Video Article

Tissue Preparation and Immunostaining of Mouse Sensory Nerve Fibers Innervating Skin and Limb Bones

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URL: https://www.jove.com/video/3485
DOI: doi:10.3791/3485

Keywords: Neuroscience, Issue 59, pain, immunostaining, sensory nerve fiber, skin, bone, plantar punch, CGRP, NF200, TRPV1, Tubulin

Date Published: 1/26/2012

Citation: Shepherd, A.J., Mohapatra, D.P. Tissue Preparation and Immunostaining of Mouse Sensory Nerve Fibers Innervating Skin and Limb Bones. *J. Vis. Exp.* (59), e3485, doi:10.3791/3485 (2012).

Abstract

Detection and primary processing of physical, chemical and thermal sensory stimuli by peripheral sensory nerve fibers is key to sensory perception in animals and humans. These peripheral sensory nerve fibers express a plethora of receptors and ion channel proteins which detect and initiate specific sensory stimuli. Methods are available to characterize the electrical properties of peripheral sensory nerve fibers innervating the skin, which can also be utilized to identify the functional expression of specific ion channel proteins in these fibers. However, similar electrophysiological methods are not available (and are also difficult to develop) for the detection of the functional expression of receptors and ion channel proteins in peripheral sensory nerve fibers innervating other visceral organs, including the most challenging tissues such as bone. Moreover, such electrophysiological methods cannot be utilized to determine the expression of non-excitable proteins in peripheral sensory nerve fibers. Therefore, immunostaining of peripheral/visceral tissue samples for sensory nerve fivers provides the best possible way to determine the expression of specific proteins of interest in these nerve fibers. So far, most of the protein expression studies in sensory neurons have utilized immunostaining procedures in sensory ganglia, where the information is limited to the expression of specific proteins in the cell body of specific types or subsets of sensory neurons. Here we report detailed methods/protocols for the preparation of peripheral/visceral tissue samples for immunostaining of peripheral sensory nerve fibers. We specifically detail methods for the preparation of skin or plantar punch biopsy and bone (femur) sections from mice for immunostaining of peripheral sensory nerve fibers. These methods are not only key to the qualitative determination of protein expression in peripheral sensory neurons, but also provide a quantitative assay method for determining changes in protein expression levels in specific types or subsets of sensory fibers, as well as for determining the morphological and/or anatomical changes in the number and density of sensory fibers during various pathological states. Further, these methods are not confined to the staining of only sensory nerve fibers, but can also be used for staining any types of nerve fibers in the skin, bones and other visceral tissue.

Video Link

The video component of this article can be found at https://www.jove.com/video/3485/

Protocol

1. Animal Perfusion

All animal procedures performed in this study are approved by the Institutional Animal Care and Use Committee of the University of Iowa, and follow NIH guidelines for the use of animals in research.

1. On the day before perfusion, prepare 1 L of phosphate buffer (0.2 M PB in double distilled H_2O, pH 7.4), and store at 4°C. This will be used for perfusion and post-fixation processes.
2. On the day of perfusion, prepare 500 ml of 4.0% paraformaldehyde in 0.1M PB (PFA, pH 7.4) fixative solution, a volume sufficient for the perfusion of 2 mice: microwave 200 ml of ddH_2O in a glass beaker for 30 sec or until it approaches boiling. Add 20 g of granular paraformaldehyde (PFA) to the beaker with constant stirring under a fume hood. Add 5 ml of 5 N NaOH drop-wise and stir until the solution clears. When the PFA is completely dissolved, cool the solution to room temperature (RT). Slowly add 250 ml of 0.2 M PB while stirring continuously. Using progressively weaker HCl solutions (6 N to 1 N to 0.1 N), adjust the pH to 7.4. Adjust the final volume to 500 ml and chill on ice. Also prepare 400 ml of 0.1 M PB from 0.2 M stock by diluting 1:1 in ddH_2O and chill on ice.
3. Anesthetize the mouse by intraperitonial injection of an overdose of anesthetic (sodium pentobarbital, 80 mg/kg, administered with a 27G needle).
4. During perfusion, 50 ml of 0.1 M PB (dilute 0.2 M PB stock 1:1 with ddH_2O) and 150 ml of PFA will be passed sequentially into the mouse circulation. Set up the peristaltic pump by filling the tubing with PB and fixing a $23^{1/2}$G butterfly needle at one end. Immerse the other end of the tubing into the beaker containing 0.1 M PB. Set the speed of the peristaltic pump to 10 ml/min, and pump through a sufficient amount of the solution to ensure that there are no air bubbles in the tubing.

