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Protocol

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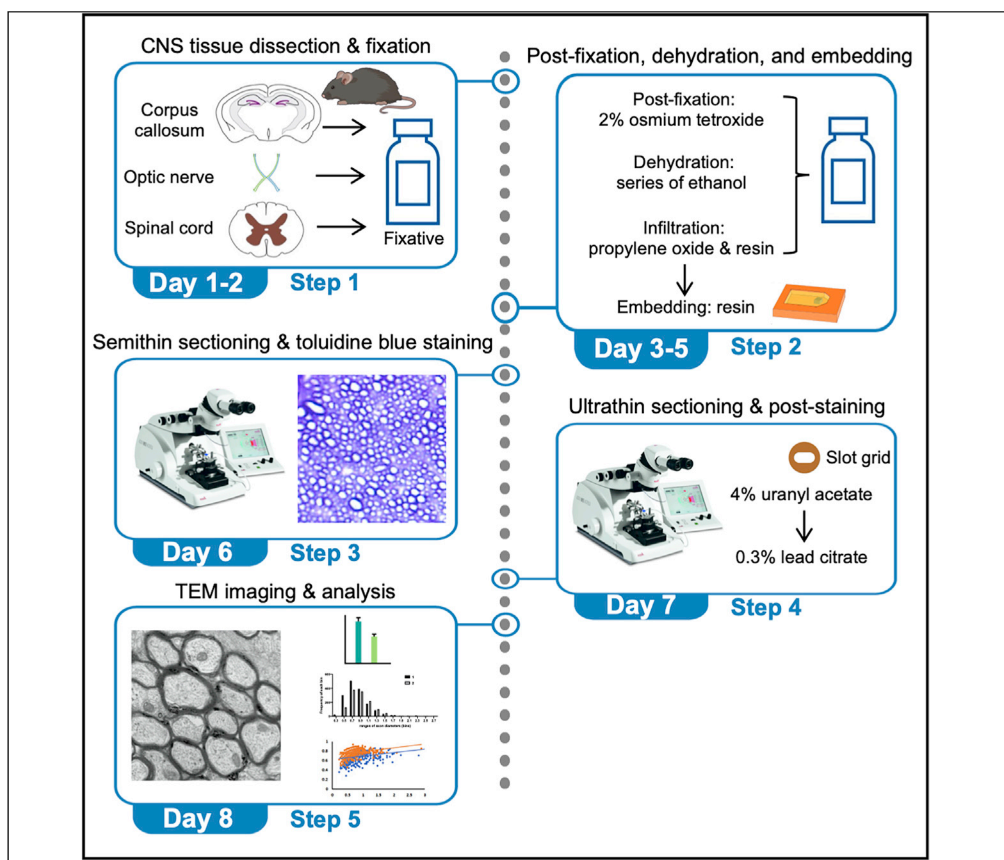

Myelin provides physical, neurotrophic, and metabolic support for axonal integrity. The thickness of CNS (Central Nervous System) myelin sheath is usually < one micrometer, which is under or near the detection threshold of the conventional light microscopy. Here, we present a high-resolution transmission electron microscopy-based protocol to assess myelination at the ultrastructural level. We describe sample preparation from mouse tissue, followed by electron microscopic imaging and CNS myelination analysis. This protocol is also useful for analyzing murine PNS myelination.

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Highlights

Protocol for CNS myelination assessment using transmission electron microscopy (TEM)

Samples preparation of the mouse brain, spinal cord, and optic nerve for TEM

Characterization and quantification of CNS myelination and myelin thickness

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Transmission electron microscopic analysis of myelination in the murine central nervous system

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SUMMARY

Myelin provides physical, neurotrophic, and metabolic support for axonal integrity. The thickness of CNS (central nervous system) myelin sheath is usually < one micrometer, which is under or near the detection threshold of the conventional light microscopy. Here, we present a high-resolution transmission electron microscopy-based protocol to assess myelination at the ultrastructural level. We describe sample preparation from mouse tissue, followed by electron microscopic imaging and CNS myelination analysis. This protocol is also useful for analyzing murine PNS myelination.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2021).

BEFORE YOU BEGIN

Myelination is the process by which oligodendrocytes produce a lipid-rich substance myelin that surrounds the neuronal axons. Myelin is essential for neurological functions (Góis Almeida, 2018). Defects of myelination are the hallmark of some neurodegenerative autoimmune diseases, such as periventricular leukomalacia, multiple sclerosis, and leukodystrophy. Hence, understanding myelin formation provides insights into developing treatment for myelin regeneration and remyelination in the central nervous system (CNS) demyelinating diseases (Höftberger and Lassmann, 2018).

The protocol below describes the specific steps for studying central nervous system myelin structure and myelination using transmission electron microscopy (TEM). This method is applicable to detect developmental myelination and demyelination induced by mouse models of demyelinating diseases.

Solutions are prepared following the recipes in the materials and equipment section. A complete list of materials and equipment required is given in the key resources table.

Institutional permissions

All the mouse procedures performed were approved by Institutional Animal Care and Use Committee at the University of California, Davis. Mice are 2 months old C57BL/6J strain. Researchers will need to acquire permissions from the relevant institutions.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sodium Chloride 0.9%	Fisher Scientific	Z1377
10× PBS	K-D Medical	Cat#RGF-3210
Sorensen's phosphate buffer, 0.2 M, pH7.4	Electron Microscopy Sciences	Cat#11601-10
50% Aqueous glutaraldehyde solution	Electron Microscopy Sciences	Cat#16320
16% Formaldehyde aqueous solution	Electron Microscopy Sciences	Cat#15700-5G
Sodium cacodylate buffer, 0.2 M	Electron Microscopy Sciences	Cat#11653
2% (w/v) aqueous osmium tetroxide	Electron Microscopy Sciences	Cat#19152
Propylene oxide	Electron Microscopy Sciences	Cat#20401
Ethyl Alcohol	Electron Microscopy Sciences	Cat#15058
Toluidine blue	Ted Pella Inc	Cat#19451
4% Uranyl acetate solution	Electron Microscopy Sciences	Cat#22400-4
Zetamine (katamine hydrochloride injection, 100 mg/mL)	VetOne	Cat#13985-584-10
Xylazine injection sterile solution (20 mg/mL)	AnaSed Injection	Cat#59399-110-20
50% Glutaraldehyde	Electron Microscopy Sciences	Cat#16130
Methyl-5-norbornene-2,3-dicarboxylic anhydride (NMA)	Electron Microscopy Sciences	Cat#19000
Benzyldimethylamine (BDMA)	Electron Microscopy Sciences	Cat#11400
Dodecenyl succinic anhydride (D.D.S.A.)	Electron Microscopy Sciences	Cat#13710
EMbed-812	Electron Microscopy Sciences	Cat#14900
Lead nitrate	Electron Microscopy Sciences	Cat#17900
Sodium citrate	Electron Microscopy Sciences	Cat#21140
Sodium hydroxide 1 N solution	Electron Microscopy Sciences	Cat#21170-01
Distilled water, laboratory reagent grade	Research Products International	W20525-4000.0
Critical commercial assays		
EMbed 812 Kit for electron microscopy embedding	Electron Microscopy Sciences	Cat#14120
Experimental models: Organisms/strains		
2 months old C57BL/6J mice (male and female)	The Jackson Laboratory	Cat#000664
Software and algorithms		
ImageJ	(Schindelin et al., 2012)	https://imagej.nih.gov/ij/
GraphPad Prism 8	GraphPad Software	https://www.graphpad.com/
Microsoft Excel	Microsoft Office	https://www.microsoft.com
Other		
Leica EM UC6 ultramicrotome	Leica Microsystems	Leica EM U6
Ultra diamond knife	DiATOME US	MD 601
Talos L120C TEM equipped with CETA 16 MP camera	Thermo Scientific	Talos L120C
Olympus BX61 Upright Metallurgical Microscope	Olympus	BX61
Nikon SMZ460 Stereo Zoom Microscope	Nikon	SMZ460
Grids support plate	Electron Microscopy Sciences	71560-32
Modified plate holder	Electron Microscopy Sciences	71560-20
Flat embedding mold	Electron Microscopy Sciences	70901-CB
Embedding capsule	Electron Microscopy Sciences	70000-B
Formvar carbon film coated single slot copper grid (2 × 1 mm)	Electron Microscopy Sciences	FCF2010-CU
Dissecting pan	Home Science Tools	DE-FLEXPAN
25-gauge winged infusion needle	Monoject	8881225315
Castroviejo micro dissecting spring scissors	Roboz Surgical Store	RS-5658
Iris scissors, curved	World Precision Instruments	501759
Iris scissors, supercut, straight	World Precision Instruments	14225-G

