $NaPO_4$ buffer for the osmium tetroxide.

	Volume	
Chemicals	500 ml	1000 ml
NaH_2PO_4	3.41 g	6.82 g
Na_2HPO_4	8.66 g	17.32 g

Primary fixative (1% formaldehyde plus 1% glutaraldehyde in 0.086 *M* $NaPO_4$, pH 7.2)

1. To 90 ml of distilled H_2O at 80 ° C add 2.0 g of paraformaldehyde; stir until dissolved (in a fume hood). If needed, add a *few* drops of 1.0 *N* NaOH to help the paraformaldehyde go into solution. After it is completely dissolved, cool to room temperature (use this formaldehyde in solution in the next step).
2. Add 100 ml of stock sodium phosphate buffer (0.172 *M* $NaPO_4$, pH 7.2) to the formaldehyde in solution.
3. Add 2.86 ml of 70% glutaraldehyde (or a sufficient amount of a different percentage glutaraldehyde to bring the final volume of 200 ml up to 1% glutaraldehyde].
4. Bring the final volume up to 200 ml. This yields a 1.0% formaldehyde–1.0% glutaraldehyde solution in 0.086 *M* $NaPO_4$ buffer, pH 7.2.

Paraffin primary fixative (4% formaldehyde in 0.086 *M* $NaPO_4$, pH 7.2)

1. To 90 ml of distilled H_2O at 80°C add 8.0 g of paraformaldehyde; stir until dissolved (in a fume hood; if needed, add a *few* drops of 1.0 *N* NaOH to help the paraformaldehyde go into solution. After it is completely dissolved, cool to room temperature (use this formaldehyde in solution in the next step).
2. Add 100 ml of stock sodium phosphate buffer (0.172 *M* $NaPO_4$, pH 7.2) to the formaldehyde in solution.
3. Bring the final volume up to 200 ml. This yields a 4.0% formaldehyde solution in 0.086 *M* $NaPO_4$ buffer, pH 7.2

Glutaraldehyde fixative (approximately 2% glutaraldehyde in 0.086 *M* $NaPO_4$, pH 7.2)

To 25 ml of stock sodium phosphate buffer (0.172 *M* $NaPO_4$, pH 7.2), add 23.5 ml of distilled H_2O and 1.5 ml of 70% glutaraldehyde.

Note: This fixative is used to fix the antibodies to each other and to the tissue section before silver enhancing; consequently it is not critical that it be exactly 2% glutaraldehyde.

Fixative wash buffer (0.137 *M* $NaPO_4$, pH 7.2, 315 mOsm)

To 4 parts stock sodium phosphate buffer (0.172 *M* $NaPO_4$, pH 7.2) add 1 part distilled H_2O. This will yield a 0.137 *M* $NaPO_4$ buffer, pH 7.2, of approximately 315 mOsm (thus isotonic with mammalian blood) and will be used after primary fixation of the tissue.

Maleate wash buffer (0.05 *M* Na–H–maleate–NaOH buffer, pH 5.2)

Stock solution (0.2 *M* maleate buffer): 100 ml of distilled H_2O (final volume), 2.32 g of maleic acid (or 1.96 g of maleic anhydride), 0.8 g of NaOH

For the pH 5.2 buffer (200 ml): 142.8 ml of distilled H_2O, 50.0 ml of stock solution from above, 7.2 ml of 0.2 *M* NaOH; adjust to a final pH of 5.2

Note: Maleate buffer is used here to wash out the phosphate buffer, because the uranyl acetate will precipitate in phosphate buffers. It is also used to buffer the uranyl acetate because ". . .the maleate buffer buffers the uranyl solution better than veronal acetate buffer. . .and may form a weak complex with the uranyl ions, mitigating against precipitation" (Karnovsky, 1967).

Uranyl acetate 2% in maleate buffer (0.05 *M* pH 4.75)

Note: The uranyl acetate is mildly radioactive; follow the recommendations of the manufacturer (Electron Microscopy Sciences, Fort Washington, PA) for handling and dispose of the solutions in radioactive waste.