5. Wait until the anesthetized mouse no longer shows any reflex activity, such as toe/tail pinch and blink reflex. Lay the animal on a dissection tray ventral side up inside a fume hood and secure the paws with tape. Spray the fur with 70% ethanol. Make an incision through the skin along the midline to expose the ribcage and the uppermost quarter of the abdominal wall. Then, cut through the abdominal wall at the base of the ribcage. After making this incision, the diaphragm and lower end of the sternum should be visible. Carefully cut from left to right through the margin of the diaphragm, and along the full length of the sternum, taking care not to damage the lungs. Some minor bleeding from the sternum at this stage is normal and will not compromise perfusion. Once the incision along the sternum has been made and the diaphragm cut, it should be possible to lift each half of the ribcage upwards and outwards, such that the heart is exposed. Move aside the lungs if necessary. Both halves of the ribcage can be held in this position with the use of hemostatic clamps. Insert the butterfly needle ($23^{1/2}$G) attached to the peristaltic pump 3-4 mm into the left ventricle, parallel to the long axis of the animal. This placement minimizes the risk of the needle becoming dislodged. Make a small incision in the right atrium to allow the return circulation to flow out of the heart.
6. Begin perfusion with 0.1 M PB at 10 ml/min for 4-5 min. If there is still a significant amount of blood emanating from the right atrium at this point, continue until the flow is clear.
7. Stop the flow of PB and switch the inlet on the peristaltic pump to the 4% PFA solution. Reduce the flow rate to 5 ml/min and resume pumping PFA solution for 20-25 min. As the PFA enters the circulation, the muscles go into spasm and, after a few minutes, the animal should be literally "fixed" in position. This rigidity can be tested by gently pushing against the hindpaws, taking care not to move the animal and dislodge the cannula. Good perfusion will prevent the limb from flexing in response. Once perfusion with PFA is complete, the cannula and hemostatic clamps can be disconnected and the cadaver prepared for tissue dissection. The dissection approach used depends on the specific tissue.
8. Prepare 4% PFA / 5% (v/v) picric acid (PA; for collection and post-fixation of plantar punch only) by adding saturated picric acid solution to 4% PFA. Inclusion of PA significantly improves antigen detectability in peripheral neuronal tissues[1]
9. Prepare bone/cartilage tissue decalcifying/cryoprotectant solution - 10% EDTA in 0.1 M PB with 0.07% glycerol and 15% sucrose. EDTA-based solutions decalcify tissue whilst preserving epitopes[2].
10. Prepare the cryoprotectant solution - 30% sucrose in 0.1 M PB.

