

Protocol

Protocol to image and analyze the morphology of mouse peripheral nerves using transmission electron microscopy



Hiromi Nakai-Shimoda, Tatsuhito Himeno, Mikio Motegi, ..., Hiroki Mizukami, Jiro Nakamura, Hideki Kamiya

thimeno@aichi-med-u.ac.jp

Highlights

Morphological analysis of mouse peripheral nerves using transmission electron microscopy

Step-by-step guide for sampling, fixation, and embedding of peripheral nerves

Ultrathin sectioning by glass knife and imaging using transmission electron microscopy

Image analysis of unmyelinated and myelinated nerve fibers using ImageJ and AxonSeg

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Morphological analysis of peripheral nerves in mouse models can be used to characterize the pathophysiology of peripheral nerve disease, but obtaining high-quality electron micrographs can be challenging. Here, we present a protocol to obtain electron micrographs of mouse peripheral nerves. We detail the procedures of sampling, fixation, and embedding of peripheral nerves. We then outline the steps for ultrathin sectioning and transmission electron microscopy imaging. Finally, we describe morphological evaluation of nerve fibers in these images using ImageJ and AxonSeg.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol to image and analyze the morphology of mouse peripheral nerves using transmission electron microscopy

Hiromi Nakai-Shimoda,^{1,7} Tatsuhito Himeno,^{1,2,8,9,*} Mikio Motegi,^{1,7} Norio Ozeki,¹ Rieko Inoue,¹ Tomohide Hayami,¹ Emiri Miura-Yura,¹ Yuichiro Yamada,¹ Yoshiaki Morishita,¹ Shin Tsunekawa,¹ Yoshiro Kato,¹ Yusuke Seino,³ Masaki Kondo,¹ Keiko Naruse,⁴ Koichi Kato,⁵ Hiroki Mizukami,⁶ Jiro Nakamura,^{1,2} and Hideki Kamiya¹

¹Division of Diabetes, Department of Internal Medicine, Aichi Medical University School of Medicine, Nagakute 480-1185, Japan

²Department of Innovative Diabetes Therapy, Aichi Medical University School of Medicine, Nagakute 480-1185, Japan

³Division of Endocrinology and Metabolism, Department of Internal Medicine, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan

⁴Department of Internal Medicine, Aichi Gakuin University School of Dentistry, Nagoya 464-0821, Japan

⁵Department of Medicine, Aichi Gakuin University School of Pharmacy, Nagoya 464-8650, Japan

⁶Department of Pathology and Molecular Medicine, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan

⁷These authors contributed equally

⁸Technical contact

⁹Lead contact

*Correspondence: thimeno@aichi-med-u.ac.jp
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SUMMARY

Morphological analysis of peripheral nerves in mouse models can be used to characterize the pathophysiology of peripheral nerve disease, but obtaining high-quality electron micrographs can be challenging. Here, we present a protocol to obtain electron micrographs of mouse peripheral nerves. We detail the procedures of sampling, fixation, and embedding of peripheral nerves. We then outline the steps for ultrathin sectioning and transmission electron microscopy imaging. Finally, we describe morphological evaluation of nerve fibers in these images using ImageJ and AxonSeg.

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

BEFORE YOU BEGIN

First, the protocol describes the collection and fixation of peripheral nerves. Second, the protocol describes the embedding, sectioning, and imaging of the nerves. Third, the protocol describes the procedure for quantification of the nerve images.

The specialized equipment for the protocol is an electron microscope and ultramicrotome. For the usage of these devices, training and practice are required. The laboratory should be equipped with a chemical fume hood for the safe usage of fixatives.

This protocol does not offer the only correct method but introduces one method as an option. Therefore, the equipment and reagents used in this protocol are not always up-to-date or best. Users can replace them with available equipment and reagents. This protocol can be applied not only to the sural and sciatic nerves but also to other peripheral nerves.



Institutional permissions

Animal studies were approved by the Institutional Animal Care and Use Committee of Aichi Medical University. Readers should acquire permissions from the relevant institutions before the experiment.

Solution preparation for tissue collection, fixation, and embedding

⌚ Timing: 1–2 h

Prepare below solutions.

1. For perfusion, 10 mL/mouse of PBS(-).
2. For rinsing after primary fixation, prepare 5 mL/specimen of ice-cold PBS(-).
3. For rinsing after post-fixation, prepare 3 mL/specimen of PBS(-) at room temperature (RT) (15°C–30°C).
4. For primary fixation, 15 mL/mouse of 1.6% PFA + 1% glutaraldehyde in PBS(-).
5. For post-fixation, 1 mL/specimen of 2% osmium tetroxide.
6. For dehydration, 2 mL/specimen of wet ethanol with a range from 70% (v/v) to 95% (v/v) of ethanol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Mouse sciatic nerves	This paper	N/A
Mouse sural nerves	This paper	N/A
Chemicals, peptides, and recombinant proteins		
99.5% Ethanol solution	Wako	WK05700456
10 × Dulbecco's Phosphate Buffered Saline (-) (PBS (-))	Wako	WK04829805
2.5% Glutaraldehyde (GA) solution	Muto Pure Chemicals	86311
Sodium hydroxide	Wako	192-15985
Paraformaldehyde	Wako	30525-89-4
4% Osmium tetroxide (OsO ₄) solution, 2mL/vial	Nissin EM	NV-3020-4
Uranyl acetate	Electron Microscopy Sciences	541-09-3
Sodium dihydrogen citrate	Wako	18996-35-5
Lead nitrate	Wako	10099-74-8
Propylene oxide	Nissin EM	NV-311
0.05% Toluidine blue solution (pH 7.0)	Wako	92-31-9
Midazolam solution, 10 mg/2mL/vial	Astellas Pharma	SZ-233-010746
Butorphanol solution, 5mg/1mL/vial	Meiji Seika Pharma	99-VETLF5 44
Medetomidine solution, 1mg/mL	Orion	06043/4003
Neoprene W (0.5% neoprene rubber in toluene)	Nissin EM	605
0.5% Formvar solution (Ethylene dichloride solution of polyvinyl formal)	Nissin EM	604
Critical commercial assays		
Plain Resin Kit™ (Epoxy resin)	Nissin EM	NV-395
Experimental models: Organisms/strains		
Mouse: C57BL6/J (age range: 8–36 weeks; gender: male/female)	The Jackson Laboratory	Cat# JAX:000642; RRID:IMSR_JAX:000642
Software and algorithms		
ImageJ 1.52a software	National Institutes of Health (Schneider et al., 2012)	https://imagej.nih.gov/ij/
AxonSeg software	MATLAB	GitHub - neuropoly/axonseg: Segment axon and myelin from microscopy data. Written in Matlab

