



Immunohistochemistry Instruction Manual

ImmunoStar, Inc.
P.O. Box 488 Hudson, Wisconsin 54016-0488
Toll-free Phone: 866-386-3500 Toll-free Fax: 866-386-4500
info@immunostar.com
www.immunostar.com

TABLE OF CONTENTS

1.0 INTRODUCTION	1
2.0 TISSUE PREPARATION	1
2.1 Standard Tissue Preparation Procedure in Pathology	
2.11 Specimen Requirements	1
2.12 Standard Tissue Fixation	1
Fixation for Paraffin-Embedded Tissue	1
Clinical Specimens Other Than Paraffin-Embedded Tissue	1
2.13 Paraffin Embedding	1
Dehydration	1
Infiltration with Molten Paraffin	1
2.14 Paraffin-Embedded Section Preparation	2
Sectioning	2
Deparaffinization	2
2.2 Standard Method for Perfusion Fixation	2
2.21 Perfusion Technique	2
2.3 Frozen and Vibratome Tissue Sectioning	3
2.31 Cryostat Sections	3
2.32 Vibratome Sections	3
2.33 Microtome Frozen Sections	3
2.4 Additional Tissue Treatments	3
2.41 Mercury Removal	3
2.42 Trypsin Digestion	3
2.43 Endogenous Peroxidase Deactivation	3
2.44 Free Aldehyde Reduction	4
3.0 IMMUNOSTAINING TECHNIQUES	4
3.1 Indirect Immunofluorescence	4
3.11 Method Description	4
3.12 Materials Required	4
Reagents	4
Supplies	4
Equipment	4
3.13 Protocol	4
3.14 Specificity Control	5
3.2 Peroxidase Antiperoxidase Immunostaining	5
3.21 Method Description	5
3.22 Materials Required	5
Reagents	5
Supplies	5
Equipment	5
3.23 Protocol	6
Preparation	6
Immunostaining Steps	6
3.24 Specificity Control	7
3.3 Avidin/Biotin Peroxidase Immunostaining	7
3.31 Method Description	7
3.32 Materials Required	7
Reagents	7
Equipment	7
3.33 Protocol	8
Preparation	8
Immunostaining Steps	8
3.34 Specificity Control	8
3.4 Free Floating Technique with Peroxidase Antiperoxidase or Avidin/Biotin Peroxidase Immunostaining	9

3.41	Method Description.....	9
3.42	Materials Required.....	9
	Reagents	9
	Supplies	9
	Equipment.....	9
3.43	Protocol.....	9
3.5	Nickel Ammonium Sulfate-Glucose Oxidase Intensification	9
3.51	Method Description.....	9
3.52	Materials Required.....	9
3.53	Protocol.....	10
3.6	Silver Intensified Immunogold Staining.....	10
4.0	IMMUNOSTAINING TECHNIQUES FOR USE WITH THE ELECTRON MICROSCOPE	10
4.1	Pre-Embedding Peroxidase Antiperoxidase Immunostaining...10	
4.11	Method Description.....	10
4.12	Materials Required.....	10
	Reagents	10
	Supplies	10
	Equipment.....	10
4.13	Protocol.....	11
	Specimen Preparation	11
	Immunostaining Steps	11
4.2	Post-Embedding: Dehydration of Tissue and Embedding With Epon/Araldite Resin.....	12
4.21	Method Description.....	12
4.22	Materials Required.....	12
	Reagents	12
	Supplies	12
	Equipment.....	12
4.23	Protocol.....	12
4.3	Post-Embedding Peroxidase Antiperoxidase Immunostaining	12
4.31	Method Description.....	12
4.32	Materials Required.....	12
	Reagents	12
	Supplies	12
	Equipment.....	12
4.33	Protocol.....	13
	Immunostaining Steps	13
4.4	Post-Embedding Electron Microscope Immunogold Staining.....	14
5.0	TROUBLESHOOTING	14
5.1	High Background.....	14
5.2	Absence of Staining.....	14
6.0	FORMULAS	15
6.1	Buffer Formulas.....	15
6.2	Perfusion Wash Formulas	15
6.3	Fixative Formulas	15
6.4	Resin Formula	16
6.5	Chromogen Formulas.....	16
	REFERENCES	16

1.0 INTRODUCTION

Immunohistochemistry is a versatile tool currently under widespread use in biological research to understand and visualize molecular distribution and expression in tissue. Immunostar has been at the forefront of antibody development towards antigens such as proteins and small molecules (i.e. serotonin) in order to further our understanding of their role in tissue. In addition to basic research, immunohistochemistry is now increasingly used in fields of pathology, such as the neurosciences and cell biology, to assess and diagnose disease states. For example, in oncology, immunohistochemistry is now routinely used to distinguish sarcomas from carcinomas; malignant lymphomas from epithelial neoplasms; and malignant melanomas from both carcinomas and sarcomas. As antibodies continue to be developed and used towards these goals of understanding molecular expression in biological research, immunohistochemistry will continue to be a helpful tool to supplement traditional morphological and developmental studies.

This booklet is intended to serve as a guide to some of the conventional techniques in tissue processing and immunohistochemistry. We hope this will help researchers using Immunostar products in their initial experiments. If we can be of further assistance, please call our Technical Service Department at: 1-866-386-3500.

2.0 TISSUE PREPARATION

2.1 STANDARD TISSUE PREPARATION PROCEDURE IN PATHOLOGY

ImmunoStar histochemical antisera are useful for localization of their respective antigens in fixed tissue. Tissues that have been stored as paraffin blocks for several decades have given successful results with ImmunoStar antisera. However, given the variety of normal and pathological tissue specimens one might investigate, coupled with the wide range of tissue processing methods employed, results cannot be guaranteed. Proper controls should be run on adjacent sections.

2.11 Specimen Requirements

Tissue should be fixed within several minutes of anoxia to prevent antigen degradation and to provide optimal staining. Autolysed, necrotic and poorly fixed tissue should not be used in the procedure.

2.12 Standard Tissue Fixation

Fixation is a critical factor for successful immunohistochemical staining. Problems can occur due to either over- or under-fixation. The length of fixation must be timed such that antigens are retained, good cellular morphology is produced and minimal masking occurs. Some types of fixatives will harm antigens and give inconsistent results as will the use of high fixative concentrations or prolonged exposure of tissue to fixative.

Fixation for Paraffin-Embedded Tissue

Cut removed tissue into small blocks no more than 2 cm square and 5 mm thick. Immerse tissue immediately in fixative.

Recommended fixatives and fixative times are: B5 (2 to 5 hours), Bouin's or 10% phosphate buffered neutral formalin (2 to 24 hours). (Please refer to Fixative Formulas, section 6.3).

B5 is the most highly recommended fixative for immunoglobulins. However, during histochemical processing, a black precipitate of mercuric pigment will occur on tissues fixed in this mercury-contained fixative. Mercury should be removed after deparaffinization (please refer to Mercury Removal Procedure, section 2.41).

Formalin fixation has a tendency to produce excessive cross-linkage in tissue. In more severe cases, tissue should be treated with trypsin to "unmask" antigen sites (please refer to Trypsin Digestion Procedure, section 2.42).

If tissue must be stored prior to sectioning or embedding, it should be placed in aqueous buffer containing 0.1% sodium azide. Long-term storage in ethanol- and methanol-containing solutions can extract peptides.

Clinical Specimens Other Than Paraffin-Embedded Tissues

Cell smears, frozen tissue sections, cytopins and imprints should be fixed with cold acetone (4°C) for approximately 10 minutes, followed with several phosphate buffered saline or Tris buffer baths (3 changes, 10 minutes each).

CAUTION: WE DO NOT RECOMMEND USING HYDROGEN PEROXIDE WITH THESE TYPES OF SPECIMENS.

2.13 Paraffin Embedding

Dehydration

Dehydration through increasing concentrations of ethanol or methanol must be accomplished rapidly due to extraction of peptides in intermediate concentrations of alcohols.

Clearing of tissue in either xylene or toluene is compatible with subsequent immunostaining.

Infiltration with Molten Paraffin

Infiltration of tissue with molten paraffin is done quickly to avoid prolonged exposure of tissue to elevated temperature. Infiltration is best accomplished in a vacuum oven. (Avoid temperatures over 60°C which may destroy protein and result in structurally damaged antigen.)

2.14 Paraffin-Embedded Section Preparation

Sectioning

Tissue blocks should be sectioned on a rotary microtome and mounted from water onto gelatinized or Elmer's Glue-All™ treated microscope slides. Heat adhere sections at 55-60°C for several hours.

CAUTION: ANTIGENS MAY BE DESTROYED BY EXPOSURE TO HIGH TEMPERATURES. [Adhesion of the section to the slide is essential to prevent tissue loss during subsequent incubations and washes. If time is not a major factor, prolonged drying of slides (in a vertical position) at 40°C for 24 to 48 hours is recommended.]

