Chapter 3

Degenerating Processes Identified by Electron Microscopic Immunocytochemical Methods

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Abstract

The application of electron microscopic immunolabeling techniques to the identification and analysis of degenerating processes in neural tissue has greatly enhanced the ability of researchers to examine apoptosis and other degenerative disease mechanisms. This is particularly true for the early stages of such mechanisms. Traditionally, degenerating processes could only be identified at the ultrastructural level after significant cellular atrophy had occurred, when subcellular detail was obscured and synaptic relationships altered. Using immunocytochemical labeling procedures, degenerating neural and glial processes are first identified through the use of antibodies directed against a variety of degenerative markers, such as proapoptotic effectors (i.e., cytoplasmic cytochrome c), pathological components (i.e., beta amyloid deposits), or inflammatory agents (i.e., Iba1). Both the subcellular distribution of the marker within the process and the relationship of the labeled process to surrounding elements can then be carefully characterized. The information obtained can be further refined through the use of dual immunolabeling, which can provide additional data on the phenotype of the degenerating process and inputs to the process.

Key words: Immunocytochemistry, Ultrastructure, Immunogold, Pre-embedding immunoelectron microscopy, Antibodies, Quantitation

1. Introduction

The identification of degenerating processes in the brain at the electron microscopic (EM) level has traditionally relied on the use of distinct morphological criteria, such as the presence of electron-dense cytoplasm and swollen mitochondria (1, 2). For instance, we have identified apoptotic nuclei in the substantia nigra following injury to projections to the medial forebrain bundle (3), degenerating terminals in the hippocampus following lesions of afferents traveling through the fornix (4), and plaques in animal models of

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Alzheimer's disease (5, 6). The concurrent development of EM immunocytochemical labeling methods and the identification of specific markers associated with both apoptosis and degenerative disease mechanisms have allowed high-resolution analysis of the morphology of degenerating processes at early time points when subcellular relationships are preserved. For example, EM immunocytochemistry has been used to label early proapoptotic effectors (e.g., cytochrome c released into the cytoplasm) (7), markers of pathology in models of disease (e.g., amyloid beta) (5, 6), or markers of inflammation (e.g., Iba1) (8, 9). Using ultrastructural analysis, one can then determine (1) the cell type of a labeled process (e.g., neuronal vs. glial cells), (2) the relationship of the labeled process to surrounding processes (e.g., making synaptic contacts or surrounded by glial processes), and (3) the subcellular localization of the marker (e.g., mitochondria vs. multivesicular bodies). In addition, dual-label EM methods can be used to determine the phenotype of the degenerating process and the nature of inputs to the process. Experimental approaches utilizing dual-labeling EM methods can, thus, provide valuable insight into the mechanisms underlying apoptosis and degenerative disease.

2. Materials (A Complete List of Materials with Catalogue Numbers Is Available Upon Request)

2.1. General Laboratory Materials

2.1.1. Chemicals

Contrad (dilute 10% with water for cleaning glassware; Polysciences, Warrington, PA).

Hydrochloric acid (HCl; make 70%) and sodium hydroxide (NaOH; 1 N). Store near pH meter.

Water purification filtration system.

2.1.2. Large Equipment

Fume hood equipped with a vacuum connection (flow rate ≥100 ft/min).

Rotating shaker table (VWR, West Chester, PA).

2.1.3. Small Equipment and Nondisposable Supplies

pH meter and stirrer/hot plate (Corning Life Sciences, Lowell, MA).

Pipette men with disposable tips (1–1,000 µL capacity), sharpie pens, stir bars, and timer.

2.1.4. Disposable Supplies

Colored tape, kimwipes, parafilm, squirt bottles, weigh paper, and boats (VWR).

Plastic wrap (Costco Stretch-tite; for covering beakers).

Scintillation vials (RPI Corps, Mt. Prospect, IL).

Transfer pipettes (Biologixresearch, Lenexa, KS).

2.2. Materials and Solutions for Perfusion Fixation

2.2.1. Chemicals

Acrolein (Polysciences, Warrington, PA).

Normal saline with heparin (Henry Schein, Melville, NY).

Paraformaldehyde, granular (Electron Microscopy Sciences (EMS), Fort Washington, PA).

Sodium phosphate [dibasic and monobasic (VWR)].

2.2.2. Large Equipment

Balance (for weighing animals and organs).

Enclosed pan for collecting perfusate (New Pig, Tipton, PA).

Peristaltic Masterflex pump with silicon tubing (tubing, 14 gauge; the tubing should be connected to three-way stopcock; Fig. 1; Cole Parmer, Vernon Hills, IL).

2.2.3. Small Equipment and Nondisposable Supplies

Beakers (glass; 50 ml for saline; 500 ml for fixative) and plastic graduated cylinders (100 ml).

18 gauge stainless steel tubing, 2″ long (custom cut 2″; Small Parts Inc., Miami Lakes, FL).

Brain blocking mold (1 mm, stainless steel; Ted Pella, Redding, CA).

Buchner funnel, Erlenmeyer 500-ml filtration flask, and spatula with pointed end (VWR).

Connector for attaching small needle to output line (Small Parts Inc., Seattle, WA).

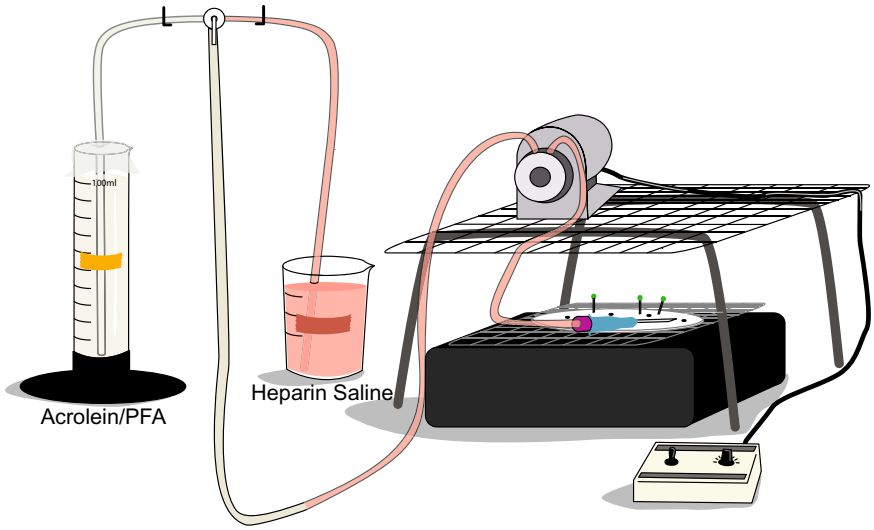

Fig. 1. Perfusion fixation setup for mice (Subheading 3.2).

Pins with large balls on ends and rack to place over pan (K-mart or Bed Bath and Beyond).

Pliers-type, vial decapper (Sigma-Aldrich, Milwaukee, WI).

Rongeurs and tissue forceps and scissors (FST, Foster City, CA). Silicon pad (remove plastic tray; EMS).

2.2.4. Disposable Supplies

Anesthesia (we use 150 mg/kg sodium pentobarbital), plastic bags (for appropriate disposal of carcasses; varies by institution), razor blades, syringes (1 cc for injecting animals; 5 cc for measuring acrolein), and Whatman #3 filter paper.

Microtubes (rinsed with heparin before collecting blood; Biologixresearch).

2.2.5. Solutions

0.2 M Phosphate Buffer Solution

1. Place 1 L of deionized water (dH_2O) into a beaker on a stir plate.
2. With the water gently stirring, add 21.8 g sodium phosphate dibasic followed by 6.4 g sodium phosphate monobasic to the beaker. Stir the solution until the phosphate buffer (PB) crystals are dissolved. The pH should be close to 7.4. DO NOT pH since this can affect the immunocytochemical reactions.
3. Make more than you need and dilute some of the 0.2 M phosphate buffer with dH_2O to make 0.1 M phosphate buffer solution for slicing. Store at 4°C.

0.1 M PB

Dilute 500 ml 0.2 M PB with 500 ml dH_2O. Alternatively, make PB as described above, except use 10.9 g sodium phosphate dibasic and 3.2 g sodium phosphate monobasic.

2% Paraformaldehyde

1. Add 250 ml dH_2O to a glass beaker containing a stir bar.
2. Heat the dH_2O to 55°C on a stir plate, and then turn the heat to a low setting.
3. Add 10 g paraformaldehyde (PFA); this does not dissolve immediately. Add 1–2.5 ml 1 M NaOH using a squirt bottle. Stir the solution until the PFA is dissolved.
4. Filter the solution using a vacuum filter with a Buchner filter and #3 filter paper.
5. Add 250 ml 0.2 M PB and adjust pH to 7.4 with HCl.

3.75% Acrolein in 2% PFA

1. Add 96.25 ml 2% PFA solution to a 100 ml graduated cylinder; you need about 250 ml/rat and 40 ml per mouse. Cover the cylinder with parafilm.
2. Fill a syringe (18- or 20-gauge, 1.5-in. long needle) with 3.75 ml acrolein. To measure the acrolein safely, hold the acrolein bottle inverted under the hood, puncture the rubber top

with the syringe needle, and remove 3.75 ml. Keep the hood sash pulled as low as possible.

3. Insert the needle through the parafilm on top of the cylinder containing the 2% PFA solution and add the acrolein slowly. Add an additional layer of parafilm and mix by inverting slowing three to four times.

2.3. Materials and Solutions for Sectioning the Brain

2.3.1. Chemicals

Agar and ethylene glycol (Sigma-Aldrich).

Sucrose (JT Baker, Phillipsburg, NJ).

2.3.2. Large Equipment

Vibrating microtome (Leica Microsystems, Deerfield, IL).

2.3.3. Small Equipment

Beakers, 1-L plastic (for storage solution).

Camel and sable hair brushes (Ted Pella Inc).

Fine forceps (for removing stray tissue and meninges; EMS).

Glass quadrant petri dishes (Corning Life Sciences).

Pipette aid (Drummond Scientific Co., Broomall, PA).

2.3.4. Disposable Supplies

Serological pipettes, 10 and 25 ml, and tissue culture dishes, 24-well (VWR).

Super glue (Loctite prism) and injector razor blades (EMS).

2.3.5. Solutions

Agar (2%)

1. Heat 100 ml water to 55°C on a stir plate.
2. Add 2 g agar to the water and stir until dissolved and bring to a gentle boil.
3. Pour about 15 ml of the agar solution into scintillation vials.
4. Store at 4°C (can keep up to 1 year).
5. To use stored agar, liquefy agar by loosening cap on vial and microwaving for 7–12 s, mix with a disposable plastic 1-ml pipette, and use.