2. Tissue Dissection/Removal, Post-fixation and Sectioning

1. **Plantar Punch**
   Place the perfused animal ventral side down on a cutting mat or other sturdy surface. Holding the foot plantar surface-up, press down firmly with the punch biopsy tool (3 mm diameter; Harris micro-punch, Ted Pella Inc.) into the middle of the foot. Turn the biopsy tool back and forth through 180 degrees to confirm the biopsy tool has cut through the entirety of the hind paw. Gently remove the biopsy tool from the foot and eject the tissue into sterile 2 ml tube with cap, containing 1 ml of 4% PFA / 5% PA solution.
2. **Limb Bone (ex. Femur)**
   Make a lateral incision along the back of the animal at the level of the pelvis, continuing down along both hind limbs. Cut into the pelvis and surrounding muscle to separate the femur from the pelvis whilst leaving the proximal head of the femur intact. Cut into the tibia/fibula to leave the distal head intact, removing the surrounding muscle/periosteum from the bone shaft. Place the femur into a 2 ml tube containing 1 ml of 4% PFA / 5% PA solution.
3. Post-fix the tissue samples in 4% PFA / 5% PA solution, with gentle mixing on a rocker for 16-18 h at 4°C
4. Decalcify the tissue as follows: *For plantar punch* (to decalcify the small bones of the foot) place the tissue punch in a sterile 2 ml tube with cap, containing 1.5 ml of 10% EDTA solution in 0.1 M PB with 0.07% glycerol and 15% sucrose. Place the tube on a rocker with mild mixing for 16-18 h at 4°C. *For limb bones* place the tissue in a sterile 2 ml tube with cap, containing 1.5 ml of 10% EDTA solution in 0.1 M PB with 0.07% glycerol and 15% sucrose. Place the tube on a rocker with mild mixing for 6-7 days at 4°C. The decalcification solution should be changed every 24 hours and the tissue monitored for loss of rigidity with a pair of forceps.
5. Transfer the tissue into a sterile 2 ml tube with cap with 1.5 ml of cryoprotectant solution (30% sucrose in 0.1 M PB), and place the tube on a rocker with mild mixing for 16-18 h at 4°C.
6. Prepare tissue for sectioning: place a bed volume of optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc.) on a cryostat tissue mounting block and allow to freeze in the cryostat chamber (typically maintained at -20°C). A cryogenic aerosol (Cyto-freeze, Control Co. USA) can also be sprayed on the OCT to accelerate the freezing process. Place tissue specimen on this bed of OCT and cover with an additional thin layer of OCT. Gently spray this OCT with cryogenic aerosol until it has hardened and the tissue within has frozen. Place specimen onto the cutting head in the cryostat chamber and allow the tissue specimen block to equilibrate to the cutting temperature - at least 1 hour. Trying to section when the embedding compound is still too cold results in brittle sections and tissue damage.
7. Once the tissue has reached optimal cutting temperature, begin cutting the specimen and generate 40 µm sections.
8. *For plantar punch* collect the cryosections into 12-well tissue culture plates containing 0.1 M PB with 10 mM sodium azide. Make sure that the sections remain submerged in this solution at 4°C. Sections can be stored in this manner for up to 4-6 months for immunostaining purposes. Alternatively, free-floating sections can be stored indefinitely in cryoprotectant solution at -20°C (500 ml 0.1 M PBS, pH 7.2, 30 g sucrose, 10 g PVP40, 300 ml ethylene glycol). Adjust final volume to 1L with distilled water)[3]. *For limb bones* collect the cryosections directly onto gelatin pre-coated slides (or Superfrost Plus slides, Fisher Scientific) and allow to air-dry for 1 h, before storing at -20°C. Bone sections on slides stored in this way can be used for immunostaining purposes within 2-3 months.

3. Immunostaining of Tissue Sections for Sensory Nerve Fibers

3.1. Plantar punch sections - floating section staining

1. Using a razor blade, cut 6-7 mm from the end of a 1 ml micropipette tip. Pre-condition the inside of the tip by aspirating 1% fetal bovine serum (FBS) in 0.1 M PB several times (this inhibits sticking of the tissue sections to the walls of the tip). Transfer plantar punch sections to be stained to a 24-well tissue culture plate. Normally 8-10 sections should be stained per well to ensure several high-quality sections are generated per immunostaining.
2. Wash plantar punch sections with 500 µl of 0.1 M PB per well for 5 min with vigorous mixing on a rocker at RT. Repeat 2 more times.
3. Discard the washing solution and incubate the plantar punch sections in blocking solution, consisting of 10% goat serum and 0.3% Triton X-100 in 0.1 M PB (500 µl of blocking solution per well), with gentle mixing on a rocker for 1 h at 4°C.

