PROTOCOL TO PREPARE PLANTAR FOOTSKIN FOR MORPHOMETRY

I. Removal and Fixation of Plantar Skin (see video)

1. Sacrifice the animal
   a. Anaesthetize the animal by placing in a closed chamber with isoflurane.
   b. Determine that the animal is unconscious by lack of response to pinching of toes and tail.
   c. Sacrifice the animal using a guillotine.

2. Place the body on its ventral surface and extend the leg.

3. Remove plantar skin from animal, attempting to remove as much of the skin and underlying connective tissue as possible.
   a. Using a razor blade, make an incision on the right and left side of the hind foot glaborous skin along the proximodistal axis.
   b. Make another incision in the skin between the digits and interdigital pads from left to right.
   c. Using dissecting forceps, hold the tissue distal to the interdigital pads at the point of the last incision, and slightly lift the skin and underlying connective tissue away from the hind foot.
   d. Continue to gently lift the tissue with forceps, while cutting the plantar glaborous skin away form the underlying fascia with a razor blade towards the heel.
   e. At the heel, finish removing the plantar tissue with dissecting scissors.

4. Lay plantar tissue flat on a sheet of plastic to prevent folding during fixation.
   a. Using a 20 gauge needle, poke holes into the bottom of a polystyrene weigh dish
   b. Cut this into squares just large enough to mount the plantar tissue.
   c. Lay the tissue, epidermis side up, onto flat side of the plastic square.

5. Immersion-fix specimen overnight in 4% buffered paraformaldehyde.

RECIPE: PREPARATION OF 20% PARAFORMALDEYDE STOCK

** Paraformaldehyde is highly toxic – WORK IN THE FUME HOOD**

i. Add 1400mL of double-distilled water to a large beaker.
ii. Heat the water, while stirring, to 60°C
   a. DO NOT allow water temperature to exceed 60°C
iii. Measure 4 groups of 100 grams of solid paraformaldehyde (*Fisherbrand* Reagent Grade)
iv. Mix one group of paraformaldehyde into the heated water until all paraformaldehyde is dissolved keeping the solution at 60°C
   Heating the polymer paraformaldehyde converts it into the monomer, formaldehyde, which is a CARCINOGEN
v. Repeat previous step with each group of 100 grams until all 400 grams of paraformaldehyde is dissolved into the heated water

vi. When the solution remains at 60°C, and all solid paraformaldehyde is dissolved, remove from heat
vii. Prepare NaOH solution:
    a. 150mL distilled water
    b. 50g NaOH
viii. Slowly pipette the NaOH into solution, with stirring, until the solution clears
ix. Place the beaker in an ice bath and allow it to cool to 25°C
x. At room temperature, measure the pH.
    a. It should be basic, around 11
xi. Adjust the pH of the solution to 7.3 by pipetting 12N Hydrochloric Acid (37% stock solution) while stirring
    a. Around pH 10.5, the solution approaches its equivalence point. Therefore, beyond this point, allow it to stir for several minutes between each addition of HCl
xii. To clarify the solution, filter it over a fine filter paper (*Fisherbrand* qualitative P2 fine filter paper).
    a. Clarification occurs through filtration, therefore filter as many times as necessary
xiii. Add double-distilled water to bring the solution to a final volume of 2 L.
xiv. Store 20% paraformaldehyde stock at 4°C.
xv. Before preparing dilutions from stock, remove solution from refrigerator, and allow stock to equilibrate to room temperature.
xvi. Prepare 4% buffered paraformaldehyde by diluting 20% paraformaldehyde stock with 0.2M sodium phosphate buffer and double-distilled water to a final concentration of 0.1M.
    a. For example, to prepare 500mL of 4% buffered paraformaldehyde, at room temperature, mix
        i. 100mL of 20% paraformaldehyde pH 7.3
        ii. 250mL of 0.2M sodium phosphate buffer pH 7.0
        iii. 150mL of double-distilled water

RECIPE: 0.2M SODIUM PHOSPHATE BUFFER

i. Prepare 0.2M sodium phosphate dibasic
    a. In 1L double-distilled water dissolve 28.4g Na_2HPO_4 (*Fisher*)
ii. Prepare 0.2M sodium phosphate monobasic
    a. In 1L double-distilled water dissolve 27.6g NaH_2PO_4 (*Fisher*)
iii. Titrate 0.2M sodium phosphate dibasic with 0.2M sodium phosphate monobasic to reach a pH of 7.4
    a. Titrate approximately 170mL of 0.2M sodium phosphate monobasic with 800mL of 0.2M sodium phosphate dibasic
iv. Prepare 0.1M sodium phosphate buffer by diluting 0.2M sodium phosphate buffer with double-distilled water 1:1.

