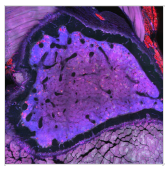

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Spatial mapping and contextualization of axon subtypes innervating the long bones of C3H and B6 mice

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ABSTRACT

Nerves in bone play well-established roles in pain and vasoregulation and have been associated with the progression of skeletal disorders including osteoporosis, fracture, arthritis and tumor metastasis. However, isolation of the region-specific mechanisms underlying these relationships is limited by our lack of precise maps of skeletal innervation. We developed an optimized workflow for imaging of nerve axons in and around the bone, resulting in novel, comprehensive maps of sympathetic adrenergic and sensory peptidergic axons within and around the full length of the femur and tibia in two strains of mice (B6 and C3H). This protocol is intended to serve as a guide during the design, implementation, and interpretation of future neuroskeletal studies and was compiled as a resource for the field as part of the NIH SPARC consortium.

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bone, nerves, immunohistochemistry, neuroskeletal, muscle, bone marrow adiposity

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MATERIALS TEXT

A	B	C
10% neutral buffered formalin	Fisher Scientific	23-245684
EDTA	Sigma-Aldrich	E5134
DAPI	Sigma-Aldrich	D9542
OCT mounting media	Fisher HealthCare	23-730-571
Triton X-100	Sigma-Aldrich	9002-93-1
Fluoromount-G	Thermo Fisher Scientific	00-4958-02
Donkey serum	Sigma-Aldrich	D9663
Acrylamide/bis-acrylamide, 40% solution	Sigma-Aldrich	A7802
Paraformaldehyde	Electron Microscopy Sciences	15710
Histodenz	Sigma Aldrich	D2158-100G
NNN'N'-Tetrakis(2-Hydroxypropyl)ethylenediamine-Quadrol	Sigma Aldrich	122262-1L
VA-044	Wako	27776-21-2
SDS Micropellets	Thermo Fisher Scientific	BP8200-500
Spinning Disk Confocal Microscope	Nikon CSU-X1	N/A

Table 1. Chemicals

A	B	C
TNT Buffer (500 mL)	Volume	Final Concentration
1M Tris-HCl, pH 7.4	50 mL	0.1 M
3M NaCl	25mL	0.15 M
Tween-20	250 µL	0.05%
diH2O	qS to 500 mL	
Filter at 0.2 µm and store at room temperature.		

Table 2. TNT Buffer recipe for 500 mL

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A	B	C	D	E
Primary Antibody (Vendor, Cat. No)	Dilution	Secondary Antibody	Fluorophore	Dilution
Anti-Calcitonin Gene Related Peptide (Bio-rad, 1720-9007)	1:1000	Donkey Anti-Goat (Jackson Immunoresearch, 705-606-147)	AlexaFluor-647	1:500
Anti-Tyrosine Hydroxylase(Abcam, UK, ab152)	1:1000	Donkey Anti-Rabbit (Jackson Immunoresearch, 711-546-152)	AlexaFluor-488	1:500
Anti-Perilipin(Progen Biotechnik, Germany, GP29)	1:500	Donkey Anti-Guinea Pig (Jackson Immunoresearch, 706-165-148)	Cy3	1:500
Neurofilament 200 (NF200) (Millipore, AB5539)	1:5000	Donkey Anti-Chicken (Jackson Immunoresearch, 703-545-155)	AlexaFluor-488	1:500

Table 3. Antibodies used for immunostaining of fixed frozen tissue

Tissue isolation and fixation 2d

1 Fully anesthetize mouse by intraperitoneal (IP) injection of ≥ 100 mg/kg ketamine + 10 mg/kg xylazine. Confirm anesthesia has been established to a surgical plane or greater depth by toe-pinch reflex. 10m

2 When the animal is fully anesthetized, open the chest cavity to expose heart and make a small incision in the right atrium to allow free flow of blood.

3 Perform cardiac perfusion by first slowly injecting 10–20 mL 1x phosphate buffered saline (PBS, Corning 46-013-CM) with a 20 gauge needle through the left ventricle of the heart until liver is cleared of blood. 5m

4 Follow by slowly injecting 10 mL 10% neutral buffered formalin (NBF, Fisher Scientific 23-245684) until fixation is achieved as evidenced by tremors in the extremities.