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Other</i>		
EM clean spray	Nissin EM	NV-6443
EM grid case	Nissin EM	NV-2731
EM grids (square grids)	Nissin EM	263 F-100 Cu
1 mL Syringe	Terumo	SS-01T
2.5 mL Syringe	Terumo	SS-02SZ10
5 mL Syringe	Terumo	SS-05SZ20
10 mL Syringe	Terumo	SS-10ESZ20
Sterile PES syringe filter 0.22µm	Hawach Scientific	NJ-SLPES2522S
22G Disposable winged needle	Terumo	SV-22CLK
20 mL Glass scintillation vial	Thermo Fisher Scientific	03-337-5
Glass petri dish, φ 9cm	N/A	N/A
Double edge razor blades, carbon steel, 1 mm thickness	FEATHER Safety Razor	FA-10
Graefe Forceps, 10cm	Fine Science Tools	11050-10
Fine Scissors	Fine Science Tools	14558-11
Soft vinyl chloride plate, 10 cm X 10 cm X 3-5 mm	N/A	N/A
Natural convection drying oven	Yamato Scientific	DY300
Glass knife maker	LKB Produkter	7800
Ultra-Diamond knife, 45°	DiATOME	MS17528
Ultramicrotome	Leica Microsystems	EM UC7
Transmission electron microscope	JEOL	JEM-1400
Soft wiper (polypropylene)	ESCO	EA929AT-41
Parafilm	Bemis	PM-996

MATERIALS AND EQUIPMENT

10N Sodium hydroxide (NaOH) solution

Reagent	Final concentration	Amount
NaOH	10 mol/L	40 g
ddH ₂ O	N/A	Adjust volume to 100 mL
Total	N/A	100 mL

Store at RT for up to 6 months.

1N Sodium hydroxide (NaOH) solution

Reagent	Final concentration	Amount
NaOH	1 mol/L	4 g
ddH ₂ O	N/A	Adjust volume to 100 mL
Total	N/A	100 mL

Store at RT for up to 6 months.

8% Paraformaldehyde (PFA) solution

Reagent	Final concentration	Amount
PFA	8%	40 g
10N NaOH solution	N/A	2-3 drops
10 × PBS(-) solution	1×PBS(-)	50 mL
ddH ₂ O	N/A	Adjust volume to 500 mL
Total	N/A	500 mL

Store at -20°C for up to 1 year.

Primary fixation solution

Reagent	Final concentration	Amount
2.5% GA solution	1%	20 mL
8% PFA solution	1.6%	10 mL
10 × PBS(-) solution	1 × PBS(-)	5 mL
ddH ₂ O	N/A	15 mL
Total	N/A	50 mL

Store at 4°C for on ice and use on the same day.

Note: Since the buffering capacity of PBS(-) is inferior to that of phosphate buffer (PB), PB may be used instead of PBS(-) to maintain a pH of 7.2–7.4 during primary fixation.

Post-fixation solution

Reagent	Final concentration	Amount
4% OsO ₄ solution	2%	4 mL
ddH ₂ O	N/A	4 mL
Total	N/A	8 mL

Store at 4°C for up to 6 months.

△ **CRITICAL:** As OsO₄ is a strong oxidant, OsO₄ solution should be stored double-bottled to avoid vapor dispersion.

Epoxy resin

Reagent	Final concentration	Amount
Plain Resin A	N/A	5.9 mL
Plain Resin B	N/A	5.9 mL
Plain Resin C	N/A	0.21 mL
Total	N/A	12 mL

Note: As these components are viscous, mix them thoroughly (at least 15 min) to get a homogeneous mixture. Store at -20°C within the syringe covered with parafilm for up to 6 months.

Anesthetic

Reagent	Final concentration	Amount
Midazolam solution, 10 mg/2mL/vial	0.4 mg/mL	2 mL
Butorphanol solution, 5mg/1mL/vial	0.5 mg/mL	2.5 mL
Medetomidine solution, 1mg/mL	0.03 mg/mL	0.75 mL
ddH ₂ O	N/A	19.75 mL
Total	N/A	25 mL

Store at 4°C for up to 1 month.

1% Uranyl acetate solution

Reagent	Final concentration	Amount
Uranyl acetate	1%	0.1 g
ddH ₂ O	N/A	10 mL
Total	N/A	10 mL

Keep sealed and shaded and store at 4°C for up to 4 months.

Reynolds lead citrate solution

Reagent	Final concentration	Amount
Component A		
Lead nitrate	1.5%	1.33 g
ddH ₂ O	N/A	15 mL
Component B		
Sodium citrate	2%	1.76 g
ddH ₂ O	N/A	15 mL
1N NaOH	0.09 N	8 mL
ddH ₂ O	N/A	50 mL
Total	N/A	88 mL

Separately make component A and B. Then mix them, add 1N NaOH, and add ddH₂O.
Store at 4°C for up to 4 months.

STEP-BY-STEP METHOD DETAILS

Sampling of nerves

⌚ Timing: 1–2 h

This section describes the perfusion fixation steps. The main purpose of this process is to ensure adequate fixation.

