PROTOCOL FOR PREPARING NERVES FOR MORPHOMETRY

I. Removal of Sciatic, Tibial, and Sural Nerves (see video)

1. Anaesthetize the animal by placing in a closed chamber with isoflurane.
2. Determine that the animal is unconscious by lack of response to pinching of toe and tail.
3. Sacrifice the animal using a guillotine.
4. Place the body on its ventral surface and extend the leg.
5. Make a 2 cm incision through the skin along the lateral aspect of the thigh and leg.
6. Carefully separate the underlying fascia and biceps femoris muscle to expose the sciatic nerve running parallel to the posterior aspect of the femur. Keep the nerve moist by applying 0.1 M sodium phosphate buffer.

   0.1 M Sodium Phosphate Buffer Recipe:

   a. Prepare 0.2 M sodium phosphate dibasic by dissolving 28.4 g of sodium phosphate dibasic (Fisher) in 1L double distilled water.
   b. Prepare 0.2 M sodium phosphate monobasic by dissolving 27.6 g of sodium phosphate monobasic (Fisher) in 1L double distilled water.
   c. Titrate a desired amount of 0.2 M sodium phosphate dibasic with 0.2 M sodium phosphate monobasic to reach a pH of 7.4 (approximately 170 ml of 0.2 M sodium phosphate monobasic with 800 ml of 0.2 M sodium phosphate dibasic)
   d. Prepare 0.1M sodium phosphate buffer by diluting 0.2 M sodium phosphate buffer with double distilled water at a 1:1 ratio.

7. Trace the sciatic nerve distally to where it divides into the sural, tibial and peroneal nerves at the popliteal fossa.
8. The tibial nerve runs deep under the gastrocnemius muscle and innervates the flexor muscles by passing between the soleus and tibialis anterior muscle to terminate in the foot.
9. The sural nerve runs through the popliteal fossa and continues downward on the dorsal surface of the gastrocnemius muscle to the foot.
10. Using fine dissection scissors, cut the sciatic nerve proximally at the sciatic notch and distally where it divides into the sural, tibial and peroneal nerves. Collect ~ 1-2 cm.

11. Taking care not to stretch the sciatic nerve, place it on a small wooden stick and immediately immerse in a vial containing 20 ml of 2.5% glutaraldehyde in 0.1M sodium phosphate buffer. Store on ice or at 4°C.

12. Cut both tibial and sural nerves at their origin in the popliteal fossa and also about 1cm distally.

13. Without stretching, place each tibial and sural nerve on a wooden stick and transfer immediately to a vial containing 20 ml of 2.5% glutaraldehyde in 0.1M sodium phosphate buffer and store on ice. Because 20 ml of fixative is enough to fix all three nerves, the vial containing the sciatic nerve can be used.

II. Tissue Processing for Plastic Sections

Primary Fixation

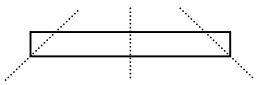
1. Fix nerves in 2.5% glutaraldehyde (in 0.1M sodium phosphate buffer) for 24 hrs at 4°C.

2. Assign an accession number to each specimen.

3. Rinse specimen twice (10 min per rinse) in 0.1 M sodium phosphate buffer, with the vial placed on a rotator during the rinse.

4. Remove connective tissue and fat around the nerve. Measure approximate nerve diameter.

5. Carefully cut nerve ends at an angle and then cut in half:



6. Rinse again in buffer (10 min).

7. If using a basket system (LYNX tissue processor baskets from Electron Microscopy Sciences), wrap nerve halves in lens paper to avoid loss of nerve while processing.

Secondary Fixation

1. Osmicate specimen in 2% osmium tetroxide (1ml 4% osmium tetroxide + 1ml 0.2 M sodium phosphate buffer). **Osmium is very toxic so work in a fume hood.** If using the basket system, use 5 ml of 2% osmium tetroxide for the first basket and add 2 ml for each additional basket.

2. Leave specimen with osmium tetroxide on the rotator. The duration of osmication is based on the nerve diameter measured above and a diffusion rate of 0.5 mm/hr.

3. Rinse specimen with distilled water twice. At this stage it can be stored in the refrigerator in 0.1 M sodium phosphate buffer for several days.

Dehydration

1. Rinse specimen in distilled water.
2. Do two quick rinses of the specimen in 30% ethanol and then leave specimen in 30% ethanol on a rotator for 10 minutes.
3. Do two quick rinses of the specimen in 50% ethanol and then leave specimen in 50% ethanol on the rotator for 10 minutes.
4. Do two quick rinses of the specimen in 70% ethanol and then leave specimen in 70% ethanol on the rotator for 10 minutes.
5. Rinse specimen in 95% ethanol twice for 15 minutes on the rotator for each rinse.
6. Rinse specimen in 100% ethanol twice for 15 minutes on the rotator for each rinse.
7. Rinse specimen in propylene oxide twice for 15 minutes on the rotator for each rinse. If the specimen will be used primarily for EM, leave in second rinse of propylene oxide for one hour.
8. Put specimen in a mixture of 50% propylene oxide and 50% resin ("half and half").
9. Leave specimen on the rotator for at least 2 hours.
10. Take out the "half-and-half" mixture and put the specimen in 100% resin.
11. Put specimen under vacuum overnight.