Tissue Storage Solution (30% Sucrose and 30% Ethylene Glycol in 0.1 M PB)

1. Place a 500-ml plastic beaker on a stir plate.
2. Add 150 g sucrose and 150 ml of ethylene glycol to the beaker.
3. Bring the solution up to 500 ml with 0.1 M PB (see Subheading 2.2.5). Stir until the sucrose is dissolved. (This takes a while.)
4. ~~Adjust the final pH of the solution to 7.4 using NaOH.~~
5. Store at −20°C (this is good for several years).

6. Prior to use, stir solution at room temperature until close to 4°C.

2.4. Materials and Solutions for Immunocytochemical Processing

Alcohol (ACS grade, 200 proof) and Trizma base (VWR).

Bovine serum albumin (BSA), diaminobenzidine (DAB), and sodium borohydride (Sigma-Aldrich).

2.4.1. Chemicals

Cold water fish gelatin, glutaraldehyde (25% EM grade) and sodium citrate, sodium hydroxide, 1 N (NaOH) (EMS).

Glycerin (glycerol), sucrose, and Triton X 100 (optional; 0.025% for EM incubations) (JT Baker).

2.4.2. Large Equipment

Plexiglass hood dedicated to DAB step containing a balance and DAB waste bucket.

2.4.3. Small Equipment

Beakers (Graduated Griffin, 1 L, 2 L), glass crystallizing dishes, glass petri dishes (9 cm), and pipette bulbs (VWR).

Beem capsules (for portioning DAB power), metal lids of staining dishes, and plastic mesh crucibles (EMS).

Camel and sable hair brushes (Ted Pella Inc).

Modeling clay (place on sides of trays to hold vials during incubations).

Punch tools for marking tissue (Small Parts Inc.).

Rubber mats (black; custom 1/32″ Neoprene; Garlock Rubber Technologies, Paragould, AR).

2.4.4. Disposable Supplies

Biotinylated secondary immunoglobulins (IgG; Jackson ImmunoResearch, West Grove, PA; Vector Laboratories, Burlingame, CA).

Gold (1 nm) conjugated IgG (EMS).

Microtubes (freestanding), microtube screw top lids with assorted colors, pasteur pipettes, 12-well tissue culture dishes, plastic beakers, 50 ml, 100 ml and plastic petri dishes, plastic petri dishes and wooden applicator sticks (VWR).

Silver IntenSE M kit (GE Healthcare, Piscataway, NJ).

Vectastain ABC kit (Vector).

2.4.5. Solutions for Immunocytochemical Processing, Part 1

Immunocytochemical processing is a multiday procedure. For all solutions, use deionized water that is 18.2 mΩ/cm at 25°C. This is especially important for the silver intensification procedure.

Sodium Borohydride Solution (Make Immediately Before Using)

1. Put 100 ml of 0.1 M PB (see Subheading 2.2b), with a stir bar, into a plastic beaker.
2. Add 1 g of sodium borohydride, and stir for 30 s. (Use immediately.)

0.1 M Tris–Saline Solution

1. To make 0.1 M Tris–saline (TS) solution, place about 975 ml dH_2O into a beaker on a stir plate.
2. With the water gently stirring, add 12.1 g Trizma base to the beaker.
3. pH the solution to 7.6 with HCl and bring the volume up to 1 L. Store the solution at 4°C.

0.5% BSA in 0.1 M TS (See Note 1)

Add 0.25 g of BSA to 50 ml of 0.1 M TS solution.

0.1% BSA in TS

Mix 10 ml 0.5% BSA with 40 ml 0.1 M TS.

Cryoprotectant Solution (for Optional Freeze–Thaw)

Mix 100 g sucrose, 40 ml of glycerin, 100 ml 0.2 M PB, and 260 ml dH_2O.

2.4.6. Solutions for Immunocytochemical Processing, Part 2

0.01 M Phosphate-Buffered Saline

1. Add 1 L of dH_2O to a plastic beaker. With the water gently stirring, sequentially add 1.09 g dibasic sodium phosphate, 0.32 g monobasic sodium phosphate, and 9 g NaCl.
2. pH the solution with HCl or NaOH to 7.4.

Washing Incubation Buffer

1. Measure 250 ml 0.01 M phosphate-buffered saline (PBS) into another beaker.
2. As the PBS is stirring, add 1.25 ml 40% gelatin stock and 2 g BSA. (Adjust the volume for the experiment.)
3. Readjust the pH to 7.4.

0.2 M Citrate Buffer

1. Measure 500 ml dH_2O to a plastic beaker. As the water is stirring, add 29.45 g sodium citrate.
2. Adjust the pH with citric acid (4.2 g of sodium citrate in 100 ml dH_2O) to 7.4.

2.5. Materials and Solutions for Embedding Tissue Sections for EM

2.5.1. Chemicals

Alcohol ACS grade, 200 proof.

2.5.2. Large Equipment

EMbed 812 embedding media kit osmium, tetroxide, 4% aqueous solution, and propylene oxide, ACS grade (EMS).

2.5.3. Small Equipment and Nondisposable Supplies

Oven, 60°C (not used for wet incubations).

Coors shallow multiwell white ceramic dishes (Sigma-Aldrich).

Large metal trays (Cole Parmer).

Rectangular steel weights (cold rolled steel type; Small Parts Inc.).

Rotary mixer (R2 model, Ted Pella).

2.5.4. Disposable Supplies

Aclar (Fluoropolymer film 33 C; Honeywell, Morristown, NJ).

Syringes, 30 cc and 60 cc (BD; Franklin Lakes, NJ).

2.5.5. Solutions

Osmium Stock Solution

1. Add 10 ml 0.2 M PB to a scintillation vial.
2. Wrap 10 ml snaptop 4% osmium vial with a paper towel, snap open under the hood, and add the osmium to the scintillation. Gently pipette up and down to mix. Osmium should be a pale yellow color.
3. Wrap the scintillation vial with foil and place in a container protected from light. Store the container at 4°C.

EMbed Resin

1. Remove the plunger of a 30-ml syringe and seal the tip with parafilm. Stand the syringe upright in a glass beaker to prevent it from tipping over. Sequentially pour the following into the syringe: 12 ml EM-bed 812 (to the 12-ml mark); 7.5 ml DDSA (to the 19.5-ml mark); 6.75 ml NMA (to the 26.25-ml mark). Wipe the lids of the bottles with a kimwipe before closing them. With a pipetman, add 600 μl DMP-30.
2. Carefully push the plunger into the syringe until it is secure, leaving a little air in the syringe so that the EMbed resin can mix.
3. Seal over the tip with another piece of parafilm and gently invert the syringe up and down ~ten times until the resin looks homogenous. Place syringe on a rotary mixer for 30 min.

Alcohols

30, 50, 70, and 95% ethanol for dehydration.

EMbed/Propylene Oxide (1:1) Mixture

1. 20 ml Embed and 20 ml propylene oxide in a conical tube.

2.6. Materials for Sectioning Tissue for EM

2.6.1. Chemicals

Acetone, ACS grade (EMS).

2.6.2. Large Equipment

Light microscope.

Ultramicrotome (Ultracut) and glass knife maker (Leica).

2.6.3. Small Equipment and Nondisposable Supplies

Antistatic gun (zerostat 3; Sigma Aldrich).

Beakers (10 ml, glass) and petri dishes (9 cm, glass) (VWR).

Diamond knife (2.7-mm edge), embedding capsule holder embedding molds, fine forceps, glass knife box, grids 400 mesh copper

thin bar, plastic partition box, for storing blocks, silicone pads with grid marks, and super glue (Loctite prism) (EMS).

Eyelash brush (eyelash mounted on a 3-in. wooden applicator stick with nail polish; see Figure 7).

Light box and scissors.

2.6.4. Disposable Supplies

Beem capsules, sand paper, diamond knife cleaning rod, glass knife strips, and extra long razor blades (EMS).

Filter paper, 9 cm and 15 cm (Whatman #1) and syringes (3 cc).

2.7. Materials and Solutions for Counterstaining EM Grids

2.7.1. Chemicals

Lead nitrate, sodium hydroxide (1 N), and uranyl acetate (EMS).

Sodium hydroxide pellets (JT Baker).

Distilled water (for uranyl acetate stain; supermarket).

2.7.2. Small Equipment and Nondisposable Supplies

Dental wax, fine forceps, and grid boxes (EMS).

Kimax media storage bottles, 100 ml (Cole-Parmer).

Glass petri dish (95 mm) with foil-covered lid (VWR).

2.7.3. Disposable Supplies

Luer lock syringe filters, 13 mm, with 0.2 μm PTFE (VWR).

Square plastic petri dish; can be reused (EMS).

2.7.4. Solutions

Before counterstaining, prepare uranyl acetate and Reynold's lead citrate solutions. These can be stored for months at 4°C.

5% Uranyl Acetate Solution

1. Add 5 g uranyl acetate to 100 ml distilled H_2O (*do not* use Millipore deionized water or uranyl acetate precipitates) in a foil-covered 100-ml bottle.
2. Stir for 18–24 h until uranyl acetate is suspended; it does not dissolve completely.

Reynolds Lead Citrate (Important: Do Not Breathe Over the Bottle as This Causes Lead to Precipitate)

1. Add 2.66 g lead nitrate to a 100-ml bottle, add 60 ml dH_2O, close lid, and shake gently for 1 min.
2. Add 3.52 g sodium citrate to the bottle, close lid, and shake gently for 1 min. Shake every 2–3 min for 30 min.
3. Add 16 ml 1 N NaOH to the bottle, bring total volume up to 100 ml with dH_2O (~24 ml), and store at 4°C. Do not use the solution if a white precipitate is visible.

3. Methods

3.1. General Comments

Before initiating any pre-embedding EM immunocytochemical study, one must consider the question that one wishes to answer. The type of question determines the experimental design. The three most common questions are: (1) Which is the cellular (e.g., neurons or glia) or subcellular (e.g., mitochondria, multivesicular bodies) localization of substance X? (2) What is the relationship of cells containing substance X with cells containing substance Y? This would include: (a) Is substance X is colocalized with substance Y? and (b) Do cellular profiles (e.g., terminals) containing substance X contact cellular profiles containing substance Y (e.g., dendrites)? (3) Does the cellular or subcellular distribution of substance X, or the relationship of profiles containing substances X and Y, change after an experimental manipulation (e.g., ischemia, or other processes inducing degeneration, potentially in combination with neuroprotective manipulations)?