4. Discard the blocking solution and incubate the tissue sections in primary antibody/antibodies diluted in 250 µl blocking solution for 18-24 h with gentle mixing on a rocker at 4°C. Based on investigator's experimental requirements, single immunolabeling (with one primary antibody against a specific protein or nerve fiber marker), or double immunolabeling (with two primary antibodies against two proteins or nerve fiber markers) can be performed on the same section. While performing double immunolabeling it is important to verify that the two primary antibodies used are raised in different host species (e.g. one raised in mouse and one raised in rabbit), or alternatively, purified monoclonal antibodies of different immunoglobulin G (IgG) isotypes (e.g. one IgG1 and one IgG2a) can be used. It is advisable to seal the plate with Parafilm for overnight incubations to avoid excessive evaporation. In order to stain the peripheral sensory nerve fibers, several specific antibodies can be used. Antibodies against the protein neurofilament 200 (NF200; Sigma) label large-diameter fibers, whereas calcitonin gene-related peptide (CGRP; Sigma) and transient receptor potential vanilloid 1 (TRPV1, Neuromics) label peptidergic, small-diameter peripheral sensory fibers. All peripheral nerve fibers can also be stained with antibodies against β3-tubulin. In skin, collagen IV (Abcam) is used to delineate the basement membrane that separates the epidermis from the dermis. As a general rule, antibodies need to be 5-10 times more concentrated than for cultured cell-based immunofluorescence assays, typically at a working concentration of 5-10 µg/ml.
5. Wash the tissue sections with 500 µl of blocking solution (with Triton X-100) per well for 5 min with vigorous mixing on a rocker at RT. Repeat 3 more times.
6. Discard the washing solution and incubate tissue sections in appropriate fluorophore-conjugated secondary antibodies (typically diluted 1:1000 in blocking solution, 500 µl) with gentle mixing on a rocker at 4°C. The plate must be wrapped in aluminium foil to avoid photobleaching of fluorophores.
7. Wash the tissue sections with 500 µl of blocking solution per well for 10 min with vigorous mixing on a rocker at RT. Then wash with 500 µl of 0.1 M PB with vigorous mixing for 10 min at RT. Finally, wash with 500 µl of 0.05 M PB with vigorous mixing for 10 min at RT.
8. Using a 1 ml micropipette with an FBS-treated tip (cut 6-7 mm from the end), aspirate the sections from the tissue culture plate and transfer onto SuperFrost Plus microscope slides. Arrange the sections and absorb excess wash buffer with a fine paintbrush. Avoid prolonged exposure to light. Incubate the slides at RT, in order to allow sections to dry sections on slide (5-30 min).
9. Apply several drops of mounting medium (ProLongGold, Vectashield or similar), slowly place a glass coverslip onto sections. Allow the slides to sit in darkness for 5 min, and then seal the coverslip edges with transparent nail polish. Allow air-drying for 15-20 min. The slides can now be visualized or stored at -20°C for several years without any significant loss of fluorescence.

3.2. Limb bone sections - on-slide staining

1. Allow the slides to return to RT from -20°C storage. Using a hydrophobic barrier pen (Super Pap Liquid Blocking Pen, Ted Pella Inc.) circumscribe the region on the slide containing the bone sections to be stained with a generous layer of solution. Allow to air dry for 10-15 min.
2. Wash the bone sections with 250 µl of 0.1 M PB per slide for 5 min and repeat for 2 more times.
3. Discard the washing solution and incubate the bone sections in blocking solution, consisting of 10% goat serum and 0.3% Triton X-100 in 0.1 M PB (250 µl of blocking solution per slide) for 1 h at 4°C.
4. Discard the blocking solution and incubate the bone sections in primary antibody (or combination of primary antibodies, in case of double immunolabeling as mentioned under 3.1.4) diluted in 250 µl blocking solution for 18-24 h at 4°C. It is very important to note that the slides must be placed in a humidified chamber with a sealed lid, in order to avoid drying of primary antibody solution. In order to stain the peripheral sensory nerve fibers, the above-mentioned set of antibodies (see section 3.1.4) can be used with similar working concentrations.
5. Wash the tissue sections with 250 µl of blocking solution (with Triton X-100) per slide for 15 min at RT and repeat for 3 more times.
6. Discard the washing solution and incubate bone sections in appropriate fluorophore-conjugated secondary antibodies (typically diluted 1:1000 in blocking solution, 250 µl) at 4°C. The staining chamber must be wrapped in aluminium foil to avoid photobleaching of fluorophores. For convenience, it is advisable to use dark-colored staining chambers (e.g. Slide incubation tray/box, RPI Corp.).
7. Wash the bone sections with 250 µl of blocking solution per slide for 15 min at RT. Then wash with 500 µl of 0.1 M PB for 15 min at RT. Finally, wash with 500 µl of 0.05 M PB for 15 min at RT. Dip briefly in ddH_2O to rinse slides and then incubate at RT in order to allow the bone sections to air dry (5-30 min). Avoid prolonged exposure to light.
8. Apply several drops of mounting medium (ProLongGold or similar) and slowly place a glass coverslip onto sections. Let the slides stand in darkness for 5 min, and then seal the coverslip edges with transparent nail polish. Allow drying for 15-20 min. Slides can be stored at -20°C for several years without loss of fluorescence.

4. Representative Results

4.1. Plantar punch sections

Plantar punch tissue sections can be visualized under epifluorescence microscope, or under a confocal microscope with a 10X, 40X or 63X objective.