1. Make a scalpel with a double-edged carbon steel razor ([Methods video S1](#)).
2. Prepare 3–4 mL of the primary fixative solution in a glass scintillation vial on ice.
3. Prepare 5 mL/tissue of PBS(-) on ice.
4. Prepare solutions for perfusion (3–4 mL of PBS (-) and 3–4 mL of primary-fixative solution) in each 5 mL syringe at RT.
5. Connect the syringe containing PBS (-) to a 22G winged needle and three-way stopcock and fill the route with the solution.
6. Place 1 mL primary fixative solution on a soft vinyl chloride plate ([Figure 1A](#)).
7. Inject 0.1 mL/10 g body weight of the anesthetic into the intraperitoneal cavity of the mouse.
8. Perfusion fixation
 - a. Make a lateral incision in the abdomen just beneath the rib cage to expose the diaphragm.
 - b. Cut both sides of the ribs through the rib cage to expose the pleural cavity.
 - c. Clamp the sternum with the forceps, then place the forceps over the head to provide a clear view of the heart.
 - d. Insert a 22G winged needle from the apex of the heart into the left ventricle.
 - e. Make an incision to the right atrium using scissors to create an outlet.
 - f. After observing a flashback of blood into the cannula, perfuse with PBS(-) for 2–3 min or until the liver is cleared of blood.
 - g. Perfuse with the primary fixative solution for 2–3 min.
 - h. As the result of fixation, tremors should be observed within seconds.
 - i. The mouse should be stiff at this stage.
9. Dissection of the sciatic and sural nerves
 - a. Place the mouse dorsal side up.
 - b. Make an incision in the skin from the femur through the popliteal fossa to the posterior ankle.
 - c. Dissect the skin from the underlying musculature, and lift away to remain out of the way during the procedure.

Note: The perfusion steps are important to achieve adequate fixation. Practice with substitute mice before mice for the research (See [Troubleshooting](#) section).

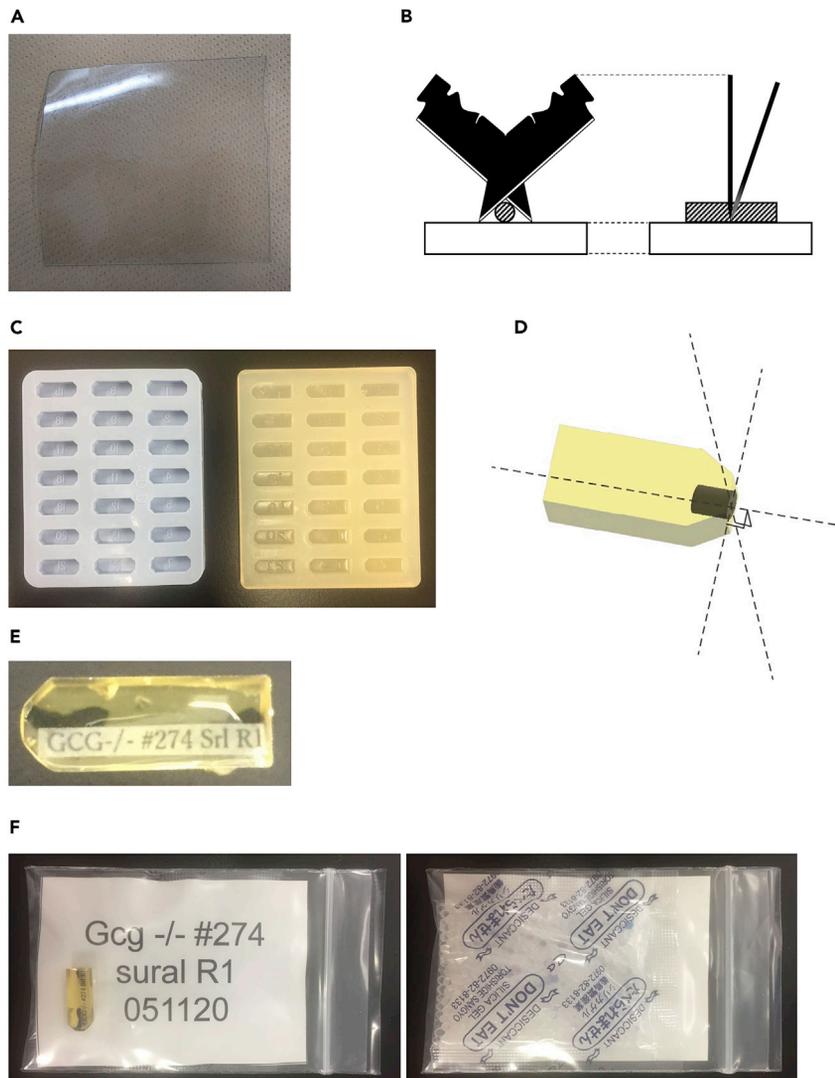


Figure 1. Photographs of epoxy-embedded blocks

- (A) A soft vinyl chloride plate for cutting nerve.
 (B) A schematic drawing how to cut the nerve using two scalpel blades.
 (C) Molds for epoxy blocks.
 (D) A schema how to put a nerve in the mold.
 (E) An epoxy block with nerves and a paper label.
 (F) An epoxy block stored in a Ziplock bag with silica gel.

- d. Open the fascial plane between the gluteus maximus and the anterior head of the biceps femoris to expose the sciatic nerve without touching it.
- e. Gently separate the sciatic nerve from the surrounding connective tissue using fine scissors (See [Troubleshooting](#) section).
- f. Cut free the sciatic nerve at the pelvic and knee ends.
- g. Carefully handling the proximal end of the nerve, put into the primary fixation solution on the soft vinyl chloride plate.
- h. Dissect the semitendinosus and biceps femoris muscles to expose the sural nerve.
- i. Gently separate the sural nerve from the surrounding connective tissue using fine scissors.
- j. Cut free the sural nerve at the knee and ankle ends.

- k. Carefully handling the proximal end of the nerve, put into the fixation solution on the soft vinyl chloride plate.

△ **CRITICAL:** If the perfusion is sluggish, ultrastructural changes will occur within min, resulting in unexpected and significant artifacts.

Tissue fixation

⌚ **Timing:** 3–4 h

This section describes the fixation steps. The main purpose of this process is to ensure the adequate fixation of the samples.

