Video Article

Preparation of Mouse Brain Tissue for Immunoelectron Microscopy

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Abstract

Transmission electron microscopy (TEM) is extremely useful for visualizing microglial, oligodendrocytic, astrocytic, and neuronal subcellular compartments (dendrite, dendritic spine, axon, axon terminal, perikaryon), as well as their intracellular organelles and cytoskeleton, in the central nervous system at high spatial resolution. Combined with TEM, pre-embedding immunocytochemistry allows the discrimination of cellular elements with few distinctive features and identification criteria (e.g., microglial perikarya and processes, when using an antibody against the microglia-specific marker Iba1 (ionized calcium binding adaptor molecule 1; as presented here)), identifying the neurotransmitter contents of cellular elements (e.g., serotonergic) and their ultrastructural localization of soluble or membrane-bound proteins (e.g., 5 HT1A and EphA4 receptors). Here, we describe a protocol for transcardiac perfusion of mice with acrolein fixative, removal and sectioning of the brain, as well as immunoperoxidase-diaminobenzidine (DAB) staining, resin embedding, and ultrathin sectioning of the brain sections. Upon completion of these procedures, the immunostained material is ready for examination with TEM. When rigorously performed, this technique provides an excellent compromise between optimal ultrastructural preservation and immunocytochemical detection.

Protocol

1. Animal perfusion

1. On the day before perfusion, prepare:
   - 2 L of phosphate buffer (PB; 100 mM, pH 7.4) and 1 L of sodium phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM PB, pH 7.4) in double distilled water. Store the PBS and 1L of PB at 4°C. These will be used for perfusion and pre-embedding immunocytochemistry.
   - 1L of 4.0% paraformaldehyde (PFA, pH 7.4) fixative solution, which will enable the perfusion of 6 mice. For that purpose, heat up 1 L of PB under a fume hood. Weigh 40 g of granular paraformaldehyde (PFA) and pour into the PB solution when it reaches 60°C. When the solution is clear, with the PFA completely dissolved, cool to room temperature (RT) and store at 4°C.
2. On the day of perfusion, prepare 500 mL of 3.5% acrolein solution in PB under a fume hood. Then, filter the 3.5% acrolein and 4.0% PFA solutions using filter paper.
3. For mouse anesthesia, inject sodium pentobarbital (80 mg/kg) into the peritoneum with a 27 gauge ½" needle.
4. During the perfusion, 50 mL of PBS, 75 mL of acrolein, and 150 mL of PFA will sequentially pass into the mouse circulation. To set up the peristaltic pump, fill the tubing with PBS and fix a 23 gauge ¾" butterfly needle at one end. Immerse the other end of the tubing into the perfusion solution (PBS, acrolein or PFA). Set the speed of the peristaltic pump to 20-25 mL/min for juvenile and adult mice. Throughout the perfusion, carefully avoid any bubbles of air forming in the tubing.
5. Wait until the anaesthetized mouse no longer responds to painful stimuli, such as a tail pinch, before proceeding. Lay the animal in a dissection tray and fix the paws using tape. With tweezers and scissors, open up the skin and chest cavity to expose the heart. To minimize brain ischemia, the perfusion needs to be started rapidly. Cut open the right atrium with small scissors and start the peristaltic pump. While holding the heart with tweezers, insert the butterfly needle into the apex of the left ventricle.
6. When changing solution, stop the peristaltic pump, transfer the tubing from one solution to another, and restart the pump immediately.
7. When 150 mL of PFA has passed, stop the peristaltic pump. Using small scissors, cut off the head, open up the skin, and break the skull between the eyes. Using small tweezers, carefully chip off small pieces of skull until the brain can be easily removed. Post-fix the brain for 2 hours at 4°C in PFA. Wash 3 times 10 minutes in PBS.
8. Immediately cut the brain in transverse (50 µm thick sections) in ice-cooled PBS using a vibratome. With a fine brush, transfer sections selected for immunocytochemistry into a glass vial containing PBS. Store the remaining sections at -20°C in cryoprotectant (30% ethylene glycol and 30% glycerol in PBS) for up to several years.

