Transmission Electron Microscopy of Oligodendrocytes and Myelin

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Abstract

In this chapter, we describe protocols to study different aspects of oligodendrocytes and myelin using electron microscopy. First, we describe in detail how to prepare central nervous system tissue routinely by perfusion fixation of the animal and conventional embedding in Epon resin. Then, we explain how, with some modifications, chemically fixed tissue can be used for immunoelectron microscopy on cryosections. Chemical fixation and Epon embedding can also be applied to purified myelin to assess the quality of the preparation. Furthermore, we describe how cryopreparation by high-pressure freezing can be used to study the fine structure of myelin in nerve, brain, and spinal cord tissue. The differences in the structural appearance of oligodendrocytes and myelin between cryopreserved and conventionally processed samples are compared using representative images. Since primary cultured oligodendrocytes are used to study structure and function in vitro, we provide protocols for chemical fixation and Epon embedding of these cultures. Finally, we explain how the cytoskeleton of cultured oligodendrocytes can be visualized by using transmission electron microscopy on platinum-carbon replicas. In this chapter, we provide a wide range of protocols that can be applied to shed light on the different biological aspects of myelin and oligodendrocytes.

Key words Electron microscopy, Immunolabeling, Oligodendrocytes, Cytoskeleton, Cryopreparation, Central nervous system, Myelin, Chemical fixation

1 Introduction

With the pace of instrumental development, imaging techniques are increasingly gaining importance, especially for the investigation of central nervous system biology and function. In combination with genetic tools and in vivo imaging techniques, electron microscopy adds valuable ultrastructural information to the related functional readouts. For the acquisition of ultrastructural data, several preparation strategies can be considered depending on the biological question. To assess general changes in cellular structures associated with the phenotype in a given animal model, chemical

fixation by perfusion and conventional Epon embedding are recommended [1]. This standard technique provides results regarding, e.g., quantification of myelin thickness and serves as basis for a more refined working hypothesis.

In the course of a study, more questions will arise that require assessment of the localization of a protein within a cell or tissue. Apart from the fact that one can learn a lot about the function of a protein by knowing its localization, the protein of interest might also be changed in abundance or localization in the mutant model. Immunoelectron microscopy is a suitable tool to study such changes [2]. Here, the antigen is visualized by gold-conjugated antibodies or protein A in the context of the subcellular environment. This so-called reference space adds valuable information, since changes occurring in mutant animals often affect not only the localization of the protein of interest but also the size and shape of organelles. Immunoelectron microscopy has always been a popular and powerful tool in cell biology, but relies on the quality of the available antibodies [3, 4]. These are crucial for the success of this technique, but often antibodies that work reliably with other techniques fail to work for immune-electron microscopy. Therefore, it is one of the most important tasks today to develop better and more reliable probes.

Apart from these *in situ* visualization strategies, electron microscopy can also complement biochemical analyses. To study the composition of myelin in transgenic mutants or different model organisms, this lipid-rich structure can be isolated biochemically due to its low density using density gradient centrifugation. To prove the purity of the myelin preparation, the content can be analyzed by electron microscopy. After chemical fixation and embedding, contaminating components of a myelin pellet, such as attached axonal membranes and mitochondria, can be identified. This helps in interpreting data, e.g., from proteome analysis.

Conventional sample preparation relies on chemical fixation. The disadvantage of this method is that the buffer conditions, such as pH value and osmotic strength, can never comply with the requirements of all components in a tissue or even in a cell. Therefore, fixation artifacts are almost inevitable and especially obvious in myelin of chemically fixed rodent tissue as lipid-rich structures are difficult to fix. To be able to discriminate effects on the myelin structure caused by the mutation in the animal model from fixation artifacts or subtle changes in the myelin architecture, a different approach in the sample preparation is required. By the application of high-pressure freezing (HPF), living samples are physically immobilized in milliseconds by freezing at approx. 2000 bar, and then later subjected to dehydration and chemical fixation during substitution of the ice with an organic solvent at low temperature [5–9]. By the application of 2000 bar during freezing, ice crystal formation is suppressed resulting in the formation of so-called

amorphous ice. In such a vitrified sample, the ultrastructure is preserved in a close-to-native state for an optimal sample preparation. Since the invention of this technique almost 50 years ago, we gained a much more detailed and complete view on the structure of living matter, especially for myelin of mice [10]. For the freeze substitution procedure, a plethora of published protocols exists. The protocol described here worked very well for mouse and rat myelin. For other tissues, other protocols might be more suitable and require testing.

Hypotheses generated from *in vivo* studies often require *in vitro* validation. For this purpose, primary oligodendrocyte cultures are useful tools. Here, we describe how such cultures can be embedded for electron microscopic inspection. In thin section electron microscopy, the architecture of the cytoskeleton is difficult to study since fibers can hardly be traced over longer distances. To overcome this disadvantage, a whole mount technique using platinum-carbon replicas [11] is described at the end of this chapter, which is also suitable to study the cytoskeleton in primary oligodendrocyte cultures.

2 Materials

2.1 Chemical Fixation and EPON Embedding

1. Mice or rats to be processed for electron microscopy.
2. Anesthesia for mice; Avertin: 2 g 2,2,2-Tribromoethanol in 2 mL tertiary amyl-alcohol, add 96.5 mL H₂O pre-warmed to 37 °C, stir for 30 min, filter with 0.2 µm filter, store 5 mL aliquots at −20 °C in the dark (light sensitive!).
3. Anesthesia for rats; 100 mg/mL ketamine (10 mL stock solution), stored at 4 °C, 20 mg/mL xylazine (50 mL stock solution), stored at 4 °C.
4. Fixative in phosphate buffer according to Karlsson-Schultz [12]: For 200 mL working solution: take 0.36 g NaH₂PO₄·H₂O, 3.1 g Na₂HPO₄·2H₂O, 1 g NaCl, add 130 mL distilled water, 20 mL 25% glutaraldehyde and 50 mL 16% formaldehyde (final concentration: 109.5 mM NaH₂PO₄·H₂O, 93.75 mM Na₂HPO₄·2H₂O, 86.2 mM NaCl, 2.5% glutaraldehyde, 4% paraformaldehyde). Filter with 0.2 µm filter to remove any particles. The salt solution can also be prepared as 2× stock in advance and stored at room temperature (RT). Fixatives are always added fresh prior to use.
5. 25% glutaraldehyde.
6. 16% paraformaldehyde (PFA) stock solution: Dissolve 16 g PFA in 90 mL double-distilled water upon heating and stirring at 65 °C. Afterward, add droplets of 5 N NaOH until solution becomes clear. Adjust volume to 100 mL. Filter cold solution

with 0.2 μm filter. Aliquot and store PFA at $-20\text{ }^{\circ}\text{C}$. Thaw aliquots in a water bath at $60\text{ }^{\circ}\text{C}$ for ~ 15 min until the solution becomes clear.