To answer questions like those posed above, we have used single- and dual-labeling EM immunocytochemical protocols. Using these protocols, antibodies can be identified using the avidin–biotin complex (ABC) peroxidase methods and/or immunogold methods (10, 11). The ABC method provides a sensitive approach to detecting antigens (12). The immunogold-silver method allows precise subcellular location of an antigen (10). For quantitative comparisons between groups, marked sections from each experimental condition are collected into a single vessel so that they are processed together through all steps. To prevent variability in immunolabeling due to day-to-day temperature variations, different reagents, etc., tissues from several groups are processed simultaneously (13). We and others have found that this is a reliable and reproducible method for quantitative comparisons (13–16).

3.2. Perfusion Fixation Methods

Sesack et al. (17) recently published a detailed perfusion fixation procedure in rats. Thus, the perfusion fixation procedure described here focuses on mice. For an illustration of the perfusion setup, see Fig. 1. All of the steps described below should be performed under a fume hood (minimum air flow 100 cycles/min) with the hood sash pulled down to 12–18 in.

1. *Make solutions*: *0.2 M PB, 0.1 M PB, 2% PFA, 3.75% acrolein*. Make enough 3.75% acrolein in 2% PFA to have about 40 ml/mouse, as well as enough 0.2 M PB to make 0.1 M PB for sectioning (Subheading 3.3).
2. *Prepare the perfusion pump and tubing*. Flush all tubing in the perfusion pump with dH_2O, and then air. The tubing should be arranged so that there are two inputs separated by a stopcock, and one output (see Fig. 1). Place one input branch in the parafilm-covered graduated cylinder containing the acrolein/PFA

solution; use scissors to make a hole in the parafilm. Prime the tubing with the acrolein/PFA to avoid losing solution. Place the output branch into acrolein/PFA cylinder and run pump until there are no air bubbles. Then, turn stopcock to close off the acrolein/PFA branch and collect the remaining acrolein/PFA solution from the output tubing. Flush the other input branch with dH_2O and then load with saline/heparin until no air bubbles are visible. Finally, close the saline line and run about 2 ml of acrolein into the saline/heparin output tube; the saline/heparin is the first 5 ml of solution used in the perfusion.

3. *Organ and blood collection.* In addition to the brain, assessment of other organs can provide insight into the effect of the experimental manipulations. This must be accomplished without compromising the quality and rapidity of the perfusion and brain collection. A checklist can be used to observe the health of the coat, eyes, nose, and genitals of the animals. Organs from the thoracic and abdominal cavity can be collected after perfusion fixation for histology, immunocytochemistry, and in situ hybridization. In particular, organ weights can be a bioassay of the degree of stress and immune responses that have occurred (see Note 2).

   Blood collection must be performed rapidly to not compromise fixation of the brain. To minimize the time needed for blood collection, calculate in advance the minimum blood volume necessary for analysis. To collect blood before beginning the perfusion, open the thoracic cavity as usual and remove the pericardium. Hold the heart with forceps, and insert a heparinized 20-gauge needle and 1-ml syringe into the right ventricle at a 1–2-mm depth. Release the pressure on the forceps slightly and slowly draw the blood from the ventricle. Volumes between 0.2 and 0.5 ml can be drawn with minimal hemolysis.

4. *Perfusion procedure.* To begin the perfusion, deeply anesthetize the mouse (we use 150 mg/kg, i.p., sodium pentobarbital, about 0.1 cc per mouse). Check that the animal is deeply anesthetized by pinching the paw to test for a reflex. Place the mouse chest up onto the silicon pad and pin the paws. Lift the chest and cut the skin under the diaphragm without injuring the underlying organs. Cut through the diaphragm and through the skin and ribs on each side. Cut the rib cage off without cutting the thymus. Cut the right atrium and grab the heart with the hemostatic forceps. Insert the needle in the left ventricle without puncturing through. Adjust the pump to a speed of about 9 ml/min and then pull the hood door down as far as possible. Perfuse transcardially a total of 35 ml.

5. *Brain removal.* When the perfusion is complete, stop the perfusion pump and remove the needle from the heart. Turn the animal over and drain out any fixative in the carcass. Remove the eyes (this makes it easier to remove the brain later) and then

decapitate the carcass. Using rongeurs, remove the skull from the region overlying the brainstem. Continue to remove the skull in small pieces from the top and sides of the brain; do not insert the rongeurs so deep that you nick the brain. Place the brain in a scintillation vial containing about 5 ml of 1.87% acrolein/2% PFA (the perfusate diluted in half with 2% PFA) and place the vial on a shaker for 30 min at room temperature (the shaker can be outside of the hood). Under the hood, decant the fixative into the perfusion tray and rinse two times with 0.1 M PB. Store the brain in 0.1 M PB at 4°C until it is cut (no more than 2 days). Flush the perfusion tubing with dH_2O.

3.3. Sectioning the Brain

1. *Preparation for sectioning (can be done the day before perfusion).* Make color-coded 24-well culture plates for each animal; mark the lid and base of each tray with colored tape. Label the top of the tray with all pertinent information (date of the experiment, animal information, experimental manipulation, investigator's name, other relevant information). Fill each well of the tray with cold 0.1 M PB and keep refrigerated until needed. Prepare a color-coded experimental data sheet (sheet for 24-well dishes *provided upon request*) to accompany each tray to record cutting and experimental information. Finally, tape one long piece of tape (about 18 in.) per animal to a bench top. Label this tape with the pertinent information. The tape is used to seal the tray before it is put in the freezer.
2. *Set up the vibratome for cutting.* Check knife holder, buffer tray, and other moving parts for buffer residue, and rinse with dH_2O. Turn on the vibratome, lower the buffer tray holder all the way down, and retract the knife holder mechanism all the way back. Set the speed between 6 and 9 and the frequency between 7 and 9. Set the section thickness to 40 μm.
3. *Blocking the brain.* Pour the brain out of the scintillation vial into a weigh boat. Do not use forceps to remove the brain as this often damages it. Place the brain ventral side up into the brain mold for blocking. Cut the brain into two or three blocks (no more than 5-mm tall) with razor blades. We usually block the brain behind the hypothalamus (see Fig. 2) so that brain is in two pieces, which are glued side by side and cut simultaneously on the vibratome. However, if you are interested in studying the ventral tegmental area or dorsal raphe, block the brain appropriately to preserve these regions.
4. *Securing the brain for slicing.* Place the specimen disc on the table and orient it so that the notch is at the 7 o'clock position. Melt the agar solution in a microwave (if you overheat it, pipette the solution up and down to cool it off; hot agar affects antigenicity). Place the blocked brain on a paper towel, and blot the part of the brain that is glued until it is completely dry on the bottom. Place one or two drops of glue in the middle of

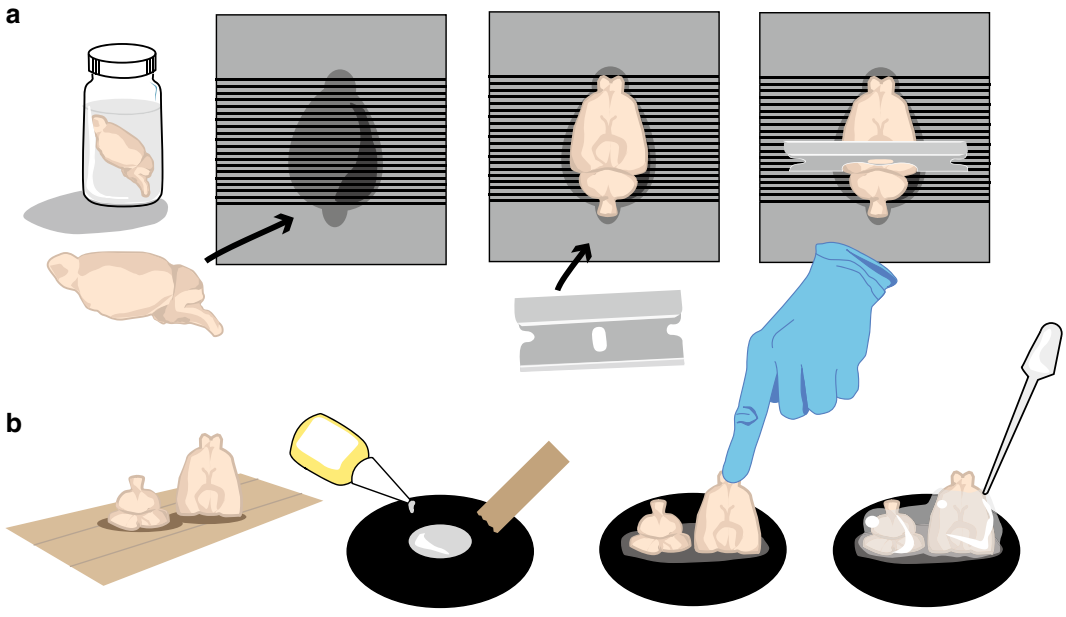

Fig. 2. Sectioning the brain on the vibratome. (**a**) Blocking the brain (Subheading 3.3, step 3). (**b**) Securing the brain for slicing (Subheading 3.3, step 4).

the stage and spread glue into a thin layer using the cardboard cover from a razor blade. Place the brain pieces close together on the glue spot, orienting them so that the cortex on the forebrain piece and ventral surface of the brainstem piece are placed away from you. Gently touch the tops of brain pieces to make sure that they are adhered completely to the specimen disc. This step is critical since the brain does not cut well if it is loose. Using a plastic pipette, surround the brain pieces with the warm agar solution.

5. *Preparing the vibratome for slicing.* Position the appropriate color-coded 24-well culture dish with chilled PB next to the vibratome and over a black background. Have the color-coded data sheet on hand. After the agar is solidified (about 1 min), place the stage with brain pieces into the buffer tray. Place the buffer tray on the tray holder. Lift the buffer tray up slightly on the holder before locking the buffer tray in place, so you do not hit the bottom of the vibratome range while sectioning. Gently pour cold PB into the buffer tray until it covers the brain.
   Eject a vibratome razor blade from the holder onto a paper towel. Squirt a small amount of 100% ethanol on the blade and gently dry it off with a kimwipe without touching the sharp edge. Put the blade in the knife holder and tighten into place. Place the knife holder in the vibratome, being careful not to hit the brain. Move the magnifying glass over the brain and adjust the position of the fiber-optic lights so that the brain is illuminated (for GFP mice, dim the lights). Move the razor blade toward

the brain with the up/down and forward/reverse knobs. Once the blade is about 2 mm above the brain, set the limits of the sectioning window.