Combine the following:

1. Distilled H_2O	123.1 ml
2. Stock solution (0.2 *M* maleate buffer)	50.0 ml
3. NaOH (0.2 *M*)	26.9 ml

Adjust the pH to 6.0 and add

4. Uranyl acetate	4.0 g

(*Note:* Addition of uranyl acetate drops the pH to about 4.75; it is not necessary to adjust.)

Uranyl acetate fixative (2% uranyl acetate in 70% methanol–H_2O)

Note: The uranyl acetate is mildly radioactive; follow the recommendations of the manufacturer (Electron Microscopy Sciences) for handling and dispose of the solutions in radioactive waste.

To prepare, add 2.0 g of uranyl acetate to 100 ml of a 70% methanol–H_2O, stir until dissolved.

(*Note:* For the alternative fixation, make each of the uranyl acetate–methanol fixatives by this same basic method.)

Aqueous uranyl acetate (1% uranyl acetate in distilled H_2O)

Note: The uranyl acetate is mildly radioactive; follow the recommendations of the manufacturer (Electron Microscopy Sciences) for handling and dispose of the solutions in radioactive waste.

	Volume	
Chemical	50 ml	100 ml
$UO_2(C_2H_3O_2–2H_2O$	0.5 g	1.0 g

Reynolds' lead citrate (Reynolds, 1963):

	Volume	
Chemical	50 ml	100 ml
Distilled H_2O	30 ml	60 ml
$Pb(NO_3)_2$	1.33 g	2.66 g

1. Stir the above well until thoroughly dissolved. Then add the following:

	50 ml	100 ml
$Na_3(C_6H_5O_7)$	1.76 g	3.52 g

2. Shake for 1 min.
3. Let stand 30 min with intermittent shaking. Then add the following:

	50 ml	100 ml
1.0 *N* NaOH	8.0 ml	16.0 ml

4. Shake twice; the solution should clear.
5. Make up to a final volume of 50 or 100 ml with distilled H_2O.
6. Keep refrigerated and tightly capped.

Basic fuchsin (Electron Microscopy Sciences)

1. Add 10 ml of 100% ethanol; to 390 ml of distilled H_2O.
2. Add, while stirring, 2 g of basic fuchsin.
3. Filter through a paper towel before use.
4. Apply to tissue sections on glass microscope slides for a few seconds up to a few minutes depending on the type of resin and tissue.
5. Rinse with distilled H_2O and dry on a warm hot plate.

Osmium tetroxide (OsO_4)

Caution: OsO_4 is extremely toxic and will fix corneas, lungs, and so on. *Open and use only in a fume hood.* Dissolve 2.0 g of OsO_4 crystals in 50.0 ml of distilled H_2O (in a fume hood, with a magnetic stirrer or sonicator).

Osmium tetroxide–sodium phosphate buffer (OsO_4–$NaPO_4$)

Caution: OsO_4 is extremely toxic and will fix corneas, lungs, and so on. *Open and use only in a fume hood.* To 50 ml of stock sodium phosphate buffer (0.172 *M* $NaPO_4$, pH 7.2) add the 50 ml of 4% OsO_4. This yields 2% OsO_4 in 0.086 *M* $NaPO_4$ buffer and is used to osmicate the tissue sections following immunocytochemistry.

Note: This solution can also be used as a secondary fixative for conventional electron microscopy, but should not be used as a fixative for immunocytochemistry. It is probably not necessary to use buffered OsO_4 for the staining of thin sections. Others have simply placed OsO_4 crystals in the container with the sections. Because this is a routine fixative in most electron microscopy laborato-

ries it is conveniently kept in buffer solution. Also, the solution is much easier and safer to handle than are the crystals.

Phosphate-buffered saline (PBS)

For a 10 × solution:

	Volume		
Chemicals	200 ml	250 ml	750 ml
NaCl	17.53 g	21.92 g	65.75 g
NaH_2PO_4	0.53 g	0.67 g	2.00 g
Na_2HPO_4	2.30 g	2.88 g	8.63 g
$(NaN_3)^a$	(1.00 g)	(1.25 g)	(3.75 g)

[a] Optional: Sodium azide, NaN_3, inhibits peroxidase, therefore do not use for diluting peroxidase-labeled antibodies as in the immunoperoxidase ABC protocol.