10. Using two scalpel blades, cut the nerves into several pieces ~2 mm length in the minor axis direction (Figure 1B).

Note: At this time, adjust the angle so that the blades of the scalpel snugly fit. That is, hold one blade vertically and tilt the other blade so that there is no gap between both blades (See [Troubleshooting](#) section).

11. Transfer the cut pieces of the nerve into the primary fixation solution in the glass vial using the Graefe forceps.

Note: At this time, do not directly pinch the nerve with the forceps, but scoop up the nerve with the fixation solution by capillarity.

12. Incubate the pieces of the nerves in the primary fixation solution for 1 h on ice.

Note: As a nerve is a lipid-rich tissue, incubation should be limited to 1 h to allow early transfer to the post-fixation solution, which is good at fixing lipid-rich tissue. For tissue other than a nerve, incubate for 2 h in the primary fixative.

13. During the incubation at step 12, prepare 1 mL/tissue of post-fixative solution inside a chemical fume hood wearing appropriate personal protective equipment.

Note: As osmium tetroxide is a strong oxidant, be careful not to inhale its vapor.

14. Replace the solution with 5 mL of ice-cold PBS(-). After gently agitating, incubate for 5 min on ice.

Note: To remove the primary fixation solution, use the 5 ml syringe attached with the 22 G needle.

15. Replace PBS(-) with 1 mL of post-fixation solution and incubate for 1 h on ice.

Note: Perform steps 14–15 inside a chemical fume hood wearing appropriate PPE.

16. Remove the post-fixation solution using a syringe attached with a 22 G needle.

17. Rinse the nerve pieces with ddH₂O.

⏸ **Pause point:** The samples can be stored in ddH₂O at 4°C for 2–3 days.

Embedding the samples

⌚ **Timing:** 4–6 h (need at least 3 days for polymerization)

This section describes the embedding steps using epoxy resin.

18. Dehydration

- a. Replace PBS(-) with 2 mL/tissue of 70% ethanol and incubate 5 min at RT.

Note: It is expected that working at RT will increase Brownian motion and promote substitution with each solution component. As an option, a shaker can be used.

- b. For dehydration of the samples, replace the solutions with 2 mL of 80%, 90%, 95%, 99.5%, 99.5%, and 99.5% ethanol in a stepwise manner every 5 min at RT.

Note: If the incubation time is too long, the tissue becomes excessively hard. Therefore, incubate the tissue for up to 15 min per solution.

- c. Replace with 2–3 mL propylene oxide and incubate 5 min at RT.
- d. Repeat step 18c for 2 more times (3 times of changes for a total time of 15 min).

Note: As propylene oxide is highly volatile, leave ~0.1 ml of propylene oxide at the bottom of the vial at the time of replacement to prevent drying.

Note: As propylene oxide can go through latex, it is important to wear nitrile gloves.

19. During the dehydration, prepare 0.5 mL/nerve of the epoxy resin embedding mixture.

Note: Being careful not to generate air bubbles, gently mix in a disposable cup for 10–15 min. To remove air bubbles, a vacuum chamber can be used.

⚠ CRITICAL: If the resin solution is not thoroughly mixed, the resin may not sufficiently cure.

20. Mix the resin mixture and propylene oxide at a ratio of approximately 1:1. Replace the propylene oxide in the sample vial with 1 mL of this mixture and incubate the sample for 1 h.

21. Replace with the same resin-propylene oxide mixture again.

22. Transfer the samples and solution to a 2.0 mL microcentrifuge tube.

Note: Tilt the vial while shaking it. If the sample remains on the bottom of the vial, place it on the tip of the needle (a fine brush can be substituted) to transfer it. If you pinch the sample with forceps, it will be crushed.

23. To separate propylene oxide from the samples, using a desktop centrifuge, intermittently centrifuge for a few seconds at $5,200 \times g$, 5–6 times over 1 h.

24. During step 23, prepare a 2 mL/sample of the 100% epoxy resin mixture.

25. Replace with 1 mL/sample of the 100% epoxy resin mixture and intermittently centrifuge for a few seconds 5–6 times over 1 h.

26. During step 25, print and cut paper labels for embedding in resin blocks, e.g., BL6#001. These labels have a font size of 5 points.

27. Pour the resin mixture into the flat plate embedding plate (Figure 1C) using a 5 mL syringe (without needle). At this time, be careful not to generate air bubbles.

28. Scoop the sample with an 18 G needle filled with the resin mixture and submerge it in the embedding plate.

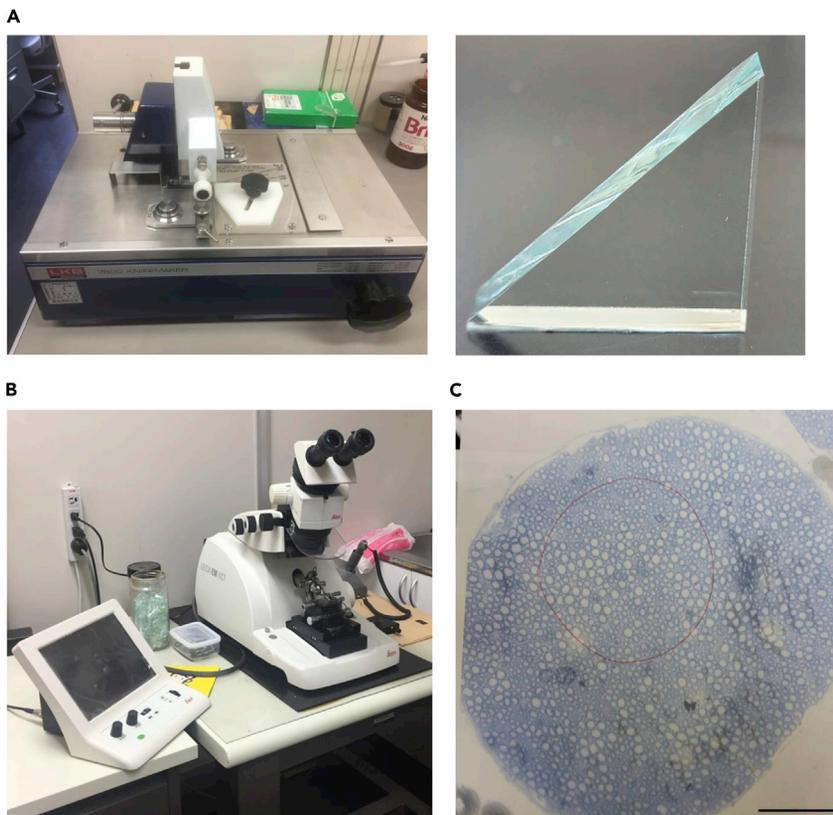


Figure 2. Photographs for thick sectioning

(A) A glass knife maker (left panel) and a glass knife (right panel).