7. HBSS.
8. Styrofoam board.
9. Butterfly needle.
10. Perfusion peristaltic pump with tubing and adaptor.
11. Schott bottles or falcons.
12. Dissection tools (forceps, scissors, clamps, needles).
13. Glass vials or falcons to store the tissue.
14. Brain slicer.
15. Platinum double-edged razor blades.
16. Leica Vibratome VT1200S.
17. Platinum-coated double-edged razor blades.
18. Plastic Pasteur pipettes.
19. Super glue.
20. Hollow biopsy punch of different diameters depending on the size of the structure of interest.
21. Cutting mat.
22. Fine brush.
23. Forceps.
24. Tea filter bags.
25. Small segment or 8-segment baskets.
26. Basket lids.
27. Lynx Tissue Processor EM sample holder.
28. Lynx Tissue Processor.
29. 0.2 M phosphate buffer (PB): Prepare solution A containing 27.6 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and solution B containing 35.7 g/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. Mixtures of these two solutions will determine the pH: 20 mL of solution A mixed with 80 mL of solution B will result in 0.2 M phosphate buffer with pH 7.3.
30. 2% osmium tetroxide (OsO_4) in 0.1 M PB: Dissolve 2 g OsO_4 in 50 mL distilled water and dilute 1:1 with 0.2 M PB.
31. Acetone
32. Epon resin: 21.4 g glycid ether 100, 14.4 g 2-dodecenylsuccinic acid anhydride (DDSA), 11.3 g methyl-nadic anhydride (MNA). Stir the solution for 10 min and then add 0.84 mL 2,4,6-Tris(dimethyl-amino-methyl)-phenol (DMP-30) stirred for additional 20 min before use.

33. Embedding molds or gelatine capsules and Parafilm™-covered glass slides.

Caution: Osmium tetroxide, uranyl acetate, and some components of the Epon resin are extremely toxic or carcinogens and should be handled only under the fume hood with great care and appropriate personal protection (nitrile gloves, safety goggles, and lab coat).

2.2 Chemical Fixation and Embedding for Immunoelectron Microscopy

1. Animals to be processed for electron microscopy.
2. Karlsson-Schultz phosphate buffer for immunoelectron microscopy: Final concentration: 109.5 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 93.75 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 86.2 mM NaCl, 0.25% glutaraldehyde, 4% paraformaldehyde. Filter with 0.2 μm filter to remove any particles. The salt solution can also be prepared as 2 $\times$ stock in advance and stored at RT. Fixatives are always added fresh prior to use.
3. 25% glutaraldehyde
4. 16% PFA stock solution.
5. HBSS.
6. Butterfly needle.
7. Perfusion pump with tubing and adaptors.
8. Schott bottles or falcons.
9. Glass vials.
10. Cutting mat.
11. Fine brush.
12. Forceps.
13. Toluidine blue.
14. 10% gelatine (for bakery).
15. Ice bucket.
16. 2.3 M sucrose in PB.
17. Whatman™ filter paper.
18. Aluminum pins.
19. Cryovials.
20. Rubber-coated forceps.
21. Liquid nitrogen.
22. Leica UC6 cryo-ultramicrotome.
23. Cryoimmuno diamond knife 35°.
24. Pick up solution: 1% methylcellulose, 1.15 M sucrose.
25. Perfect loop™.
26. Carbon and Formvar-coated grids.

27. Parafilm™.
28. Plastic Pasteur pipettes.
29. PBS.
30. Glycine.
31. Bovine serum albumin.
32. Primary antibody (if possible raised in rabbit).
33. Rabbit secondary antibody, if the primary was not raised in rabbit.
34. Protein A gold, conjugated with 10 nm or 15 nm gold particles.
35. Neutral uranyl acetate: 2% uranyl acetate, 150 mM oxalic acid. Adjust the pH to 7 with 25% ammonium hydroxide.
36. Methylcellulose-uranyl acetate mix: dilute 4% uranyl acetate 1:10 in 2% methylcellulose.
37. Ice box.
38. Metal plate.
39. Filter paper.
40. Loops.
41. Rack to dry the loops.
42. Gridbox.

**2.3 Chemical
Fixation
and Embedding
of Myelin Preparations
for Conventional
Electron Microscopy**

1. Myelin preparations.
 2. Karlsson-Schultz phosphate buffer: 109.5 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 93.75 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 86.2 mM NaCl, 2.5% glutaraldehyde, 4% paraformaldehyde. Adjust pH to 7.4. Filtered with 0.2 μm filter. Salt solution can be prepared as 2 $\times$ stock in advance and stored at RT. Fixatives are added fresh prior to use.
 3. Tabletop centrifuge.
 4. 1.5 mL Eppendorf tubes.
 5. 2.5% low-melting agarose.
 6. Razor blade or scalpel.
 7. Needle.
 8. Cutting mat.
 9. Forceps.
 10. Segment baskets.
 11. Basket lids.
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1. Ice box with ice-cold PBS.
 2. Scissors for cutting skin and bones.

2.4 Cryopreparation by High-Pressure Freezing

3. Fine spring scissors.
4. Scalpels.
5. Brain slicer.
6. Razor blades.
7. Gelatine (for bakery).
8. Superglue.
9. Leica vibratome VT1200S.
10. 20% poly-vinyl-pyrrolidinone (MW = 10,000; PVP; in PBS)
11. Hollow biopsy punch of 2 mm diameter.
12. Specimen carrier, Type A and B.
13. Specimen holder cylinder half and middle plate.
14. Filter paper.
15. 1-Hexadecene.
16. Leica HPM100.
17. Rubber-coated forceps.
18. Perforated cryovials.
19. Styropor box filled with liquid nitrogen.
20. Tannic acid.
21. Glass-distilled acetone.
22. Rubber-coated forceps.
23. Plastic Pasteur pipettes.
24. Leica aluminum tins as containers equipped with disposable insets (flow-through rings).
25. Conical closures (used as disposable lids).
26. Leica AFS 2.
27. Osmium tetroxide.
28. 5% uranyl acetate in methanol.
29. Ice bucket.
30. Epoxy resin (Epon) (*see* recipe).

2.5 Monolayer Embedding of Oligodendrocytes

1. Cultured primary cells in small dishes (optimal: 3 cm dishes, no well plates).
2. 0.1 M Phosphate buffer (PB, *see* recipe above).
3. 1% OsO₄ (in PB).
4. Uranyl acetate.
5. Tungstophosphoric acid hydrate.
6. Epon resin.
7. Gelatine capsules.

8. Formvar-coated 100 mesh hexagonal carbon grids (*see* [13, 14] for preparation).
9. Gridbox.

2.6 Cytoskeleton Visualization of Oligodendrocytes

1. Cells cultured on glass coverslips.
2. Serum-free DMEM.
3. PEM buffer: 100 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.9.
4. Triton X-100.
5. Polyethylene glycol (35 kDa).
6. Glutaraldehyde.
7. Tannic acid.
8. Uranyl acetate.
9. Critical point dryer, e.g., Leica EM CPD 300.
10. Ethanol.
11. Sputter coater, e.g., Leica EM ACE600.
12. Needle.
13. 12 well cell culture plate.
14. Hydrofluoric acid.
15. Household detergent.
16. Perfect loops™.
17. Formvar-coated 100 mesh hexagonal carbon grids (*see* [13, 14] for preparation of formvar films).
18. Gridbox.

3 Methods

3.1 Chemical Fixation and EPON Embedding

Electron microscopy by chemical fixation (Fig. 1) has proven to be a useful tool to study the ultrastructure of oligodendrocytes (Fig. 2) and myelin in the central nervous system. With the help of conventional electron microscopy, alteration in the myelin ultrastructure of mutant mice (Fig. 3) could be revealed, such as changes in myelin thickness, altered paranodes or adaxonal space.