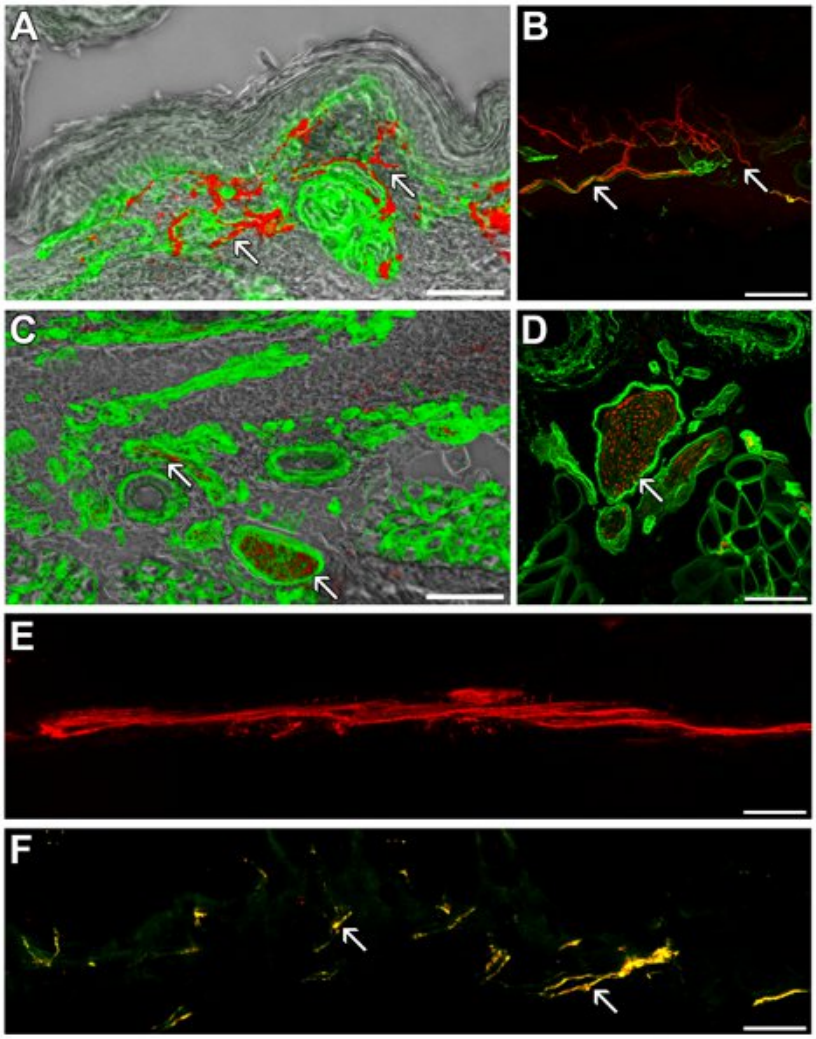

Figure 1. A-D show staining with Collagen IV antibody (green) in basement membranes, at the epidermal-dermal junction and in cartilage and muscle. Numerous CGRP- (A-B, red) and NF200-positive fibers (C-D, red) are distributed throughout the mouse skin in the plantar region (arrows). β3-tubulin is a pan-neuronal marker (E, red), whereas TRPV1 staining (F; red) is mainly confined to small-diameter fibers that are also CGRP-positive (green; arrowheads). A and C are epifluorescence images taken with a 10X objective (scale bar -500 μm); B, D, E and F are confocal image composites generated from an 11-image z-stack taken at 2 μm increments under a 60X objective (scale bar - 50 μm).

4.2. Limb bone sections

Limb bone tissue sections can also be visualized under epifluorescence microscope, or under a confocal microscope with a 10X, 40X or 63X objective.