6. *Sectioning.* Set the stroke on "continuous" and cut through the brain. Immediately after each section is cut, pick it up with a brush and place it systemically in a well of the tray. When you reach the end of the tray (e.g., well 24), mark the sheet and then start over at well 1. Repeat these steps until brain is cut as close to the stage as possible (about six passes for a full mouse brain). As you are cutting, make sure that the sections are cutting evenly and not tearing. Periodically, you need to hit the pause button and pull off any dura, blood vessels, or meninges that are sticking to the sides of the brain with a forceps. Adjust the size of the continuous sectioning window as needed.
7. *Storage of tissue sections.* Clean up vibratome and sectioning area. Once the sectioning is complete, note the well that contains the last section on the data sheet. Place the tray in a cold room or refrigerator until you are ready to add the storage solution. This should be done within a few hours of sectioning; do not leave sections overnight in PB as this severely diminishes antigenicity. To prepare the trays for storage at –20°C, pipette off PB completely a few wells at a time (without drying out the sections) and quickly add storage solution until the wells are about half full. Remove all PB or the sections freeze in storage and ruin the tissue. Once all the wells have been transferred to storage solution, seal the edges of the tray with the 18-in. color-coded, labeled tape that you placed earlier on the bench top. Let the sections sit in the storage solution for 15–30 min in the cold room before placing them flat in the –20°C freezer. Sections can be kept for at least 5 years in storage without any apparent loss in antigenicity or compromise to morphology. Sequential storage in 24-well plates allows for the selection of stereologically unbiased random systematic series of sections through a given region for analysis. For instance, selecting the tissue in one well provides a random systematic 1-in-24 series.

3.4. Immunocytochemical Processing, General Comments

For studies comparing different experimental manipulations, sections from all groups must be processed for immunocytochemistry simultaneously (13). For EM studies, a minimum of three sets of tissue should be used; each set should contain sections from the brains of animals from each experimental manipulation. The sections should be punched with different codes so that they can be pooled into single containers (see Fig. 3). Tissue from up to six different animals can be pooled into a single container.

The two-part dual-labeling EM protocol is described below: the first part involves preparing the tissue for incubation with the

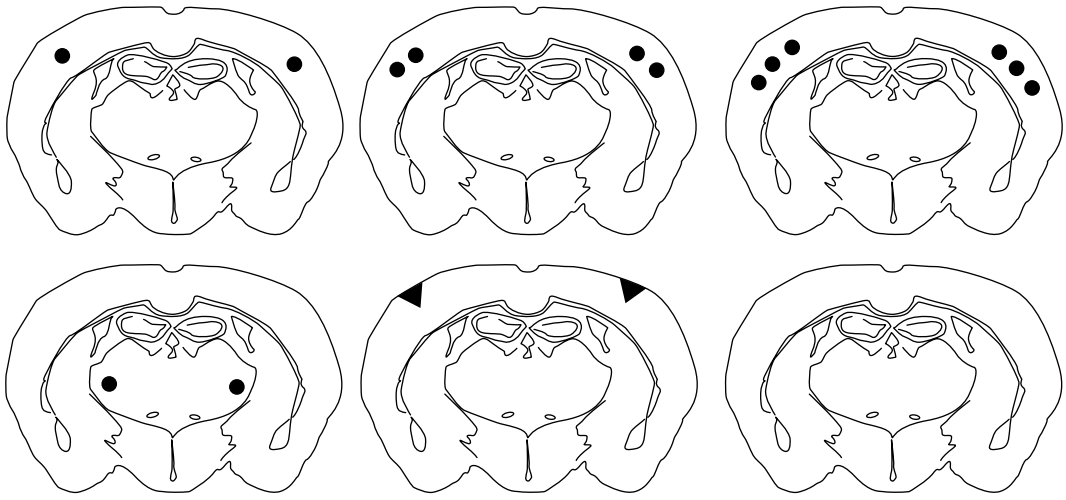

Fig. 3. Immunocytochemical processing procedures: Tissue selection. Examples of punch patterns used to distinguish groups within a crucible (Subheading 3.4).

primary antibody(s) and the second part involves secondary antibody(s) incubation and peroxidase and/or immunogold-sliver processing. Single-labeled studies can also be done for either peroxidase or immunogold-silver by processing the tissue through the appropriate sections of the dual-labeling protocol. For all steps, do not let the sections dry out when transferring them between solutions. Unless noted, all steps are carried out at room temperature with solutions at room temperature. All incubation steps and rinses are conducted on a shaker. Delicate pieces of glassware (e.g., crystallizing dishes, petri dishes, crucibles) should be cleaned in 10% Contrad solution.

3.5. Immunocytochemical Processing, Part 1

1. *Tissue selection*. First, select tissue from the brain region you wish to investigate. Remove the trays containing the stored tissue from the –20°C freezer. Gather divided petri dishes (one per tray), data sheets, "punch" dish (i.e., petri dish lined with black rubber or black electrical tape), punch tool, paint brushes, crucibles, and crystallizing dishes and make at least 1 L of PB. Sort the trays into the appropriate groups. Select tissue from one group at a time to avoid confusion. For each group of tissue, dry off the trays and carefully remove the tape sealing them. Save the long tape to reseal the tray later. Put PB into each quadrant of the divided petri. Place one petri dish in front of each tray. Fill crystallizing dishes with about 100 ml of PB (up to the bottom of the white label) and set aside. Place the tissue from the desired well into the petri dish and put the selected brain section into the adjacent division of the petri dish. Continue the selection process; each set has tissue selected

from one animal per experimental condition. Note removed sections on the data sheet. Place tissue sections from each animal into the dish lined with black rubber and mark them with a pattern (see examples in Fig. 3) using the hole-punch tool. Record your punch codes. Once all sections from one set are marked, pool them together in a color-coded crucible placed in a crystallizing dish containing PB.

2. *Removal of unbound aldehydes with sodium borohydride.* Once crucibles from all sets of animals are in the crystallizing dishes, unbound aldehydes are removed with sodium borohydride. For this, place the crystallizing dishes with crucibles on a shaker that is moving slowly. Mix the sodium borohydride solution, put it in a second crystallizing dish, transfer the crucibles into the sodium borohydride solution, and incubate on the shaker for 30 min. Gently squirt solution over the tissue during the incubation. At the conclusion of the incubation, rinse copiously with several changes of PB until there are no bubbles, and the sections sink in the crucibles. One technique is to lift the cups out of the dish and pour PB through them while gently swirling. For all immunocytochemistry steps, watch for lost sections.
3. *Optional freeze–thaw.* To enhance penetration of certain antibodies, sections can be quickly frozen and thawed (method adapted from Yoland Smith, see ref. 17). Place crucibles in a crystallizing dish containing cryoprotectant solution and incubate for 20 min at room temperature. Transfer each crucible to a 50-ml plastic beakers containing about 15 ml cryoprotectant solution, cover beakers with parafilm, and place in the −80°C freezer for 20 min. After the −80°C incubation, remove the beakers from the freezer and place them in crystallizing dishes with warm or room-temperature water. When the cryoprotectant begins to melt and the crucibles are free (less than 5 min), transfer the crucibles to a crystallizing dish with room-temperature cryoprotectant solution for a 10-min incubation. During this incubation, prepare 70, 50, and 30% cryoprotectant diluted in 0.1 M PB. Incubate crucibles for 10 min in each, and finally wash for an additional 10 min in 0.1 M PB.
4. *Rinse.* Transfer crucibles to crystallizing dish with TS. Rinse twice, 5 min each.
5. *Blocking step.* To block nonspecific staining, incubate the tissue in TS containing 0.5% serum. We use BSA to avoid cross-species reactivity with primary and secondary antibodies. However, normal goat or rabbit serums can be used instead. Add 0.5 g BSA to 100 ml TS in a plastic beaker and stir slowly. (You need about 100 ml per large crystallizing dish and 50 ml per small crystallizing dish.) Be careful not to overstir, as this cause the BSA to froth. Place the crucibles in the crystallizing dish with BSA solution and shake gently for 30 min.

6. *Rinse.* Transfer crucibles to crystallizing dish with TS. Rinse 2×, 5 min each.
7. *Make up primary antibody solutions during the rinses.* Primary antibody aliquots are diluted in 0.1% BSA in TS. For some antibodies, the addition of 0.025% Triton-X-100 to the 0.1% BSA in 0.1 M TS allows greater penetration of the antibody into the tissue. (This works best for nonmembrane-bound antigens.) To make the 0.025% Triton solution, make 0.25% Triton first and then dilute it. For factors to consider when working with antibodies and suggestions for antibodies to identify degenerating processes, see Note 3.
   Mix up the total volume of primary antibody solution in one vial and divide if necessary. Using a pipetman, add the primary antibody(s) to the diluent and mix gently. Allow 2 ml of primary antibody solution per vial. This amount should be adjusted depending on the amount of tissue being placed in the vial and the availability of the antibody.
8. *Begin the primary antibody incubation.* Add tissue sections to the vials with a clean paintbrush (rinse bristles with hot water between different antibodies). Cap each vial and gently swirl the sections to be sure that none are stuck to the sides of the vial. Press the vials into a clay-lined staining dish lid at about a 10° angle. Place the tray with the vials on a shaker table and adjust the speed of the shaker so that the sections are slightly moving. (If the shaker is too fast, the sections will break apart or even dissolve.) After 20–24 h, move the tray with the vials to a shaker table in the cold room. Continue incubating the sections in the antibody diluent for 1–4 days (times vary by antibody).

3.6. Immunocytochemical Processing, Part 2

Peroxidase Labeling Procedure (Modified from Hsu et al. (11))

1. *Remove sections from primary antibody and rinse in TS.* Using a clean brush, transfer the tissue to appropriately labeled crucibles in TS in a crystallizing dish. Alternatively, swirl tissue in the scintillation vial and immediately dump the contents into a crucible over a waste beaker. Check for missing sections, and rinse tissue in TS 3×, 10–15 min each.
2. *Prepare secondary biotinylated antibody.* (The secondary antibodies should be aliquoted (25 μl) into plastic screw-top tubes and stored at −20°C or lower until use.) In a scintillation vial, dilute the secondary antibody to 1:400 with 0.1% BSA in TS (25 μl for each 10 ml). Mix the solution gently and aliquot into scintillation vials. Usually, 2 ml per vial is sufficient but should be adjusted depending upon how many tissue sections are there.
3. *Secondary biotinylated antibody incubation.* Using a brush, transfer the tissue to the scintillation vials containing the

secondary antibody. Place the scintillation vials on the tray in the shaker for 30 min.