For this purpose, it is necessary to perfuse the animal with aldehyde-based fixatives. After postfixation in aldehyde-containing fixative, the dissected optic nerve or a segment of the spinal cord can be embedded directly. In contrast to that, when brain vibratome sections need to be cut, a region of interest is selected, excised, and then embedded. Sometimes, spinal cord samples also require vibratome sectioning. If an embedding machine is used, depending on the size of the sample, they are processed in small segment baskets of suitable size. To avoid loss of samples during

Fig. 1 Preparations strategies for transmission electron microscopy. (a) For conventional fixation of brain samples, the animal is perfused and after postfixation, vibratome sections are cut. A fine brush and a Pasteur pipette are used to handle the vibratome sections. A similar procedure is used to prepare fresh brain and

processing, small samples, such as optic nerves or punched tissue pieces, have to be wrapped in small pieces of tea filter paper. The embedding, including the contrasting and infiltration with the Epon resin, can be done manually or automatically.

**3.1.1 Perfusion
of the Animal
for Conventional Fixation
of Brain and Spinal Cord
Samples**

1. Prepare the perfusion pump by attaching the tubing and the adaptor. Flush the system with HBSS. Avoid introducing air bubbles into the system.
2. Anesthetize the animals. For mice, use 0.2 mL avertin per 10 g body weight by intraperitoneal (i.p.) injection. For rats, anesthetize with ketamine (60 mg/kg body weight) and xylazine (8 mg/kg body weight) by i.p. injection. Check that the animal is deeply anesthetized by pinching its paw and making sure that it does not twitch. The anesthesia should last 1–2 h.
3. Put the animal with the back down on a Styrofoam board. Stretch the limbs away from the body and pin them down with needles. Wet the fur with 70% ethanol.
4. Open the abdominal cavity with a transversal incision. Make a lateral incision in the diaphragm from one edge of the chest to the other. Cut laterally along the chest and flip that part of the rib cage up. Fix it with needles to gain good access to the heart.
5. Insert a 27G butterfly needle in the left ventricle and fix it with clamps or needles to the Styrofoam board. Cut the right atrium immediately.
6. Perfuse the animal with HBSS until the liver becomes yellow (10 mL) at very low speed of perfusion (1 mL/min, *see Note 1*).
7. Exchange the solution to the fixative without introducing air bubbles and perfuse for 10 min with fixative. The animal will twitch and then become stiff.

Fig. 1 (continued) spinal cord slices for high-pressure freezing. **(b)** The area of interest is punched out using a hollow biopsy punch (2 mm diameter). To avoid sample loss upon processing, the tissue sample is packed in tea filter paper by folding over twice from each side (*see right panels in (b)*). **(c)** The tools used for punching the area of interest in brain vibratome sections are displayed on a cutting mat. **(d)** The sample has to be placed with the flat side on one of the 8-segment baskets shown in on the left side (black arrow). Tissue pieces, such as spinal cord, can be placed directly into the basket shown on the right side (asterisk). **(e)** The loading station for preparing the baskets used for processing of the samples in an embedding machine, such as the Lynx Tissue Processor. **(f)** Embedding of spinal cord samples in gelatine for vibratome sectioning and subsequent cryopreparation by high-pressure freezing. **(g)** The optic nerve has to be cut into two pieces to fit into a carrier. The nerve piece is placed with fine forceps into specimen holder. **(h)** Specimen carrier with loaded spinal cord, rostral corpus callosum, and optic nerve samples (from left to right). **(i)** Embedding of optic nerve samples and spinal cord pieces in the flat, blue embedding molds. Brain tissue is embedded flat on Parafilm™-coated glass slides with Epon-filled gelatine capsules (*seen on the right side*). The polymerized resin blocks are shown in the middle (spinal cord on the top, optic nerve on the left and brain tissue on the right side)

Fig. 2 Oligodendrocytes in the mouse corpus callosum. (a) and (b) Oligodendrocytes are shown in cryopreserved and conventionally fixed samples. (a) In the cryoprepared sample, the nucleus contains a lot of heterochromatin and is surrounded by a narrow rim of pale cytoplasm. The free ribosomes and microtubules are abundant in the cytoplasm, but no glycogen or glial fibrillary acidic protein bundles can be detected. (b) In conventionally prepared samples, the cytoplasm of oligodendrocytes appears darker than the cytoplasm of other cell types. The oligodendrocyte nucleus shows a patchy pattern of hetero- and euchromatin with a large proportion of condensed chromatin. Sometimes, the fine processes can be found in attachment to myelin sheaths. Scale bar 2 μ m

8. Dissect the brain immediately as described below and immerse the tissue in the same fixative overnight. The spine is roughly dissected as described in **step 3** and immersed in fixative before dissecting the spinal cord.

3.1.2 Preparation of Brain Samples After Conventional Fixation

1. Wet the skin of the head with 70% ethanol. Cut the skin with a scalpel from the nose to the neck and flip it to either side.
2. In case the optic nerves should be prepared as well, insert small scissors carefully into the eye socket behind the eye ball and cut the optic nerve. Avoid stretching the nerves by pulling the eyes.
3. Cut the skull with scissors from the brainstem to the coronal suture.
4. Perform a lateral cut to either side and remove that part of the skull.
5. Cut the skull from the coronal suture to the ears along the brain.
6. Cut the bone in between the eyes and remove the skull by flipping it to either side.
7. Remove the brain from the skull by inserting angled forceps beneath the brain and gently pulling it out. The optic nerves should remain attached to the brain.

Fig. 3 Mouse optic nerve from different myelin mutants in transmission electron microscopy using cryopreparation and conventional fixation. (a) and (b) Representative electron micrographs of the optic nerve from wild type (WT), (c) and (d): proteolipid protein (PLP)-deficient ($Plp^{null/y}$) and (e) and (f): myelin basic protein (MBP)-deficient mice (*shiverer*, $Mbp^{shiv/shiv}$) prepared by cryopreparation (HPF, left panel) and conventional fixation (CF, right panel). Cryopreparation results in well-preserved myelin with smooth and tightly attached myelin lamellae. In conventional fixation, a typical artifact is the loosening of the myelin membranes in focal areas (see white asterisk in (b) and (d)). In $Plp^{null/y}$ mice, large cytosolic channels within single myelin membranes appear frequently (black arrow). Due to the cryopreparation that preserved the sample in a close-

8. Continue fixation of the brain by immersion in Karlsson-Schultz fixative overnight at 4°C. For prolonged storage, replace the fixative by 1% PFA in PB.
9. In order to embed the region of interest, cut 1 mm slices with a brain slicer matrix and razor blades or prepare vibratome sections of the fixed brain as described in the next steps.
10. For vibratome slices, cut the brain with a razor blade sagittal along the sagittal suture or coronal depending on region of interest. To speed up processing time, the tissue can be trimmed in such a way that the part of the tissue that is not of interest is removed beforehand.
11. Use superglue to mount the flat side of the tissue block on the vibratome plate. Wait 30 s for the glue to harden. Attach the plate to bottom of the cutting chamber filled with ice-cold PBS. Attach a platinum double-edged razor blade to the vibratome head and set the cutting window in such a way that it contains the whole tissue.
12. Cut the brain in 200 µm thick slices using an amplitude of 1 mm and speed of 1 mm/s with the cortex facing the razor blade first. Gently hold the top of the tissue with a fine brush and push a stream of buffer with a plastic Pasteur pipette onto the razor blade (Fig. 1a).
13. Select two sections containing the region of interest and transfer them to a cutting mat with a fine brush. Punch out a piece of tissue containing the area of interest using a hollow biopsy punch (Fig. 1b and c).
14. If an embedding machine, such as the Lynx II, is used, the punch of brain tissue is packed in a small piece of tea filter paper like a small packet so that the tissue is contained within the folded filter paper (Fig. 1b). Put the flat side of the tea bag into the 8-segment basket filled with 0.1 M phosphate buffer (PB) and cover it with the basket lid after filling up all segments (Fig. 1d and e). The filter paper packet will prevent the loss of small samples, such as optic nerves and tissue pieces by floating out of the basket segments (*see Note 2*).
15. The tissues can then either be processed automatically with an embedding machine or manually by incubating the samples with the different solutions. The protocols used for either automated or manual embedding are shown in Tables 1a and 1b. The steps are the same, but the incubation times vary.