5 Carefully remove tibias and femurs keeping most of the surrounding musculature and soft tissue intact to preserve the periosteum.

6 Post-fix tissues in 10% NBF for 24 h at 4 ˚C. 1d

7 Wash tissues for 2 h in diH_2O. Store in PBS at 4 ˚C or proceed to sample preparation. 2h

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Frozen sample preparation 1w 5d

8 Fully decalcify bones in 20x volume of 14% EDTA (Sigma-Aldrich E5134), pH 7.4 with gentle shaking at 4 ˚C for 10 days, replacing with fresh 14% EDTA, pH 7.4 every 2–3 days. 1w 3d

9 Wash tissues 3 x 30 min in diH_2O. 1h 30m

10 Cryoprotect tissues by infiltrating with 20x volume 30% sucrose in PBS for 24–48 h at 4 ˚C. 2d

11 Embed bones in optimal cutting temperature compound (OCT, Fisher HealthCare 23-730-571) in deep disposable tissue molds (molds come with VWR Mega Cassettes, VWR 18000-286). Orient the bone so that the side touching the short edge of the mold is the side that will be sectioned first. For transverse sections of the tibia or femur, the knee should touch the short edge of the mold. Fill the mold completely with OCT and gently push the tissue with forceps to ensure that the bottom surface of the tissue is level and the tissue is located in the center of the mold lengthwise. 5m

12 Use an appropriate cold source to freeze down the tissue in the OCT block relatively quickly and evenly. Store blocks at -80 ˚C or proceed to cryosectioning. 15m

13 For cyrosectioning, in a cryostat with chamber set to -20 ˚C, orient the OCT block on the chuck so that the knee will be sectioned first in a transverse plane. Allow to set until frozen. 3m

14 Mount the chuck and adjust so that the block surface is parallel to the blade. Face the block so that sectioning results in an even layer across the entire surface of the block. Continue until the tissue sample is exposed. 5m

15 Section sample at 50 µm slowly but steadily using the anti-roll plate or a brush to keep the section flat as it comes off the blade. 1m

16 Collect section (up to three) on Colorfrost Plus glass slides (Fisher Scientific 12-550-18). Check sections under a light microscope to assess correct plane by bone morphology. Adjust angle of the block holder if necessary to achieve cross-section. 15m

17 Continue sectioning through the length of the bone. 1h 30m

18 Store slides at -80 ˚C or proceed to immunohistochemistry.

Immunohistochemistry 2d

19 Air dry slides for 30 min at room temperature (RT). 30m

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20 Wash sections 2 x 5 min with 1x PBS. 10m

21 Block with 10% donkey serum and permeabilize with 0.3% Triton X-100 in PBS for 1 h at RT. 1h

22 Incubate with primary antibodies (Table 3) in 1% normal donkey serum in TNT buffer (Table 2) overnight at RT in humidified chamber. 1d

23 Wash 3 x 5 min in TNT buffer. 15m

24 Incubate with secondary antibodies (Table 3) in 1% normal donkey serum in TNT buffer for 3 h at RT or 24 h at 4 ˚C. 3h

25 Wash sections 3 x 5 min in TNT buffer. 15m

26 Incubate with DAPI (Sigma-Aldrich, D9542) 1:1000 in TNT buffer for 5 min at RT. 5m

27 Wash sections 3 x 5 min in TNT buffer. 15m

28 Coverslip (Fisher Scientific 12-545-M) with Fluoromount-G (Thermo Fisher Scientific 00-4958-02). Air dry and seal with clear nail polish before imaging. 1d

Imaging

29 Capture serial-tiled images through the depth of the section at step size 2.5 µm with a 10x objective on a confocal microscope. For subsequent neural analysis, the resolution should reach at least 1.5 pixels/µm to adequately capture axons of 1 µm in size or larger. A higher resolution is needed to accurately visualize axons smaller than 1 µm. Tissue clearing may be performed to improve visibility in optically dense regions such as the bone marrow.

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