Note: To use the 10× PBS or 10× PBS–NaN_3, stir well before measuring and dilute with distilled H_2O to the desired concentration (1:9) and volume. *Adjust the pH to 7.4.*

Phosphate-buffered saline–bovine serum albumin–sodium azide (PBS–BSA–NaN_3)

Supplement the diluted PBS–NaN_3 with 0.5% BSA. Use this PBS–BSA–NaN_3 to dilute the antibodies and to wash the sections after the primary and secondary antibody incubations.

Dilute blocking serum:

Use serum from the species in which the secondary antibody was produced. For example, if the secondary antibody was produced in a goat, then use normal goat serum. This blocking serum serves three purposes: (1) if there are any free aldehydes on the tissue sections, they will react with the proteins in this serum and not with the primary antibody thus decreasing nonspecific primary antibody "labeling"; (2) the proteins in the serum adsorb to the sections, thus blocking nonspecific adsorption; and (3) if there happens to be any antigenic sites recognized by the goat serum, these will be blocked by this serum and will reduce background labeling with the specific secondary antibody.

Dilute the normal serum (e.g., goat) with PBS–BSA–NaN_3 (150 μl of serum diluted into 10.0 ml of buffer, 1:67, works well).

Dilute primary antibody and nonimmune IgG (primary antibody and IgG)

The primary antibody used clearly depends on the antigen of interest. For example, we were interested in the intermediate filaments of glial cells (Müller

cells and astrocytes), which are usually composed of (at least) glial fibrillary acidic protein (GFAP). We bought a commercially available anti-GFAP (Dako Corp., Carpenteria, CA); GFAP that was purified from bovine CNS was injected into rabbits and the rabbits made the polyclonal anti-GFAP. Dako Corporation has characterized this anti-GFAP and shown it to be specific for GFAP and no other intermediate filament protein. We characterized the antibody/tissue specificity with Western blots of whole-retina homogenates. This characterization showed that this antibody is not only specific for GFAP, but also does not label any other detectable retinal antigens by Western analysis, and can be used for immunocytochemistry with retina tissue.

The dilution that yields the optimum signal-to-noise ratio (specific labeling to nonspecific background) for each primary antibody used for tissue immunocytochemistry will have to be determined empirically. For example, with the polyclonal rabbit anti-GFAP, we ran a dilution series of 1:50, 1:100, 1:200, 1:300, 1:400, 1:500, and 1:750 (antibody to buffer).

For the experimental controls, run a dilution series with nonimmune IgG (if preimmune IgG is not available), nonspecific positive antibodies, and nonspecific negative antibodies. Buffer without primary antibody (PBS–BSA–NaN_3) should also be run as a control.

Once the optimum concentration of primary antibody (e.g., 1:400) has been determined, and the controls are clean (i.e., no background labeling), then it is possible to start cutting back on the number of control experiments required when repeating the immunolabeling. For example, just run the primary antibody at 1:400, nonimmune IgG (e.g., 1:400), and PBS–BSA–NaN_3. However, continue using a nonspecific positive antibody in case primary antibody labeling does not work for some unknown reason. A positive control that *does* work at least indicates the protocol is still working.

Mixture of primary antibodies

Determine the optimum concentration for *each* of the primary antibodies [e.g., rabbit–anti-GFAP (1:400) and mouse–anti-vimentin (1:300); then to a single container of PBS–BSA–NaN_3 add the following.

1. Enough rabbit–anti-GFAP to bring the final concentration up to 1:400
2. Enough mouse–anti-vimentin to bring the final concentration up to 1:300

Dilute secondary antibody

Which secondary antibody is used depends on the species and class of the primary antibody. For example, if the primary antibody is an IgG from rabbit, then an anti-rabbit IgG is used. To continue with the previous example for a primary antibody that is an IgG rabbit–anti-GFAP, the secondary antibody could be a goat–anti-rabbit IgG (GAR). This secondary antibody can be purchased from a number of vendors (e.g., Amersham) and is complexed to various sizes of gold spheres [e.g., 5 nm (GAR-G5)].