II. Tissue Processing for Paraffin Sections

1. Assign each tissue an accession number according to the sequence in accession logbook.

2. Wash tissue for 10 minutes by immersing in 0.1M sodium phosphate buffer with gentle agitation, 3 times to replace all fixative in tissue with buffer.
    i. Tissue can be stored in 0.1M sodium phosphate buffer until embedded.

3. Place tissue in a cassette labeled with the tissue's accession number

4. Load cassettes into paraffin tissue processor (*Leica* model TP1020) for dehydration and infiltration.
    a. Dehydrate by immersion with gentle agitation
        i. 70% EtOH, 113min
        ii. 95% EtOH, 113 min
        iii. 95% EtOH, 113 min
        iv. 100% EtOH, 113 min
        v. 100% EtOH, 113 min
        vi. 100% EtOH, 113 min
    b. Clear by immersion with gentle agitation
        i. Citrisolv (*Fisherbrand*), 113 min
        ii. Citrisolv (*Fisherbrand*), 113 min
        iii. 100% Xylene (*Fisher*), 113 min
    c. Infiltrate by immersion with gentle agitation
        i. Paraffin (*Fisherbrand* paraplast plus embedding medium), 113 min
        ii. Paraffin, 113 min
        iii. Paraffin, 113 min

5. Embed tissue in paraffin (*Fisherbrand* paraplast plus embedding medium).
    a. Trim off any excess tissue from the proximal and distal ends, to leave only the glaborous center of the plantar skin
        i. Usually only between 5mm and 10mm for mouse and rat is necessary.
    b. Cut plantar skin in half along the proximodistal axis
    c. Embed tissue, orienting the cut edge toward what will be the face of the paraffin block.
        i. This cleanly exposes the epidermis, dermis, hypodermis, and any underlying tissue for sectioning.

6. Cut 6µm thick sections using a rotary microtome.

7. Expand paraffin sections by floating in 37°C water bath for a few minutes, and mount on microscope slides (*Fisherbrand* SuperforstPlus).

8. Evaluate sections at 40x magnification to ensure the epidermis and dermis are included in the entire section of tissue.
    a. Continue cutting if necessary to reach the appropriate depth within the tissue, until both the epidermis and dermis span the entire length of the section.

III. Peroxidase Method Immunohistochemistry against Protein Gene Product 9.5 for visualization of skin innervation

(*AbD Serotec* PGP9.5, Rabbit Anti-Human, Polyclonal IgG: and *Vectastain* ABC kit)

1. Deparaffinize
    a. Incubate slides at 60°C for 1 hour. Allow slides to equilibrate to room temperature.
    b. Clear sections by immersing slides in CitriSolv (*Fisherbrand*) for 3 minutes with gentle agitation, 3 times.

2. Rehydrate sections by immersion with gentle agitation
    a. 100% EtOH, 3 minutes
    b. 100% EtOH, 3 minutes
    c. 95% EtOH, 3 minutes
    d. 50% EtOH, 3 minutes
    e. 25% EtOH, 3 minutes
    f. 10% EtOH, 3 minutes
    g. double distilled water, 3 minutes

3. Quench endogenous peroxidase by immersing slides in 3% H_2O_2 for 15 minutes with gentle agitation.

4. Rinse slides by immersing in double distilled water for 3 minutes with gentle agitation, 3 times.

5. Prepare 1x PBS (phosphate buffered saline) pH7.5.
    a. Dilute 10 x PBS 1:10
    b. pH to 7.5 with HCL

RECIPE: 10 x PBS

Dissolve into 1L of double-distilled water

i. 12g Na_2HPO_4 (*Fisher*)
ii. 2.2g NaH_2PO_4 (*Fisher*)
iii. 85g NaCl (*Fisher*)

6. Rinse slides by immersing in 1 x PBS for 3 minutes with gentle agitation.

7. Circle sections on slides with a hydrophobic marker (*Sigma* 5mm Pap Pen).

8. Prepare diluted goat serum (for about 20 slides)
    a. 10mL of 1xPBS
    b. 3 drops of normal goat serum (*Vectastain* ABC kit).