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
20 mL disportable scintillation vial	Kimble Chase	#FS74511-20
Acrylic mouse brain matrix, coronal, 1 mm	Braintree Scientific, Inc	BS-A-5000C
Stainless steel mouse spinal cord matrix, 0.5 mm	Braintree Scientific, Inc	BS-SS-SC 5005
High precision straight tapered ultra-fine point forceps	Fisherbrand	12-000-122
Personna platinum double edge razor blades	Personna	X000T0BTX9
Single edge razor blades	Genesee Scientific	38-101
Eyelash with handle brush	Ted Pella, Inc.	No. 113
Hot plate stirrer	Corning	PC-4200
Permount mounting medium	Fisher Chemical	SP15-100
0.2 μm syringe filter	Corning	431229
Quantitative grade filter paper	Fisherbrand	09-709-2G
Disposable petri dish	Fisherbrand	09-720-500

MATERIALS AND EQUIPMENT

Ketamine/xylazine anesthesia solution		
Reagent	Concentration	Amount
Zetamine (katamine hydrochloride injection, 100 mg/mL, VETone, #13985-584-10)	66.7 mg/mL	2 mL
Xylazine injection sterile solution (20 mg/mL, AnaSed Injection, #59399-110-20)	6.67 mg/mL	1 mL
Total		3 mL
Store at 4°C for a maximum of 2 weeks.		

Note: The anesthesia solution is injected intrapetritoneally 1.5 μL/g body weight (0.1 mg/g body weight for ketamine and 0.01 mg/g body weight for xylazine).

⚠ CRITICAL: Ketamine is a Schedule III substance under the Controlled Substances Act and should be used under regulations.

4% Formaldehyde aqueous solution		
Reagent	Concentration	Amount
16% Formaldehyde aqueous solution (Electron Microscopy Sciences #15700-5G)	4%	75 mL
0.2 M Sorensen's phosphate buffer (Electron Microscopy Sciences #11601-10)	0.1 M	150 mL
Deionized water (Electron Microscopy Sciences #22800-05)		75 mL
Total		300 mL
Prepare immediately before use. Adjust PH to 7.3–7.4. Store at room temperature (RT; 20°C–22°C). Filter using 0.22 μm filter before use.		

⚠ CRITICAL: Formaldehyde is a highly toxic systemic poison, exposure to which can be irritating to the eyes, nose, upper respiratory tract. Skin exposure can cause sensitization that may result in allergic dermatitis.

3% Glutaraldehyde		
Reagent	Concentration	Amount
50% Glutaraldehyde (Electron Microscopy Sciences #16130)	3%	18 mL
0.2 M Sorensen's phosphate buffer (Electron Microscopy Sciences #11601-10)	0.1 M	150 mL
Deionized water (Electron Microscopy Sciences #22800-05)		132 mL
Total		**300 mL**
Prepare one day before use. Adjust PH to 7.3–7.4. Store at 4°C. Filter using 0.22 μm filter before use.		

⚠ CRITICAL: Glutaraldehyde solution is noxious, which is a contact irritant, dermal sensitizer, and potential respiratory sensitizer.

Embedding resins		
Reagent	Concentration	Amount
Methyl-5-norbornene-2,3-dicarboxylic anhydride (NMA, Electron Microscopy Sciences #19000)	28.6%	14.3 g
Benzyldimethylamine (BDMA, Electron Microscopy Sciences #11400)	3%	1.5 g
Dodecenyl succinic anhydride (D.D.S.A., Electron Microscopy Sciences #13710)	32%	16 g
EMbed-812 (Electron Microscopy Sciences #14900)	58%	29 g
Total		**50 g**
Prepare immediately before use at RT.		

⚠ CRITICAL: All the components of epoxy resin components are toxic, irritating, potentially carcinogenic, particularly when they are hot. They are not safe until completely polymerized. The waste residual must be fully polymerized at 60°C at least overnight (12–16 h) before disposal.

0.3% lead citrate		
Reagent	Concentration	Amount
Lead nitrate (Electron Microscopy Sciences #17900)	0.0266 g/mL	1.33 g
Sodium citrate (Electron Microscopy Sciences #21140)	0.0352 g/mL	1.76 g
Sodium hydroxide 1 N solution (Electron Microscopy Sciences #21170-01)	0.2–0.28 g/mL	5–7 mL
Distilled deionized water (Millipore Sigma #101262)		Add to 50 mL
Total		**50 mL**

Add 1.33 g lead nitrate and 1.76 g sodium citrate to ~30 mL of boiled distilled water in a 50 mL conical tube. Shake vigorously for 1–2 min and continue shaking 3–4 times over 30 min to ensure complete conversion of lead nitrate to lead citrate. Add 5–7 mL 1 N NaOH to the mixture and add distilled water to a final volume of 50 mL. Store in sealed bottle at RT for 6 months. Filter before use.

⚠ CRITICAL: Lead citrate is extremely toxic. It is harmful if swallowed, inhaled, or absorbed through the skin. It may cause cancer and reproductive and fetal effects. Use and prepare under a fume hood.

⚠ CRITICAL: When preparing or using the hazardous substances mentioned above, always work under a chemical fume hood to prevent release of the hazardous substances into

general laboratory space and avoid inhalation. Wear lab coat, gloves, and safety goggles to control exposures to skin and eye irritants. Wash hands carefully at the end of the experiment. Make sure to place the wastes in the appropriate waste bottles for disposal. Clean the experimental area.

STEP-BY-STEP METHOD DETAILS

Perfusion fixation

Timing: 2.5 h

Mice transcardiac perfusion (Methods video S1) and tissues fixation.

1. Prepare the NE-1000 programmable single syringe pump. Fill 20 mL of ice cold 0.9% saline in a 60 mL syringe and install the syringe in the syringe pump. Flush the system with 0.9% saline without air bubbles. Adjust pump to a flow rate of 1 mL/min.
2. Deeply anesthetize mouse by intraperitoneally injecting 1.5 μL/g body weight of ketamine (0.1 mg/g body weight) and xylazine (0.01 mg/g body weight) mixture. Check the mouse anesthesia by pinching its paw, until it does not twitch.
3. Transcardiac perfusion of the mouse to prevent coagulation of the blood.
   a. Stretch out the mouse with the back down on a dissecting pan with needles. Wet the fur with 70% ethanol.
   b. Use tweezers and operating/dissecting scissors to open the skin and expose the chest cavity.
   c. Cut open the diaphragm.
   d. Grab at the base of the sternum using tweezers. Cut through the ribcage to expose the heart.
   e. Insert a 25-gauge winged infusion needle into the left ventricle of the heart. Control the position of the needle without injuring the aorta.
   f. Cut the right atrium immediately to prevent recirculation of the solution.
   g. Perfuse with 0.9% saline solution until the liver becomes clear (approximately 20 min) at low speed of perfusion (1 mL/min).