It is also important to do a dilution series with the secondary antibody, because gold spheres, if too concentrated, will be seen everywhere (nonspecific binding; this can occur even without a primary antibody). We usually use dilutions ranging from 1:25 to 1:100; consider a dilution series around these concentrations, but remember that using less will save money as long as the protocol works.

Secondary fluorescent antibody

Follow the guideline presented in the previous section (Dilute secondary antibody), but substitute a secondary antibody that has a fluorochrome attached rather than a gold sphere. There are numerous vendors of secondary antibodies with fluorescent tags; for single labeling, we have been using the Cappel fluorescein- or rhodamine-conjugated, affinity-purified goat–anti-mouse (or rabbit) IgG, heavy and light chain specific. Newer, brighter fluorochromes are now being offered that promise greater sensitivity (e.g., Cy3). For double labeling, see the next section (Mixture of secondary antibodies), especially the caveat regarding cross-reactivity. Also see other articles in this volume.

Mixture of secondary antibodies

Once the optimum concentration for each of the secondary antibodies has been determined [e.g., GAR-G5 (1:25), labeling rabbit–anti-GFAP; GAM-G15 (1:40) labeling mouse–anti-vimentin] and each of the secondary antibodies is a different size (i.e., 5 and 15 nm) then add the following to a single container of PBS–BSA–NAN_3:

1. Enough GAR-G5 to bring the final concentration up to 1:25
2. Enough GAM-G15 to bring the final concentration up to 1:40

Note: One caveat regarding double labeling is the possibility of cross-reactivity between secondary antibodies directed toward different primary antibodies. For example, goat–anti-mouse IgG can recognize epitopes on rat IgG antibodies and goat–anti-rabbit IgG can recognize epitopes on guinea pig IgG antibodies. To determine if the secondary antibodies to be used have this characteristic (undesirable for double labeling), apply the inappropriate secondary antibody in a single labeling experiment (e.g., use a primary antibody made in mouse with a secondary antibody that detects rabbit primaries but supposedly does not detect mouse primaries). If cross-reactivity is detected between the inappropriate primary and secondary antibodies, try secondary antibodies that have been further purified by absorption against the primary antibody species to be used in double labeling.