Deparaffinization

Deparaffinize and rehydrate tissue sections using standard methods. Paraffin residue in tissue sections will increase background and obscure results. It is necessary to completely remove the embedding material. The xylene and alcohol bath solutions should be replaced frequently.

Suggested Reagents and Times for Deparaffinization

Reagent Bath	Suggested Time
Xylene	2 to 3 changes, 5 minutes each
100% alcohol	2 changes, 3 minutes each
95% alcohol	2 changes, 3 minutes each
80% alcohol	3 minutes
70% alcohol	3 minutes
Phosphate buffered saline or Tris buffer	2 changes, 3 minutes each

PRECAUTION: Once the tissue sections have been rehydrated, do not allow them to dry.

Dry the slide around the tissue section with an absorbent wipe. Using a diamond pencil, china marking pencil or fingernail polish, draw a circle on the microscope slide around the section. This circle will help retain solution on the section during subsequent incubations with reagents.

2.2 STANDARD METHOD FOR PERFUSION FIXATION

Perfusion fixation allows tissue to be fixed via the vascular system. This results in further control of experimental conditions by exposing tissue to a minimal period of anoxia. The procedure commonly washes blood from the vasculature before introducing the fixative.

2.21 Perfusion Technique

Step 1 Anesthetize the animal whose tissue will be perfused.

Step 2 Restrain the anesthetized animal in a supine position with animal's abdomen, thorax and neck region well exposed.

Step 3 **OPTIONAL:** Artificially breathe the animal. (Do this for rats and all other mammals if a time delay may occur between the opening of the diaphragm and the introduction of fixative.)

Step 4 Open the abdomen/thorax region so that the heart is well exposed.

Step 5 Cannulate the aorta via the left ventricle using a large gauge, beveled needle or catheter.

Step 6 Open the right atrium or right ventricle to allow an efflux of blood and perfusate.

Step 7 Wash the vasculature with one of the suggested washes:

- Oxygenated calcium free tyrodes
- Phosphate buffered saline
- 0.9% saline

(Please refer to Perfusion Wash Formulas, section 6.2)

Over a time interval of a few minutes, flush the vascular system with the wash solution. (For a small animal, such as a rat, the suggested perfusion time is 2 minutes or less using a wash volume of 50 to 100 mL.)

Step 8 Connect the cannula to a reservoir of fixative which is either elevated four feet above the animal or is connected to a motor pump. Adjust the flow rate to perfuse the vasculature with 500 to 700 mL of fixative solution over a time span of 30 minutes unless otherwise specified. (This is appropriate for a rat or other comparably sized animal.)

The following fixatives are suggested:

- 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (detection of peptides and hormones)
- 4% paraformaldehyde in phosphate buffer, pH 6.5, followed by 4% paraformaldehyde in borate buffer, pH 11.0 (detection of peptides and hormones)
- 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (detection of GABA and CHAT with ImmunoStar antibodies)
- 5% glutaraldehyde in 0.1 M cacodylate buffer with 1% sodium metabisulfite (detection of dopamine with ImmunoStar antibody)
- 4% carbodiimide in phosphate buffered saline (detection of histamine with ImmunoStar antibody)

(Please refer to Fixative Formulas, section 6.3)

Step 9 Remove tissue that is to be examined.

Step 10 Immerse tissue in fixative and continue fixation at 4°C for 1.5 hours (or otherwise noted in specification sheet).

Step 11 Transfer and store tissue in:

- Phosphate buffer containing preservatives, for tissue which is to be paraffin embedded.
- 5% to 30% sucrose in phosphate buffer, for tissue which is to be frozen (store for 12 to 72 hours, or as otherwise noted in specification sheet).

2.3 FROZEN AND VIBRATOME TISSUE SECTIONING

2.31 Cryostat Sections

- Several tissue freezing techniques are used for cryostat sectioning. A suggested method is to freeze tissue on the surface of liquid nitrogen or in isopentane cooled by liquid nitrogen. (For this technique, it is best to freeze the tissue in a plastic mold surrounded with tissue paste of similar organ consistency or O.C.T. embedding medium.)
- Frozen tissue is bound to a chuck and sections (3 to 15 microns) are cut at temperatures from -12°C to -20°C. (The optimal cutting temperature must be determined for each particular type of tissue.)
- Collect a section onto a cold (cryostat temperature), gelatinized slide using either static force (gently place the slide flat against the section) or a small, soft brush (such as camel's hair). Warm the back side of the slide by slowly moving your finger across it; this will melt the tissue section onto the slide. The warming should continue for 1 to 2 minutes.
- Store the mounted tissue at -20°C.

2.32 Vibratome Sections

- Adhere the tissue block to the cutting stage with cyanoacrylate adhesive. Fill the cutting well with cold (4°C) phosphate buffer, phosphate buffered saline, or Tris buffer. Cut the sections 30 to 300 microns thick and collect them with a soft brush.
- Store the sections in buffer prior to immunostaining. (Staining is conducted by incubating free floating sections in immunochemicals. Please refer to Free Floating Technique, section 3.4.)

2.33 Microtome Frozen Sections

- Place the tissue on the stage of the microtome and freeze with dry ice snow. Section at a thickness of 30 to 75 microns and collect the sections from the knife blade (room temperature) with a buffer-moistened brush.
- Store the sections in buffer prior to immunostaining. (Staining is conducted by incubating free floating sections in immunochemicals. Please refer to Free Floating Technique, section 3.4.)

2.4 ADDITIONAL TISSUE TREATMENTS

2.41 Mercury Removal

Tissue that was fixed with a mercury-containing fixative should be treated to remove mercuric pigments.

Suggested Reagents and Times for Mercury Removal

<u>Reagents</u>	<u>Suggested Time</u>
0.5% Gram iodine in 80% alcohol	5 minutes
Gently running tap water	5 minutes
5% sodium thiosulfate	5 minutes
Gently running tap water	5 minutes
Buffer (phosphate buffered saline or Tris)	5 minutes

2.42 Trypsin Digestion

Trypsin digestion may "unmask" antigenic sites that have become inaccessible by excessive cross-linkage from formalin fixation.

Suggested Reagents and Times for Trypsin Digestion

<u>Reagent Bath</u>	<u>Suggested Time</u>
0.1% Trypsin and 0.1% Calcium Chloride in Tris buffer	10-30 minutes
Tris buffer	2 changes, 5 minutes each

2.43 Endogenous Peroxidase Deactivation

If an immunoperoxidase labeling method is to be used (such as peroxidase anti-peroxidase or avidin/biotin peroxidase), it is common practice to denature the endogenous peroxidase activity in the tissue prior to immunostaining.

Suggested Reagents and Times for Peroxidase Deactivation

<u>Reagent Bath</u>	<u>Suggested Time</u>
3% Hydrogen peroxide in distilled/deionized water	5-20 minutes
Buffer (phosphate buffered saline or Tris)	2 changes, 5 minutes each

2.44 Free Aldehyde Reduction

Tissues fixed in glutaraldehyde may have false positive labeling due to free aldehydes. This can be eliminated by reducing agents such as sodium borohydride before beginning immunostaining procedures.

Suggested Reagents and Times for Free Aldehyde Reduction

Reagent Bath	Suggested Time
1% sodium borohydride in distilled/deionized water	30 minutes
Buffer (phosphate buffered saline or Tris)	3 changes, 5 minutes each

3.0 IMMUNOSTAINING TECHNIQUES

(For immunostaining techniques for use with the electron microscope, please refer to section 4.0).

3.1 INDIRECT IMMUNOFLUORESCENCE

3.11 Method Description

The indirect immunofluorescent technique involves the use of a primary antibody directed against a specific antigen in the tissue followed by a secondary antibody which binds to the primary and is fluorescently tagged with a substance such as fluorescein- or rhodamine-isothiocyanate (Coons, 1958; Hartman, 1973; Hokfelt et al., 1975). WE DO NOT RECOMMEND THE USE OF PARAFFIN SECTIONS WITH THIS TECHNIQUE.

3.12 Materials Required

Reagents

- **ImmunoStar Primary Histochemical Antibody**
See specification sheet for recommended working dilution.
- **Secondary Antibody**
Fluorescein or rhodamine conjugated antibodies directed against the host species of the primary histochemical antibody. Use at dilution recommended by manufacturer.
- **Negative control**
Normal serum (serum prior to antibody production) from the same animal (or species) providing the primary antiserum. Prepare same as primary antiserum; use same diluent and working dilution.
- **Antisera diluent**
Triton X-100 (0.3% v:v) in phosphate buffered saline (PBS).
- **Rinsing buffer**
PBS
- **Mounting media**
Glycerin/PBS (3:1; v:v)

Supplies

- Histological staining dishes
- Wash bottle
- Absorbent wipes
- Nail polish, china marking pencil or diamond pencil
- Coverslips (No. 1½)

Equipment

- Incubation chamber capable of retaining moisture (plastic or metal box with tight fitting lid)
- Fluorescent microscope (transmitted or reflected light) with filters appropriate for fluorescein-isothiocyanate excitation (excitation 440-490 nm; emission 520-560 nm) or rhodamine excitation (excitation 546 nm; emission 580-600 nm)
- **OPTIONAL:** Laboratory oven or incubator capable of maintaining 37°C

3.13 Protocol

Step 1 Bring the frozen tissue sections to room temperature.