Note: Perfuse the mouse at a low speed. High perfusion pressure will rupture blood vessels and allow blood cells into the surrounding tissues.

4. Fill a new 60 mL syringe with 60 mL of 4% formaldehyde. Take the infusion needle out of the mouse heart. Remove the infusion tubing from the syringe with 0.9% saline and remove the syringe from pump. Install syringe with 4% formaldehyde in the pump, connect the infusion tubing to the syringe and remove air bubble. Insert needle into left ventricle and perfuse for 60 min (1 mL/min). The animal will twitch and then become stiff.
5. Exchange the flushing solution to 3% glutaraldehyde and perfuse for another 60 min (1 mL/min). The exchange method is the same as step 4.

Note: Perfusion fixation allows the access of the fixative to cells in a rapid manner so that cells can be quickly fixed. The fixative hardens tissues and decreases tissue damage and morphological change during subsequent tissue collection.

Note: Perfusion time depends on the weight of mice. A total perfusion time of 2 h ensures that the largest mice (~30 g) are adequately fixed.

⚠ CRITICAL: Preserving myelin sheath integrity by appropriate fixation methods is critically important for a successful assessment of the CNS myelination by TEM. Inappropriate fixation of myelin sheath during this step usually results in myelin sheath rupture or damage (Figure 6).

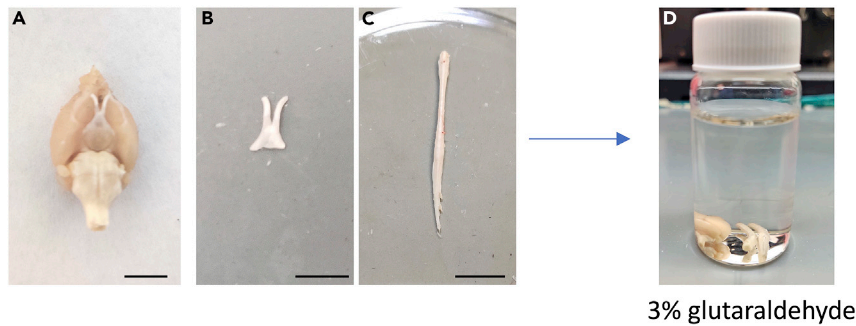

Figure 1. Illustration of tissue collection
(A) Adult mouse brain attached with optic nerve.
(B) Optic nerve.
(C) Spinal cord.
(D) Immersion fixation of tissues in 3% glutaraldehyde at 4°C overnight. Scale bars: (A and B) 0.5 cm; (C) 1 cm.

Tissue dissection and post-fixation

⏲ Timing: 30 min for tissue dissection and overnight for immersion fixation

Dissect the optic nerves, brain or spinal cord immediately as described below and immerse the tissue in 3% glutaraldehyde for fixation overnight at 4°C.

6. Dissection of spinal cord (Methods video S2).
   a. Lay down the mouse with the back facing up on a dissecting pad. Wet the fur with 70% ethanol. Remove dorsal skin from head to tail.
   b. Remove the muscle from the entire spine and expose the vertebrae using fine curved scissors with the blade tips facing up.
   c. Decapitate the mouse head.
   d. Gently hold the dorsal spine, insert straight supercut scissors laterally at the side of the spine canal and cut both sides of the spine. Pay attention not to damage the spinal cord. Continue cutting in this way along the entire spine, until the whole spinal cord is exposed.
   e. Gently lift the cervical spinal cord, cut the attached dorsal root ganglia on both sides (Figure 1C).
   f. Place the spinal cord on a Petri dish and cut it into two pieces at the thoracic region.
   g. Place the spinal cord in a 20 mL disportable scintillation vial containing ~ 15 mL of 3% glutaraldehyde for fixation overnight at 4°C in a refrigerator (Figure 1D).
7. Dissection of optic nerves and brain (Methods video S3).
   a. Expose the skull by removing the skin.
   b. Remove the connective tissue around the eyes to gain access to the eye socket.
   c. Insert micro dissecting spring scissors carefully into the eye socket behind the eyeball, cut the optic nerve and remove the eye.

   Note: Avoid stretching the nerves by pulling the eyes.

   d. Cut the skull laterally on both sides using a scissor.
   e. Cut the skull from the brainstem to the coronal suture along the sagittal suture. Then cut the coronal suture.
   f. Cut the skull along the lambdoid suture.
   g. Remove the skull to expose the brain.
   h. Insert forceps in a 45° angle into the forebrain and gently pull the brain out without stretching the optic nerve. The optic nerves should stay attached to the brain (Figure 1A).

i. Remove the optic nerve by cutting with micro dissecting spring scissors at the optic chiasm (Figure 1B).
j. Pick up the optic nerves gently at the chiasm with forceps and transfer into the same scintillation vial containing 3% glutaraldehyde overnight at 4°C in a refrigerator (Figure 1D).
k. Place the brain into the same vial containing 3% glutaraldehyde for fixation for overnight at 4°C in a refrigerator (Figure 1D).

Note: The entire tissue fixation overnight at 4°C in a refrigerator after the perfusion fixation is to fully fix the tissues and make sure tissues will not be damaged when sectioning.

Note: To separate tissues from different mice, place the optic nerve, brain, and spinal cord from one mouse in one vial for fixation.

⚠ CRITICAL: After fixation, carefully dissect the optic nerve, spinal cord, or brain without bending or distorting the tissues. Mechanic distortion of the fixed spinal cord or brain will likely result in myelin sheath damage, which can be seen in TEM images, particularly at the very high resolution.

Pause point: The tissues can be stored at 4°C in a refrigerator for one week.

Tissue slices preparation and trimming

Timing: 30 min for tissue preparation and overnight for immersion fixation

Prepare tissue slices using brain or spinal cord matrix and then trim the slices to retain the region of interest.

8. Corpus callosum section preparation.
   a. Place the brain dorsal side up in the brain matrix.
   b. Place the first blade in the channel, which lies at the true lambda (Figure 2A1).
   c. Place three more blades in the anterior three consecutive channels. The brain matrix allows slicing of coronal sections through the mouse brain at an interval of 1 mm (Figure 2A2).

   Note: Use the double-edge razor blades, which are thinner than the normal single-edge blades, thus minimizing the movement of the brain within the matrix and enabling accurate cutting.

   d. Lift out the three slices between these two blades. Transfer the brain section #2 in Petri dish for the following tissue trimming (Figure 2A3). Store the other two sections in 3% glutaraldehyde. Identification of the corpus callosum structure in the coronal sections was based on the annotation from Allen Mouse Brain Atlas (http://mouse.brain-map.org/static/atlas).
   e. Perform middle sagittal sectioning of the brain along the midline, divide the brain into left and right sides.
   f. For both sides, remove the cortex by cutting the section along the dorsal side of corpus callosum under stereo zoom microscope.
   g. Cut the section again along the ventral side of corpus callosum parallel to the dorsal cutting. The width of the two cuts is approximately 0.5 mm (Figures 2A4 and 2A5).
   h. Flip the corpus callosum to let the wider face lying on the Petri dish using ultrafine point forceps and cut a bevel at the outer edge of the corpus callosum as an embedding mark (Figure 2A6).
   i. Transfer the corpus callosum in a new disposable scintillation vial containing 3% glutaraldehyde on ice.