4. *ABC solution preparation.* When the tissue is in the secondary antibody, prepare the ABC solution from an Elite Vectastain kit. Add two drops of solution A and two drops of solution B to each 10 ml of TS (no serum!) and mix *immediately* by vortexing or rapidly pipetting up and down. Let the solution sit undisturbed for 30 min prior to use.
5. *Rinse in TS.* At the conclusion of the secondary antibody incubation, transfer tissue to crucibles in TS in a crystallizing dish. Wash the tissue in TS 3×, 10–15 min each.
6. *ABC incubation.* Aliquot the ABC solution into scintillation vials, allowing about 2 ml/vial. Transfer the sections to vials and place the vials on a tray on the shaker. Incubate the sections in ABC solution for 30 min exactly. Time this carefully; overincubation in the ABC solution increases the background peroxidase labeling.
7. *Defrost DAB aliquots during ABC incubation.* (We weigh out the DAB and place it into Beem capsules. The capsules are stored in a jar with desiccant in the –20°C freezer.) Defrost for 30 min; protect aliquot from light during thaw using a foil-covered beaker.
8. *Rinse in TS.* Once the ABC incubation is complete, transfer the sections to crucibles in TS in a crystallizing dish. Wash the tissue in TS 3×, 10 min each. Prior to the last wash, replace the crucibles with clean ones. Otherwise, you get ABC–DAB precipitate on your sections.
9. *Prepare the DAB solution under the hood during the last rinse.* DAB is carcinogenic and should be handled with gloves. All liquid and solid DAB waste should be collected and disposed of according to institutional guidelines. The first TS rinse after the DAB step should be considered DAB waste. To make the DAB solution, get 100 ml TS gently stirring in a disposable beaker. Carefully open a capsule containing DAB (22 mg) and release the DAB; then, drop the capsule into the solution. Use a stir bar dedicated to DAB (we use a yellow one). DAB is light sensitive, so cover the beaker with a larger foil-covered beaker. Just before using the DAB solution, add 10 µl of 30% H_2O_2 with a pipetman. Drop the pipette tip into the solution.
10. *DAB peroxidase labeling procedure.* Move the crystallizing dishes containing the crucibles under the DAB hood. Lay disposable plastic petri dishes (one per four crucibles) next to the crystallizing dishes. Pour the DAB solution into the petri dishes, place the crucibles into the DAB solution, and start the dedicated DAB-hood timer counting up. This can be done sequentially at timed intervals or four crucibles at a time. As

soon as the crucibles go into the DAB solution, start gently squirting the solution over tissue using a plastic disposable pipette. Continue squirting the tissue with the DAB solution until the conclusion of the incubation. The dilution of the primary antibody should be such that the optimum time for the tissue in the DAB solution is 6 min. However, DAB times can vary from different experiments due to room temperature, age of the ABC kit, or antibody concentrations. Thus, the reaction should be checked by wet mounting a section on a slide and looking at it under a microscope. The section can then be put back in the DAB solution and incubated longer if necessary.

11. *Rinse in TS.* Once the DAB reaction is complete, wash the tissue in TS 3×, 2 min each. Be sure to discard the first rinse as DAB waste.
12. *Rinse in PB.* Transfer the crucibles to PB and rinse 3×, 5 min each. If you are proceeding directly to the embedding step (3.7), be sure and rinse sections thoroughly in PB. Saline contamination ruins the osmium reaction.

Immunogold-Silver Labeling Procedure

1. *Rinse in 0.01 M PBS.* Wash the tissue in 0.01 M PBS for 5 min.
2. *Washing incubation buffer.* Place the crucibles containing the tissue sections in the washing incubation buffer solution on shaker for 10 min.
3. *Prepare gold-conjugated secondary.* While the tissue is incubating in the washing buffer, prepare gold-conjugated IgG solution. For this, add 20 μl IgG per ml incubation buffer (i.e., 1:50 dilution) in a scintillation vial. Two milliliter per vial is usually ideal.
4. *Gold-conjugated secondary incubation.* Using a brush, transfer the tissue into the scintillation vials containing the secondary antibody. Incubate the tissue for 2 h in the secondary antibody. During the incubation step, bring the silver intensification kit to room temperature on the bench top.
5. *Washing incubation buffer.* Transfer the tissue into crucibles in a dish containing the washing buffer. Wash the tissue in buffer 1× for 5 min.
6. *Rinse.* Wash tissue in 0.01 M PBS, 3× for 5 min.
7. *Glutaraldehyde incubation.* Under the fume hood, place the crucibles in a crystallizing dish containing 2% glutaraldehyde in 0.01 M PBS for 10 min. (To make the glutaraldehyde solution, add 1 ml 25% EM grade glutaraldehyde to 12.5 ml 0.01 M PBS.) Agitate the crucibles periodically; the tissue does not need to be on a shaker.
8. *Rinse in 0.01 M PBS.* Wash tissue in PBS for 1×, 2 min.
9. *Rinse in 0.1 M PB.* Wash tissue in 0.1 M PB for 2×, 2 min.

10. *Citrate buffer*. Transfer the tissue to a crystallizing dish containing citrate buffer and place the dish on a bench top next to the silver kit.
11. *Silver intensification procedure*. This step is tricky (see Fig 4). Do not use brushes to transfer tissue to and from the silver solution. Instead, use pointed sticks made by breaking applicator sticks in half (use your hands to break the sticks not razor blades as metal contaminates the silver solution, see Fig. 4a). Open a *sterile* 12-well plastic petri dish and fill the top and bottom row with 0.2 M citrate buffer. When you are ready to begin, place ten drops of reagent A followed by ten drops of reagent B (i.e., a 1:1 ratio) into one well in the center row. This solution can be used at least once. Watch for precipitate that appears as a silver film on top of the solution. Work on a dark surface so that you can watch for this precipitation and do not use a well that has precipitated.
    Test one section from each antibody condition first. Using the stick, transfer test section to intense solution. With the tissue culture plate flat on the bench top, gently swirl the plate so

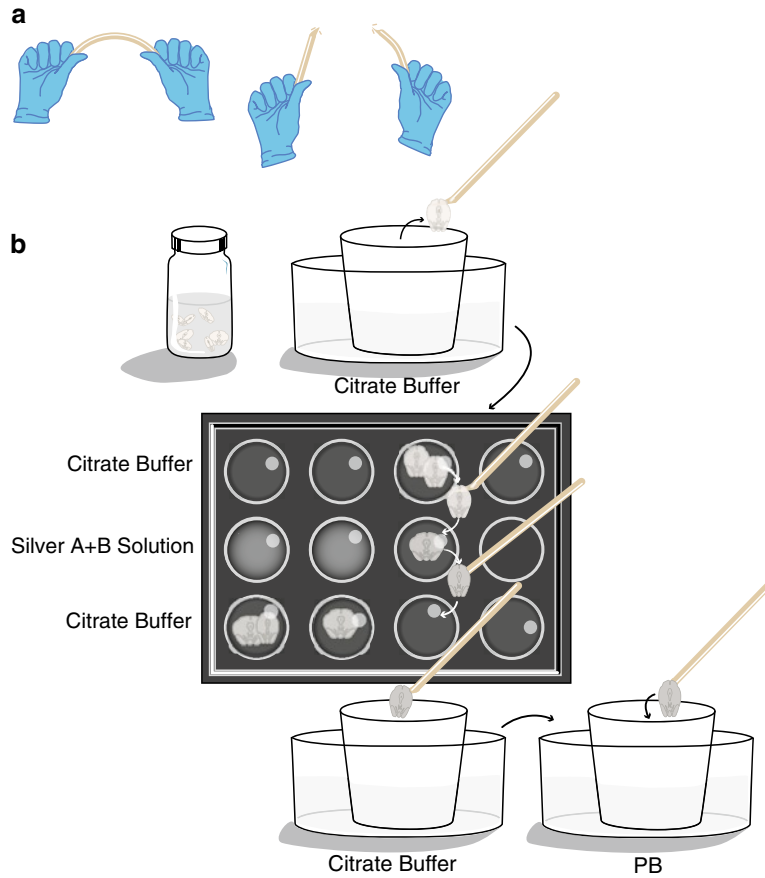

Fig. 4. Immunocytochemical processing procedures: Silver intensification. (**a**) Breaking wooden applicator sticks. (**b**) Silver intense procedure (Subheading 3.6, part 2 "immunogold-silver labeling procedure"steps 11–13).

that the section is constantly moved in the intense solution during the entire incubation step (usually, 4–7 min). To stop the intensification reaction, transfer the tissue citrate buffer, and note the length of the intensification. Wet mount the test section on a slide and exam it under the light microscope. Ideally, you should see small black dots (barely visible) over the cells that you expect to be labeled. If you intensify too long (over 8 min), nonspecific gold particles appear over the entire tissue section. If needed, the test section can be placed back in the silver solution to increase the incubation time. Once the right incubation time has been identified, repeat the procedure with the rest of the sections.

12. *Transfer tissue back to citrate buffer*. After all of the tissue sections have undergone silver intensification, use a wooden stick to transfer tissue to the crucibles in a crystallizing dish containing citrate buffer.
13. *Rinse in 0.1 M PB*. Transfer crucibles to 0.1 M PB for 3× 10-min washes. Saline contamination in the PB ruins the osmium reaction.

3.7. Embedding Tissue Sections for Electron Microscopy

All osmication and embedding procedures should be performed under a fume hood. Glassware contaminated with osmium should be deactivated with 70% ethanol prior to removing it from the hood. All liquid and solid osmium waste should be collected and disposed of according to institutional guidelines. Glassware used for EM embedding should be cleaned with Contrad.

1. *Separate tissue according to animal and/or group before beginning osmication*. Sections may crack or break during this step, making it impossible to separate groups. Label Coors dishes with tape to keep animals and/or groups separate. Place under the hood, add a small amount of PB into each well, and put two to four sections in each well. It is okay if the sections overlap, but it is important to unfold and flatten the sections in each well with a brush.
2. *Osmium incubation*. Slowly draw off the PB from each well with a glass pipette and replace with 2% osmium tetroxide in PB stock solution using another glass pipette. If necessary, use wooden applicators that have been broken in half to form a point to manipulate sections. Protect Coors dishes from light (we use the bottoms of plastic food storage containers that have been covered with foil) and incubate the sections for 1 h undisturbed.
3. *Embed preparation*. While the tissue is incubating in osmium, prepare the EMbed resin.
4. *PB rinse*. After the 1-h osmium incubation, uncover the Coors dishes, remove the osmium, and immediately add PB with a fresh pipette. Place the used osmium solution, as well as the

first PB rinse, in osmium waste. Rinse sections with PB, 3× 3 min each.