Step 2 Using fingernail polish, a diamond pencil or china marking pencil; draw a circle on the microscope slide around the section. Apply a drop of PBS to cover the tissue and allow the drop to remain in place for 10 to 30 minutes.

Step 3 Tap off the excess buffer and wipe around the section with an absorbent wipe. **CAUTION: DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.**

Step 4 Cover each section with one to two drops (approximately 50 to 100 µL) of the diluted primary antisera or negative control (please refer to section 3.12). Incubate the slides at 4°C for 12 to 24 hours in a closed incubation chamber.

Step 5 Remove the slides from the incubation chamber and rinse the excess antiserum from the tissue with a gentle stream of cold PBS from a wash bottle. Continue washing sections by immersing in cold PBS for 15 minutes.

Step 6 Blot the excess PBS from the slide with an absorbent wipe. **CAUTION: DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.** Apply one to two drops (50 to 100 μ L) of diluted fluorescein or rhodamine-labeled secondary antibody (please refer to section 3.12). Incubate the slides in an incubation chamber at room temperature for one hour or 37°C for 30 minutes.

Step 7 Remove the slides from the incubation chamber and rinse the excess antiserum from the slide with a gentle stream of room temperature PBS from a wash bottle. Continue washing sections by immersing in room temperature PBS for 15 minutes.

Step 8 Drain and blot the excess PBS from all areas of the slide, except that area containing the tissue. Place a drop of glycerin/PBS over the tissue and apply a coverslip.

Step 9 Observe the tissue staining using a darkfield microscope with filters appropriate for the fluorescein or rhodamine labeling.

Step 10 If slides will not be viewed immediately; freeze them horizontally in a dark environment.

3.14 Specificity Control

Antibody specificity is tested by using a negative control serum and/or absorbed primary antiserum on adjacent sections. Any staining which occurs using the negative control is due to nonspecific background and helps differentiate specific positive staining from nonspecific background staining on the specimens under investigation. In addition, the primary antibody may be pretreated for 12 hours with the same antigen used to generate the antibody (100 μ g antigen/mL diluted antiserum). Structures that are stained using untreated antiserum, but unstained with pretreated antiserum are considered "specific" localizations by present standards. For further information, refer to Petrusz et al., 1976; and Swaab et al., 1977.

3.2 PEROXIDASE ANTIPEROXIDASE IMMUNOSTAINING

3.21 Method Description

The peroxidase antiperoxidase technique, developed by Sternberger and coworkers (Sternberger et al., 1970; Sternberger, 1979), is a series of immunological reactions utilizing a soluble species-specific horseradish peroxidase immune complex.

Specific antigenic sites in the tissue are bound by the applied primary antibody; an antibody directed against the antigen under investigation. This is followed by the addition of the secondary antibody which serves as a "bridge" for the primary antibody and the peroxidase reagent. The addition of the peroxidase reagent (peroxidase antiperoxidase complex) completes the sequence.

The antigenic site is made visible by the addition of a chromogenic substrate, such as diaminobenzidine (DAB) or aminoethylcarbazole (AEC). The chromogenic substrate reacts with the peroxidase reagent in the presence of the substrate reagent, hydrogen peroxide, to form a colored precipitate.

3.22 Materials Required

Reagents

- **ImmunoStar Primary Histochemical Antibody**

See specification sheet for recommended working dilution.

- **Secondary (Bridge) Antibody**

An antibody against a species immunoglobulin that matches the host species of the primary antibody. Use at a dilution recommended by manufacturer.

- **Peroxidase Antiperoxidase Complex (PAP)**

A complex of horseradish peroxidase enzyme and antibodies to peroxidase. The antibodies to peroxidase are generated in the same species as the host species of the primary antibody. Use at a dilution recommended by manufacturer.

- **Negative Control**

Normal serum (serum prior to antibody production) from the same animal or species providing the primary antiserum. Prepare same as primary antiserum; use same diluent and working dilution.

- **Antisera Diluent**

Use to dilute primary and secondary antisera. Contains normal serum from the host species providing the bridge antibody. If using frozen tissue sections, diluent should also contain 0.3% Triton X-100.

– Phosphate buffered saline (PBS), pH 7.4 with 1% normal serum

– Tris/Saline buffer, pH 7.6 with 1% normal serum

- **Pre-blocking Agent**

A reagent solution that serves to block nonspecific antigenic sites found on tissue. Contains normal serum from host species providing second antibody. If using frozen tissue sections, the reagent solution should also contain 0.3% Triton X-100.

– 10% normal serum in PBS, pH 7.4 or Tris/Saline buffer, pH 7.6

– 1% normal serum in combination with 0.25% bovine serum albumin and 0.1% gelatin in PBS, pH 7.4 or Tris/Saline buffer, pH 7.6

- **Rinsing Buffer**

– PBS, pH 7.4

– Tris/Saline buffer, pH 7.6

- **Chromogen**

– DAB

DAB-4-HCl (3,3' Diaminobenzidine tetrahydrochloride) dissolved in Tris/Saline buffer, pH 7.6 (please refer to Chromogen Formulas, section 6.5).

– AEC

3-amino-9-ethylcarbazole in N,N-dimethylformamide dissolved in acetate buffer, pH 5.2 (please refer to Chromogen Formulas, section 6.5).

- **Substrate Reagent**
 - Hydrogen peroxide
- **Dehydration Reagents (for DAB only)**
 - Ethanol baths of increasing concentration: 70, 80, 95 and 100% ethanol and xylene baths
- **Mounting Media**
 - Water based mounting media (can be used with either DAB or AEC)
 - Permanent organic based mounting media (for DAB only). Tissue must be dehydrated prior to use.

Supplies

- Histological staining dishes
- Wash bottle
- Absorbent wipes
- Coverslips (No. 1½)

Equipment

- Incubation chamber capable of retaining moisture (plastic or metal box with tight fitting lid)
- Brightfield microscope
- **OPTIONAL:** Laboratory oven or incubator capable of maintaining 37°C

3.23 Protocol

Preparation

- **Paraffin Tissue Sections**
Please refer to Standard Tissue Preparation Procedure in Pathology, section 2.1
- **Frozen Tissue Sections**

Please refer to Cryostat Sections, section 2.31.

Bring the frozen tissue to room temperature. Using fingernail polish, a diamond pencil or china marking pencil, draw a circle around the section on the microscope slide. Apply a drop of PBS or Tris/Saline buffer to cover the tissue section and allow the drop to remain in place for approximately 10 minutes.

Immunostaining Steps

Step 1 Tap off excess buffer and wipe around the tissue section with an absorbent wipe. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.

Step 2 Apply one or two drops (50 to 100 µL) of pre-blocking agent (please refer to section 3.22) to the tissue section and allow it to remain in place for 10 to 20 minutes.

Step 3 Tap off pre-blocking agent and wipe away any excess around the tissue. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.

Step 4 Cover each section with one or two drops (50 to 100 µL) of diluted primary antibody or negative control (please refer to section 3.22). Incubate the slides at 4°C for 12 to 24 hours in a closed incubation chamber.

Step 5 Remove the slides from the incubation chamber and rinse the excess antiserum from the slide with a gentle stream of cold PBS or Tris/Saline buffer from a wash bottle. Continue washing sections by immersing in cold buffer for 2 changes of 5 to 10 minutes each.

Step 6 Blot the excess buffer from the slide with an absorbent wipe. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY. Cover each section with one to two drops (50 to 100 µL) of diluted secondary antibody (please refer to section 3.22). Incubate the slides in an incubation chamber at room temperature for 20 to 60 minutes or 37°C for 15 to 30 minutes. **CAUTION:** RECOMMENDED INCUBATION TIMES SHOULD BE CHECKED WITH THE SECONDARY ANTIBODY MANUFACTURER.

Step 7 Remove the slides from the incubation chamber and rinse the excess antiserum from the tissue with a gentle stream of room temperature PBS or Tris/Saline buffer. Continue washing sections by immersing in room temperature buffer for 2 changes of 5 to 10 minutes each.

Step 8 Blot excess buffer from the slide with an absorbent wipe. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY. Cover each section with one to two drops (50 to 100 µL) of diluted PAP complex (please refer to section 3.22). Incubate the slides in an incubation chamber at room temperature for 20 to 60 minutes or 37°C for 15 to 30 minutes. **CAUTION:** RECOMMENDED INCUBATION TIMES SHOULD BE CHECKED WITH THE PAP COMPLEX MANUFACTURER.