(B) An ultramicrotome.

(C) A thick section stained with toluidine blue. The red circle indicates the region of interest. Scale bar: 100 μm .

Note: At this time, place the samples so that the nerve fibers are perpendicular to the cutting surface in both the vertical and horizontal directions (Figure 1D). Keep the sample as close to the cutting surface as possible (See Troubleshooting section).

29. Float the paper labels on the resin mixture (Figure 1E).

Note: The paper labels can be submerged to prevent label displacement during curing.

30. Cure the molded resin at 60°C in the drying oven for 3 days.

31. Store the resin blocks in a small zipper bag containing silica gel to keep them dry (Figure 1F).

▣ **Pause point:** The samples can be stored at RT for decades.

Sectioning of the epoxy blocks

⌚ **Timing:** 1–2 weeks

This section describes the steps to obtain semi-thin and ultrathin sections from the epoxy resin blocks using the ultramicrotome with a glass or diamond knife. As the sectioning procedure requires much detailed know-how, expert guidance is essential.

32. Make glass knives using a glass knife maker (Figure 2A).

Note: Two knives can be obtained from one glass square. One knife will be of higher quality than the other. The knives can be stored in a box to prevent dust.

33. Place the glass knife onto the knife holder of the ultramicrotome ([Figure 2B](#)).
34. Place the resin block onto the chuck of the ultramicrotome.

Note: To reduce rattling, insert the block firmly to the back of the chuck.

35. Using a razor blade, trim the top of the block into a trapezoid shape with 4–5 sloping sides.
36. Using a low-quality glass knife, trim the surface of the block until the entire surface of the sample is exposed on the block surface.
37. To use a fresh glass knife edge, move the glass knife sideways to an unused area.
38. Section the block with a thickness of 1–2 μm (semi-thin section).
39. Drop a drop of distilled water on a glass slide.
40. Using the forceps or metal loop, place the semi-thin section on the drop on the glass slide.
41. Dry the glass slide on an 80°C hot plate.
42. Drop toluidine blue solution on the section and incubate it on the 80°C hot plate for 6–8 min. Do not let stain dry down or it could overstain sections.
43. Wash under running water.
44. Under a light microscope, take an image of the whole nerve including a scale bar for analysis of the section (See [Troubleshooting](#) section).
45. Select the region of interest of the sample ([Figure 2C](#)).

Note: The region stained well by toluidine blue often gives good quality images with an electron microscope.

46. Trim the block to the smaller containing only the region of interest ([Methods video S2](#)).

Note: To reduce wear of the diamond knife, the cutting width of the resin block is preferably less than 0.3 mm.

47. Preparation of the grids coated with Formvar
 - a. Add 100 mL of 0.5% Formvar in ethylene dichloride into a 300 mL beaker.
 - b. Carefully wipe the glass slide with a soft polypropylene wiper.
 - c. Soak half to two-thirds of the glass slide in the solution.
 - d. Take out the glass slide from the solution (Take a few seconds).
 - e. After drying the slide, make a square cut on the glass slide film with a razor blade.
 - f. Float off the film by immersing the glass slide into the water.

Note: Tilt the glass slide about 30 degrees and immerse at a speed of 2–5 mm/sec.

Note: A film thickness of 50–60 nm is appropriate (silver in color). Increasing the film thickness (gold in color) improves resistance to electron beams, but decreases the contrast of the TEM image.

- g. Put the grids with the smooth side down on the film. To pick up the grid and membrane floating on the surface of the water, cover them with a piece of release liner of parafilm, which will allow them to stick to the liner. After picking up, dry them (See [Troubleshooting](#) section).
48. For ultrathin sectioning, switch to a diamond knife ([Figure 3A](#)).

Note: As the handling of a diamond knife is very delicate, follow the guidance of an expert including handling of the ultramicrotome and finer adjustments of the alignment between the block and the knife edge.