5. *Dehydration*. Dehydrate sections through 30, 50, 70, and 95% ethanol, 5 min each. Remove PB and add the alcohols with fresh glass pipettes, one row at a time, moving quickly so that the tissue does not dry out. Transfer the sections to scintillation vials preloaded with about 5 ml of 100% ethanol. (The 100% ethanol should be from a bottle opened within 1 week, as opened bottles can absorb moisture from the air.) From this point on, replace solutions one vial at a time and keep the vials sealed. Any moisture in the solutions can ruin the hardening of the EMbed resin. If the room is above 40% humidity, use a dehumidifier.

   Incubate the sections in two changes of 100% ethanol, 10 min each. Then incubate the sections in two changes of propylene oxide, 10 min each. During the incubation steps, dilute the EMbed resin 1:1 with propylene oxide in a conical tube and invert to mix.

6. *EMbed/propylene oxide (1:1) mixture*. Working one vial at a time, remove all propylene oxide with a fresh glass pipette and add the 1:1 mixture to the vials. Try to keep the sections flat in the vials, as they are very fragile. Once the EMbed/propylene oxide mixture is added, gently swirl the vials so that the sections are suspended in the solution. Place vials on a slowly rotating mixer. Incubate the sections in the EMbed/propylene oxide mixture overnight at room temperature. The rotating mixer can be on the bench top.

7. *EMbed incubation*. The next day, prepare fresh EMbed (5–10 ml per vial). One vial at a time, replace the EMbed/propylene oxide with straight EMbed. Be sure and pull off all the EMbed/propylene oxide since propylene oxide can affect EMbed hardening. Gently rotate the vials by hand to suspend the sections in the EMbed. Replace the vials on the rotary mixer and allow the tissue to incubate in the EMbed for 2–4 h. (Do not go longer than 4 h as this makes the sections brittle and the EMbed does not cure properly.)

8. *Preparation for flat embedding*. While the sections are incubating in the EMbed, prepare for the flat embedding step. For this, clean the metal trays and with 100% ethanol. Forcefully wipe the Aclar sheets with 100% ethanol (this helps the Aclar peel off the Epon later) and let dry completely. Break wooden applicator sticks so that they form sharp points. Put the trays on blue pads under a hood. Set up a box of kimwipes, cotton applicators, and a plastic bag to collect waste near the trays.

9. *Flat embedding*. One vial at a time, tilt the vial and use the stick to remove the sections from the vial (see Fig. 5). Gently wipe off the EMbed from the sections on the lip of the vial as you

remove them. Place the sections about 1 cm apart on an Aclar sheet. Gently touch the tops of the sections with a cotton applicator or kimwipe to soak up excess EMbed. Once the sections from one vial are on the Aclar, slowly cover them with a smaller Aclar sheet. Use small pieces of Aclar as it is expensive. Once the sections are covered, gently roll your finger on each section several times to squeeze out EMbed and push the air bubbles off the sections. (Do not push too hard or the sections break). Try to squeeze out as much EMbed as possible as this makes the subsequent ultrathin sectioning much easier.
Place pieces of colored tapes identifying the tissue from a particular vial near, but not on top of the Aclar cover. Do not use marker as it is dissolved by any EMbed resin that oozes out of the cover. Place steel weights on top of the sections. Bake the sections in a 60°C oven for between 3 and 5 days.

10. *Storage of flat-embedded tissue*. Remove the tray from the oven and let cool a few minutes before removing the steel weights. To remove the weights, hold the top Aclar sheet in place and

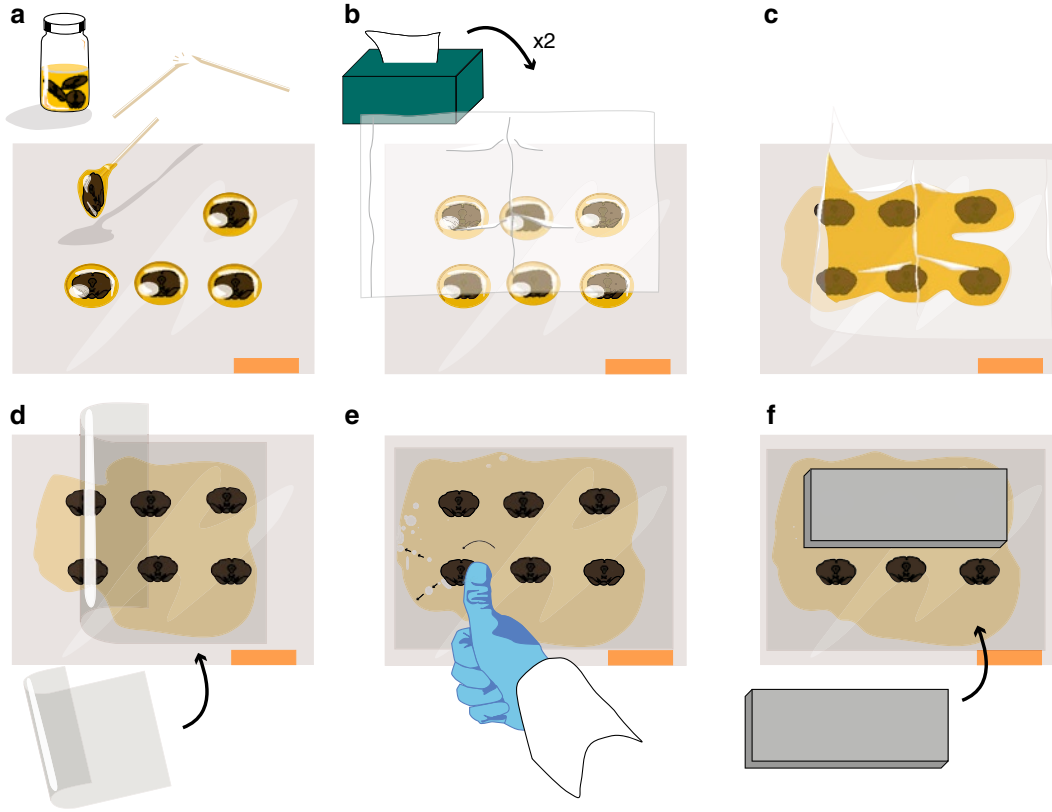

Fig. 5. Embedding tissue sections for electron microscopy: Flat embedding. (**a**–**f**) Sequence of steps for flat embedding (Subheading 3.5, step 9).

carefully lift the weights off. If the weights stick, gently rock them back and forth to dislodge them. Do not pull the weights off too quickly; this can pull the Aclar sheet and the sections off. Store the flat-embedded tissue in envelopes labeled with all the pertinent experimental details. The flat-embedded sections can be stored indefinitely.

3.8. Sectioning Tissue for EM

The ultratome should be on an anti-vibration table. The table should be in a room that has 30–40% humidity, little foot traffic, and away from direct ventilation. All items that contact the EM sections should be as clean as possible. The steps described below do not have to be performed on the same day. However, before preparing large quantities of tissue to be examined under the electron microscope, examine a test section to insure that the morphology and immunocytochemical labeling are satisfactory. Detailed instructions on use of the ultratome as well as videos can be found on the Leica Web site (http://www.leica-microsystems.com).

1. *Preparing EMbed chucks.* Make EMbed resin. We make two types of EMbed chucks. For the first, cut out preprinted labels (laser-printed text on white paper) and place them label side down in embedding molds. Fill the molds with EMbed and place in a 60°C oven until they are set (about 2 days). For the second, cut off the pointed ends of Beam capsules, close the lids, and place lid side down in an embedding capsule holder. Insert preprinted labels, label side out, into the top of the beam capsule (wrap the label around a syringe top to insert). Fill the capsules with EMbed, let them sit about a half hour to allow the bubbles to rise to the top, and bake at 60°C for 3–4 days. For both methods, do not take the capsules out until they are hard; they cannot be rebaked once they have cooled. Prior to gluing tissue on the blocks, remove the chucks from the molds and lightly sand the tops (do this under the hood).
2. *Mounting sections on EMbed chucks.* Select the region of the desired region of the brain section for EM analysis using a light microscope. Mark the section with a marker. Slowly peel one of the Aclar sheets off the "flat-embedded" sections. The sections may stick to the cover, bottom, or both sides of the Aclar. (Make note of which side they stick.) Lay the "flat-embedded" sections, sanded EMbed chucks, forceps (use a pair dedicated to gluing), and long razor blade on a light box (see Fig. 6). (Place the peeled EMbed/section side down.) Use the end of the razor blade to cut an irregular four-sided shape (about 2–3-mm wide) containing the desired tissue piece out of the flat-embedded tissue. (This way, if the tissue flips over, you can tell which side is up). Put a drop of glue on the EMbed chuck and thin it out with a piece of cardboard. Take the forceps and

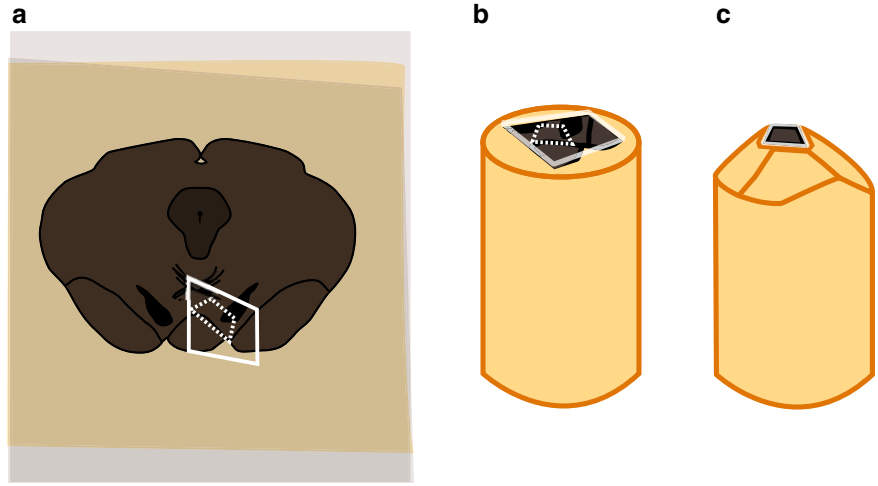

Fig. 6. Sectioning tissue for EM: Mounting sections on EMbed chunks (Subheading 3.6, steps 1–4). (**a**) Marking a trapezoid. (**b**) Gluing tissue to block. (**c**) Trimming tissue blocks on the ultratome

pick up the tissue section and place it on the glue (make sure that the EMbed side of the tissue, not Aclar, apposes the glue). If the Aclar falls off during the gluing process, do not touch the tissue surface with the forceps while placing it on the chuck. Use a light microscope to check that the glue completely surrounds the tissue. If not, add a little glue to the sides of the tissue. Do not worry if glue gets on top of the block; this comes off when the Aclar is peeled off later. Tissue blocks can be stored indefinitely.