Step 9 Remove the slides from the incubation chamber and rinse the PAP complex from the tissue with a gentle stream of room temperature PBS or Tris/Saline buffer. Continue washing sections by immersing in room temperature buffer for 2 changes of 5 to 10 minutes each.

Step 10 Immerse the tissue sections in a freshly prepared chromogen/substrate reagent solution such as DAB/H₂O₂ or AEC/H₂O₂ for 2 to 30 minutes (please refer to Chromogen Formulas, section 6.5). Remove slides periodically to check for staining development with a low-power microscope.

Step 11 **OPTIONAL:** Counterstain.

Step 12 If organic based mounting media is to be used, dehydrate sections with increasing concentrations of ethanol followed by xylene as in conventional histology (please refer to section 3.22). **CAUTION:** AEC IS ORGANICALLY SOLUBLE, DO NOT DEHYDRATE SECTIONS.

Step 13 Coverslip.

3.24 Specificity Control

Antibody specificity is tested by using a negative control serum and/or absorbed primary antiserum on adjacent sections. Any staining which occurs using the negative control is due to nonspecific background and helps differentiate specific positive staining from nonspecific background staining on the specimens under investigation. In addition, the primary antibody may be pretreated for 12 hours with the same antigen used to generate the antibody (100 µg antigen/mL diluted antiserum). Structures that are stained using untreated antiserum, but unstained with pretreated antiserum, are considered “specific” localizations by present standards. For further information, refer to Petrusz et al., 1976; and Swaab et al., 1977.

3.3 AVIDIN/BIOTIN PEROXIDASE IMMUNOSTAINING

3.31 Method Description

The avidin/biotin peroxidase technique incorporates a biotinylated secondary antibody and a “universal” avidin horseradish peroxidase complex (Bayer and Wilcheck, 1980; Hsu et al., 1981) as part of the labeling procedure. The technique utilizes the powerful binding affinity of biotin and avidin.

Specific antigenic sites in the tissue are bound by the applied primary antibody, an antibody directed against the antigen under investigation. This is followed by the addition of a biotinylated secondary antibody which serves as a “bridge” for the primary antibody and the avidin peroxidase complex. The addition of the avidin peroxidase complex completes the sequence. With this technique, the avidin peroxidase complex binds to the biotin of the secondary antibody.

The antigenic site is made visible by the addition of a chromogenic substrate, such as diaminobenzidine (DAB) or aminoethylcarbazole (AEC). The chromogenic substrate reacts with the peroxidase reagent in the presence of the substrate reagent, hydrogen peroxide, to form a colored precipitate.

3.32 Materials Required

Reagents

- **ImmunoStar Primary Histochemical Antibody**

See specification sheet for recommended working dilution.

- **Biotinylated Secondary (Bridge) Antibody**

A biotinylated antibody against a species immunoglobulin that matches the host species of the primary antibody. Use at a working dilution recommended by the manufacturer.

- **Avidin Peroxidase Complex**

A complex of peroxidase enzyme and avidin. Use at working dilution recommended by the manufacturer. If the manufacturer does not suggest a particular diluent, the use of sodium carbonate buffer is recommended (please refer to Buffer Formulas, section 6.1).

- **Negative Control**

Normal serum (serum prior to antibody production) from the same animal or species providing the primary antiserum. Prepare same as primary antiserum; use same diluent and working dilution.

- **Antisera Diluent**

Used to dilute primary and secondary antisera. Contains normal serum from species providing the bridge antibody. If using frozen tissue sections, diluent should also contain 0.3% Triton X-100.

- Phosphate buffered saline (PBS), pH 7.4, with 1% normal serum
- Tris/Saline buffer, pH 7.6, with 1% normal serum

- **Pre-Blocking Agent**

A reagent solution that serves to block nonspecific antigenic sites found on tissue. Contains normal serum from the host species providing the secondary antibody. If using frozen tissue sections, the reagent solution should contain 0.3% Triton X-100.

- 10% normal serum in PBS, pH 7.4, or Tris/Saline buffer, pH 7.6.
- 1% normal serum in combination with 0.25% bovine albumin and 0.1% gelatin in PBS, pH 7.4, or Tris/Saline buffer, pH 7.6.

- **Rinsing Buffers**

- PBS, pH 7.4
- Tris/Saline Buffer, pH 7.6

- **Chromogen**

– DAB
DAB-4-HCl (3,3' Diaminobenzidine tetrahydrochloride) dissolved in Tris/Saline buffer, pH 7.6 (please refer to Chromogen Formulas, section 6.5).

– AEC

AEC (3-Amino-9-Ethylcarbazole in N,N-dimethylformamide) dissolved in acetate buffer, pH 5.2 (please refer to Chromogen Formulas, section 6.5).

- **Substrate reagent**

Hydrogen Peroxide

- **Dehydration Reagents (For DAB only)**

Ethanol baths of increasing concentration: 70, 80, 95 and 100% ethanol and xylene baths

- **Mounting Media**

- Water based mounting media (can be used with either DAB or AEC).
- Permanent organic based mounting media (for DAB only). Tissue must be dehydrated prior to use.

Supplies

- Histological staining dishes
- Wash bottle
- Absorbent wipes
- Coverslips (No. 1½)

Equipment

- Incubation chamber capable of retaining moisture (plastic or metal box with tight-fitting lid)
- Brightfield microscope
- **OPTIONAL:** Laboratory oven or incubator capable of maintaining 37°C.

3.33 Protocol

Preparation

- **Paraffin Tissue Sections**

Please refer to Standard Tissue Preparation Procedure in Pathology, section 2.1.

- **Frozen Tissue Sections**

Please refer to Cryostat Sections, section 2.31.

Bring the frozen tissue to room temperature. Using fingernail polish, a diamond pencil or china marking pencil, draw a circle around the section on the microscope slide. Apply a drop of PBS or Tris/Saline buffer to cover the tissue section and allow the drop to remain in place for approximately 10 minutes.

Immunostaining Steps

Step 1 Tap off excess buffer and wipe around the section with an absorbent wipe. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.

Step 2 Apply one or two drops (50 to 100 µL) of pre-blocking agent (please refer to section 3.32) to the tissue section and allow it to remain in place for 10 to 20 minutes.

Step 3 Tap off pre-blocking agent and wipe away any excess around the tissue. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.

Step 4 Cover each section with one or two drops (50 to 100 µL) of diluted primary antibody or negative control (please refer to section 3.32). Incubate the slides at 4°C for 12 to 24 hours in a closed incubation chamber.

Step 5 Remove the slides from the incubation chamber and rinse the excess anti-serum from the tissue with a gentle stream of cold PBS or Tris/Saline buffer from a wash bottle. Continue washing sections by immersing in cold buffer for 2 changes of 5 to 10 minutes each.

Step 6 Blot the excess buffer from the slide with an absorbent wipe. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY. Cover each section with one to two drops (50 to 100 µL) of diluted biotinylated secondary antibody (please refer to section 3.32). Incubate the slides in an incubation chamber at room temperature for 20 to 60 minutes or 37°C for 15 to 30 minutes. **CAUTION:** RECOMMENDED INCUBATION TIMES SHOULD BE CHECKED WITH THE SECONDARY ANTIBODY MANUFACTURER.

Step 7 Remove the slides from the incubation chamber and rinse the excess anti-serum from the tissue with a gentle stream of room temperature PBS or Tris/Saline buffer. Continue washing sections by immersing in room temperature buffer for 2 changes of 5 to 10 minutes each.

Step 8 Blot the excess buffer from the slide with an absorbent wipe. **CAUTION:** DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY. Cover each section with one to two drops (50 to 100 µL) of diluted avidin peroxidase complex (please refer to section 3.32). Incubate the slides in an incubation chamber at room temperature for 20 to 60 minutes or 37°C for 15 to 30 minutes. **CAUTION:** RECOMMENDED INCUBATION TIMES SHOULD BE CHECKED WITH THE AVIDIN PEROXIDASE MANUFACTURER.

Step 9 Remove the slides from the incubation chamber and rinse the excess antiserum from the tissue with a gentle stream of room temperature PBS or Tris/Saline buffer. Continue washing sections by immersing in buffer for 2 changes of 5 to 10 minutes each.

Step 10 Immerse the tissue sections in a freshly made chromogen/substrate reagent solution such as DAB/H₂O₂ or AEC/H₂O₂ for 2 to 30 minutes (please refer to section 6.5). Remove slides periodically to check for staining development with a low-power microscope.

Step 11 **OPTIONAL:** Counterstain

Step 12 If an organic based mounting media is to be used, dehydrate sections with increasing concentrations of ethanol followed by xylene as in conventional histology (please refer to section 3.32). **CAUTION:** AEC IS ORGANICALLY SOLUBLE, DO NOT DEHYDRATE SECTIONS.

Step 13 Coverslip.