2. Pre-embedding immunocytochemistry

1. For immunocytochemistry, sections are processed freely floating in glass vials. Throughout the procedure, one needs to carefully avoid letting sections dry out.
2. First, remove the PBS using a transfer pipette and immediately replace with a fresh solution of 0.1% sodium borohydride in PBS for 30 minutes at RT.
3. Rinse sections with PBS 3 times 10 minutes, removing all bubbles, and incubate for 2 hours at RT in a blocking solution of PBS containing 0.5% gelatin and 5% normal serum of the animal in which the secondary antibody was generated (normal goat serum in the present example of Iba1-immunostaining).
4. Incubate for 48 hours with primary antibody in blocking solution. For Iba1-immunostaining, use rabbit anti-Iba1 antibody (1:1000).
5. Rinse sections with PBS 3 times 10 minutes and incubate in secondary antibody conjugated to biotin (1:1000) in blocking solution for 2 hours at RT.
   For Iba1-immunostaining, use goat anti-rabbit IgGs.

6. Rinse sections with PBS 3 times 10 minutes and incubate in streptavidin-horseradish peroxidase (1:1000) in blocking solution for 1 hour at RT.
7. Rinse sections with PBS 10 minutes and with Tris/HCl-buffered saline (TBS; 50 mM, pH 7.4) 2 times 10 minutes. To reveal the labeling, incubate sections with 0.05% DAB and 0.01% hydrogen peroxide in TBS (solution freshly prepared). When the staining is dark-brown (see example in Figure 1), stop the reaction by rinsing in TBS. In any case, end the reaction after a maximum of 5 minutes. Rinse sections with TBS 10 minutes and with PB 2 times 10 minutes.

3. Processing for electron microscopy

1. Transfer sections into PB in a multiwell culture plate using a fine brush. Prepare 1% osmium tetroxide solution in PB under a fuming hood. Remove PB from the wells of the culture plate and spread the sections flat with a fine brush. Immediately immerse sections in 1% osmium for 30 minutes at RT.
2. Transfer the sections into glass vials, rinse with PB 3 times 10 minutes, and dehydrate through 2 minutes immersions into ascending concentrations of ethanol: 2 times 35%, 1 time each of 50%, 70%, 80%, 90%, and 95%, 3 times 100%, followed by 3 times propylene oxide.
3. To prepare the Durcupan resin, combine 20 g of component A, 20 g of component B, 0.6 g of component C, and 0.4 g of component D into a disposable beaker, mix well until the color becomes uniform, and pour into an aluminum weigh dish. If needed, resin blocks can be prepared by pouring resin into embedding molds and curing in a 55°C oven for 48-72 hours.
4. Using a fine brush rinsed with propylene oxide, remove the sections from the propylene oxide and immerse into the Durcupan resin for impregnation overnight at RT.
5. The following day, put the aluminum weigh dish into a 55°C oven for 10 minutes to soften the resin. Using a brush rinsed with propylene oxide, coat an ACLAR embedding film with a thin layer of resin, lay down the sections, cover with another embedding film, and evenly distribute light weights (about 2 g; for example the plastic caps of glass vials) on top to help resin spreading. For resin polymerization, incubate in the oven for 48-72 hours (at 55°C).
6. After polymerization, remove the light weights and embedding film on the top of the sections. Under a binocular microscope, select square areas of interest (about 2x2 mm) and carefully excise them from the film using a razor blade.
7. Glue the areas of interest at the tip of resin blocks using Superglue and cure in the 55°C oven for 1 hour.
8. In preparation for sectioning, trim the resin block to the shape of an isosceles trapezoid with a razor blade.
9. Using an ultramicrotome and a glass knife, remove the glue and resin at the surface of the tissue. Fill the boat of the glass knife with double distilled water and cut a few semi-thin sections (0.5-1 µm thick).
10. Transfer the sections to a SuperFrost slide with a perfect loop. Dry the sections by placing the slide on a heating plate at 80°C for 1 minute. Cover with a few drops of 0.1% toluidine blue stain (2 g sodium borate and 0.2 g toluidine blue in 200 mL double distilled water) for 1 minute. Rinse the excess stain with double distilled water. Examination of the sections with a light microscope will enable to distinguish the stained tissue from the resin (Figure 2).
11. Resume cutting until the border between tissue and resin reaches the middle of the sections. Note that the following procedure requires training. Replace the glass knife with a diamond knife and fill the boat with double distilled water. Cut silver to silver-gold ultrathin sections (60-80 nm thick) and carefully collect them on copper mesh grids using fine inverted tweezers. Dry the grids on a filter paper.
12. To enhance contrast, ultrathin sections can be stained with lead citrate (0.03 g lead citrate and 0.1 mL 10N sodium hydroxide in 10 mL double distilled water[1]). Using fine inverted tweezers, transfer a grid to a drop of lead citrate for 2 minutes. Remove the grid and delicately rinse in 3 successive baths of double distilled water. Lastly, dry the grids on a filter paper and store them in a grid box until electron microscopic examination (Figures 3-6).