Fig. 3 (continued) to-native state, the cytosolic channels are more apparent than in conventional fixed samples. Astrocytic processes (as) are also visible. In *shiverer* mice (*Mbp*^{shiv/shiv}), compaction of myelin is impaired due to the lack of MBP. Thus, instead of myelin lamellae microprocesses are formed (black asterisk). These microprocesses are only clearly visible in cryofixed samples. as, astrocyte, scale bar: 500 nm

Table 1a
Automated embedding protocol for conventional electron microscopy

Solution	Duration	Temperature
PB buffer	10 min	4 °C
PB buffer	10 min	4 °C
PB buffer	10 min	4 °C
2% OsO ₄	4 h	4 °C
PB buffer	10 min	RT
PB buffer	10 min	RT
PB buffer	10 min	RT
30% acetone	20 min	RT
50% acetone	20 min	RT
70% acetone	20 min	RT
90% acetone	20 min	RT
100% acetone	10 min	RT
100% acetone	10 min	RT
100% acetone	10 min	RT
100% acetone	10 min	RT
acetone: Epon mix (2:1)	2 h	RT
acetone: Epon mix (1:1)	2 h	RT
acetone: Epon mix (1:2)	4 h	RT
Fresh pure Epon	4 h	RT

16. In brief, for automated embedding, wash the samples with 0.1 M PB three times for 10 min at 4°C. After postfixation and staining in 2% OsO₄ in 0.1 M PB for 4 h at 4 °C, wash the tissue again with 0.1 M PB for 10 min at RT. Dehydrate the samples with increasing concentrations of acetone in water (30, 50, 70 and 90%) for 20 min each. Incubate the samples in 100% acetone for 10 min four times. Infiltrate the tissue with increasing concentrations of Epon resin mixed with acetone (2:1, 1:1 for 2 h each and 1:2 for 4 h) prior to incubation with pure Epon resin for 4 h.
17. After infiltration with Epon resin, remove the samples from the baskets and the tea filter packets. Place the samples in

Table 1b
Protocol for manual embedding for conventional electron microscopy

Solution	Duration	Temperature
PB buffer	15 min	RT
PB buffer	15 min	RT
PB buffer	15 min	RT
2% OsO ₄	4 h	4 °C
PB buffer	15 min	RT
PB buffer	15 min	RT
PB buffer	15 min	RT
50% acetone	20 min	RT
70% acetone	20 min	RT
90% acetone	20 min	RT
100% acetone	20 min	RT
100% acetone	20 min	RT
100% acetone	20 min	RT
100% acetone	20 min	RT
acetone: Epon mix (2:1)	2 h	RT
acetone: Epon mix (1:1)	2 h	RT
acetone: Epon mix (1:3)	Overnight	RT
Fresh pure Epon	2 h	RT

embedding molds (for optic nerves and spinal cord) or flat on Parafilm™-covered glass slides with Epon-filled gelatine capsules upside down on the samples (Fig. 1i and see **Note 3**).

- 18. Polymerize the resin for 24 h at 60 °C.
- 19. Boil resin blocks with gelatine capsules in water to remove the gelatine.

3.1.3 Preparation
of the Spinal Cord After
Conventional Fixation

- 1. For the dissection of the spinal cord after the perfusion of the animal, lay down the animal with the back facing up on a Styrofoam plate. Wet the skin with 70% ethanol. Cut the skin along the spinal cord and flip to either side.

2. Cut away all the muscle tissue and the organs attached to the spine. Leave some parts of the rib cage attached to the spine to facilitate the dissection of the spinal cord.
3. After prolonged fixation in Karlsson-Schulz fixative, pin the extracted spine to the Styrofoam plate using needles. Gently hold the upper side of the spine and insert strong, fine spring scissors on one side of the spinal cord. Cut a piece of the bone and then insert the scissor on the other side of the spinal cord and continue cutting the spine. Proceed to expose the spinal cord by alternately lifting one part of the spine and cutting the bones around the exposed spinal cord.
4. Use forceps to lift the cervical part of the spinal cord and remove it from the underlying spine. Gently pull the spinal cord up and cut the attached dorsal root ganglia.
5. Put the extracted spinal cord on a petri dish and cut a piece of tissue containing the area of interest. Small pieces of the spinal cord, as well as vibratome sections of the spinal cord, can be processed for conventional electron microscopy. In general, tiny samples are better infiltrated by chemicals and resins than large tissue blocks. Therefore, thin vibratome sections are recommended. In some cases, however, a larger piece of spinal cord is required for analysis. Vibratome sections (*see* protocol above subheading [3.1.2](#), **step 14** onward) are packed into tea filter packets and placed into 8-segment baskets for embedding (as shown for brain samples in [Fig. 1b](#), *see* **Note 2**). Wrapping into tea filter packets prevents bending or loss of the small tissue slices during the embedding process. Larger pieces of spinal cord can directly be transferred into a small segment basket ([Fig. 1c](#) and [d](#)) and be processed for embedding as described above.
6. After dehydration and resin infiltration, vibratome sections are placed flat on Parafilm™-covered microscope glass slides ([Fig. 1i](#) and *see* **Note 3**). Then, Epon-filled gelatine capsules are placed upside down on top of the samples. Spinal cord pieces are placed into embedding molds and covered with Epon resin ([Fig. 1i](#)). Include small paper labels for identification of the samples into the resin. The blocks are polymerized at 60 °C for 24 h.

3.1.4 Preparation of the Optic Nerve After Conventional Fixation

1. Kill the animal (mouse or rat) by cervical dislocation. In case of rats, or according to local animal handling regulations, the animal needs to be anesthetized before.
2. Cut off the head of the animal.
3. Make an incision with a small, sharp scissor behind the eyes. The eye ball should loosen from the eyecup and the end of the optic nerve attached to the eye ball should be cut.

4. Wet the skin with 70% ethanol. Cut the skin with a scalpel from the nose to the neck and flip it to either side.
5. Cut the skull with strong scissors from the brainstem to the coronal suture.
6. Cut a lateral cut to either side and remove the skull.
7. Cut the skull from the coronal suture to the ears along the brain.
8. Cut the bone in between the eyes and remove the skull by flipping to either side.
9. Remove the brain from the skull by inserting angled forceps beneath the brain and gently pulling the brain out.
10. Flip the brain over without stretching the optic nerve (*see Note 4*).
11. Cut the optic nerve at the chiasm where it is attached to the brain.
12. Pick up the optic nerves at the chiasm with angled forceps and pull them up gently.
13. Place the optic nerve into Karlsson-Schulz fixative overnight (or at least for 2 h).
14. Pack the optic nerve in tea filter packets and place in 8-segment baskets (as shown for brain sample in Fig. 1b and d). Close with lid and process for embedding as indicated in Table 1a or 1b.
15. Embed the samples in embedding molds (Fig. 1i), include a label indicating the experimental conditions and polymerize the blocks for 24 h at 60 °C.