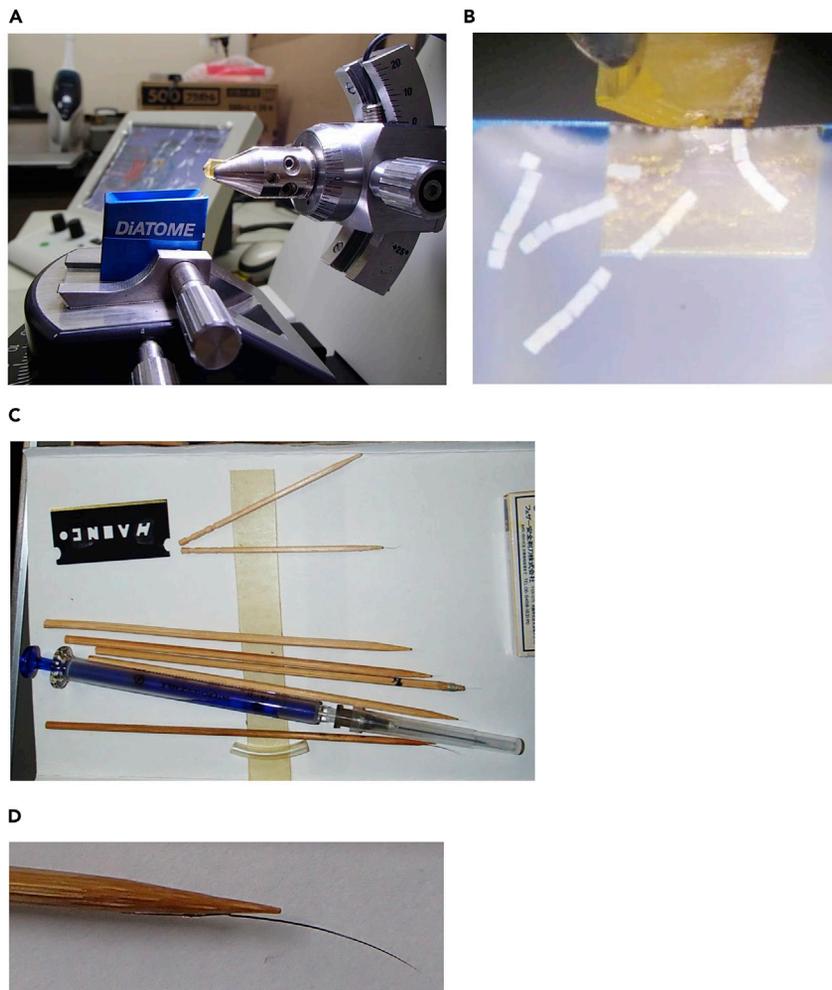


Figure 3. Photographs for ultra-thin sectioning

- (A) A epoxy block held by chuck and a diamond knife.
(B) Floating ultra-thin sections on the water surface of the diamond knife.
(C) Tools for picking up the sections.
(D) An eyelash brush.

49. Fill the boat of the knife with deionized water and adjust the water level to the knife edge ([Methods video S3](#)).

Note: If this protocol was done properly, the blocks should be consistently hard. However, if the block is soft, adjust the water level to a slightly lower position.

50. Section the block with an ultrathin thickness (60–100 nm) ([Figure 3B](#), [Methods video S3](#)).

51. Using an eyelash brush, detach the sections from the knife-edge and mount them onto EM grids ([Figures 3C](#) and [3D](#)).

Note: There are two ways to mount a section onto the EM grid: 1) Using the forceps, insert the grid into the water surface near to the section and bring the section to the support film containing side of the grid using the eyelash brush, and 2) Cover the floating section with the grid ([Methods video S4](#)) (See [Troubleshooting](#) section).

52. Allow to dry, then store the grids in an EM grid case.

Staining the sections

⌚ Timing: 1–2 h

This section describes the steps to stain the ultrathin sections for the observation by TEM.

Note: The uranyl acetate solution and the lead citrate solution are sensitive to light or CO₂, respectively, and will precipitate if exposed. Therefore, during these staining steps, these solutions should be protected from light or CO₂.

53. Place one drop of 1% uranyl acetate solution on a parafilm in a glass petri dish and put the grid upside down on the drop for 10 min ([Figure 4A](#)).

Note: Cover with the foil covered lid of the petri dish to protect from light and CO₂ and to prevent dust during the staining.

54. During the 10-min staining, cut a filter paper into 16 wedges ([Figure 4B](#)).
55. Prepare three drops of distilled water next to the drop of uranyl solution on the parafilm.
56. Pick up the grid from the uranyl solution and remove the solution using the wedged filter paper.
57. Wash the grid with the film-supported side down on the drops of distilled water on a parafilm three times.
58. Put a piece of parafilm in a glass petri dish and scatter 10–20 pellets of NaOH around the film.
59. Place one drop of Reynolds lead citrate solution on the parafilm and put the grid upside down on the drop for 2 min.

Note: Cover with the lid of the petri dish to prevent dust during the staining.

60. Repeat the same steps as for uranium staining.
61. Dry the grids in the dish for at least 30 min on filter paper.

Note: During the rainy season when the humidity is high, it may take 24 h to completely dry. Do not breathe onto grids while staining as this can cause precipitate to form.

62. Dry the grids on filter paper. Store them in an EM grid case ([Figure 4C](#)).

Caution: The Formvar films are delicate and can easily break.

Image analysis

⌚ Timing: 4–5 days for each nerve

Imaging with an electron microscope requires assistance from a well-trained person. Therefore, this section mainly describes image analysis using ImageJ ([Schneider et al., 2012](#)) and AxonSeg software ([Zaimi et al., 2016](#)).

63. Imaging
 - a. Prepare the TEM according to the manufacturing manual (imaging at 80 kV) ([Figure 5A](#)).
 - b. Place the sample into the microscope.
 - c. Locate the region of interest under low magnification.
 - d. For the analysis of myelinated fibers, select the magnification (600- to 1,000-fold) that includes 10%–20% of the nerve. Take multiple images to cover more than two-thirds of the nerve.

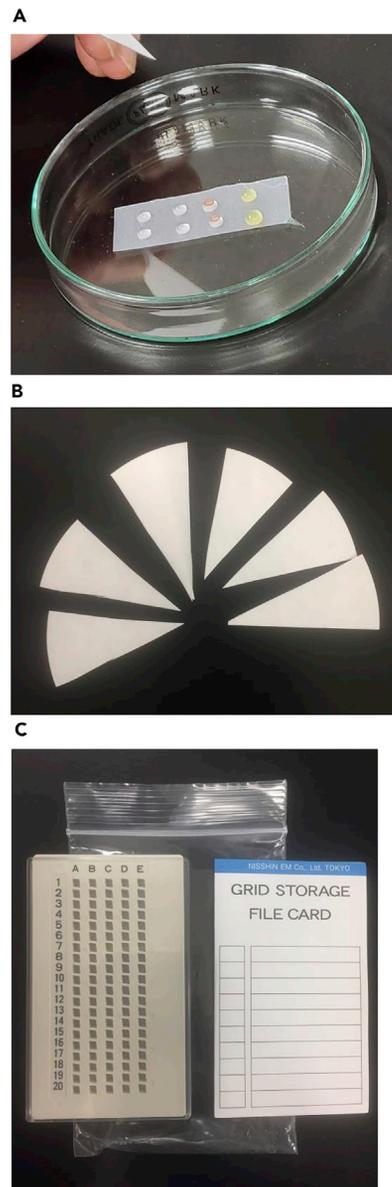


Figure 4. Photographs for staining

(A) Drops of uranyl acetate solution (yellow) and ddH₂O on a parafilm in a glass petri dish. The grids are being washed with the drops of ddH₂O.