⚠ CRITICAL: Place the blades close to the edge of the corpus callosum when trimming and just keep the region of interest to make corpus callosum easy to be found under EM imaging. Remove the tissue that is not of interest.

⚠ CRITICAL: Slice brains from different mice at the same location to maintain consistency across different mice.

9. Separate the right and left optic nerves using a razor blade and transfer them in the same vial containing 3% glutaraldehyde on ice (Figure 2B).
10. Spinal cord section preparation:
    a. Place the spinal cord containing lumbar segments in the spinal cord matrix.
    b. Insert the first blade in the lumbar enlargement.
    c. Cut spinal cord into 1 mm sections by placing more blades in the anterior three consecutive channels (Figures 2C1 and 2C2).
    d. Transfer the spinal cord slices in 3% glutaraldehyde on ice (Figure 2D).
11. Fix optic nerves, corpus callosum and spinal cord slices from one mouse in a new disposable scintillation vial containing 15 mL of 3% glutaraldehyde at 4°C overnight (Figure 2D).

⚠ CRITICAL: Drop 3% glutaraldehyde on the tissues when slicing tissues. Do not let tissues dry during the whole processes.

Tissue embedding in epoxy resin

Timing: 3 days

Day 1: Tissue post-fixation, dehydration, and infiltration.

12. Decant 3% glutaraldehyde, add cold 0.2 M sodium cacodylate buffer and wash 2 × 10 min in a refrigerator at 4°C. 0.2 M sodium cacodylate buffer is stored at 4°C in a refrigerator.

⚠ CRITICAL: Sodium cacodylate is considered particularly hazardous by ingestion, inhalation, and skin absorption. It may cause disturbances of the blood, kidneys, and nervous system.

13. Place tissues for post-fixation in scintillation vials containing 2% osmium tetroxide for 1–1.5 h at RT in a chemical fume hood. Wrap the vials using aluminum foil to protect the samples away from direct light.

Note: Osmium tetroxide is light sensitive.

⚠ CRITICAL: Osmium tetroxide is acutely toxic and capable of fixing the mucous membranes of the eyes and nose causing irreversible damage. Skin exposure will result in black dots. It causes long-term toxicity to liver and kidneys.

14. Perform 2 × 10 min wash in cold 0.2 M sodium cacodylate buffer in a 4°C refrigerator.
15. Wash 2 × 5 min in cold distilled H_2O.
16. Dehydrate tissues in increasing concentrations of ethanol:
    a. Quick wash tissues with 50% ethanol and 2 × 10 min wash in freshly prepared 50% ethanol at 4°C.
    b. Quick wash tissues with 70% ethanol and 2 × 10 min wash in freshly prepared 70% ethanol at 4°C.
    c. Quick wash tissues with 90% ethanol and 2 × 10 min wash in freshly prepared 90% ethanol at 4°C.

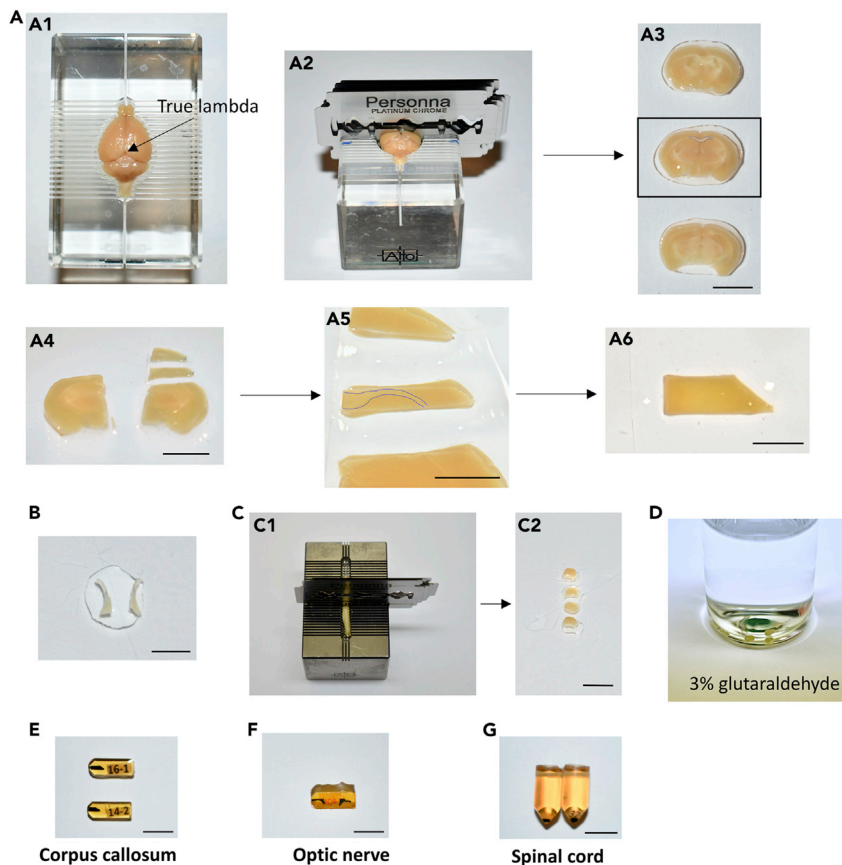

Figure 2. Illustration of tissue sectioning and embedding

(A) Brain sectioning. (A1) Brain in a brain slicer matrix. Arrow shows the true lambda (Benskey and Manfredsson, 2016). (A2) Brain was cut into 1 mm slices with a brain slicer matrix and razor blades. (A3) Three coronal sections of the mouse brain. The second section was used for corpus callosum analysis. The line shows the edge of corpus callosum. (A4) Trimming of the brain to obtain the region of interest, corpus callosum. (A5) Enlarged picture of Figure A4. The line shows the edge of corpus callosum. (A6) Embedding mark cut at the outer edge of the corpus callosum.

(B) Separated right and left optic nerves using a razor blade.

(C) Spinal cord sectioning. (C1) Spinal cord was cut into 1 mm slices at the region of lumbar enlargement with a spinal cord slicer matrix and razor blades. (C2) Four coronal sections of spinal cord.

(D) Immersion fixation of tissues sections in 3% glutaraldehyde at 4°C in a refrigerator overnight.

(E–G) Resin embedding of the corpus callosum, optic nerve and spinal cord. Small, printed labels are included in the blocks to identify the sample. Scale bars: (A3, A4, B and C2) 0.5 cm; (A5 and A6) 0.25 cm; (E–G) 1 cm.

d. Quick wash tissues with 100% ethanol and 3 × 20 min wash in 100% ethanol at RT (tissues acclimate to RT).

Note: Completely dehydrate the tissues, otherwise water remaining in the specimen will not allow epoxy resin to completely infiltrate the specimen and fail to polymerize.

17. Quick wash tissues with propylene oxide and then wash 3 × 30 min in propylene oxide at RT.
18. Overnight infiltration in freshly made mixture of propylene oxide and embedding resin (1:1) at RT with rotation.

Note: Agitate gently to prevent samples to attach the vial surface.

Day 2: Tissue infiltration.