3.2 Chemical Fixation and Embedding for Immunoelectron Microscopy

A powerful tool to study the subcellular localization of proteins is immunoelectron microscopy. Here, antibodies directed against the protein of interest are employed to identify its localization in relation to the ultrastructure of the tissue. Since the antigenicity of a protein is negatively influenced by the strength and duration of the chemical fixation, care has to be taken regarding the concentration of the fixative and the incubation time. Therefore, the animals have to be perfused with a fixative containing 4% PFA and 0.25% glutaraldehyde (or less). Dissected tissue should be exposed to fixatives only for a limited time of 2–4 h or maximal overnight (depending on the sample size), and then transferred into 1% PFA that is suitable for prolonged storage at 4 °C. Small tissue pieces (such as optic nerve or vibratome sections of the spinal cord or brain) are then infiltrated with 2.3 M sucrose as a cryoprotectant for 20–22 h. The samples are then mounted on aluminum pins and frozen in liquid nitrogen. After cutting cryosections according to Tokuyasu, the sections are first blocked and then stained with primary antibodies to detect the protein of interest. The sections are then

incubated with either gold-conjugated Protein A that binds to rabbit antibodies or other secondary gold-conjugated antibodies. The antibody-gold complexes are then fixed and the samples are contrasted in a two-step protocol with neutral uranyl acetate first and secondly with uranyl acetate and methylcellulose (for further reading, *see* [13–15]). At the electron microscope, the subcellular localization of the protein is apparent as black dots on the background of the general ultrastructure of the tissue (Fig. 4).

1. Perfuse the animal as described in Protocol 1 with a Karlsson-Schultz phosphate buffer for immunoelectron microscopy containing 0.25% glutaraldehyde or less (*see* **Note 1**).
2. After removal of the tissue, store the samples in 1% PFA at 4 °C until further processing.
3. Cut vibratome sections of the spinal cord and brain at 200 µm thickness (*see* protocol 1 for details).
4. For brain and spinal cord sections, infiltrate the vibratome sections with 2.3 M sucrose for 20–22 h at 4 °C on a rotating wheel. For optic nerve, stain the nerve briefly in 1% Toluidine blue and embed it in small blocks of 10% gelatine prior to infiltration with sucrose.
5. Place a small petri dish on a big petri dish filled with ice. Add a drop of 2.3 M sucrose on the dish and transfer the section or the optic nerve-gelatine blocks into it.
6. Use a scalpel to cut a small piece containing the area of interest from the vibratome sections (size around: 1 mm × 1 mm).
7. Mount the tiny tissue piece or optic nerve-gelatine block on an aluminum pin with a droplet of sucrose. Remove carefully all the excessive sucrose with Whatman™ filter paper.
8. Freeze the pin with the attached sample in liquid nitrogen using rubber-coated forceps. Samples can be stored in liquid nitrogen or processed for cutting immediately.
9. Trim the block and cut ultrathin cryosections with a Leica UC6 cryo-ultramicrotome equipped with a cryoimmuno diamond knife and a trimming diamond knife (Diatome, Biel, Switzerland). Use 1.15 M sucrose and 1% methylcellulose to pick-up and mount the sections on formvar- and carbon-coated copper grids using a Perfect loop™. Store samples on a paper-covered glass slides in a petri dish at 4 °C.
10. To perform immunolabeling of the sections with gold-conjugated Protein A, cover the bench top with Parafilm™ and place droplets of the incubation solutions with a plastic Pasteur pipette directly on the Parafilm™. Always float one grid upside down on each droplet. Move the grids from droplet to droplet with forceps by touching the grid only at the rim.

Fig. 4 Cryo-Immunoelectron microscopy of mouse optic nerve. **(a)** and **(b)** Immunogold labeling of proteolipid protein (PLP) in optic nerve cryosections of adult WT mice using a polyclonal rabbit antibody directed against PLP and protein A-gold. In panel **(b)**, the nerve is sectioned longitudinally. Here, an adjacent microglia cell (mg) containing numerous lysosomes (lys) can serve as an internal negative control for the labeling. **(c)** Immunogold labeling of PLP on optic nerve sections of a PLP-deficient mouse ($Plp^{null/y}$) that serves as a negative control. A few gold particles represent background. **(d)** shows labeling of td Tomato in a transgenic reporter mouse (TDTO tg) expressing this reporter protein only in oligodendrocytes. This protein is expressed in the cytosol and appears in the outer and inner tongue of the myelin sheath. Here we used a polyclonal antibody directed against RFP (Rockland, US, # 600–401-379) and protein A-gold. The sample was fixed with only 4% formaldehyde. This is insufficient to stabilize the lipid-rich myelin structure resulting in loss of material from the section (indicated by black asterisks). lys, lysosomes; mg, microglia cell, scale bar: 200 nm

Make sure to clean the forceps with water and tissue paper in between different samples and steps (*see Note 5*).

11. Wash off the pick-up solution from the grids by incubation in droplets of PBS for 2 min. Repeat this step twice.
12. Incubate with PBS containing 0.1% glycine to block free aldehyde groups from fixative for 2 min. Repeat step twice.
13. Block the samples using 1% BSA in PBS for 3 min.

14. Incubate the sections with the primary antibody of choice (in appropriate dilution) in 1% BSA in PBS for approximately 1 h. The optimal dilution and incubation time has to be determined beforehand. Usually, the concentration of antibody solution has to be higher than for immunohistochemistry. Antibody concentrations between 0.2 and 2 $\mu\text{g}/\text{mL}$ should be tested. If the antibody concentration is unknown, a dilution series has to be performed. For the incubation with the primary antibody, droplets of 5–7 μL of antibody solution are used per grid. As a negative control, the primary antibody is omitted, and the grid is floated only on blocking solution. Cover the droplets during the antibody-incubation with a flat box or petri dish to avoid evaporation of the antibody solution and deposition of dirt particles on the samples (*see Note 6*).
15. Wash the grids in 0.1% BSA in PBS for 2 min. Repeat this step four times.
16. If the primary antibody is not derived from rabbit, incubate the samples with a suitable bridging antibody. This rabbit antibody has to bind antibodies derived from the species of the primary antibody. Float the grids on the secondary antibody for 30 min. Then, wash the grids in 0.1% BSA in PBS for 2 min. Repeat this step four times.
17. Incubate the samples with gold-conjugated Protein A (CMC, Utrecht, Netherlands) at the indicated dilution for 20 min.
18. Wash the grids in PBS for 2 min. Repeat this step four times.
19. Fix the antibody-gold-complexes and denature the Fc-domain of the antibody using 1% glutaraldehyde in PBS for 5 min.
20. Wash the sections with distilled water for 1 min. Repeat this step nine times.
21. Stain the samples with neutral uranyl acetate for 5 min at RT.
22. Continue staining and protecting the samples with a layer of methylcellulose by incubation with 0.4% uranyl acetate in 2% methylcellulose for 5 min on ice (*see Note 7*). Use a metal plate covered with Parafilm™ for a flat surface.
23. After the incubation of the samples with methylcellulose-uranyl acetate mix, take a stainless steel wire loop of 3 mm diameter and insert it into the droplet underneath the grid in such a way that the grid resides in the middle of the loop and gently lift the loop upward. Dry the grid by striking the loop over Whatman™ filter paper until no more liquid is released.
24. Place the loops upright into a rack and let the grids dry for ~20 min. Place a box above the loops to avoid contamination with particles in the air.

25. To remove the grids from the loops, place the loop with the grid upside down on a filter paper. Insert forceps in between the grid and the edge of the loop and keep repeating this step in a rotating motion around the grid. This way the grid can be released from the loop without breaking the methylcellulose layer.
26. Store the grid in a grid box until imaging.