4. Representative Results

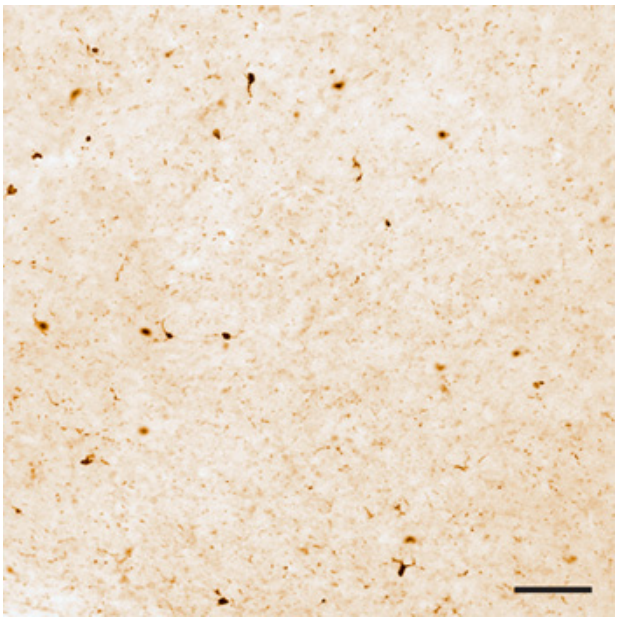

Figure 1. Iba1-stained section at the light microscopic level. The cellular distribution of Iba1 is restricted to microglia, which shows the specificity of the immunostaining. Scale bar=100 µm.

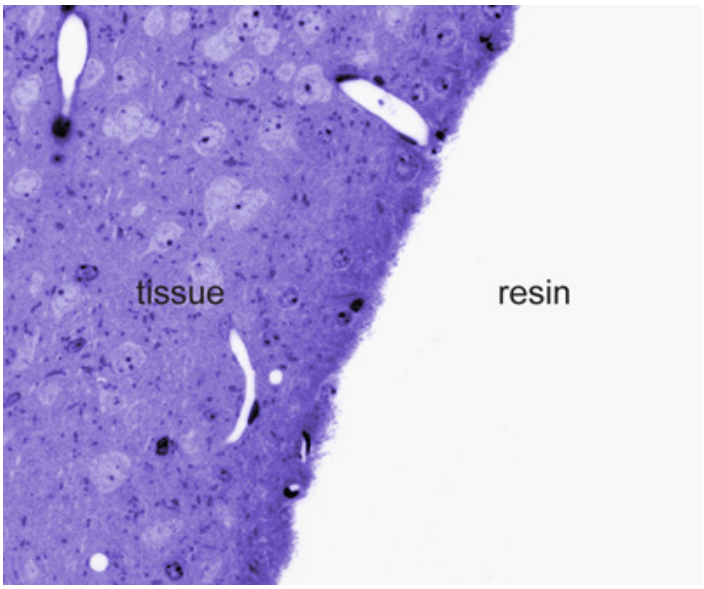

Figure 2. Toluidine blue-stained semi-thin section at the light microscopic level. Note the border between tissue and resin where the immunostaining will appear most intense at the electron microscope.