19. 8 h infiltration in freshly made propylene oxide: embedding resin mix 1:3 at RT with rotation.
20. Overnight infiltration in freshly made pure embedding resin at RT with rotation.

Note: Agitate gently to prevent samples to attach the vial surface.

Day 3: Tissue embedding and polymerization.

21. Place tissues in appropriate embedding molds with freshly prepared embedding resin. Add printed labels in the mold for identification.

Note: Write the label for each specimen with a pencil or with a laser printer. Do not use ink as it would be dissolved by reagents.

⚠ CRITICAL: Thoroughly and slowly mix embedding resin ingredients prior to use and eliminate bubble formation. Remove bubbles from the embedding mold or capsules using pipette tips. Bubbles will make cutting difficult and make samples unstable in the electron beam.

   a. Place corpus callosum at the bottom of the flat embedding mold with the medial corpus callosum close to the cutting edge of the mold.
   b. Place optic nerve at the bottom of the flat embedding mold with the anterior optic nerve close to the cutting edge of the mold.
   c. Place the spinal cord segment near the extremities of the embedding capsule.

⚠ CRITICAL: Carefully position the tissues to favor the sectioning and experimental purpose when placing them in embedding mold or capsule. During the process of resin polymerization, check and adjust the position of tissues in case of slight movement of tissues using a tapered ultra-fine point tweezers.

22. Polymerize the embedding resin block in an oven at 60°C overnight. Block can remain in the oven for a longer period for sufficient hardness. Remove the block from the mold and store in plastic bags at RT. (Figures 2E–2G).

Note: Use the same scintillation vials with caps from step 8 throughout the whole processes of tissue post-fixation, dehydration, and infiltration. Decant the vials and fill up with a new solution.

⚠ CRITICAL: All the reagents and solutions are prepared immediately before use and used one time.

Do not allow the tissues to dry out during dehydration and embedding.

⏸ **Pause point:** The polymerized embedding resin block can be stored at RT to be sectioned later.

Semi-thin sectioning and toluidine blue staining

⏲ **Timing: 1 h**

Trim the block to 1 mm^2 size to accommodate the grid. Next, expose the nerve to make it visible and correct the orientation of the specimen using semi-thin sectioning and toluidine blue staining.

23. Trim the cutting surface of polymerized resin blocks down to approximately 1 mm^2 size using single edge razor blade.

Note: Razor blades are easy to be worn out when trimming the hard blocks, so change the razor blades occasionally.

24. Semithin sectioning. Appropriately orientate the tissue blocks, make sure the sections (semithin and ultrathin) transect across the longitudinal axonal bundles. For detailed processes, please refer to Methods video S4.
    a. Cut resin blocks to 0.5 μm semithin sections in filtered distilled H_2O using Leica EM UC6 ultramicrotome and diamond knife.
    b. Collect and place the semi-thin sections on a slide with water droplets using the eyelash with handle brush.
    c. Place the slide on a hotplate stirrer at ~ 70°C and allow the distilled H_2O to evaporate. Circularly move the slide to flatten the sections.
25. Toluidine blue staining.
    a. When the sections dry out, add drops of toluidine blue staining solution on them and incubate until the edge of the drops begin to dry.
    b. Remove the slide from the hot plate, rinse under tap water and dry them on the top of hot plate. Mount the slide with a coverslip and Permount mounting medium.
    c. Visualize the sections using Olympus BX61 upright motorized microscope combined with oil immersion (100×) objective lens. Take microscopic images in bright field.

Note: The purpose of semithin sections stained by toluidine blue is to observe myelin for further analysis of electron microscopy. Although semithin sections stained by toluidine blue can be technically used to identify the number of myelinated nerve fibers and gross alterations in myelination, ultrathin sections imaged by electron microscopy is required for a more detailed myelination analysis, such as myelin thickness, the process of myelination (ensheathment, wrapping and compaction), axonal degeneration and regeneration, and node of Ranvier.

Ultrathin sectioning and post-staining

Timing: 1.5 h

Cut ultrathin sections using an ultramicrotome and post-staining with uranyl acetate and lead citrate.

26. Ultrathin sectioning (Methods video S4):
    a. Use Leica EM UC6 ultramicrotome and diamond knife to cut ultrathin sections in filtered distilled H_2O using 0.2 μm syringe filter. The gold color indicates approximate 100 nm of thickness.
    b. Directly collect section on formvar carbon film coated single slot copper grid. Check it under a microscope to make sure the section is in the right area. Allow the sections to dry up on filter paper.

Note: Formvar carbon film coated grid can stabilize material and make the grid more resistant to the handling during preparation.

27. Post-staining using grid staining pad (Methods video S5).
    a. Carefully insert the edge of grid into the grid staining pad (Figures 3A and 3B). Place the grid staining pad on a piece of parafilm. Drop uranyl acetate solution to fully cover the gird, which forms a solution dome covering the grid. Stain for 20 min away from direct light by covering the grid using a black lid.

Note: Use of a grid staining pad can increase efficiency and reduce handling processes and potential mechanical damage to grids. Comparable grid staining pad is available for purchase (Electron Microscopy Sciences, # 70901-CB, and 70000-B) (Hiraoka, 1972; Seifert, 2017).

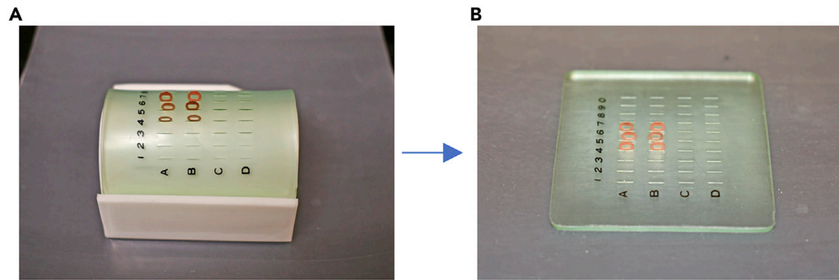

Figure 3. TEM post-staining using grid staining pad
(A) Inserting the grids into the grid staining pad.
(B) Take the grid pad off from the plate holder ready for staining.

Note: The uranyl ions combine in large quantities with phosphate groups in nuclei acids as well as phosphate and carboxyl groups on the cell surface including phospholipids and phosphoproteins (Wyffels, 2001).

⚠ CRITICAL: Centrifuge an appropriate volume of 4% aqueous uranyl acetate at 5,000 g for 10 min at 4°C and filter with a 0.22 μm syringe before use. The uranyl acetate crystallizes as needles or rhombi-formed crystals or granular aggregates.

⚠ CRITICAL: Uranyl acetate is sensitive to light and will precipitate if exposed (Figure 6B). Store at 4°C in a refrigerator in the dark.

⚠ CRITICAL: Uranyl acetate is both radioactive and toxic. It has mild radioactivity that is not sufficient to be harmful while the material remains external to the body. However, uranyl acetate is very toxic if ingested, inhaled or contacted if skin is cut or abraded. Please follow the law regarding how to work with and how to dispose of the radioactive waste material.

b. Discard uranyl acetate solution. Rinse staining pad briefly using distilled water.
c. Rinse the grid staining pad in three disposable beakers filled with distilled water. Allow the grids to dry.

⚠ CRITICAL: Rinse the grid thoroughly with water, as the uranyl acetate forms crystal precipitate if not removed from the section.

d. Stain with lead citrate for 1 min. Rinse staining pad briefly using distilled water.