3.3 Chemical Fixation and Embedding of Myelin Preparations for Conventional Electron Microscopy

Due to the high lipid content of myelin, it can be purified biochemically via density gradient centrifugation [16]. Often not only Western Blotting, but also electron microscopy is performed to assess the purity of the preparation. The following protocol allows an easy and rapid quality control of sample preparation and gives first insights into the ultrastructure of myelin preparations in mutant animals as well (Fig. 5).

1. Centrifuge myelin preparations in a 1.5 mL Eppendorf tube at maximum speed in an Eppendorf centrifuge for 10 min.
2. Fix the pellet with Karlsson-Schultz fixative for 1 h at RT.
3. Cover the pellet with 2.5% low-melting agarose (pre-warmed to 60 °C) and place on ice for several minutes to allow the agarose to solidify.
4. Cut the bottom of the Eppendorf tube with a scalpel or a razor blade.
5. Remove the pellet from the remaining tube with a needle.
6. Cut the pellet in half on a cutting mat and place in a small segment basket. Cover with basket lid.
7. Perform the embedding protocol as described in protocol 1.
8. Embed the samples by placing the flat side of the pellet on a Parafilm™-coated glass slide and cover it with an Epon-filled gelatine capsule.
9. After polymerization for 24 h at 60 °C, remove the gelatine by boiling the samples in water for 15 min.

3.4 Cryopreparation by High-Pressure Freezing

The state-of-the-art preservation of the ultrastructure of nervous tissue employs high-pressure freezing of the tissue sample. This method of cryopreservation quickly and physically immobilizes the sample in a close-to-native state. The tissue is fully hydrated as the water within the sample is rapidly transformed into amorphous ice. In contrast to chemical fixation, the samples are not fixed with aldehydes, but subjected to freezing in live state. During freeze substitution, the ice is replaced by organic solvents, while fixing and contrasting the samples at low temperatures.

Therefore, fresh tissue needs to be extracted and either a piece of tissue (e.g., optic nerve) or a piece of a vibratome slice of the brain or spinal cord is cryopreserved (Fig. 1 f–h). For that, the

Fig. 5 Myelin preparations of different myelin mouse mutants. **(a)** Myelin preparation from WT mice. **(b)** Area indicated by a white box in **(a)** at higher magnification. Myelin lamellae are clearly visible in the preparation (see also inset in **(b)**). **(c)** The overview image of the myelin preparation from *shiverer* mice (*Mbp^{shiv/shiv}*) shows that compact myelin cannot be detected. **(d)** In contrast to this, the myelin from PLP-deficient mice (*Plp^{null/y}*) is still compacted. Scale bars: 1 μm in **(a)**, 500 nm in **(b)** and **(d)**, 2 μm in **(c)**

sample needs to be loaded in the specimen carrier prior to freezing with liquid nitrogen at 2000 bar in a Leica HPM100 (Fig. 1g and h) (for further reading, see [17]). The subsequent freeze substitution is carried out in a semiautomated fashion in a Leica AFS 2. The dehydration and fixation is performed at -90°C with different cocktails containing tannic acid followed by osmium tetroxide and uranyl acetate to stabilize and contrast the specimen. Thereafter, the samples are embedded in resin after infiltration with increasing amounts of Epon (in acetone, modified from [18], Fig. 1i).

3.4.1 High-Pressure Freezing of Fresh Dissected Tissue

1. Cool down the high-pressure freezer (e.g., Leica HPM100) according to the manufacturer's instructions with liquid nitrogen until the internal storage dewar is full. Refill the machine after 20 min again and allow the machine to cool down for another 30 min before some test freezing cycles with empty

assemblies of middle plate and cylinder halves (without carrier). Check that all freezing parameters are in the normal range such as: rise time: 4.1 ms, shift p/T: 1.3 ms, hp dwell: 376.5 ms, and cooling rate: 21505 °C/s.

2. Kill the animal (mouse, rat or other) by cervical dislocation (in case of rats, anesthetize the animal before).
3. Cut off the head of the animal.

3.4.2 Cryopreservation of the Optic Nerve

1. Dissect the optic nerve fresh as described in protocol 1 (*see Note 4*).
2. Place the optic nerve in ice-cold PBS. Transfer the nerve into drop of 20% PVP (in PBS) on the cutting mat with a fine brush or angled forceps.
3. Cut the nerve with a scalpel in ~2 mm long pieces.
4. Submerge the specimen carrier in 20% PVP and transfer the tissue into the carrier by placing the specimen carrier underneath the tissue and moving it up. Make sure to avoid touching the sample. If necessary only touch the rim of the tissue. For the optic nerve, two pieces can be processed in one specimen carrier (Fig. 1g and h). For tiny nerves of young animals, the 0.1 mm carrier (Type A) can be used, for all other samples the 0.2 mm carrier (Type A, other side) is preferred. Always use the smallest carrier possible, since smaller samples with less space freeze better than bulky samples.
5. Remove putative air bubbles, blot the bottom side of the carrier containing the sample on filter paper and transfer it into the middle plate on top of the lower cylinder half with the sample facing upward. Wet the second carrier (0.3 mm, Type B, flat side facing down onto the sample) with hexadecene and close the specimen carrier.
6. Remove excessive liquid with filter paper.
7. Close the specimen sandwich with the upper cylinder half.
8. Freeze the sample.
9. Remove the samples from the sample dewar and transfer the carriers into a Styrofoam box with liquid nitrogen (*see Note 8*).
10. Remove the carrier from the middle plate. Remove the lid of the carrier.
11. Transfer the carrier with the frozen tissue with rubber-coated forceps into perforated cryovials under liquid nitrogen atmosphere. The cryovials should be perforated in the upper half of the vials to prevent floating in liquid nitrogen. Make sure to label the tubes with the sample details.
12. Store the samples in liquid nitrogen until freeze substitution.

3.4.3 Cryopreservation of the Brain

1. Dissect the brain fresh according to the protocol for fixed tissue as described in protocol 1.
2. Wash the brain in ice-cold PBS and put it on a petri dish.
3. Cut the brain with a razor blade sagittal along the sagittal suture or coronal depending on region of interest. To speed up processing time, the tissue can be trimmed in such a way that only the relevant brain area remains.
4. Mount the flat side of the brain on the vibratome plate using superglue. Wait for the glue to harden and then attach the plate to bottom of the cutting chamber filled with ice-cold PBS. Cut the brain in 200 μm thick slices using an amplitude of 1 mm and speed of 0.6–0.7 mm/s with the cortex facing the razor blade first (*see* Fig. 1a).
5. Select two sections containing the region of interest that are of good quality (equal thickness, no cuts or damages in the area of interest). Transfer them to a petri dish and then to the cutting mat using a fine brush.
6. Punch out a piece of tissue containing the area of interest using a 2 mm hollow biopsy punch.
7. Load the tissue in the specimen carrier as described in Sub-heading 3.4.2, steps 4–12 for the preparation of the optic nerve (Fig. 1h) and proceed as described in the following steps of the protocol.