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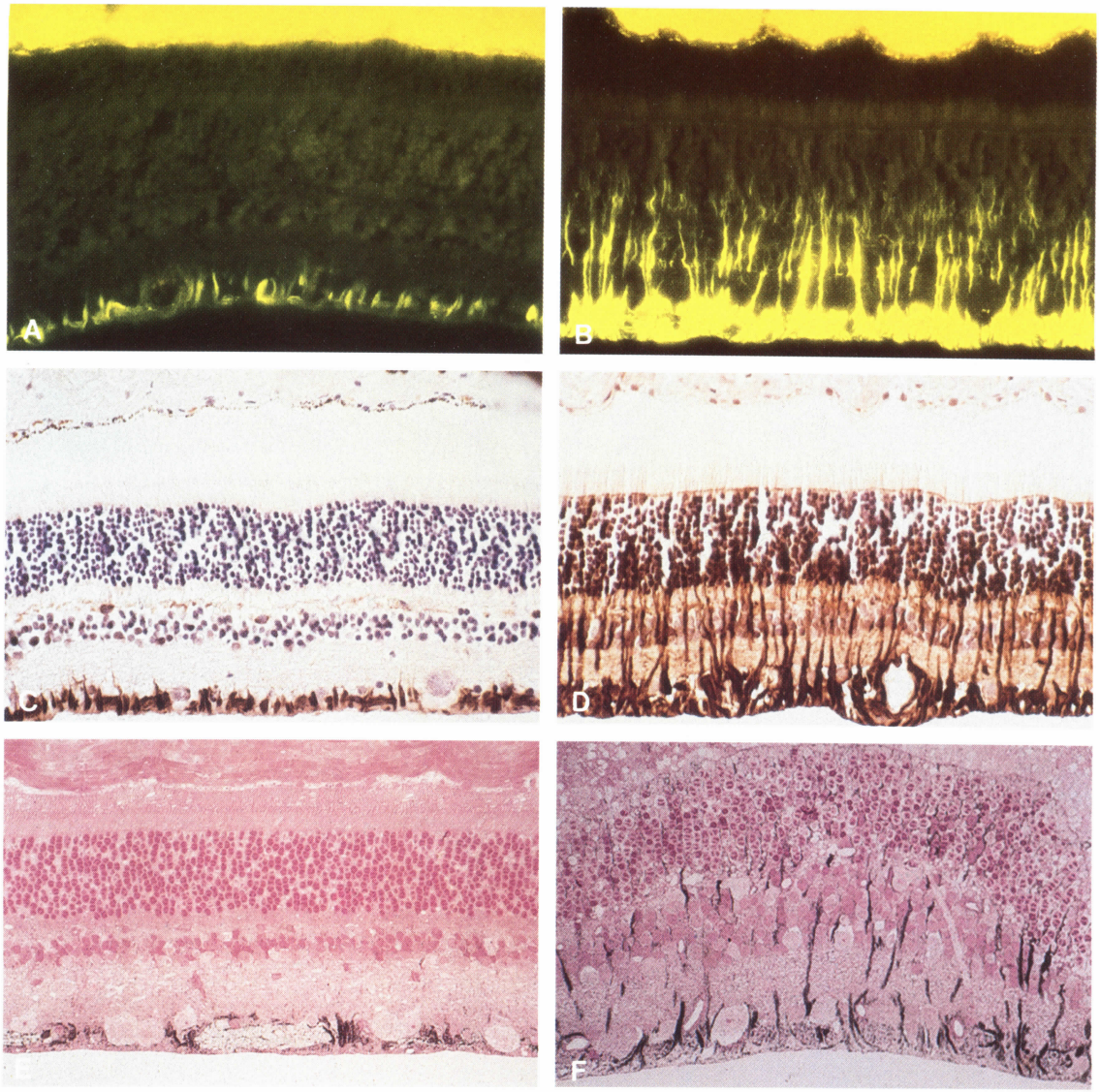
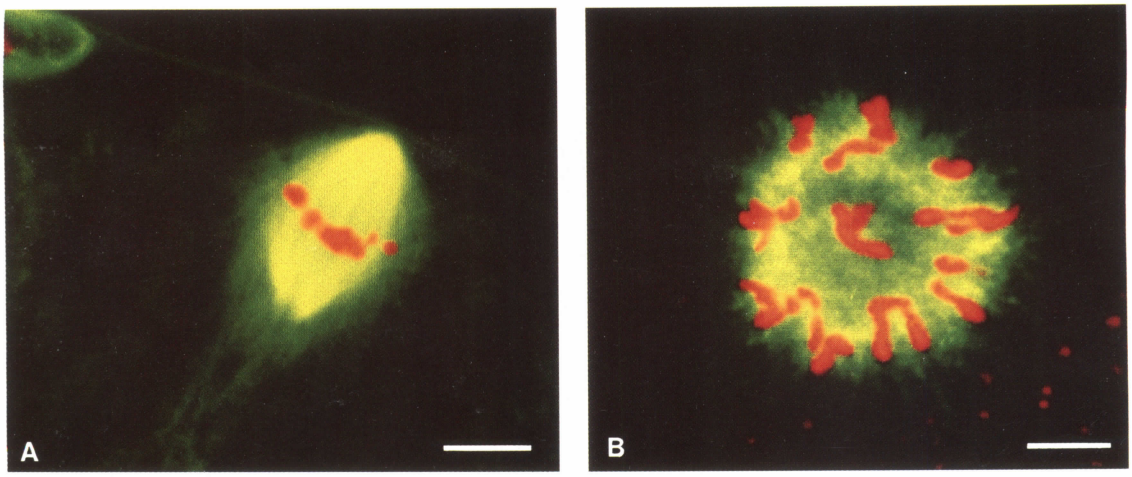
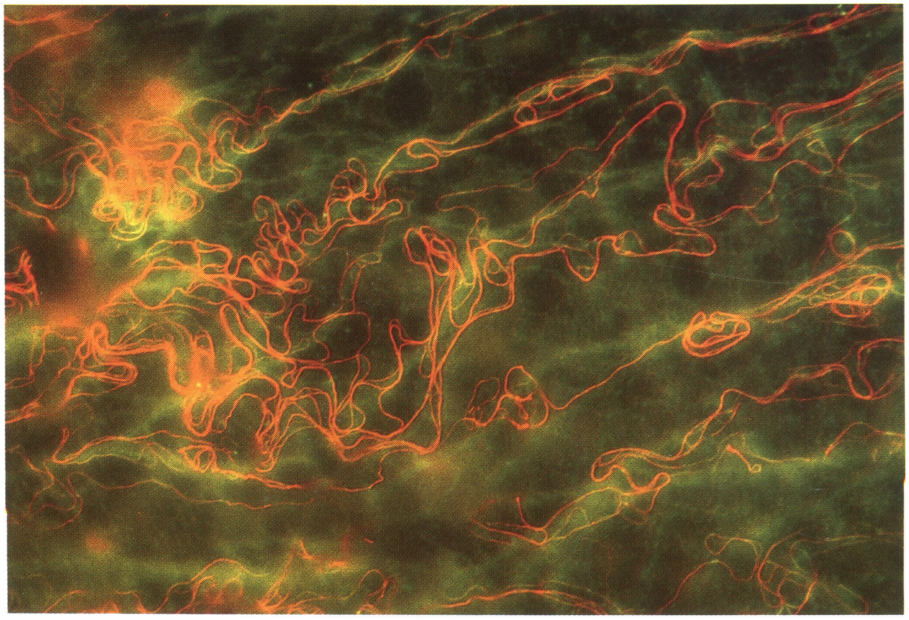