3.34 Specificity Control

Antibody specificity is tested by using a negative control serum and/or absorbed primary antiserum on adjacent sections. Any staining which occurs using the negative control is due to nonspecific background and helps differentiate specific positive staining from nonspecific background staining on the specimens under investigation. In addition, the primary antibody may be pretreated for 12 hours with the same antigen used to generate the antibody (100 µg antigen/mL diluted antiserum). Structures that are stained using

untreated antiserum, but unstained with pretreated antiserum, are considered "specific" localizations by present standards. For further information, refer to Petrusz et al., 1976; and Swaab et al., 1977.

3.4 FREE FLOATING TECHNIQUE WITH ANTIPEROXIDASE OR AVIDIN/BIOTIN PEROXIDASE IMMUNOSTAINING

3.41 Method Description

This technique creates more tissue surface area for immunochemicals to penetrate. Tissues are thick-sectioned (30 to 300 microns) and placed in reagents.

3.42 Materials Required

Reagents

See reagent section for Peroxidase Antiperoxidase Immunostaining (section 3.22) or Avidin/Biotin Peroxidase Immunostaining (section 3.32). **NOTE:** Primary and secondary antibodies must also contain 0.3% Triton X-100 when performing a free floating technique.

Supplies

- Pasteur pipettes
- Incubation wells (such as tissue culture slides fitted with wells or small Petri dishes)
- Camel's hair brush
- Glass microscope slides
- Coverslips (No. 1½)

Equipment

- Shaker table or similar device
- Brightfield microscope
- Slide warmer
- Vibratome or microtome for frozen sections

3.43 Protocol

NOTE: Use a shaker table or similar device to mildly agitate sections during incubations and washes.

We recommend that tissue sections be placed in a minimum of 300 microliters of diluted antibody for 4 small sections or 2 large sections. Greater volumes should be used for wash buffers and preincubation reagents.

Step 1 Cut sections with vibratome or microtome for frozen sections (please refer to section 2.32 or 2.33).

Step 2 Collect sections with a soft brush and place in incubation wells containing PBS.

Step 3 Remove buffer from the wells by siphoning or decantation and replace with a pre-blocking agent containing 0.3% Triton X-100. Incubate tissue sections for 30 minutes at room temperature.

Step 4 Remove pre-blocking agent and replace with PBS. Wash tissue with two 15 minute PBS buffer baths.

Step 5 Replace buffer with diluted primary antibody. (Antibody diluent should contain 0.3% Triton X-100.) Incubate for 12 to 48 hours at 4°C.

Step 6 Remove primary antibody and replace with PBS. Wash tissue with two 15 minute PBS buffer baths.

Step 7 Replace buffer with diluted secondary antibody or biotinylated secondary antibody. (Antibody diluent should contain 0.3% Triton X-100.) (We suggest using a more dilute solution than that used for tissue on slides.) Incubate for one hour at room temperature.

Step 8 Remove secondary antibody and replace with PBS. Wash tissue with two 15 minute PBS buffer baths.

Step 9 Replace buffer with diluted peroxidase reagent (peroxidase antiperoxidase or avidin peroxidase). (We suggest using a more dilute solution than that used for tissues on slides.) Incubate for one hour at room temperature.

Step 10 Remove peroxidase reagent and replace with PBS. Wash tissue with two 15 minute PBS buffer baths.

Step 11 Replace buffer with freshly prepared chromogen/substrate reagent, DAB/H₂O₂ (please refer to Chromogen Formulas, section 6.5). Incubate for 5 to 20 minutes until tissue turns a light caramel color. Remove tissue periodically to check for staining development with a low-power microscope.

Step 12 Terminate the reaction by replacing the chromogen solution with PBS. Wash tissue with three 5 minute PBS buffer baths.

Step 13 Mount sections on gelatinized slides and dry for 3 to 12 hours on a slide warmer.

Step 14 Dehydrate sections with increasing ethanol concentrations followed by xylene as in conventional histology.

Step 15 Coverslip.

3.5 NICKEL AMMONIUM SULFATE- GLUCOSE OXIDASE INTENSIFICATION

3.51 Method Description

This procedure utilizes glucose oxidase and nickel ammonium sulfate to enhance the DAB chromogen of peroxidase immunohistochemistry. The result is a distinct purple/ black reaction product, which is easily visualized.

3.52 Materials Required

Please refer to Peroxidase Antiperoxidase Immunostaining, section 3.22; Avidin/Biotin peroxidase Immunostaining, section 3.32; or Free Floating Technique with Anti-peroxidase or Avidin/Biotin Peroxidase Immunostaining, section 3.42

Additional Reagents

- Nickel Ammonium Sulfate
- 0.1 M Sodium Acetate
- 0.2 M Acetic Acid
- Beta-D-Glucose
- Ammonium Chloride
- Glucose Oxidase, type VII

3.53 Protocol

Please refer to Peroxidase Antiperoxidase Immunostaining, section 3.23; Avidin/Biotin Peroxidase Immunostaining, section 3.33; or Free Floating Technique with Anti-peroxidase or Avidin/Biotin Peroxidase Immunostaining, section 3.43. **The following steps are to be used in place of Step 10 (in sections 3.23 and 3.33) and in place of Step 11 (in section 3.43).**

Step 1 Dissolve 1.5 g of nickel ammonium sulfate in 50 mL of 0.1 M sodium acetate, pH 6.0. Adjust the pH of the sodium acetate by the addition of 0.2 M acetic acid.

Step 2 Dissolve 50 mg of DAB-4-HCl in 50 mL of distilled water.

Step 3 Combine and stir the nickel ammonium sulfate solution with the DAB-4-HCl solution.

Step 4 Add to the above the following in sequential order: 200 mg of beta-D-glucose, 40 mg of ammonium chloride and 2 mg of glucose oxidase.

Step 5 Immerse the slides or tissue sections in the solution prepared in Step 4 and wait for a purple/black color reaction (1-5 minutes). Remove slides or sections periodically to check for staining development with a low-power brightfield microscope.

Step 6 Terminate the color reaction by washing the slides or sections with 0.1 M sodium acetate, pH 6.0.

Step 7 Wash the slides or sections with three 10 minute distilled water baths.

Step 8 **OPTIONAL:** Counterstain.

Step 9 If organic based mounting media is to be used, dehydrate sections with increasing concentrations of ethanol followed by xylene, as in conventional histology (please refer to section 3.32).

For additional information, please refer to Shu, et al., 1988.

3.6 Silver-Intensified Immunogold Staining

Incorporated in electron microscopy for over a decade, immunogold has recently gained importance at the light microscopic level with the addition of a silver-intensified step.

Please refer to the following reference articles for methods and additional information: Holgate, et al., 1983; Mey, J.E., 1983; and Van den Pol, 1986.

4.0 IMMUNOSTAINING TECHNIQUE FOR USE WITH THE ELECTRON MICROSCOPE

4.1 PRE-EMBEDDING PEROXIDASE ANTIPEROXIDASE IMMUNOSTAINING

4.11 Method Description

This technique is useful for electron microscopy. Tissues are immunostained prior to plastic embedding and ultrathin sectioning (Priestly and Cuello, 1983).

4.12 Materials Required

Reagents

- **ImmunoStar Primary Histochemical Antibody**
- **Secondary (Bridge) Antibody**

An antibody against a species immunoglobulin that matches the host species of the primary antibody. Use at a dilution recommended by the manufacturer. (We suggest using a dilution greater than that used for tissue on slides.)

- **Peroxidase Antiperoxidase Complex (PAP)**

A complex of horseradish peroxidase enzyme and antibodies to peroxidase. The antibodies to peroxidase are generated in the same species as the host species of the primary antibody. Use a dilution recommended by the manufacturer.

- **Antisera Diluent**

Used to dilute primary and secondary antibodies. Phosphate buffered saline (PBS), pH 7.4, containing 1% normal serum (from the species providing the bridge antibody). Diluent should contain 0.3% Triton X-100 for primary antibody. (If Triton X-100 is omitted, please refer to section 4.13, Step 1).

- **Pre-Blocking Agent**

A reagent solution that serves to block nonspecific antigenic sites found on tissue. A suggested solution is 10% normal serum (from the species providing the bridge antibody) in PBS. Diluent should contain 0.3% Triton X-100. (If Triton X-100 is omitted, please refer to section 4.13, Step 1.)

- **Rinsing Buffer**

- Phosphate buffered saline (PBS), pH 7.4
- 0.1 M phosphate buffer, pH 7.4

- **Chromogen**

- DAB
- DAB-4-HCl (3,3' diaminobenzidine tetrahydrochloride)

- **Substrate Reagent**

Hydrogen peroxide

- **Osmium tetroxide (OsO₄)**

- **Dehydration Reagents**

Ethanol baths of increasing concentration: 70, 95 and 100% ethanol

- **Propylene oxide-anhydrous, E.M. grade**

- **Resin**

- **Cyanoacrylate adhesive**

Supplies

- Small tipped pipettes
- Incubation wells
- Camel's hair brush
- Dimethyldichlorosilane dipped slides
- Razor blades
- Blank resin blocks

Equipment

- Shaker table or similar device
- Vibratome
- Transmission electron microscope

4.13 Protocol

Specimen Preparation

- Perfuse animal with 4% paraformaldehyde and 0.1 to 0.3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Continue fixation by immersing tissues of interest in the same fixative for an additional 3 hours at 4°C.
- Vibratome sections in cold phosphate buffered saline (PBS) at a 50 to 100 micron thickness. Collect sections and place in incubation wells containing cold PBS.