3.4.4 Cryopreservation of the Spinal Cord

1. Dissect the spinal cord freshly as described above for fixed tissue.
2. Put the extracted spinal cord on a petri dish and cut a piece of tissue of 5 mm length containing the area of interest.
3. Immerse the spinal cord in Karlsson-Schultz fixative for 1–5 min to fix the rim of the tissue.
4. Put a drop of 10% gelatine (pre-warmed to 45 °C) in an embedding mold in an ice bucket. Gently blot the spinal cord on tissue paper to remove excess liquid and transfer it into the gelatine. Adjust the orientation according to the purpose, e.g., for longitudinal sections lay the spinal cord down flat longitudinally, for cross-sections of the spinal cord put the sample upright. Allow the gelatine to harden for 30 s to 1 min (Fig. 1f).
5. Cover the tissue with gelatine solution and wait for the gelatine to solidify.
6. Remove the block of gelatine.
7. Use superglue to attach the block of gelatine containing the tissue on the vibratome plate (Fig. 1f).
8. Continue to cut and freeze the tissue as described above.

3.4.5 Freeze Substitution
of the High-Pressure
Frozen Samples

1. Fill the Leica AFS 2 with liquid nitrogen until the internal dewar is full. Precool the chamber to -90°C .
2. Prepare 0.1% tannic acid fresh in glass-distilled acetone.
3. Equip the Leica-provided aluminum tins with disposable insets (called flow-through rings) for ten specimens and label the individual flow-through rings with notches made using a razor blade. Pipette the tannic acid solution into the tins until the flow-through rings are half-full. Freeze the tannic acid / acetone mix in liquid nitrogen. Avoid spilling of liquid nitrogen into the tins.
4. Remove the sample from the liquid nitrogen container and put them into a Styrofoam box filled with liquid nitrogen.
5. Take out the specimen carrier from the cryovials and transfer them upside down with rubber-coated forceps into the insets under liquid nitrogen atmosphere (*see Note 8*).
6. After all samples are loaded into the inserts, transfer the tins into the Leica AFS 2. Cover the tins then with disposable plastics lids (conical closures).
7. Start the freeze substitution according to the protocol shown in Table 2 in a semiautomated fashion. The optic nerves and the spinal cord samples are incubated with the tannic acid for 100 h, whereas brain tissue is only incubated with tanning agent for 24 h (*see Note 9*).
8. After the incubation with the tannic acid solution, the samples are washed with acetone. Aspirate the solution with a plastic Pasteur pipette and quickly replace it with acetone precooled to -90°C . Perform four washes of 30 min each and make sure that the samples are always covered with liquid.
9. During the fourth wash, prepare the contrasting cocktail containing 2% OsO_4 and 0.1% uranyl acetate (from a 5% stock solution in methanol) in 25 mL acetone. Handle the chemicals under the fume hood with complete personal protection (lab coat, safety goggles, mask). Dissolve 0.5 g OsO_4 crystals in 25 mL acetone in a 50 mL Falcon tube with plastic Pasteur pipettes and then add 500 μL 5% uranyl acetate. Transfer the cocktail into a closed glass vial and then into the AFS to allow the contrasting solution to cool down. When the fourth wash is completed, incubate the samples in the OsO_4 and uranyl acetate mix according to the protocol shown in Table 2 (7 h at -90°C , 14 h temperature raise to -20°C with an increment of $5^{\circ}\text{C}/\text{h}$, 16 h at -20°C followed by a 2.4 h temperature raise of $10^{\circ}\text{C}/\text{h}$ to 4°C). The waste (including the washing steps) should be handled carefully and disposed of according to the appropriate regulations.

Table 2
Automated freeze substitution protocol (modified from [16])

Solution	Duration	Temperature
0.1% tannic acid in acetone	24 h (for brain samples) 100 h (for optic nerves and spinal cord samples)	−90 °C
Ice-cold acetone	4 × 30 min	−90 °C
2% osmium tetroxide, 0.1% uranyl acetate in acetone	7 h 14 h 16 h 2.4 h 1 h	−90 °C Warm up to −20 °C, in 5 °C/h −20 °C Warm up to 4 °C, in 10 °C/h 4C
Cold acetone	4 × 30 min	4C
Cold acetone	1 h	RT
Acetone: Epon mix (2:1)	2 h	RT
Acetone: Epon mix (1:1)	2 h	RT
Acetone: Epon mix (1:2)	2 h	RT
90% Epon in acetone	Over night	RT
Fresh pure Epon	Over night	RT

10. When the freeze substitution protocol reached 4 °C, remove the tins from the AFS and place on ice under the fume hood. Wash out the contrasting cocktail four times with acetone. Then, allow the samples to warm up to RT.
11. Infiltrate the tissue with increasing concentrations of Epon in acetone (2:1, 1:1, 1:2 acetone/Epon) by incubation for 2–3 h for each step.
12. Incubate the samples with 90% Epon in acetone at RT overnight.
13. Infiltrate the samples with fresh Epon resin for 4 h.
14. Remove the flow-through rings from the tins and the samples from the rings using a needle and forceps (*see Note 3*).
15. To embed the optic nerve, place the tissue in flat embedding molds. For cross-sections of the spinal cord and the brain, place the samples on Parafilm™-covered glass slides with Epon-filled gelatine capsules turned upside down on the sample. Include labels in the resin to identify the samples (Fig. 1i).
16. Polymerize the Epon resin at 60 °C for 24 h.
17. Remove the gelatin capsule by boiling the resin blocks in water for 15 min.

3.5 Monolayer Embedding of Oligodendrocytes

With the help of this method, cultured cells can be fixed and embedded in Epon to assess their morphology on the ultrastructural level. After cutting the Epon blocks either parallel to the culture substrate or in transverse orientation, the samples can be imaged at the transmission electron microscope. With this method, the morphology of subcellular structures, such as organelles, can be examined from “top-view” (Fig. 6) or “side view.” Hence, the morphology of the whole cells cannot be assessed with this method.

1. Grow primary cells in small cell culture dishes (optimal: 3 cm dishes).
 - (a) **Note:** Do not use multi-well plates as they would need to be broken apart during the sample preparation.
2. Fix the cells with Karlsson-Schultz fixative for 15 min at RT.
3. Wash the cultured cells thrice with 0.1 M phosphate buffer (PB). All steps are carried out in the cell culture dish directly (*see Note 10*).
4. Fix the cells with 1% OsO₄ in 0.1 M PB at 4 °C for 1 h.
5. Wash the samples three times with double-distilled water.
6. Dehydrate with 50% ethanol for 10 min, repeat this step.
7. Incubate samples with 70% ethanol for 10 min, repeat this step.
8. Stain the cultured cells with a freshly prepared mix of 1.5% uranyl acetate and 1.5% tungstophosphoric acid hydrate in 70% ethanol for 90 min at RT.
9. Dehydrate with 90% ethanol for 10 min, repeat this step.
10. Incubate samples with 99.9% ethanol for 5 min, repeat this step five times.

Fig. 6 Monolayer embedding of primary mouse oligodendrocytes in culture. (a) Representative image of a mature primary mouse oligodendrocyte (5 days in vitro) with the nucleus (nu) and primary processes (p). (b) Branching processes in the periphery of the cell. Scale bars: 5 μm in (a) and 2 μm in (b)

11. Infiltrate the cells with increasing concentrations of Epon in ethanol (1:2, 1:1, 2:1 Epon/ethanol) by incubation for 30 min for each step at RT.
12. Incubate the samples in pure Epon resin for 1 h at RT.
13. Remove as much Epon resin from the cell culture dish as possible.
14. Fill gelatine capsules with Epon resin and include a label indicating all relevant information for the experiment. Put the capsule upside down on the monolayer.
15. Polymerize the resin at 60 °C for 24 h.
16. The capsules need to be removed by applying a bit of force. The monolayer covers the smooth surface of the block.