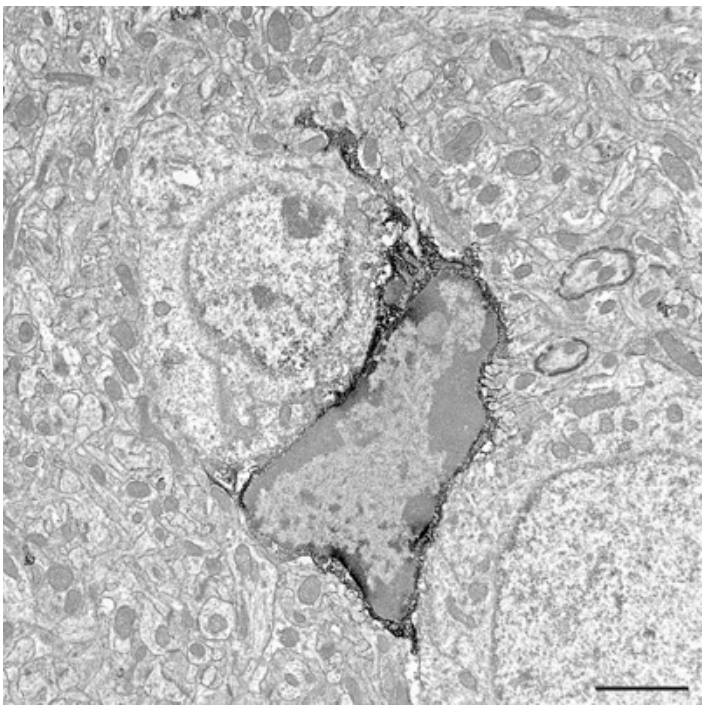

Figure 3. Ultrathin section showing Iba1-immunopositive microglial perikaryon and processes at the electron microscopic level. Iba1-positive structural elements are recognized by their immunoperoxidase-DAB electron-dense precipitate. Scale bar=2 µm.

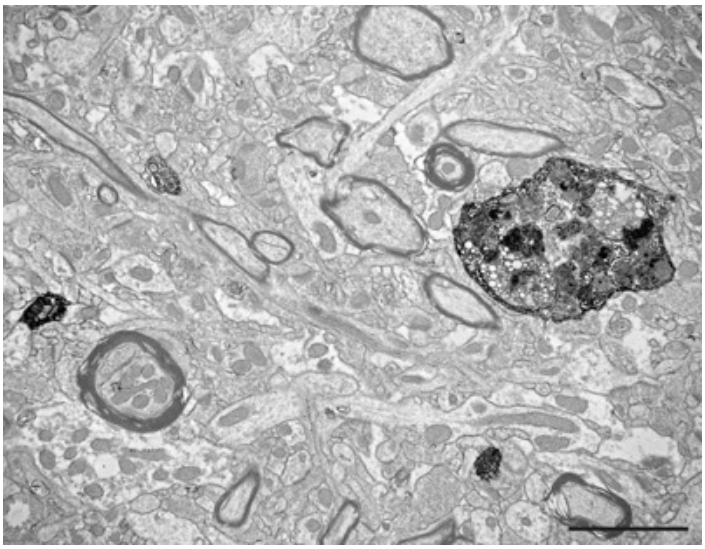

Figure 4. Ultrathin section displaying Iba1-immunopositive microglial processes of different sizes and shapes at the electron microscopic level. Scale bar=2 µm.

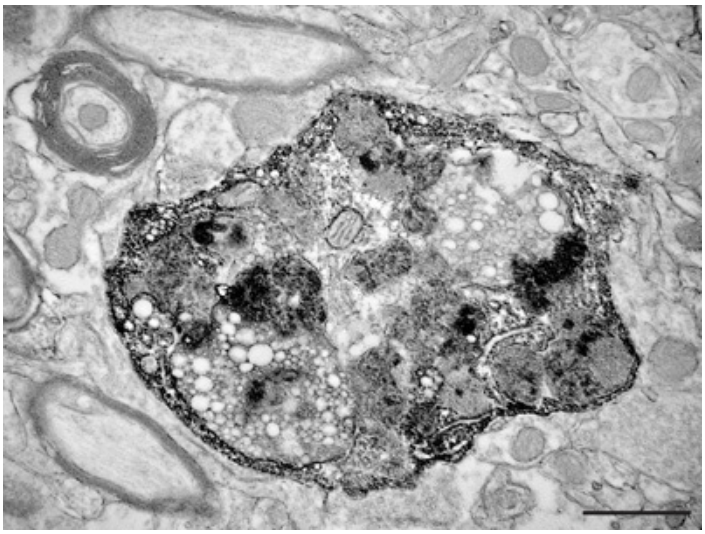

Figure 5. Ultrathin section showing at higher magnification an Iba1-positive microglial process from Figure 4, enabling identification of its intracellular organelles. Scale bar=1 µm.