Immunostaining Steps

NOTE: Use a shaker table or similar device to mildly agitate sections during incubations and washes.

Step 1 Remove buffer by decanting or siphoning solution from the wells. Incubate tissue with pre-blocking agent (please refer to section 4.12).

NOTE: Detergents such as Triton X-100 may damage the morphology of the tissue as seen through the electron microscope. In place of adding detergents to the diluents, tissues can be subjected to two minute incubations in a series of graded ethanols: 10%, 25%, 50%, 25% and 10%, followed by a 15 minute rinse in 0.1 M phosphate buffer, pH 7.4 before pre-blocking agent is applied.

Step 2 Remove pre-blocking agent from the wells and replace with PBS. Wash tissue with one or two 15 minute PBS buffer baths.

Step 3 Replace buffer with diluted primary antibody (please refer to section 4.12). Incubate sections at 4°C for 12 to 48 hours.

Step 4 Remove the primary antibody and replace with PBS. Wash tissue with three 15 minute PBS buffer baths.

Step 5 Remove buffer and replace with secondary antibody (please refer to section 4.12). Incubate for one hour at room temperature.

Step 6 Remove secondary antibody and replace with PBS. Wash tissue with three 15 minute PBS buffer baths.

Step 7 Remove buffer and replace with PAP complex diluted in PBS at a dilution recommended by the manufacturer (please refer to section 4.12). Incubate for one hour at room temperature.

Step 8 Remove the PAP complex and replace with PBS. Wash tissue with three 15 minute buffer baths.

Step 9 Remove buffer and replace with 0.05% DAB-4-HCl in PBS (please refer to section 6.5). Incubate the tissues in DAB for 15 minutes.

Step 10 Remove 0.05% DAB-4-HCl and replace with DAB/H₂O₂ (please refer to section 6.5). Incubate the tissue until it turns a light caramel brown color (usually within several minutes). It is recommended that the reaction be monitored with a low-power brightfield microscope. **Do not allow the incubation to exceed 30 minutes.**

- Step 11** Terminate the reaction by replacing the DAB/H₂O₂ solution with PBS. Continue washing the tissue by rinsing several times in buffer.
- Step 12** Trim tissues to no more than 3 mm square. Rinse in PBS.
- Step 13** Place tissue in 1% osmium tetroxide in 0.1 M phosphate buffer for two hours.
- Step 14** Remove osmium tetroxide and replace with phosphate buffer. Wash tissue in phosphate buffer for two 15 minute baths.
- Step 15** Remove buffer and dehydrate tissues in a graded series of alcohols: 70, 90 and 100% anhydrous, E.M. Grade ethanol. For each alcohol concentration use two baths, 10 minutes each.
- Step 16** Remove ethanol and replace with anhydrous, E.M. Grade propylene oxide. Use two baths, 10 minutes each.
- Step 17** Replace propylene oxide with propylene oxide/resin (1:1) for 30 minutes.
- Step 18** Replace propylene oxide/resin with resin (Spurr's resin) for several hours (please refer to Resin Formulas, section 6.4). Replace with fresh plastic and allow an additional 12 to 16 hours to elapse.
- Step 19** Embed sections. We suggest using slides that have been dipped in dimethyldichlorosane (DDS) and allowed to air dry for at least 10 minutes. Place a tissue piece on the slide with a small drop of resin. Cover with another slide dipped in DDS.
- Step 20** Polymerize.
- Step 21** Pop the slides apart with a razor blade.
- Step 22** Glue the sections to blank resin blocks using a cyanoacrylate adhesive.

4.2 POST-EMBEDDING IMMUNOSTAINING: DEHYDRATION OF TISSUE AND EMBEDDING WITH EPON/ARALDITE RESIN

4.21 Method Description

This procedure ensures proper infiltration and embedding of tissue in epon/araldite resin, which is used for post-embedding electron microscopic immunostaining (please refer to Resin Formulas, sections 4.3 and 4.4).

4.22 Materials Required

Reagents

- Graded ethanols - 50, 70, 95, 100% (E.M. Grade)
- Propylene oxide (E.M. Grade)
- Epon/araldite resin (with and without DMP-30) (please refer to section 6.4).

Supplies

- Disposable plastic beakers
- Foil embedding pans
- Embedding molds
- Molecular sieves

Equipment

- Oven capable of maintaining 60°C
- Rotating table or similar device

4.23 Protocol

Please refer to Section 2.2 for Perfusion Fixation.

- Perfuse animal with 4% paraformaldehyde and 0.1 to 0.3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Continue fixation by immersing tissues of interest in the same fixative for an additional 3 hours at 4°C. After post fixation, immerse the tissue in PBS at 4°C for 12 to 16 hours before beginning plastic embedding protocol.

Step 1 Cut fixed tissue into 2 to 3 mm square pieces.

Step 2 Place tissue pieces in a graded series of ethanols: 50, 70 and 95%, for two 2-minute incubations each.

Step 3 Place tissue pieces in anhydrous, 100% ethanol (E.M. Grade) for three 2-minute incubations. (Pretreat 100% ethanol with oven dried molecular sieves.)

Step 4 Place tissue pieces in anhydrous, propylene oxide (E.M. Grade) for three 2-minute incubations. (Pretreat propylene oxide with oven dried molecular sieves.)

Step 5 Place tissue pieces in a mixture of 1:1 (v:v) un-accelerated resin (resin without DMP-30) and propylene oxide. Incubate for 3 hours at room temperature using continuous, gentle rotation.

Step 6 Place tissue pieces in full strength, un-accelerated resin (resin without DMP-30) using sealed vials. Incubate for 12 to 16 hours at room temperature using continuous, gentle rotation.

Step 7 Place tissue pieces in accelerated resin (resin with DMP-30), in foil embedding pans. Incubate in a 60°C oven for two 45-minute incubations using fresh changes of accelerated resin.

Step 8 Embed tissue in accelerated resin using an appropriate embedding mold. Polymerize in a 60°C oven for 48 hours.

4.3 POST-EMBEDDING PEROXIDASE ANTIPEROXIDASE IMMUNOSTAINING

4.31 Method Description

The peroxidase antiperoxidase labeling is performed on plastic embedded (epon/ araldite), ultrathin sections, mounted on nickel mesh grids. The following technique was developed by Dr. Mark Brownfield at the University of Wisconsin-Madison, School of Veterinary Medicine.

4.32 Materials Required

Reagents

- **ImmunoStar Primary Histochemical Antibody**

- **Secondary (Bridge) Antibody**

An antibody against a species immunoglobulin that matches the host species of the primary antibody. Use at a dilution recommended by the manufacturer.

- **Peroxidase Antiperoxidase Complex (PAP)**

A complex of horseradish peroxidase enzyme and antibodies to peroxidase. The antibodies to peroxidase are generated in the same species as the host species of the primary antibody. Use at dilution recommended by the manufacturer.

- **Negative Control**

Normal serum (serum prior to antibody production) from the same animal (or species) providing the primary antiserum. Prepare the same as primary antiserum; use same diluent and working dilution.

- **Antisera Diluent**

Used to dilute primary and secondary antisera. Contains normal serum from the host species providing the bridge antibody.

– Phosphate buffered saline, pH 7.4, with 1% gelatin and 1% normal serum. (Please refer to PBS Gelatin Buffer Formula, section 6.1.)

- **PAP Diluent**

Used to dilute Peroxidase antiperoxidase complex.

– Phosphate buffered saline, pH 7.4, with 1% gelatin. (Please refer to PBS Gelatin Buffer Formula, section 6.1.)

- **Pre-Blocking Agent**

A reagent solution that serves to block nonspecific antigen sites found on tissue. A suggested solution is 1% normal serum (from the species providing the bridge antibody) in PBS-gelatin buffer. (Please refer to PBS Gelatin Buffer Formula, section 6.1.)

- **Wash Buffers, for E.M.**

– 0.01 M PBS, pH 7.4

– 0.1 M Tris Buffer, pH 5.6

– distilled water

- **Chromogen**

– DAB

DAB-4-HCl (3,3' Diaminobenzidine tetrahydrochloride) dissolved in 0.1 M Tris Buffer, pH 7.6, for E.M.