3.6 Cytoskeleton Visualization of Oligodendrocytes

The cytoskeleton of the oligodendrocytes defines its shape, endocytosis and intracellular transport. The distribution of actin and microtubule bundles can easily be visualized in fluorescence microscopy, but to examine the ultrastructural architecture of the cytoskeleton the resolution of an electron microscope is needed (Fig. 7). This brings about the problem that cytoskeletal elements are hardly contained over longer distances within the volume of an ultrathin section and cannot be imaged in total.

Here, we present a “whole mount technique,” where the cultured cells are examined as a whole [11, 19]. Therefore, the cells are fixed, and all lipids and cytosolic proteins are extracted with detergents. The remaining cytoskeleton is then fixed and contrasted during dehydration. After critical point drying, the cells are then sputter coated. The resulting replicas are subsequently detached from the coverslips and mounted on grids. Often, the remaining cell material and cytoskeleton around the nucleus is too dense to be examined by electron microscopy, whereas the peripheral areas of the cells can be imaged at high resolution to obtain insight into the higher-ordered structures of the cytoskeletal filaments [11, 19] (Fig. 7).

1. Grow cells in small cell culture dishes (optimal: 3 cm dishes).
2. Wash the cultured cells three times with pre-warmed 0.1 M PB. All steps are carried out in the cell culture dish (*see Note 10*).
3. Extract lipids and proteins using one of the following protocols for 5–7 min at RT.
 - *General cytoskeletal visualization:* 1% Triton X-100 and 2% PEG in PEM buffer.
 - *Visualization of labile, easily extractable components:* 0.5% Triton X-100 and 0.25% glutaraldehyde in PEM buffer.
4. Wash twice for 1 min in PEM buffer.

Fig. 7 Cytoskeletal visualization of primary mouse oligodendrocytes in culture. (a), (c) and (e): Representative images of replicas generated from primary mouse

5. Fix the cytoskeletal proteins using 2% glutaraldehyde in PEM buffer for 20 min at RT.
6. Incubate with freshly prepared 0.1% tannic acid in PEM buffer.
7. Wash three times with double-distilled water. Then, incubate with water for 5 min at RT to remove the tannic acid completely before contrasting.
8. Contrast the samples with 0.1% uranyl acetate in water for 20 min at RT.
9. Incubate with increasing concentrations of ethanol for 10 min at each step: 30, 50, 70, 90% and anhydrous ethanol.
10. Add contrast by staining with 0.2% uranyl acetate in 100% ethanol for 20 min at RT.
11. Wash three times with 100% ethanol.
12. The samples need to be transferred into the coverslip-sample holder of the automated critical point dryer. Keep track of the sample position in the holder (Fig. 8a). Then, transfer the samples to the critical point dryer (e.g., Leica EM CPD 300). Set the device according to the manufacturer's instructions to perform 12 exchanges with liquid CO₂ at 5–10 °C prior to critical point drying (slow heating, slow speed during gas out).
13. Stick the dried glass coverslips on sticky notes (label them with the sample name) and adhere the sticky notes with double-sided tape onto the sample table in the sputter coater, such as Leica EM ACE600 (Fig. 8b and *see* **Note 11**).
14. Deposit 2.7 nm platinum at 1 mPa vacuum during 45° angle rotation (*see* manufacturer's protocol for setting up the protocol for sputter coating). Monitor the thickness of the coating with a quartz. Set the working distance to 45 mm and the sample height to 2 mm.
15. Deposit 5 nm carbon layer at 90°.

Fig. 7 (continued) oligodendrocytes at day 3 and **(b)**, **(d)** and **(f)**: 5 in vitro (DIV3 and DIV5 respectively) at different magnifications. The area in the white box is magnified in the panel below. The white arrows mark clathrin-coated pits and allow quantification of endocytosis. **(g)** Fluorescent confocal images of younger **(h)** and more mature primary mouse oligodendrocytes stained for actin with phalloidin in green and for tubulin in red, whereas the nuclei of the cells are shown in blue by a DAPI stain. The actin filaments are generally found at the leading edge of the cells and the processes, whereas tubulin is only found in the processes. The underlying architecture of the cytoskeleton cannot be determined with these confocal images and hence, replicas can reveal the ultrastructure of the cytoskeleton. Scale bars: 5 µm in **(a)** and **(b)**, 1 µm in **(c)** and **(d)** and 250 nm in **(e)** and **(f)**, 25 µm in **(g)** and **(h)**

Fig. 8 Critical point drying and platinum-carbon coating for generation of replicas for cytoskeletal imaging in transmission electron microscopy. **(a)** Image of the coverslip-sample holder of the critical point dryer immersed into anhydrous ethanol. The 12 mm coverslips are placed in the holder and the sample position is noted down. The inset on the right side shows in detail how the coverslips are positioned in the holder. **(b)** The dried glass coverslips are placed with double-sided tape onto the sample table of the sputter coater. The image shows the coverslips before (left) and after sputter coating (right) with platinum-carbon

16. Scratch horizontal and vertical lines into the coating of the coverslips in such a way that the resulting pieces of replicas fit on a 3 mm copper grid (*see Note 12*).
17. Float the coverslips on 10% hydrofluoric acid in a 12 well cell culture plate. The coverslips will sink down to the bottom of the plate and the replicas will float.
18. Use a Perfect loop™ to transfer the replicas to the next well filled with water and a droplet of highly diluted detergent.

19. Pick up the replicas from below to avoid them breaking apart and wash them in the next well with water (*see* **Note 13**).
20. Pick up the replicas with cells (*see* **Note 14**) on copper grids by pushing the replicas onto the grids with an eyelash attached to a wooden stick. Dry the grid and store them in a gridbox until imaging. No further contrasting is necessary.
21. Image by TEM. The resulting micrographs will appear with a 3D aesthetics after inverting the contrast.

4 Notes

1. Make sure to perfuse the animals at low speed to keep the ultrastructure as intact as possible. A good perfusion is critical for a good outcome of the electron microscopy.
2. Make sure to pack the tissue well in tea bag filter paper in such a way that the tissue cannot slip out of the baskets during transfer between different solutions (Fig. 1b). Furthermore, the flat end of the packet should be facing the wall of the 8-segment basket to generate a flat tissue for embedding as this avoids loss or crumbling of the sample (Fig. 1d).
3. Handle the samples after dehydration and infiltration with Epon resin (prior to embedding) with great care as they can easily break.
4. When dissecting the optic nerve fresh, avoid stretching the nerve as it induces artifacts.
5. Always clean forceps in between steps when performing an immunolabeling to avoid cross-contamination between samples and dirt particles on the sections.
6. Include a no-primary antibody control to detect nonspecific binding of the bridging antibody and Protein A.
7. Do not process more than 6–8 samples at a time as otherwise the methylcellulose layer becomes too thick, and hence makes it more difficult to image since the electron beam has to pass through.
8. Make sure to only touch the samples with forceps that are precooled to liquid nitrogen temperature as this avoids thawing, and hence freeze damage of the samples.
9. Do not let the samples fall dry during the freeze substitution.
10. Do not let the cells dry out.
11. Do not affix the coverslips too strongly on the sample table for the sputter coater as they will break upon detachment.

12. When edging the replicas, the squares in between the lines should not become too small as the replicas will break easily, when transferring them between the wells.
13. Try to immerse and remove the replicas with the loop on an angle as they will break into small pieces otherwise.
14. Replicas that contain cells can be identified due to the reflection of the cells in a binocular microscope.

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