Note: Lead citrate binds to osmium and uranyl acetate. It is used as enhancer for heavy metal staining in electron microscopy, thereby enhances the contrast of several cellular structures.

⚠ CRITICAL: Filter lead citrate with a 0.22 μm syringe filter before use.

⚠ CRITICAL: Lead citrate precipitates easily in the presence of carbon dioxide (Figure 6B). To prevent precipitation of the lead citrate by exposure to CO_2, we used lead citrate in syringe and got rid of air bubble to exclude atmospheric CO_2. In addition, the water used for preparing the stain or for rinsing should be distilled CO_2 free.

⚠ CRITICAL: Quickly transfer grids during preparation, not breathe on grids, which can cause lead-carbonates precipitates formation.

e. Rinse the grid staining pad in three disposable beakers filled with distilled water.

f. Dry the grids out using filter paper. Store grids in a grid storage box for EM imaging.

Alternatives: Post-staining (place grid on drops of stain).

g. Place a piece of parafilm on a Petri dish and drop uranyl acetate solution on the parafilm. Place the grid on the drop of uranyl acetate solution and incubate for 20 min away from direct light by covering the grid using a black lid. The side with the samples should be facing the staining solution.
h. Remove the grid after incubation using forceps and rinse it by dipping the grid for 15s into three 50 mL Falcon tubes containing distilled H_2O. Drain off excess distilled H_2O by gently touching filter paper to the edge of the grid.
i. Place a piece of parafilm in a new Petri dish and place the grid on the parafilm. Drop 0.3% lead citrate in 0.1 N sodium hydroxide on the grid. Incubate for 1 min keeping the Petri dish covered with lid.
j. After staining, rinse the grid 4 times for 15 s each using distilled water. Drain off excess distilled H_2O and place the grid on a clean filter paper to dry. Store the grid in a grid box for EM imaging.

Pause point: Samples can be stored at room temperature to be imaged later.

TEM imaging

Timing: 1 h

Use FEI Talos L120C TEM equipped with CETA 16 MP camera or a TEM with similar configuration to obtain images of ultrathin sections (Figures 4B and 4C).

28. Mount the grid in a standard TEM holder. Place the specimen facing down inside the specimen carrier. Then insert the holder into the TEM (Figure 4A).
29. Turn the magnification down to find the stained sheets. Scan the grid at low magnification (×1250) to get an overall image of the myelinated regions. The acceleration voltage is 80 kV.
30. The Stage^2 control panel provides functionality for storing positions and tracks (Figure 4D). We firstly use the Stage^2 control panel to label the outline of the myelinated region of a tissue. Then we randomly acquire 5–30 images per section (5 images for optic nerve and corticospinal tract, 30 images for corpus callosum) at higher magnification. To avoid taking repeated images, we use the Stage^2 control panel to mark the stage position of different images.
31. Remove the grid from the TEM.

EXPECTED OUTCOMES

In this study, we chose the corticospinal tract of the spinal cord as an example to characterize and analyze the CNS myelin. The corticospinal tract is a white matter motor pathway that conveys motor commands from the brain to spinal motoneurons (Moreno-Lopez et al., 2021).

Toluidine blue is an acidophilic metachromatic dye, discovered by William Henry Perkin in 1856 (Sridharan and Shankar, 2012). It selectively stains acidic tissue components like sulfates, carboxylates, and phosphate radicals (Epstein et al., 1992). Toluidine blue staining of resin embedded semithin nerve sections is a gold standard for light microscopic imaging of myelinated nerve fibers, providing high quality and detailed images of nerve structures (Ghnenis et al., 2018). As shown in Figure 5A, toluidine blue staining can preserve nerve structure in its natural form, as well as dark staining of myelin sheaths in the corticospinal tract in spinal cord. The staining of myelin sheaths is due to osmium tetroxide post-fixation but not toluidine blue (Di Scipio et al., 2008). Quantification results show a significant decrease in the myelinated axon density in gene knockout (KO) mice compared with wildtype (WT) mice (Figure 5B).

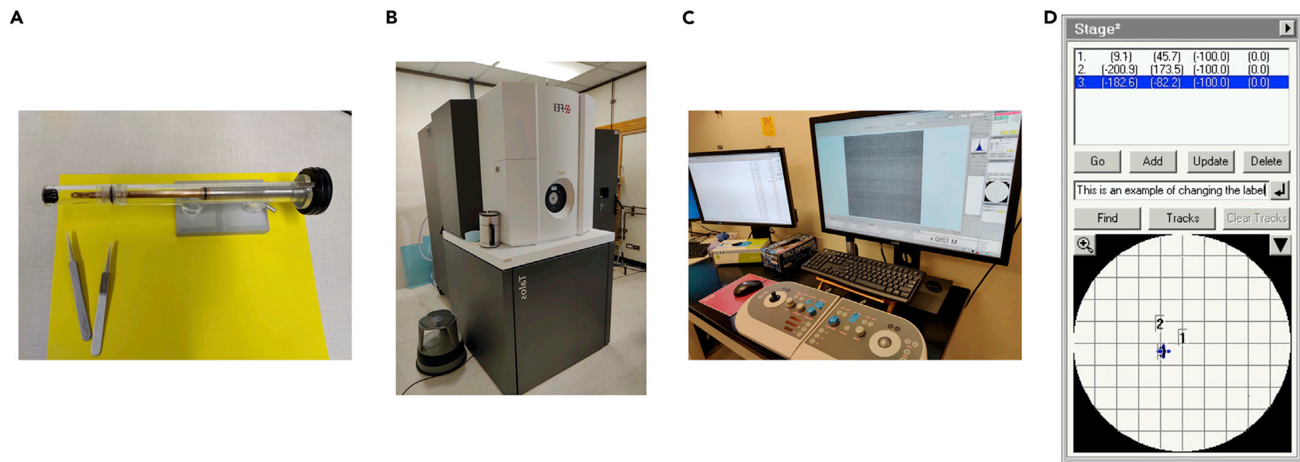

Figure 4. Illustration of TEM image taken

(A) Mount the grid in a standard TEM holder.

(B and C) FEI Talos L120C TEM equipped with CETA 16 MP camera to obtain images.

(D) Stage^2 Control Panel provides functionality for controlling the CompuStage, storing positions and tracks.

TEM images at low magnification (×1250) show the distribution pattern of myelinated axons in the corticospinal tract of spinal cord (Figure 5C). To visualize the structural details of myelin sheaths and quantify the myelin thickness, we observed myelinated axons at higher magnification (×6500) (Figure 5D). The myelin sheaths and axon ultra-structure of WT and gene KO mice were in general well preserved. The myelinated axons could be identified and counted (Figure 5E). The axon diameter (d) and myelinated fiber diameter (D) were clearly identifiable and therefore could be quantified as described below (Figure 5F). Figure 5G shows g-ratio calculation of elongated myelinated axons (see quantification and statistical analysis section). Among the myelinated axons, the myelin sheath was significantly thinner in gene KO corticospinal tract as evidenced by a greater g-ratio than that of WT (Figure 5H). We then plotted the myelinated axon size distribution of WT and gene KO mice. As shown in Figure 5I, their axon size patterns are nearly identical, and most of the axon diameters are in the range of 0.5–0.9 μm in the corticospinal tract. The typical average g-ratio of normal control mice is 0.67 $\pm$ 0.013 in the corticospinal tract. We then examined the correlation of g-ratio distribution and myelinated axon diameter. The g-ratio of each sample overlaps extensively for axons with 0.5–1 μm in diameter, which is consistent with the axon size distribution (Figure 5J).