(B) Filter papers cut into 16 wedges.

(C) An EM grid case.

- e. For the analysis of unmyelinated fibers, select higher magnification (3,000- to 5,000-fold) so that individual unmyelinated fibers can be clearly identified. Take multiple images to analyze more than 300 fibers/nerve.
64. Download ImageJ software (<http://rsb.info.nih.gov/ij/download.html>).
65. For the analysis of light microscope images, open the image of the semi-section stained with toluidine blue.
66. Trace basal membrane of the whole nerve using the polygon selection tool.
67. Measure the traced area and convert the number of the area from pixels to μm^2 .

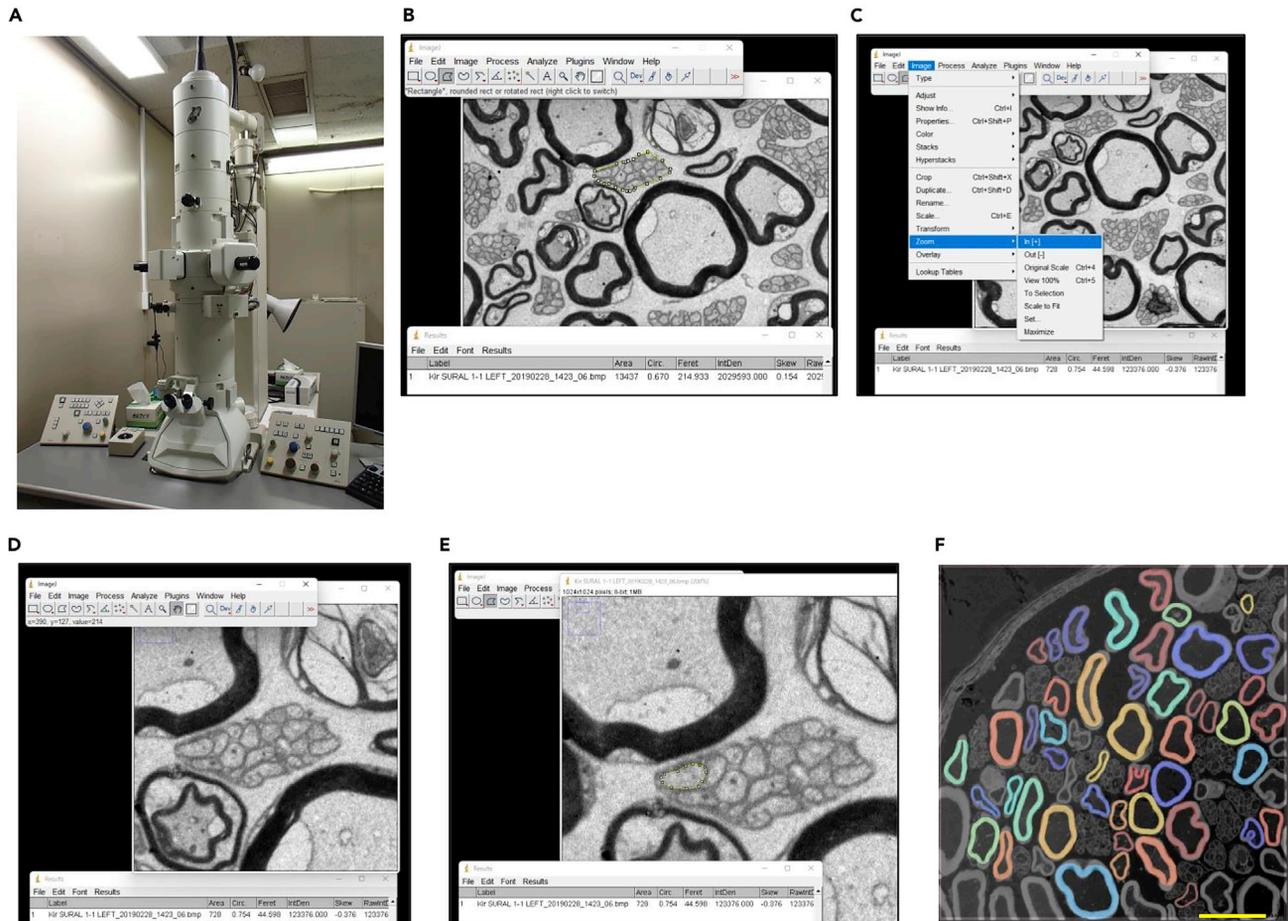


Figure 5. Photographs for image analysis

- (A) A transmission electron microscopy.
 (B) A screen image of ImageJ during analysis of Remak bundle (yellow dotted polygon).
 (C and D) A screen image using Zoom In command and the scrolling tool.
 (E) A screen image of Image J for tracing individual unmyelinated fiber (yellow dotted polygon).
 (F) An image of AxonSeg. Color-labeled rings are myelin area. Scale bar: 10 μm .

Note: To convert the area from pixels to μm^2 , take a picture with a scale bar, measure the length of the bar in pixels, and convert from pixels to μm^2 .

68. Analysis of unmyelinated fibers (using TEM images)
 - a. Open the TEM image for unmyelinated fiber analysis.
 - b. Analysis of Remak bundles
 - i. Trace basal membrane of Remak bundle using the polygon selection tool (Figure 5B).
 - ii. Measure the traced area using the "Ctrl"+"M" keyboard shortcut and copy the results to an Excel file.
 - iii. Trace and measure all Remak bundles in the image except for the bundles on the edge of the image.
 - c. Analysis of individual fiber
 - i. Using the *Image>Zoom>In[+]* command, zoom in the image so that individual fibers can be accurately traced (Figure 5C).
 - ii. Using the scrolling tool, or use the corresponding shortcut "spacebar" + the left mouse button, set one Remak bundle at the center of the image (Figure 5D).