Embedding in 100% Resin

1. Prepare identification labels for each specimen using lowercase letters to identify pieces that belong to the same specimen (3.1a, 3.1b etc).
2. Using PELCO flat embedding molds (Ted Pella Inc.), place the label on the right lower corner of the mold.
3. Fill the embedding mold with 100% resin using a plastic syringe.
4. Using a straightened paper clip puncture all bubbles trapped in the resin.

5. Place the specimen in the mold by gently pushing it down to the bottom of the mold using a straightened paper clip.

6. Orientate the specimen according to the type of section (cross or longitudinal section) needed for study. For nerves, orient the straight end of each nerve, which is the middle of the nerve, towards the trapezoidal side of the mold face and the diagonal end pointing straight away from it.

7. The resin should be covering the specimen. Add resin if necessary.

8. Put mold into the oven overnight at 60^{o}C.

Block Preparation for Sectioning

1. Take the mold out of the oven and allow the blocks to cool for 30 minutes before placing in the block holder for trimming.

2. On the block face (i.e. where the tissue is located) define a trapezoid shape containing the sample. The height of the trapezoid should be parallel to the thickness of the block.

3. Using a WecPrep carbon steel blade (57.4 mm L x 13.4 mm W x 0.279 mm: Electron Microscopy Sciences) or similar single-edged blade, trim the resin surrounding the trapezoid containing the sample.

4. While trimming, create a slope of 45 degrees to the surface of the block. Too steep an angle causes the block to break when cutting with a microtome.

5. With a new blade, gently trim the block face to expose the surface of the tissue. Remove block from block holder.

6. Section the trimmed block using a glass knife and microtome to between 0.5µm (semi-thin) to 1.3µm (thick). Thickness of the section can be evaluated by its interference color (0.5µm = pink-bluish, 1µm = blue-green with some pink in it, 1.3µm = green) as it floats on the water-filled knife trough.

III. Resin

- Resin components are highly toxic substances. Use nitrile gloves, changing often to prevent skin contact.

- Always use fresh resin for infiltration. For embedding, one can use resin prepared the day before or freshly made resin.

To Prepare 100 mL of Resin

1. Take a 100 mL plastic beaker and mark 50 mL, 80 mL, and 100 mL.
2. Add the following components (Electron Microscopy Sciences) in sequence: 50 ml DDSA resin (hardener) + 30 ml Araldite resin (monomer) + 20 ml Polybed 812 resin (monomer).
3. Stir for 5 minutes.
4. Add 1.6 mL of DMP-30 (accelerator) using a plastic syringe.
5. Stir for 15 minutes.

IV. Staining Slides with *P*-Phenylene Diamine (1,4 D Diamine Benzene *P* -Phenylenediamine)

(PPD stains lipids so it is used to stain myelin in thick sections)

1. Prepare 2% of PPD stain (Sigma) in 50% ethanol and stir for 3 hours.
2. Keep stain at room temperature in a dark glass jar. Cover the jar with foil paper to protect it from the light, and cover the lid with parafilm to avoid ethanol evaporation.
3. Keep the stain at room temperature for 5 or more days before use.
4. Filter PPD with a 0.2µm nylon sterile filter.
5. Put slides in a staining jar and pour in filtered stain. Cover the staining jar with its glass cover to prevent ethanol evaporation.
6. Leave slides in the stain at room temperature for approximately 20 minutes. Vary staining time to achieve desired stain intensity, which can be assessed by examining "wet" slides with light microscope.
7. Pour stain out of the staining jar. **Stain is reusable.**
8. Slowly rinse slides twice in tap water.
9. Slowly rinse slides twice in deionized water.
10. Slowly rinse slides twice in distilled water.
11. Use a stain rack to dry the slides. The rack can be placed in the oven for at least 15 min at 60^{o}C.

12. When dry, take slides out of the oven when dry and allow to cool at room temperature for 5 minutes.
13. Place a drop of Cytoseal-60 (Thermo Scientific) on the stained slides and cover with cover glass.

V. Nerve Morphometry

1. Code slides to obscure identity.
2. Select a single section on each slide that is well stained and free from artifact, such as folds, tears and knife marks. Mark the section for future reference.
3. Myelinated fibers are analyzed with morphometry program if:
    - Myelin sheath is intact
    - Axon and myelin sheath have circular or oval shape
    - Adequate fixation
    - No evidence of mechanical artifact
    - Transverse cross-section
    - Cross-section is not paranodal
    - Cross-section does not include Schmidt-Laterman clefts
    - Cross-section does not include Schwann-cell nucleus
4. Myelinated fibers in a nerve fascicle are sampled using a systematic serpentine sampling scheme that assures equal sampling of sub-perineurial and centrifascicular regions.