QUANTIFICATION AND STATISTICAL ANALYSIS

All images were saved as TIFF format to obtain quantitative measurements in Image J (NIH).

Quantification of semithin sectioning with toluidine blue staining

Quantification of the density of myelinated axons. This method is based on counting the number of myelinated axons in the defined unit area. The density of myelinated axons is calculated as the number of myelinated axons per square millimeter.

The following criteria are applied for the TEM analysis:

1. Include axons with diameter >0.3 mm for analysis (Lee et al., 2012), include myelinated axons that intersected the right or the lower border of the sampling area, discard the myelinated axons with awkward shape in which a diameter could not be defined.
2. Use at least 3 mice per group. The number of pictures taken depends on the size of myelinated region in different tissues. For optic nerve and corticospinal tract, we take 5 images per section. For corpus callosum, we take 30 images per section. We analyze all the images we take in the following three analysis.

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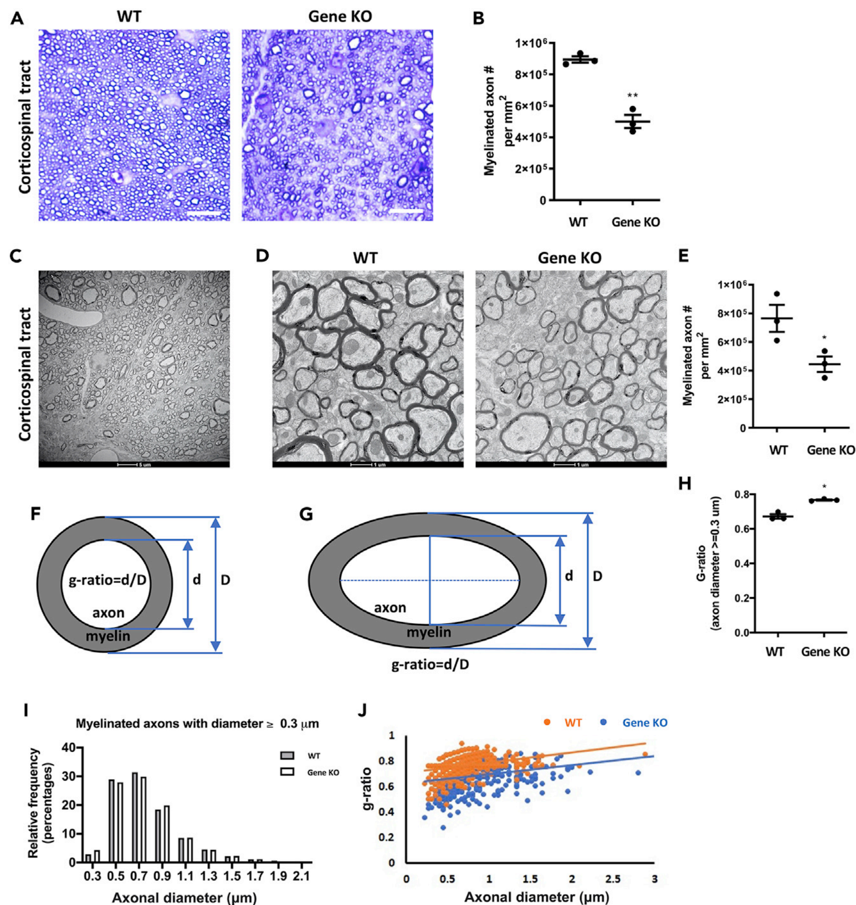

Figure 5. Quantitative analysis of myelination of TEM samples

(A) Toluidine blue myelin staining of the corticospinal tract in the spinal cord on semithin (500 nm) sections of WT and gene KO mice. Scale bar, 10 μm.

(B) Quantification of myelinated axon density in toluidine blue staining. Data are represented as mean ± s.e.m. **$p < 0.01$.

(C) Representative picture of myelin at low magnification (×1250) in the corticospinal tract. Scale bar, 5 μm.

(D) Representative pictures of myelin at higher magnification (×6700) in the corticospinal tract. Scale bar, 1 μm.

(E) Myelinated axon density (mean number of myelinated axons per square millimeter) in WT and gene KO mice. Data are represented as mean ± s.e.m. *$p < 0.05$.

(F) Diagram and calculation of g-ratio of myelinated axons.

(G) Diagram and calculation of g-ratio of elongated myelinated axons.

(H) Quantification of g-ratio. Data are represented as mean ± s.e.m. *$p < 0.05$.

(I) Percentage distribution of myelinated axons size.

(J) Scatter plot of individual g-ratio versus axons. Axons with diameter > 0.3 mm were included for analysis. Dots in panel (B, E, and G) represent different mice.

TEM analysis and quantification

Myelinated axon density

Count the number of myelinated axons in the defined unit area. For normal corticospinal tract, we count 50–60 axons in a typical area of 70 μm^2. The density of myelinated axons is calculated as the number of axons per square millimeter (Figures 5D and 5E).

Myelin sheath thickness (g-ratio)

The myelin g-ratio is calculated as the ratio between the inner (d) and the outer (D) diameter of the myelin sheath: g-ratio = d/D. g-ratio is widely utilized as a functional and structural index of myelin sheath thickness of individual axon fibers (Figure 5F). The smaller g-ratio demonstrates the thicker myelin sheath layer.

In the case that the angle of plane of the section produces elliptic or elongated myelinated axons, first identify the axis producing the longest diameter of the axon and then measure the inner and outer diameter perpendicular to this (Figure 5G).

Print each image, measure manually and calculate according to scale bar.

Size of myelinated axons

The diameter of each myelinated axon was measured within the inner borders of the myelin sheath, excluding the myelin sheath. Then frequency distribution of the diameters of myelinated axons is analyzed according to their diameters (Figure 5I).

Scatter plots of g-ratio versus axon diameter

Use Excel to analyze the relation between axon diameter and g-ratio (Figure 5J).

Statistical analysis

Quantification was performed by observers blind to animal genotype and treatment. Data were presented as mean ± s.e.m. Unpaired two-tailed Student's t test was used for statistically analyzing two groups of datasets. One-way ANOVA followed by Tukey's post-test was used for the statistical analysis of three or more groups of datasets. All data graphing and statistical analyses were performed using GraphPad Prism version 8.0. *P* value less than 0.05 was considered as significant. ns stands for not significant with *P* value greater than 0.05.

LIMITATIONS

When considering TEM as a research tool for the CNS myelin study, researchers must appreciate its limitations. One of the most troublesome limitations is the lengthy procedure and laborious preparation of samples. The experience of the researchers plays a very important role in preserving the quality of the samples and preventing potential artifacts from sample preparation. Operation of the TEM and analysis of the data also require special training.

TROUBLESHOOTING

Problem 1

Poor sample and/or image quality (Figure 6; steps 28–30).

Potential solution

Good perfusion, fixation and tissue collection are critical for a good outcome of TEM imaging.

- Perfuse the mouse at a low speed to keep the tissue ultrastructure intact. Insert the perfusion needle correctly into the left ventricle to let saline or fixative distribute throughout the vascular system properly. A successful perfusion can be judged by the blanching liver and twitching tail, indicating that the flush and fixative are flowing unimpeded through the circulate system. If the perfusion is not successful, the mouse tissues will not be properly fixed. In this case, the perfusion needle should be repositioned. Improper perfusion at this initial step will cause tissues not properly preserved failure for the entire process (Methods video S1).
- Insufficient fixation will probably result in myelin sheath rupture or damage. After 2 h perfusion fixation, we do 2 days immersion fixation of isolated tissues and slices.
- After fixation, carefully dissecting the spinal cord, brain, and optic nerve without damaging, bending, or distorting the tissues. Mechanic distortion of the fixed tissues will also cause myelin sheath damage.