- **Substrate Reagent**

– Hydrogen Peroxide

Supplies

- 0.2 µm Millipore filters

- Microtiter plates

- Parafilm

- Fine forceps

- Nickel mesh grids

Equipment

- Incubation chamber capable of retaining moisture

- Shaker table or similar device

- Transmission electron microscope

4.33 Protocol

Please refer to section 4.2 for plastic embedding protocol (epon/araldite resin).

- Ultrathin section tissue and mount sections on nickel mesh grids.

- All solutions used in post-embedding labeling must be Millipore filtered through 0.22 µm filters and stored in sterile glass bottles. The solutions should be fresh (no more than 2 weeks old). To avoid contamination, never place excess solutions back into Millipore filtered solutions.

- For incubating grids we recommend using a microtiter plate covered with parafilm to which small depressions have been made over the wells to hold solutions.

- Touch only the edge of the grid with the forceps. Be careful not to touch the tissue while handling the grid.

- The grids are washed by successively touching them to a drop of wash solution and wicking them off on filter paper several times. When wicking off the grids be careful to only touch the edge of the grid to the filter paper, not the tissue.

- Do not let the grids dry out between incubation and wash steps.

- During the incubation steps, gentle agitation is recommended by using a shaker table at a very low speed.

- Incubations should be done in a humidified chamber.

Immunostaining Steps

- Step 1** Float grids in 10% hydrogen peroxide in distilled water for 10 minutes at room temperature. Use gentle agitation.
- Step 2** Wash grids with three successive drops of distilled water.
- Step 3** Wash grids with one drop of 0.01 M PBS, pH 7.4. (Please refer to Buffer Formulas, section 6.1.)
- Step 4** Incubate grids in pre-blocking agent (please refer to section 4.32) for 30 minutes at room temperature.
- Step 5** Incubate grids in diluted primary antisera or negative control (please refer to section 4.32) for 12-24 hours at 4°C.
- Step 6** Wash grids with three successive drops of 0.01 M PBS, pH 7.4.
- Step 7** Incubate grids in diluted secondary antisera (please refer to section 4.32) for 30 minutes at room temperature.
- Step 8** Wash grids with three successive drops of 0.01 M PBS, pH 7.4.
- Step 9** Incubate grids in diluted PAP complex (please refer to section 4.32) for 30 minutes at room temperature.
- Step 10** Wash grids with three successive drops of 0.1 M Tris buffer, pH 7.6. (Please refer to Buffer Formulas, section 6.1).
- Step 11** Incubate grids in DAB/hydrogen peroxide solution (please refer to section 6.5 for chromogen formula) for 10 minutes.
- Step 12** Terminate the reaction by washing grids with three successive drops of 0.1 M Tris buffer, pH 7.6.
- Step 13** Wash grids with three successive drops of distilled water.
- Step 14** Allow grids to dry for at least 15 minutes in an appropriate grid storage container.
- Step 15** Incubate grids in 2% osmium tetroxide in distilled water for 10 minutes.
- Step 16** Wash grids with three successive drops of distilled water.
- Step 17** Allow grids to dry for at least 15 minutes in an appropriate grid storage container.

NOTE: Osmication will intensify the PAP labeling. However, counterstaining with lead citrate/uranyl acetate is not recommended since it may mask the specific staining.

4.4 POST-EMBEDDING ELECTRON MICROSCOPE IMMUNOGOLD STAINING

Immunogold labeling employs an immunoglobulin conjugated to colloidal gold which is used as the secondary antibody. The immunogold labeling is performed on plastic embedded, ultrathin sections, mounted on nickel mesh grids.

Please refer to the following reference articles for methods and additional information: Varndell, et al., 1982; Childs, et al., 1986.

5.0 TROUBLESHOOTING

5.1 HIGH BACKGROUND

The following are common remedies for background problems:

- Check the dilution of your antiserum; a higher dilution may eliminate some (or all) of the background problem while maintaining specific staining.
- Incubate your tissue with a non-immune serum to “pre-bind” nonspecific sites. Serum from the species providing the secondary antibody is normally used. A recommended concentration varies from 1 to 10% normal serum in rinses and diluted antisera.
- Preincubate tissue with bovine serum albumin (BSA) or gelatin. Each of these proteins has reactive amino groups which can “pre-bind” non-specific sites.
- Preabsorb the primary antisera with its carrier molecule if you are using antibodies generated against haptens. For example, ImmunoStar anti-serotonin was generated against serotonin conjugated to BSA; therefore, anti-serotonin would be preabsorbed with BSA. The preabsorption should take place at 4°C for at least 12 to 24 hours prior to actual staining with the antiserum.
- If a problem with autofluorescence is encountered using the fluorescein-isothiocyanate method, switch to a rhodamine or peroxidase label. Autofluorescence is in the same emission range as fluorescein.
- If using a peroxidase label, you may need to remove the endogenous peroxidase present. Treat tissue with either of the following:
 - 3% H₂O₂ in distilled/deionized water at room temperature for 5 to 20 minutes
 - 0.01% phenylhydrazine at room temperature for 15 to 30 minutes
- Up to 0.5 M sodium chloride may be added to the primary antiserum diluent to reduce background.
- Treatments that will increase penetration of the sample, improve signal and simultaneously reduce background:
 - Use smaller or thinner pieces of tissue.
 - Remove any penetration barriers from the tissue, such as pieces of dura or sheath.
 - Increase Triton concentration.
 - Remove lipids with solvent extraction.
 - Use proteases.
- Preabsorb the primary antiserum with tissue from the same animal which you are studying, but which does not contain the antigen of interest.
- Change fixatives. **Never use glutaraldehyde with fluorescein-isothiocyanate label.**
- Change buffers. In some systems, cacodylate buffer causes high background.
- Check the storage conditions of the antibodies. Antiserum that has been frozen and thawed excessively may deteriorate and result in increased background. Avoid frost-free freezers.
- Increase the number and length of washes between antisera incubations to reduce background.

- Make sure paraffin is completely removed from the paraffin-embedded tissue sections. Warm slides to 56-60°C and immediately transfer to a fresh xylene or toluene bath. Change baths frequently.

5.2 ABSENCE OF STAINING

- If you do not see immunoreactivity, but have reason to believe the antigen is present in your tissue:
- Try a different fixation; you could be over-fixing and thereby losing antigenicity, or under-fixing and thereby losing the antigen itself during rinses and incubations.
- Try a lower antibody dilution (more concentrated antibody). Caution should be used here, as antiserum that is too concentrated can also give negative results.
- Try proteolysis to “unmask” antigenic activity (Finely and Petrusz, 1982).
- Eliminate any treatment that can sometimes damage antigenicity:
 - Dehydration/extraction before immunocytochemical processing
 - Proteolysis
- Try different stocks of ancillary antibodies.
- If using the DAB reaction, check the potency of DAB and hydrogen peroxide. (Hydrogen peroxide is a common offender.)
- Examine procedure for proper antibody sequence.
- Do not allow tissue to dry during the experiment.
- Eliminate preservatives such as sodium azide in the wash buffer baths. Sodium azide and mercury containing preservatives are harmful to the peroxidase enzyme. Always use a buffer that is fresh and free of preservative.
- Do not allow tissues to be exposed to temperatures greater than 60°C. Excessive heat destroys proteins.
- Check the solubility of the chromogen. If using the chromogen AEC, do not use organic based solutions after the chromogen has been applied. AEC is soluble in organic based solutions; therefore, tissue cannot be dehydrated through alcohol and xylene, stained with a counter stain based in an organic solvent, or mounted with an organic based mounting media.

6.0 FORMULAS

6.1 BUFFER FORMULAS

PHOSPHATE BUFFERED SALINE, pH 7.4

Add 13.40 g of sodium phosphate dibasic, heptahydrate and 8.00 g of sodium chloride to 900 mL of distilled water. Stir until dissolved. Obtain a pH of 7.4 by slowly adding 2 N hydrochloric acid to the mixture. Bring to a final volume of 1 L with distilled water. Store at 4°C.

TRIS BUFFER, pH 7.6

Add 6.10 g Tris base (trihydroxymethyl) amino methane to 500 mL of distilled water. Stir until dissolved. Add 1 N hydrochloric acid (approximately 37 mL) until pH 7.6 is reached. Bring to a final volume of 1 L with distilled water. Store at 4°C.

TRIS/SALINE BUFFER, pH 7.6

Refer to Tris buffer formula. Replace distilled water with 0.9% sodium chloride in distilled water. Store at 4°C.

0.2 M PHOSPHATE BUFFER, pH 7.4

Add 8.06 g potassium phosphate and 37.75 g sodium phosphate, heptahydrate to 900 mL distilled water. Stir until dissolved. Adjust with 2 N hydrochloric acid or 2 N sodium hydroxide until a pH of 7.4 is reached. Bring to a final volume of 1 L with distilled water. Store at 4°C.

0.1 M PHOSPHATE BUFFER, pH 7.4

Add together: 500 mL of 0.2 M phosphate buffer, pH 7.4, and 500 mL distilled water. Stir until mixed. Store at 4°C.