Color Plate 3 Three different immunocytochemical detection methods. A comparison is made using an antibody against GFAP on normal (A,C,E) and 3-day-detached cat retinal sections (B,D,F). (A,B) *Immunofluorescence technique on paraffin-embedded tissue.* With the immunofluorescence technique, the FITC-tagged secondary antibody appears as a yellow-green color. (The yellow at the top of the sections is autofluorescence from a specialized reflecting layer in the cat retina, the tapetum.) (C,D) *Immunoperoxidase technique on paraffin-embedded tissue.* The anti-GFAP was detected with a peroxidase-conjugated secondary antibody which results in a brown reaction product. These sections have also been counterstained with hematoxylin which stains the nuclei blue. (E,F) *Immunogold silver-enhanced technique on LR White-embedded tissue.* The gold-conjugated secondary antibody is enhanced with silver which produces a black reaction product. These sections have been counterstained with basic fuchsin. The paraffin-embedded tissue (A,B,C,D) results in a higher labeling density but less tissue resolution as compared to the tissue embedded in LR White resin (E,F). A comparison of results from all three techniques in normal retina, to the retinas that were detached for 3 days, shows how the distribution of GFAP dramatically increases after the detachment. The use of electron microscopic immunocytochemistry shows that the label is associated with intermediate filaments in a specific class of retinal glial cells, the Müller cells (see Chapter 15, Fig. 3). Magnification ×218.



Color Plate 1 Dual fluorescence of microtubules and chromosomes in methanol-fixed *Xenopus* eggs. (A) Longitudinal section of a second meiotic spindle (the first polar body is apparent in the upper left corner) in an unfertilized *Xenopus* egg fixed with methanol. Upon closer examination, microtubules can be seen to be fragmented and collapsed into bundles. Cytoplasmic microtubules are very poorly preserved under these conditions. (B) Cross-section of the metaphase plate of a similar meiotic spindle. Eggs were fixed and stained with antitubulin (green) and propidium iodide (red) as described in Chapter 9, Table III, and were examined using the dual wavelength filter set (Texas red: fluorescein) provided with the MRC-600. Scale bar is 10 μm in A, and 5 μm in B. Reprinted from Gard, 1992. (For more information see Chapter 9.)



Color Plate 2 Double label immunofluorescence microscopy of calmodulin and tubulin in the green alga *Ernodesmis verticillata.* Rabbit anticalmodulin and mouse monoclonal antitubulin were followed by fluorescein-tagged goat anti-rabbit IgG and rhodamine-tagged goat anti-mouse IgG and were photographed via a double exposure. Courtesy of Russell H. Goddard and John W. La Claire II, University of Texas, Austin. (For more information see Chapter 10.)