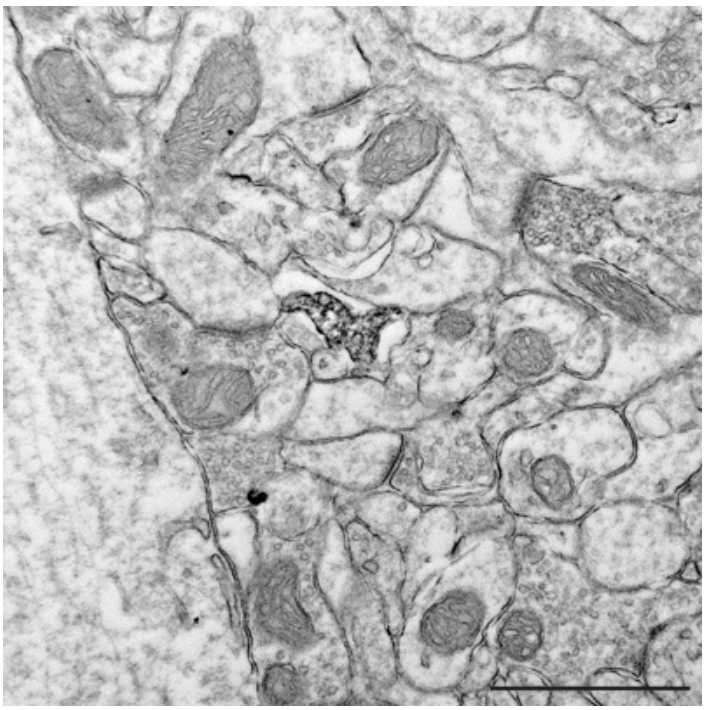

Figure 6. Ultrathin section revealing at higher magnification the ultrastructural relationships between an Iba1-positive microglial process and nearby elements of neuropil including synapses. Scale bar=1µm.

Discussion

Here we have described a protocol for preparation of mouse brain tissue for immunoelectron microscopy that provides an excellent compromise between optimal ultrastructural preservation and immunocytochemical detection, when the procedures are rigorously performed.

Combined with TEM, this method enables to distinguish cellular elements with few distinctive features and identification criteria. In particular, the immunoperoxidase-DAB staining of Iba1 enables to identify microglial perikarya and processes, within the neuropil, as well as to analyze their morphology, intracellular organelles, and ultrastructural relationships with other cellular elements (Figures 3-6). Additionally, this protocol allows analyzing the ultrastructural locations of soluble or membrane-bound proteins in glial or neuronal elements, as well as their association with intracellular organelles. Indeed, using this protocol with specific antibodies, we have previously revealed the ultrastructural localization of 5-HT1A and 5-HT1B serotonergic receptors in the neuronal perikarya, dendrites, dendritic spines, unmyelinated axons, and endothelial cells of adult rat raphe dorsalis nucleus, substantia nigra, globus pallidus, and hippocampus[2]. We have also shown the ultrastructural localization of EphA4 and EphB2 tyrosine kinase receptors in clathrin-coated vesicles and different astrocytic and neuronal elements in mouse and rat hippocampus and cerebral cortex, during postnatal development and adulthood[3,4,5,6]. Lastly, this protocol can be combined with pre-embedding immunogold to analyze the ultrastructural relationships between two simultaneously labeled proteins, for example the co-localization of Vglut1 glutamatergic transporter and EphA4 receptor in axon terminals of adult mouse cerebral cortex[6]. Therefore, this technique can be successfully applied, alone or in combination with immunogold labeling, for the ultrastructural analysis of various proteins within different regions and cell types of the central nervous system, throughout postnatal life, in health and disease.

Disclosures

No conflicts of interest declared.

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