Problem 2

Resin fails to polymerize properly (step 22).

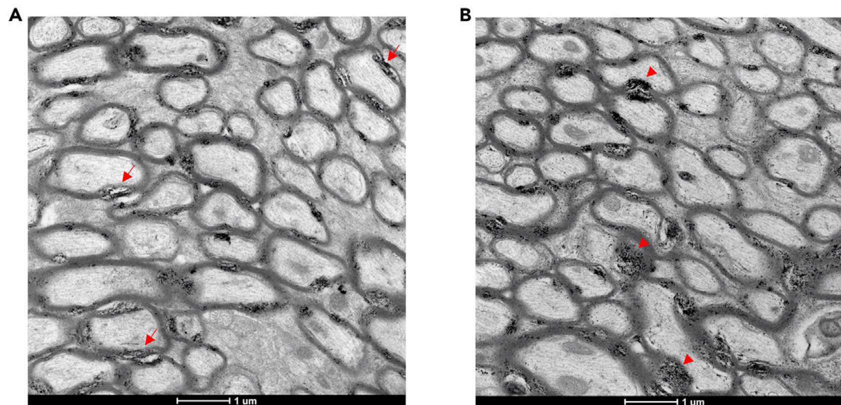

Figure 6. TEM images with potential artifacts
(A and B) Poor image quality and elongated myelinated axons. (A) Arrows, myelin sheath rupture or damage. (B) Arrowheads, myelin sheath rupture with uranyl acetate or lead citrate precipitates. Scale bar, 1 μm.

Potential solution

- Totally dehydrate samples using ethanol series before transferring to propylene oxide and embedding. The epoxide resin is intolerant of water. Also, water remaining in the sample will not allow resin to completely infiltrate the sample. These result in rubbery blocks, which are impossible to be sectioned. In this case, retrieve tissues to correct the issue.
- Uneven mixing of embedding mixture ingredients, wrong proportions, insufficient time for mixing and incubation may prevent proper embedding mixture infiltration and polymerization.

Problem 3

Myelin sheath resembles an ellipse in shape, but not of circular form (Figure 6; steps 28–30).

Potential solution

- Carefully orientate and position the tissue in embedding blocks to favor the sectioning. For corpus callosum preparation, cut the brain section parallelly along with the corpus callosum nerve fibers. For spinal cord, cut the 1 mm sections vertically to spinal cord nerve fibers. When embedding, orientate the axonal bundles vertically to the sectioning surface. Check and adjust the position of nerves during resin polymerization using ultra-fine forceps.
- Appropriately orientate tissue blocks when sectioning, and make sure the sections transect across the longitudinal axonal bundles (Methods video S4).
- Another reason for ellipse myelin sheath shape is insufficient tissue fixation. Perfuse the mouse at low speed for 2 h with fixative to ensure sufficient fixation, harden tissues and decrease tissue damage during collection. Carefully hold tissue using forceps with wide tip and keep the original shape of the tissues. Do not pull, distort, or bent spinal cord or optic nerve during collection. Otherwise, cellular, or subcellular shape of the tissue will be changed (Methods videos S2 and S3).

Problem 4

No myelinated axons exist in the specimen of corpus callosum (steps 28–30).

Potential solution

It is easy to observe the myelinated axons in optic nerve and corticospinal tract, but researchers often cannot find myelin in the corpus callosum. The reason is improper brain slice preparation and corpus callosum isolation.

- Use brain matrix for brain slicing and cut the brain at the right location (true lambda for 1st cut) (Figure 2A).
- Use stereomicroscope to cut and isolate corpus callosum and make the tissue sample as small as possible (Figure 2A).
- Embed corpus callosum in the right direction (Figure 2E).

Problem 5

Image is distorted or cannot be focused (steps 28–30).

Potential solution

- Specimen holder contamination. Take the specimen holder out, clean or replace a clean one.
- Grid or specimen contamination. Take picture of the non-contaminated area. Use filtered distilled water for ultrathin sectioning collection and use clean grid in the future.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Fuzheng Guo (fzguo@ucdavis.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101302.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.W. and F.G.; Investigation, Y.W., B.Sun., and B.Shibata; Writing, Y.W.; Review & Editing, F.G.; Funding Acquisition, Y.W. and F.G.

DECLARATION OF INTERESTS

The authors declare no competing interest.

REFERENCES

Benskey, M.J., and Manfredsson, F.P. (2016). Intraparenchymal stereotaxic delivery of rAAV and special considerations in vector handling. Methods Mol. Biol. *1382*, 199–215.

Epstein, J.B., Scully, C., and Spinelli, J. (1992). Toluidine blue and Lugol's iodine application in the assessment of oral malignant disease and lesions at risk of malignancy. J. Oral Pathol. Med. *21*, 160–163.

Ghnenis, A.B., Czaikowski, R.E., Zhang, Z.J., and Bushman, J.S. (2018). Toluidine blue staining of resin-embedded sections for evaluation of peripheral nerve morphology. J. Vis. Exp. *137*, 58031.

Góis Almeida, R. (2018). The rules of attraction in central nervous system myelination. Front. Cell Neurosci. *12*, 367.

Hiraoka, J.I. (1972). A holder for mass treatment of grids, adapted especially to electron staining and autoradiography. Stain Technol. *47*, 297–301.

Höftberger, R., and Lassmann, H. (2018). Inflammatory demyelinating diseases of the central nervous system. Handb. Clin. Neurol. *145*, 263–283.

Lee, S., Leach, M.K., Redmond, S.A., Chong, S.Y., Mellon, S.H., Tuck, S.J., Feng, Z.Q., Corey, J.M., and Chan, J.R. (2012). A culture system to study oligodendrocyte myelination processes using engineered nanofibers. Nat. Methods *9*, 917–922.

Moreno-Lopez, Y., Bichara, C., Delbecq, G., Isope, P., and Cordero-Erausquin, M. (2021). The corticospinal tract primarily modulates sensory inputs in the mouse lumbar cord. Elife *10*, e65304.

Di Scipio, F., Raimondo, S., Tos, P., and Geuna, S. (2008). A simple protocol for paraffin-embedded myelin sheath staining with osmium tetroxide for light microscope observation. Microsc. Res. Tech. *7*, 497–502.

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Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, S., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9* (7), 676–682.

Seifert, P. (2017). Modified Hiraoka TEM grid staining apparatus and technique using 3D printed materials and gadolinium triacetate tetrahydrate, a nonradioactive uranyl acetate substitute. J. Histotechnol. *4*, 130–135.

Sridharan, G., and Shankar, A.A. (2012). Toluidine blue: A review of its chemistry and clinical utility. J Oral Maxillofac Pathol. *16* (2), 251–255.

Wang, Y., Zhang, Y., Zhang, S., Kim, B., Hull, V.L., Xu, J., Prabhu, P., Gregory, M., Martinez-Cerdeno, V., Zhan, X., et al. (2021). PARP1-mediated PARylation activity is essential for oligodendroglial differentiation and CNS myelination. Cell Rep. *37*, 109695.

Wyffels, J.T. (2001). Principles and techniques of electron microscopy: biological applications, Fourth Edition, by M. A. Hayat. Microsc. Microanal. *7*, 66.