3. *Cleaning grids and collection supplies.* Pour grids into a 10-ml beaker that contains about 2 ml of acetone. Swirl the grids around about ten times and then tilt the beaker so that the grids clump together. Carefully pick up the grids with forceps and lay them on two pieces of #1 filter paper in a glass petri dish. To prevent the grids from moving around due to static, tape a piece of filter paper on the top of the petri dish and moisten it with water. This is critical if the air is dry, e.g., in the winter. Next, take a rubber grid mat, squirt it with 100% ethanol and wipe it off with a paper towel (kimwipes leave lint), and place inside another clean petri dish. Pour about 10 ml of 100% ethanol in a scintillation bottle; this can be reused for several weeks. Prior to cutting and in between specimens, dip the eyelash brush and forceps in the 100% ethanol.
4. *Trimming tissue blocks on the ultratome.* Examine the block and glued section by placing it in the ultratome block holder under the microscope to check that the section is glued. If there are air bubbles under the tissue, add more glue and let dry. Draw (or photograph) the tissue piece in a logbook. Note distin-

guishing features, like blood vessels, cracks, or axon fields. Take the block and secure it in the ultratome block holder. Wear a disposable mask covering your nose and mouth for trimming process. Trim the block to a 1–1.5-mm long and 1-mm wide trapezoid shape (this shape helps orient the tissue on the electron microscope; Fig. 3.6). Do not touch the top of the block with your fingers while you are trimming; this can introduce contamination into the electron microscope later. If the Aclar piece covering the tissue falls off during trimming, gently push it off with the forceps before finagling your shape. Use a fresh razor blade to insure nice, clean edges on the tissue section. The top and bottom of the trapezoid should be parallel. If you are not going to section the block immediately, make the trapezoid slightly larger and do the final trim immediately before you section (otherwise, the sections may not form a ribbon during cutting).

5. *Aligning tissue block with the ultratome knife.* Immunoreaction products usually penetrate about 1–2 μm into a tissue section. Thus, ultrathin sections should be collected from the surface (i.e., tissue–plastic interface) of the vibratome section. The trapezoid should be aligned so that the block face hits the knife evenly during the sectioning procedure. Place the block in the specimen arm, and place a disposable glass knife in the knife holder. This knife can be reused several times. Illuminate the block with the top light only. Place the knife about 2 mm away from the face of the block. Using the shadow from the knife on the block face to guide you, align the left and right sides of the blade until they appear parallel to the block face. Do not touch the up and down control yet. Once the left/right alignment appears parallel, retract the knife 2 or 3 mm and move the block up and down with the handwheel. Using the shadow, get the top and bottom to look equidistant from the knife. Move the knife a little closer and repeat these steps until the shadow of the knife on the block face looks even at all points. If you saw a thick layer of EMbed on top of the tissue while you were trimming the block, cut 1 or 2 μm off the top of the block with the glass knife. This saves time later. If not, back up the knife holder about 2 cm from the block face. Remove the glass knife and replace it with the diamond knife.
6. *Cutting tissue sections.* Illuminate the block with both the top and bottom lights. Move the knife edge within about 0.5 mm from the block face. Fill the knife boat with dH_2O, adjust the water level so that it is just below the knife edge (the water should have a light silver reflection), and let sit for 5 min. Set the cutting window and then approach the block 1 μm at a time until you are as close as possible to the knife without taking a section (do not cut 1-μm sections with the diamond knife as this dulls it quickly). Decrease the approach interval to

Fig. 7. Sectioning tissue for EM: Collection supplies. Eyelash brush made for collecting thin sections (Subheading 3.6, step 7).

0.5 μm, then 0.4 μm, etc. until you cut the first section. At this point, turn on the automatic sectioning button (start with 70-nm thick) and allow the ribbon of sections to float in the boat (the sections should be of a silver or silver/gold color). Keep cutting until the vibratome ridges are no longer apparent (if the embedded vibratome sections are flat, this should be about 20 sections). Turn off the automatic sectioning button and place the block face below the knife edge. The tissue sections should stick together to create a ribbon. As the ultratome cuts, use the eyelash brush (see Fig. 7) to position the floating sections for collection.

7. *Collecting thin tissue sections.* Set up your workspace with one petri dish containing the acetone-cleaned grids, another dish with the rubber tissue mat, two pieces of 9-cm #1 filter paper, several more pieces of filter paper cut into one-eighth wedges, a pair of clean sharp forceps, and an eyelash brush. Pick up the most superficial sections first (the ones farthest from the knife-edge). Hold the forceps in your dominant hand and the eyelash brush in the other. Pick up a grid by the edge with the forceps, and hold it dull side up. Bring the grid to the knife boat and gently break the surface tension to submerge it in an area of the boat away from the sections you wish to collect. Once the grid is under the water, orient the grid about 15° from vertical, with the dull side facing up. Slowly move it under the floating sections using the eyelash brush to move the sections if necessary. Hold the sections in place with the brush and lift the grid up out of the water, maintaining the angle of the grid. As the top of the grid emerges from the water, adjust the angle of the grid to catch your sections. Remove the grid to the stacked filter paper, and blot gently on the bottom. Use

a wedge of the cut filter paper to push the grid onto the filter paper. Move the forceps holding the grid over the rubber mat. Without letting go of the forceps, insert the filter paper wedge between the tips of the forceps. Slowly push the wedge into the tips while releasing pressure on the forceps. This allows the grid to come off the forceps and land on the mat. Repeat these steps until all the sections are collected. Once the sectioning is complete, remove the knife, rinse with water, and dry with a canned duster. Take the specimen block and turn it upside down in a beam capsule (this way, you can cut more, if needed, at a later time). Wipe off and cover the ultratome.

3.9. Counterstaining EM Grids

Counterstain only every other grid or every third grid. This preserves at least one set of grids in case something goes wrong during the staining procedure. Stain only four to six grids at first to get a sense of the proper timing; if grids sit for longer than 7 min in the lead citrate, you will see precipitate on the tissue.

1. *Preparation for uranyl acetate step*. Take small amount (about 0.5 ml) of the uranyl acetate and lead citrate solutions out of the bottle with a 3-ml syringe. Cover the syringe containing the uranyl acetate with foil. Put a luer lock filter (13 mm, 0.2 μm, cellulose acetate membrane) on each syringe. Let the solutions warm up to room temperature (about 30 min).
2. *Uranyl acetate step*. Place a clean piece of dental wax into a foil-covered petri dish. Uranyl acetate is light sensitive, so keep the lid closed as much as possible during the incubation. Place small drops of uranyl acetate solution on dental wax, one drop for each grid to be stained. Float the grids, section side down, on drops. Wipe the forceps off with a paper towel in between grid transfers. Incubate the grids in the stain for 20 min, protected from light.
3. *Preparation for lead citrate step*. When there are 2 min left in the uranyl acetate incubation step, prepare for the lead citrate incubation step. Place a clean piece of dental wax in a petri dish and surround the wax with NaOH pellets (usually, 15–20 pellets). Place small drops of the lead citrate solution on the wax. Keep the lid closed as much as possible and try not to breathe over the lead citrate drops, as this causes the lead to precipitate. Arrange five 10-ml beakers filled with dH_2O between the uranyl acetate dish and the lead citrate dish. During the uranyl incubation, prepare for the drying step. Place a fresh set of five 10-ml beakers with dH_2O between the lead citrate and a stack of two pieces of Whatman (22 cm) #1 filter paper. Cut up eight wedges of 9-cm filter paper and set in a petri dish next to the stacked paper.
4. *First rinse and movement of grids from uranyl acetate to lead citrate*. Move grids to the lead citrate in the same order they

were placed in the uranyl acetate solution. Grab the first grid with the forceps. Rinse each grid by submerging it in the first beaker and moving it up and down about ten times while keeping the grid under the surface. Move through the series of five beakers in this way. At the end of the rinsing series, place the grid section side up inside the lead citrate drop. Close the lid of the petri dish between transfers. Incubate the grids in the lead citrate stain for 5–7 min.

5. *Second rinse and drying.* Remove the grids one by one and rinse them in the series of beakers as described in **step 3**. Minimize the transition time between the lead citrate and the first rinse to avoid lead precipitate on the grids. After rinsing, blot the bottom of each grid on filter paper and use a filter paper wedge to push the grid off of the forceps tips onto a filter paper-lined petri dish. Dry the grids for 30 min before putting into grid boxes. (If the room is dry, shoot the grid boxes with an antistatic gun prior to loading the grids. Otherwise, the grids will fly out of the box).
6. *Clean up.* Dispose of the drops and unused stains in the appropriate waste bottles. Rinse the beakers and wax well with dH_2O and set aside to dry.

3.10. Sampling Tissue Sections

1. *Controlling for the effects of antibody and label penetration*
The immunolabeling protocols described preserve ultrastructural membranes through avoidance (or reduced use) of detergents, like Triton-X-100 (see Note 3). Because membranes are preserved, labeling is limited to a depth of 1–2 μm on either side of a section. Within that region, the extent of labeling decreases markedly deeper in the tissue. It is, therefore, critically important to control for the effects of penetration when sampling, both to ensure optimum labeling and to allow quantitative comparisons between different tissue samples. The best approach is to restrict sampling to the region that is immediately adjacent to the plastic/tissue interface (14). Every effort should be made to embed tissue sections as flat as possible, with relatively little EMbed plastic resin covering the tissue surface (see Subheading 3.5). However, tissue sections are never perfectly flat. As thin sections are cut through a block, tissue will likely begin to appear as islands surrounded by plastic (i.e., plastic/tissue interface). The interface is ridge-like or jagged in appearance due to the vibration of the Vibratome blade (see Fig. 8). Sampling should be limited to grid squares that are adjacent to this border and which contain no visibly damaged tissue. To select which grid squares to sample, first capture a low-magnification image of the entire thin section on the grid with an electron microscope and use this as a map. Then, increase magnification, adjust the contrast level so that

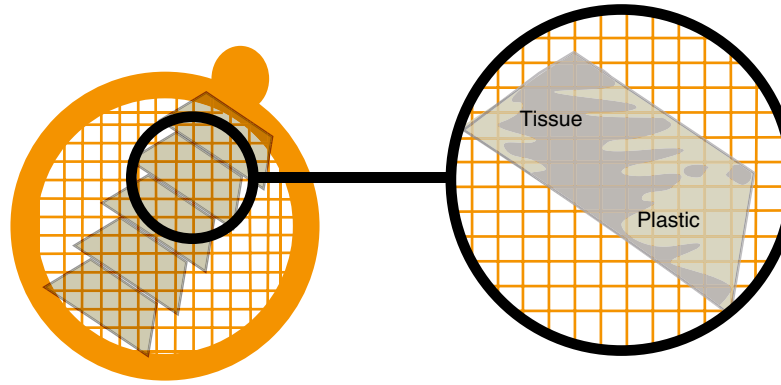

Fig. 8. EM analysis: Sampling. Detail of tissue/plastic interface (Subheading 3.8, step 1).

the plastic/tissue interface is apparent, and select a grid square that is next to the plastic/tissue interface. For systematic random sampling (18), identify the interface, randomly select a grid square, and then analyze a defined series of grid squares along the interface (e.g., every third grid square). Finally, increase magnification again, adjust contrast, and begin collecting micrographs.