ACETATE BUFFER, pH 5.0

Add 2.72 g of sodium acetate and 0.6 mL of glacial acetic acid to 900 mL of distilled water. Stir until dissolved. Adjust to a pH of 5.0 by slowly adding 2 N hydrochloric acid or 2 N sodium hydroxide. Bring to a final volume of 1 L with distilled water. Store at 4°C.

SODIUM CARBONATE BUFFER, pH 9.2

Add 7.19 g of sodium carbonate and 16.5 g of sodium bicarbonate to 900 mL of distilled water. Stir until dissolved. Adjust to a pH of 9.2 by slowly adding 2 N hydrochloric acid or 2 N sodium hydroxide. Bring to a final volume of 1 L with distilled water. Store at 4°C.

0.01 M PHOSPHATE BUFFERED SALINE, pH 7.4 (* FOR E.M.)

Add 50 mL of 0.2M phosphate buffered saline, pH 7.4 to 900 mL of distilled water. The pH should remain at 7.4; if not, adjust by slowly adding 2 N hydrochloric acid or 2 N sodium hydroxide. Bring to a final volume of 1 L with distilled water. Millipore filter through a 0.22 µm filter and store at room temperature in a sterile glass bottle.

PBS-GELATIN BUFFER, pH 7.4 (*FOR E.M.)

Add 50 mL of 0.2M phosphate buffered saline, pH 7.4 and 1.0 g of gelatin (from swine skin) to 900 mL of distilled water. Gently heat the mixture to dissolve the gelatin. The pH should remain at 7.4, if not, adjust by slowly adding 2 N hydrochloric acid or 2 N sodium hydroxide. Add 20 mg of neomycin and 100 mg of thimerosal as a preservative. Bring to a final volume of 1 L with distilled water. Millipore filter through a 0.22 µm filter and store at 4°C in a sterile glass bottle.

0.1 M TRIS BUFFER, pH 7.6 (*FOR E.M.)

A 12.2 g of Tris HCl and 2.78 g of Tris Base to 900 mL of distilled water. Stir until dissolved. Obtain a pH of 7.6 by slowly adding 2 N hydrochloric acid or 2 N sodium hydroxide to the mixture. Bring to a final volume of 1 L with distilled water. Millipore filter through a 0.22 µm filter and store at room temperature in a sterile glass bottle.

6.2 PERFUSION WASH FORMULAS

CALCIUM FREE TYRODES

Add 6.80 g sodium chloride, 0.40 g potassium chloride, 0.32 g magnesium chloride, 0.10 g magnesium sulfate, heptahydrate, 0.17 g monobasic sodium phosphate, monohydrate, 1.00 g glucose and 2.20 g sodium bicarbonate to 800 mL distilled water. Stir until dissolved. Bring to a final volume of 1 L with distilled water. Store at 4°C. Oxygenate 10 minutes prior to actual perfusion.

0.9% SALINE

Add 9.00 g of sodium chloride to 1000 mL of distilled water. Stir until dissolved. Store at 4°C.

6.3 FIXATIVE FORMULAS

CAUTION: FIXATIVE SOLUTIONS SHOULD BE MADE AND USED IN A VENTED HOOD.

B5 FIXATIVE

Add 6.00 g of mercuric chloride and 1.25 g of sodium acetate, anhydrous to 90 mL distilled water. Right before use, add nine parts of the above to one part 40% buffered formalin.

40% BUFFERED FORMALIN

Add together: 1000 mL of 40% formalin, 6.5 g dibasic sodium phosphate, anhydrous and 4.0 g of monobasic sodium phosphate, monohydrate. Stir until dissolved.

BOUIN'S FIXATIVE

Add 750 mL filtered, saturated aqueous picric acid to 250 mL of 40% formalin and 50 mL of glacial acetic acid. Stir until mixed. Store at room temperature.

10% PHOSPHATE BUFFERED NEUTRAL FORMALIN

Add 100 mL of 40% formalin, 6.5 g of dibasic sodium phosphate, anhydrous and 4.0 g of monobasic sodium phosphate, monohydrate to 900 mL distilled water. Stir until dissolved. Store at 4°C.

8% PARAFORMALDEHYDE STOCK SOLUTION

Add 80.00 g of powdered paraformaldehyde to 1000 mL of distilled water. Heat and stir. When the solution reaches 55 to 60°C, carefully add 2 N sodium hydroxide until the solution clears. Cool and filter with Whatman No. 1 paper. Store at 4°C.

4% PARAFORMALDEHYDE IN 0.1 M PHOSPHATE BUFFER, pH 7.4

Add 500 mL of 8% paraformaldehyde stock solution and 500 mL 0.2 M phosphate buffer, pH 7.4 (please refer to section 6.1). Stir until mixed. Store at 4°C.

4% PARAFORMALDEHYDE IN 0.1M PHOSPHATE BUFFER, pH 6.5

Add 500 mL of 8% paraformaldehyde stock solution and 500 mL of 0.2M phosphate buffer, pH 6.5. Stir until mixed. Store at 4°C.

– 0.2M Phosphate Buffer, pH 6.5: Add 9.00 g of potassium phosphate and 8.05 g of dibasic sodium phosphate, heptahydrate to 500 mL of distilled water. Stir until dissolved. Adjust pH to 6.5 with 2 N hydrochloric acid. Store at 4°C.

4% PARAFORMALDEHYDE IN BORATE BUFFER, pH 11.0

Add 500 mL of 8% paraformaldehyde stock solution to 500 mL of borate buffer, pH 11.0. Stir until mixed. Store at 4°C.

– Borate Buffer, pH 11.0: Dissolve 6.19 g of boric acid into 50 mL of 1 N sodium hydroxide. Bring volume to 400 mL with distilled water. Add 2 N sodium hydroxide until a pH of 11.0 is reached. Bring the final volume to 500 mL with distilled water.

4% PARAFORMALDEHYDE AND 0.1% GLUTARALDEHYDE IN 0.1 M PHOSPHATE BUFFER

Add 499 mL of 8% paraformaldehyde stock solution to 499 mL of 0.2 M phosphate buffer, pH 7.4. Mix and add 2.00 mL of 50% glutaraldehyde. Use solution immediately. (All the reagents should be at room temperature.)

5% GLUTARALDEHYDE IN 0.1 M CACODYLATE AND 1% SODIUM META-BISULFITE, pH 7.5-7.8

Add 16.0 g of sodium cacodylate and 10.0 g of sodium metabisulfite to 800 mL of distilled water. Stir until dissolved. Add 100.00 mL of 50% glutaraldehyde. Stir until mixed. pH and bring to a final volume of 1 L with distilled water. Use solution immediately.

5% CARBODIIMIDE IN PHOSPHATE BUFFERED SALINE

Add 5.00 g of carbodiimide [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] to 100 mL of 4°C phosphate buffered saline. Stir until dissolved. Use immediately.

4% CARBODIIMIDE IN PHOSPHATE BUFFERED SALINE

Follow the above procedure, but use 4.00 g of carbodiimide.

5% GLUTARALDEHYDE IN PHOSPHATE BUFFERED SALINE

Add 100.00 mL of 50% glutaraldehyde to 900 mL of phosphate buffered saline. Stir until mixed. Use immediately.

6.4 RESIN FORMULAS

SPURR'S RESIN

Add together: 10.0 g of vinylcyclohexane dioxide, 6.0 g of diglycidyl ether of polypropylene glycol, 26.0 g of nonenyl succinic anhydride and 0.4 g of dimethylamino-ethanol. Mix for 15 minutes. Cure mixture in a 65°C oven for 12 to 16 hours.

EPON/ARALDITE RESIN

Add together: 37.8 g of epon 812, 69.8 g of dodecyl succinic anhydride (DDSA), 21.7 g of araldite 502, 3.8 g of dibutylphthalate (DBP), and 2.5 g of DMP-30. Mix for 5 minutes. (**NOTE:** DMP-30 is added only for accelerated resin. Please refer to section 4.2 for embedding protocol.) This resin is for use in post-embedding electron microscope immunostaining, but may also be used for other plastic embedding.

6.5 CHROMOGEN FORMULAS

0.05% DAB Add 50 mg of DAB-4-HCl to 100 mL Tris/Saline buffer or PBS. Mix and filter.

DAB/H₂O₂ Add 33 µL of 30% H₂O₂ to 100 mL of 0.05% DAB and stir. Use immediately.

AEC Stock Solution (2.5% AEC) Add 2.5 g of 3-amino-9-ethylcarbazole to 100 mL of dimethyl formamide. Mix. This stock solution can be stored at 4°C.

0.05% AEC Add 2 mL of AEC Stock Solution to 98 mL of acetate buffer (please refer to section 6.1) and stir.

AEC/H₂O₂ Add 33 µL of 30% H₂O₂ to 100 mL of 0.05% AEC and mix. Use immediately.