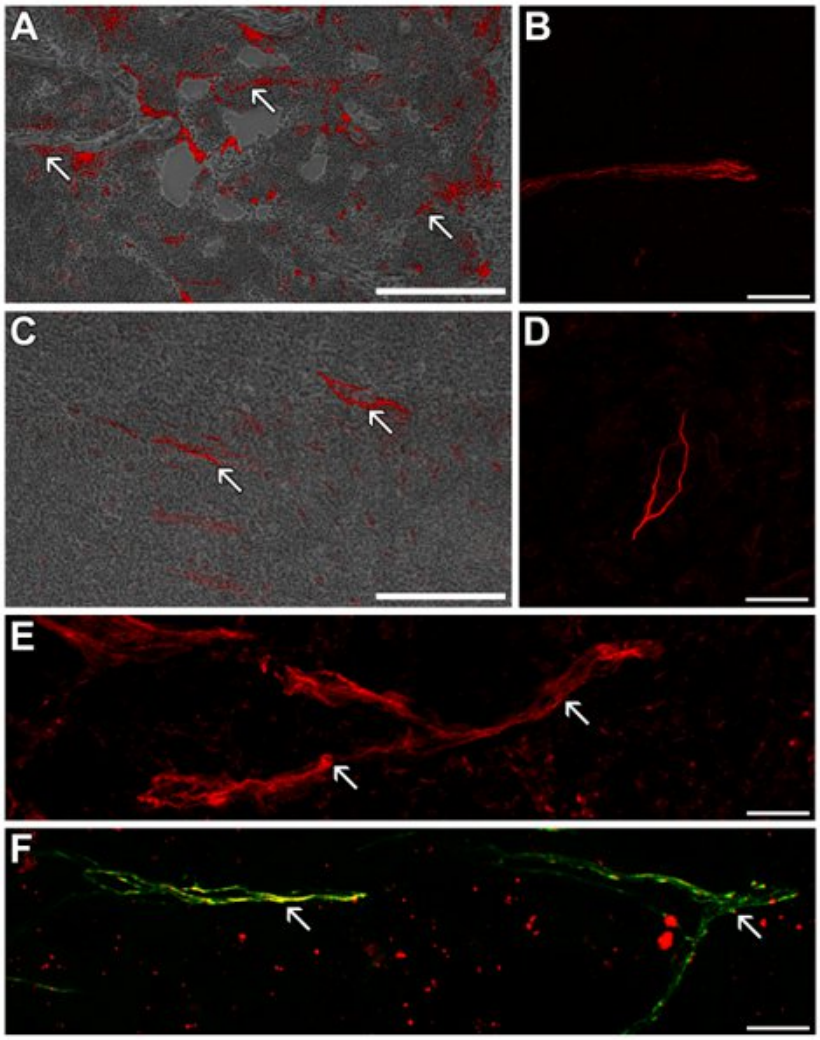

Figure 2. shows immunostaining with anti-CGRP (A-B, red; arrows), anti-NF200 (C-D, red; arrows), anti-β3-tubulin (E; red) and anti-TRPV1 (F; red) co-stained with anti-CGRP antibody (green; arrows). These images show subtypes of sensory nerve fibers distributed throughout the bone matrix in the spongy head region of mouse femur. A and C are epifluorescence images taken with a 10X objective (scale bar -500 μm); B, D, E and F are confocal image composites generated from an 11-image z-stack taken at 2 μm increments under a 60X objective (scale bar - 50 μm).

Discussion

Here we have detailed the methods for preparation of mouse skin and bone tissue sections for immunostaining and detection of peripheral sensory nerve fibers. The sections produced from plantar punch biopsies contain both glabrous and hairy skin, which means the protocol can be used on any skin type. These techniques can also be employed to stain other cell types in these tissues (e.g. leukocytes, vascular endothelia, smooth muscle among others). These methods provide an excellent compromise between optimal ultrastructural preservation (which is achieved by glutaraldehyde fixation, but frequently results in disruption of epitopes and diminished immunostaining staining quality) and immunocytochemical detectability, if the procedures are followed step-by-step in a rigorous manner.

Detection of sensory nerve fibers in these tissues can aid in our understanding of the regulation of peripheral neurite outgrowth and sprouting[4], as well as anatomical changes in peripheral sensory afferents under different pathological conditions. Furthermore, changes in the expression of neurotransmitters, receptors, ion channels or other phenotypic markers in normal developmental or pathological conditions can also be studied[3,5-10]. Along with appropriate electrophysiological, biochemical and behavioral testing, such changes in peripheral sensory neuron staining patterns can be used to test hypotheses related to various pain states[6,9], inflammation[11] and neuropathies[5,12]. In conclusion, these techniques provide an invaluable source of *in vivo* data that complements and reinforces other anatomical, structural and functional data acquired through additional approaches, furthering our understanding of the regulation and acquisition of plasticity in peripheral sensory nerve fibers in health and disease.

Disclosures

No conflicts of interests declared.

Acknowledgements

We thank Dr. Yuriy M. Usachev for his help in the initial standardization of confocal microscopy/imaging of mouse plantar punch biopsy immunostaining; and Dr. Donna L. Hammond for her continued help and constructive criticism in this work. This work was funded by grants from the NINDS/NIH (NS069898), and an Idea Development Grant Award from the Department of Defense Prostate Cancer Research Program (DoD-PCRP-101096) to D.P.M.

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