Table 1. Example of raw data: Remak bundles

Bundle ID	Fiber (n)	Area (pixels)	Area (μm^2)	Circ.
1	20	15268	9.8	0.644
2	7	3376	2.2	0.785
3	12	7041	4.5	0.77
4	11	6945	4.5	0.77

- iii. Trace each unmyelinated fiber using the polygon selection tool (Figure 5E).
 - iv. Measure the traced area and copy the results to an Excel file.
 - v. Trace and measure all fibers in the Remak bundle.
 - vi. Repeat the analysis of the Remak bundle and unmyelinated nerve fibers over the entire image.
- d. Using Excel application, calculate the sizes of bundles and unmyelinated fibers, fiber density, a circularity of bundles and fibers, and the ratio of fiber area per bundle.

Note: In order to evaluate circularity, the specimen must be carefully submerged perpendicular to the cutout surface during resin embedding. If the axons in the image are oval in the same direction, the distortion may be due to the misorientation of the sample and knife during sectioning, and such images should be excluded from the analysis.

69. Analysis of myelinated fibers

- a. Download AxonSeg software (<https://github.com/neuropoly/axonseg>).
- b. Segment axon and myelin in each image using an automatic algorithm of AxonSeg software (Figure 5F).

Note: As the automatic segmentation is not always reliable, check the results of segmentation, then manually reselect nerve fibers for reanalysis.

- c. Using Excel application, calculate the areas of myelin and axon, the diameters of nerve fiber and axon, g-ratio (axon diameter/fiber diameter), myelin thickness.

70. Perform histogram analyses to examine the overall distribution of myelinated or unmyelinated axon sizes.

EXPECTED OUTCOMES

This protocol will support obtaining good quality samples of mouse peripheral nerves for TEM. These samples will provide stunning electron microscope images. These images enable an objective and definitive pathological evaluation of peripheral nerve diseases. At low magnification, the presence or absence of demyelination, axonal de-/re-generation as well as the number, density, and morphology of myelinated fibers can be evaluated. At high magnification, the number, density, and morphology of unmyelinated fibers and the presence or absence of de-/re-generation of these fibers can be evaluated. Additionally, it is useful to evaluate the distribution of axons by size to understand the pathological condition.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the quantification of the findings in nerve fibers, use the raw data tables as below (Tables 1 and 2).

For the statistical analysis, unpaired Student's t-test can be used after the determination of the normality distribution using the Kolmogorov-Smirnov test. F-test is used to test equivalence of variances.

Table 2. Example of raw data: Unmyelinated fibers

Fiber ID	Area (pixels)	Area (μm^2)	Circ.
1	5298	0.1365	0.927
2	10875	0.2802	0.917
3	2713	0.0699	0.893
4	8772	0.2260	0.703

LIMITATIONS

As this protocol is simple and well-established, this can be applied to various fields including peripheral and cranial nerves other than the sural and sciatic nerves. However, the supervision of an expert is indispensable for the ultrathin sectioning steps and the analysis and evaluation of the TEM images. Considerable experience with these procedures is vital to correctly interpret the images.

TROUBLESHOOTING

Problem 1

Steps 9 and 10: Sections stained with toluidine blue show damaged or crushed tissue under a light microscope.

Potential solution

Rough handling of tissue can cause damage to the tissue. Avoid stretching the nerve while separating it from the surrounding connective tissue. Additionally, the excision of the nerve using a scalpel made by a razor may cause damage. To obtain a sharp cut without twisting force, it is important to ensure that there is no gap between the two blades. Replacing the soft vinyl chloride plate with a softer plate might improve the integrity of the tissue.

Problem 2

Step 8: Sections under TEM show loosened myelin sheaths or obscure unmyelinated fibers.

Potential solution

The fixation is likely inadequate. If blood cells are found in the blood vessels, perfusion fixation may not have been successful. It may be effective to accelerate the flow of the perfusate to wash out the blood cells. It may also be useful to prefilter the fixative through a 0.22 μm filter. As inadequate immersion fixation for EM embedding is often caused by gradient fixation, the sample size should be kept small ($\sim 1 \text{ mm} \times 2 \text{ mm}$).

Problem 3

Steps 28 and 44: Sections stained with toluidine blue show that nerve fibers appear elliptical.

Potential solution

It is difficult to place the samples so that the nerve fibers are perpendicular to the cutting surface in both the vertical and horizontal directions. Adjust the angle of the resin on the chuck so that the elliptical nerve fibers are closer to a perfect circle. If the nerve is embedded in a non-adjustable orientation, cut it out from the resin, and re-embed in fresh resin to desirable orientation.

Problem 4

Step 47: At the steps of the grids coating with Formvar, the film does not adhere to the grids.

Potential solution

To create an adhesive layer, drop 0.5% neoprene solution on the grids before the film is applied.

Problem 5

Step 51: The section on the grid is wrinkled.

Potential solution

For mounting an ultrathin section on a grid, insert the grid into the water surface near the section, bring the section to the grid using the eyelash brush, and then very slowly pull up the grid. Additionally, it can be helpful to avoid wrinkle formation that, prior to collection of a section, the grid is dipped in ethanol and distilled water (Dittmayer et al., 2021).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tatsuhiro Himeno (thimeno@aichi-med-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any datasets and code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101591>.

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AUTHOR CONTRIBUTIONS

Conceptualization, T. Himeno, Y.S., S.S., and H.K.; methodology, T. Himeno, N.O., M.M., E.M.-Y., Y.S., and H.M.; investigation, H.N.-S., T. Hayami, and R.I.; writing – original draft, H.N.-S., M.M., and T. Himeno; writing – review & editing, M.K., Y.Y., J.N., and H.K.; funding acquisition, T. Himeno and J.N.; supervision, T. Hayami, Y.M., S.T., Y.Y., K.N., K.K., J.N., and H.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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