9. Cover sections on each slide with prepared diluted goat serum and incubate at room temperature in a humidifier for 30 minutes.

10. Prepare primary antibody (for about 20 slides)
    a. Dilute PGP9.5 Ab (*AbD Serotec*) 1:1000 in 5mL 1x PBS pH 7.5
    b. Add 1 drop of normal goat serum (*Vectastain* ABC kit).

11. Remove goat serum from all sections except the negative control

12. Cover sections on each slide with prepared primary antibody, and incubate at room temperature for 3 hours in a humidifier.

13. Rinse slides by pipetting 1xPBS over the sections 3 times, then immerse in 1xPBS for 3 minutes with gentle agitation, 3 times.

14. Prepare secondary antibody (for about 20 slides)
    a. 10mL of 1xPBS
    b. 1 drop of normal goat serum (*Vectastain* ABC kit)
    c. 1 drop of secondary antibody (*Vectastain* ABC kit).

15. Cover sections on each slide with prepared secondary antibody, and incubate at room temperature in humidifier for 1 hour.

16. Prepare ABC solution 30 minutes prior to use (for about 20 slides)
    a. 10mL 1xPBS
    b. 2 drops from bottle A (*Vectastain* ABC kit)
    c. 2 drops from bottle B (*Vectastain* ABC kit)

17. Following secondary antibody, rinse slides by pipetting 1xPBS over the sections 3 times, then immerse in 1xPBS for 3 minutes with gentle agitation, 3 times.

18. Cover sections on each slide with prepared ABC solution, and incubate sections at room temperature in humidifier for 1 hour.

19. Rinse slides by immersing in 1xPBS for 3 minutes with gentle agitation, 2 times.

20. Rinse slides x 2 by immersing in double-distilled water for 3 min with agitation.

21. Prepare chromagen solution (*Vector* NovaRed Chromagen) (for about 20 slides)
    a. 5mL of double distilled water
    b. 3 drops from bottle 1 (*Vector* NovaRed Chromagen)
    c. 2 drops from bottle 2 (*Vector* NovaRed Chromagen)
    d. 2 drops from bottle 3 (*Vector* NovaRed Chromagen)
    e. 2 drops of H_20_2 solution (*Vector* NovaRed Chromagen)

22. Cover sections on each slide with prepared chromagen solution, and incubate at room temperature in a humidifier for 1 to 5 minutes, or until desired intensity of red stain appears in each sections.

23. Rinse slides by pipetting double-distilled water over the sections 3 times, then by immersing in double-distilled water for 3 minutes with gentle agitation, 3 times.

24. Counter-stain sections by immersing slides in 10% Gill's Hemotoxylin (*Fisher*) for 1 minute.

25. Wash slides with running double-distilled water, until water runs clear.

26. Dehydrate sections by immersing slides, with gentle agitation
    a. 50% EtOH, 30 seconds
    b. 95% EtOH, 30 seconds
    c. 100% EtOH, 30 seconds
    d. 100% EtOH, 30 seconds.

27. Clear sections by immersing slides in CitriSolv (*Fisherbrand*) with gentle agitation for 30 seconds, 3 times.

28. Mount slides using permount (*Fisher*).

IV. Quantification of Plantar Skin Innervation

1. Code slides to obscure group 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. At 400x magnification, count and record the total number of nerve profiles immunoreactive against PGP9.5
    a. Record number of epidermal nerve profiles
    b. Record number of subepidermal nerve profiles immediately adjacent to the epidermis
    c. Record number of Langerhan's cells.

4. Measure the length of each section at 200x magnification.
    a. Use Scion Image software for Windows PC to trace a freehand line along the epidermis and measure its length in micrometers.

5. Normalize immunoreactive nerve profiles to epidermal length, and record plantar skin innervation density as nerve profile per millimeter.