2. *Mapping regional borders*
   Images of individual thin sections also are useful if one wants to limit analysis to specific tissue regions or subregions. Capturing a light microscopic image of the sectioned block face can help to identify borders between regions. Illuminate the block from below, preferably after sectioning when the face is smooth, and use this image to create a map of the regions included in the block. Scale the image and use as an overlay to define regional borders within a thin section. Thin sectioning itself can produce compression in the axis perpendicular to the block face. Compensate for this by using the "free transform" function found in graphic software packages. By using an overlay to define regional borders, ideal thin sections in which the plastic/tissue interface crosses the region of interest can then be identified.
3. *Sampling within grid squares and initial analysis*
   The manner in which one samples neuropil within selected grid squares depends on the goals of the experiment and the pattern of labeling. If the density of labeled processes is sparse, acquire images of all labeled processes in a selected grid square for analysis. However, if labeling density is high, then acquire images of a smaller, random field (e.g., a corner of the grid square) and analyze labeling in that field. Labeled process "profiles" are cross-sections of a labeled structure. Profiles can be categorized

according to type (e.g., glial, or neuronal perikarya, dendrites or terminals (19)). Profiles also can be qualitatively described in terms of the pattern of labeling, including association with subcellular locations (e.g., mitochondria, endomembranes, or plasma membranes). Labeled neuronal somata and dendrites also can be classified by the type of synaptic input they receive and the presence or absence of immunoreactivity in contacting terminals. Labeled terminals can be examined for cross-sectional area, the types of synapses formed, and vesicle content. Such analysis can provide morphological evidence for the type of transmitter within the terminal (20). This type of analysis can yield information on the types of degenerating processes, their position within neural circuits, and the subcellular distribution of a specific degeneration marker.

3.11. Quantifying the Extent of Degeneration

Ultrastructural analysis can be used to quantify regional degeneration and/or to facilitate comparisons between experimental conditions or across time points. The most apparent measure is the number of labeled profiles per unit area (N_A). However, this value is dependent on both the actual number of degeneration processes and their size. Larger processes more frequently appear in random cuts through tissue than smaller ones. One solution to this problem is to apply stereological counting methods to determine N_A by using the physical disector (18). This requires the analysis of adjacent thin sections, but generates a number that is independent of size. Another approach is to determine the volume density (V_V), the fraction of the volume occupied by degenerating processes. This is equal to the fraction of neuropil area occupied by degenerating processes (A_A), the total area of labeled processes in a given field area. Of course, these types of analyses can be combined with the categorization schemes described in Subheading 3.10.8 to determine how various subgroups of degenerating processes are affected. It should be remembered that both of these measures, N_A and V_V, represent densities. If the overall volume of tissue changes, a change in density may appear because of the volume change. This could be problematic in later stages of degeneration. In this case, total tissue volume of the affected region should be analyzed.

3.12. Analysis of Dual-Labeled Material

Ultrastructural analytic approaches can be further refined through dual-labeling EM methods. For instance, immunoperoxidase labeling can be used to identify a marker of degenerating processes in parallel with immunogold labeling. The immunogold labeling can identify an antigen, which could identify the phenotype of the degenerating process, identify the phenotype of afferent inputs, or label an additional component associated with apoptosis or degenerative disease mechanisms. The selection of antigen labels influences the type of analysis that can be performed. Immunoperoxidase

labeling is particularly sensitive, and can fill labeled processes, so that even small profiles of the process can be identified (particularly useful to measure V_v, for instance). However, it can obscure subcellular detail within the labeled process. Immunogold labeling is less sensitive, but more carefully identifies the subcellular location of the antigen, and provides a quantifiable particle. Silver-enhanced immunogold (SIG) particles can be used to determine the density of labeling along the plasmalemma or a subcellular membrane (particles/μm) (21) and/or the density of labeling within the cytoplasm (particles/μm^2) (22–24). In addition, the fractional distribution of SIG particles between membranes and cytoplasm (22–26) or between specific subcellular organelles, such as mitochondria or at synapses also can be quantified (27). The relative proportions of dual-labeled versus single-labeled processes can also be determined. Methods utilizing dual-labeling EM methods provide valuable insight into the mechanisms underlying apoptosis and degenerative disease.

4. Notes

1. *Solutions with BSA*. Solutions containing BSA should be kept in the cold room and not used if they are more than 2 days old. These solutions grow bacteria quickly. Make only what you need as BSA is costly.
2. *Organ collection following perfusion fixation*. Chronic stress decreases thymus and spleen weight (28) and increases adrenal weight (29). Spleens are sensitive to immunosuppression (30) and infection (31), and can be further examined by histology (for review, see ref. 32). The liver is especially interesting because of its role in drug metabolism. Challenges to the metabolic function of the liver by drugs (33) or diet (34) can alter liver weight. Equally striking are histological changes, necrosis, and apoptosis present after liver damage (35). Gonads reflect changes in circulating hormone levels; high gonadal steroid levels correlate with increased uterine and seminal vesicle weight (36).
3. *Factors to consider while working with antibodies*
   (a) *Antibodies used for identifying degeneration*.
   Electron microscopy can be used to identify the subcellular structures associated with light-level immunolabeling. Characteristic morphological changes for apoptosis and autophagy, such as chromatin clumping, mitochondrial swelling, lysosome, endosome, or autophagosome alterations, autophagic vacuole accumulation, ubiquitin inclu-

sions, and nuclear or cellular membrane involution, can also be immunolabeled. A rise of neurodegeneration in specific cell types can be identified by a number of protein changes, such as translocation of cytoplasmic cytochrome c (7), ubiquitin inclusions (37), amyloid-beta accumulation (38), phosphorylation of Tau (38), and changes in inflammatory markers (9). Oxidative damage is associated with alterations in the levels of NAPH oxidase subunits (26). Organelles' composition changes while autophagic vacuoles are rare in the normal adult brain; calnexin-labeled autophagic vacuoles increase during neurodegeneration (39). In addition, compartmentalization of transgenes labeled with GFP can be examined with immunoelectron microscopy (8, 40, 41). A list of antibodies used to identify the degeneration-related processes by EM is presented in Table 1.

(b) *Determining optimal primary antibody parameters.* Incubation parameters vary with each antibody. For initial antibody dilutions, we typically start at 1:1,000 and incu-

Table 1
Primary antibodies for labeling degeneration-related processes

Antigen	Species	Catalog no.	Source	References
Amyloid-beta 42	Rabbit	AB507/8P	Chemicon	Takahashi et al. (38)
Calnexin (autophagic vacuoles)	Rabbit	SPA-860J	Stressgen	Nixon et al. (39)
Cytochrome c, cytoplasmic	Mouse			Alonso et al. (7)
GFP	Chicken	GFP-1020	Aves Lab	Bulloch et al. (8)
GFP	Rabbit	A11122	Invitrogen	Justice et al. (40)
Iba1 (microglia)	Rabbit	MCA497R	Wako	Bulloch et al. (8)
Tau phosphorylated at ser 202 and thr 205, clone AT8	Mouse	90343	Innogenetics	Takahashi et al. (38)
Tau phosphorylated at thr 231, clone AT180	Mouse	AT180	Endogen	Takahashi et al. (38)
NADPH oxidase P47 (ROS production)	Goat	sc-7660	Santa Cruz	Pierce et al. (26)
NADPH oxidase P22 (ROS production)	Goat	sc-11712	Santa Cruz	Pierce et al. (26)
Ubiquitin	Rabbit	Z0458	DAKO	Komatsu et al. (37)

bate the tissue for 1 day at room temperature and 1 day overnight. For antigens not located on the membrane (e.g., neuropeptides or nuclear proteins), we test the antibody in the presence and absence of 0.25% triton in the primary antibody diluent. Although *0.25% triton is not good for EM*, it is useful in determining if penetration enhancement will promote better labeling for EM studies. Thus, if better labeling is seen in the presence of 0.25% triton in the primary antibody diluent, future EM studies should use either the freeze–thaw method or 0.025% triton in the primary antibody diluent. If a primary antibody appears to yield the desired staining pattern, a dilution series should be performed with another set of test tissue to determine the optimal dilution of the antibody (see ref. 42 for details).

(c) *Antibody specificity tests.* If an antibody appears to yield the desired staining pattern, additional tests should be performed to determine specificity. These tests include (a) Western blots; (b) labeling in cells transfected with the antigen of interest; (c) lack of labeling in Western blots, cells, and/or tissue sections with primary antibody preadsorbed with the antigen; and (d) if available, absence of labeling in knock-out animals. For discussion of these issues, *see* refs. 43, 44.

(d) *Antibody storage*. Unless noted otherwise by manufacturer, primary antibodies store best in the –70°C freezer. Before freezing a "neat" solution of antibody, divide it into 25- or 50-µl aliquots into microtubes with screw-top lids. Label tubes with the name and species that the antibody was raised against, company, catalog number, aliquot date, and how much antibody is contained in the tube. We also store some antibodies diluted 1:10. For this, add 10 µl antibody to 90 µl of 1% BSA/TS. Gently mix the antibody with a vortex or by pipetting in and out, and aliquot as described above. Primary antibody diluents can also be saved and reused. For this, add 1–2 µl of 1% sodium azide solution to the diluent and store the vial at 4°C.

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