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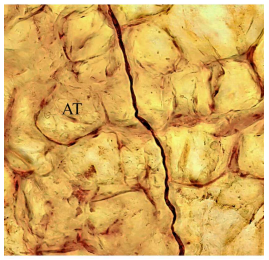
Anterograde tracing of spinal afferent innervation in rat atria flat-mounts



Forked from Anterograde tracing of spinal afferent innervation in rat stomach flat-mounts

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Abstract

This protocol describes the methods used to trace and digitize spinal afferent axons in the rat atria. A mixture of dextran conjugates was injected into the dorsal root ganglia C8-T3 of adult Sprague-Dawley rats. 16 days after the tracer injection, the animals were sacrificed, the left and right atria were prepared and processed for avidin-biotin permanent labeling and Cuprolinic Blue labeling. All flat-mounts were examined using a Zeiss M2 Imager and MicroBrightField's Neurolucida tracing and digitization system to determine the distribution and terminal structures of spinal afferent axons.

Protocol materials

- Sprague-Dawley **Envigo**
- Dextran-Biotin 3k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D7135
- Dextran-Biotin 10k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D1956
- Thin Wall Glass Capillaries **World Precision Instruments** **Catalog #**TW150-4
- Buprenorphine **McKesson Corporation** **Catalog #**42023017905
- IsoThesia (Isoflurane) Solution **Henry Schein Animal Health** **Catalog #**029405
- 10 µL Syringe **Hamilton Company** **Catalog ##**80300
- Fluorogold **Fluorochrome**
- Isoflurane **Covetrus** **Catalog #**029404
- Heparin **Henry Schein Animal Health** **Catalog #**049130
- VECTASTAIN Elite ABC HRP Kit (Peroxidase, Standard) **Vector Laboratories** **Catalog #**PK-6100
- 3,3′-Diaminobenzidine tetrahydrochloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D5905
- Cuprolinic Blue (quinolinic phthalocyanine) **American Elements**
- DPX **Merck Millipore (EMD Millipore)** **Catalog #**1.00579.0500

Animals

1 Three to five-month-old male Sprague-Dawley **Envigo** rats (n = 15) were housed in an animal room at which the dark/light cycle was set to 12/12 hours and water and food were supplied ad libitum. All procedures were carried out under the ethical guidelines of the University of Central Florida and approved by the University of Central Florida's Institutional Animal Care and Use Committee (IACUC).

Neural tracer injections

2 Overnight-fasted rats were anesthetized with IsoThesia (Isoflurane) Solution **Henry Schein Animal Health Catalog #**029405 inhalation (3% in oxygen for induction, 2% for maintenance). Hind paw pinch withdrawal reflex was checked as an indicator of the depth of anesthesia. While under anesthesia, the rat was placed in a ventral decubitus position, and a 5–6-cm long incision was made along the midline of the dorsal surface. Left paraspinal muscles were separated from the vertebrae by blunt dissection to expose the lateral aspect of the C8-T3 vertebrae. Throughout the dissection, special attention was given to avoid large blood vessels and other critical anatomical features that are located close to the vertebral bodies. This delicate procedure was carried out under a Leica surgical microscope, which provided magnification and enhanced visualization of the surgical field. DRGs (C8-T3) were then exposed by drilling holes at their corresponding intervertebral foramina using

Equipment

Micro drill	NAME
Burr for micro drill	TYPE
Fine Science Tools	BRAND
19007-21	SKU
https://www.finescience.com/en-US/Products/Bone-Instruments/Micro-Drill-Accessories/Burrs-for-Micro-Drill/19007-21	LINK
Shaft Diameter: 2.3mm, Length: 44mm, Tip Diameter: 2.1mm, Alloy/Material: Carbon Steel, Autoclave Safe: No	SPECIFICATIONS

. To locate the correct foramina for each DRG, the spinous processes of the vertebrae directly before and after the target DRG were first identified. These landmarks were used to guide the positioning of the drill and ensure that the procedure was both accurate and minimally invasive. During drilling, extreme care was taken to avoid any damages of targeted DRGs.

For the DRG injection, a 10 µL Syringe **Hamilton Company** **Catalog #**#80300 was used to aspirate 2-µl of lysine-fixable Dextran Biotin (DB) solution consisting of a 1:1 mixture of 3K and 10K MW dextrans in ultrapure water (final concentration 15% DB consisting of 7.5% Dextran-Biotin 3k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D7135 and 7.5% Dextran-Biotin 10k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D1956). This 2-µl solution was then transferred to a Thin Wall Glass Capillaries **World Precision Instruments** **Catalog #**TW150-4 (outer diameter: 1.5 mm, inner diameter: 1.12 mm) that was pulled on a

Equipment

P-87 Flaming/Brown	NAME
micropipette puller	TYPE
Sutter Instruments	BRAND
Rev. 0299c	SKU
https://www.sutter.com/MICROPIPETTE/p-87.html	LINK

with tip diameter of approximately 5-10 µm.

The micropipette was inserted at a 45-80 degree angle relative to the spine into the posterior side of DRG, and the tracer was slowly injected as the needle gradually retracted in a stepwise fashion within the ganglion. The micropipette was kept in place for 1 minute during each injection before withdrawal to ensure the infusion pressure within the DRG had dissipated. After each DRG (C8-T3) was injected, cotton-tipped applicators (Puritan, USA) were immediately used to remove any possible leakage of tracers at the injection sites ensuring the confinement of the tracer within the ganglia.

After the injection, the paraspinal muscles and skin were closed using interrupted sutures. The rats were placed on a heating pad at 37°C for recovery and regaining of their normal reflexes prior to being transferred to their home cages. To reduce post-surgical discomfort and pain,
Buprenorphine **McKesson Corporation Catalog #**42023017905 (0.01 mg/kg body weight; s.c., Par Pharmaceuticals, Chestnut Ridge, NY, USA) was administered during surgery and once a day for the following 72 h post-surgery. Also, to prevent infection, a single dose of penicillin (50,000 IU/kg body weight; i.m., NorBrook, Newry, UK) was given. Both body weight and intake of food and water were monitored throughout the duration of the recovery period to ensure that rats did not experience any postsurgical complications.

Fluoro-Gold counterstaining

3 Twelve days after survival surgery, seven animals were intraperitoneally (i.p.) injected with Fluorogold **Fluorochrome** (1 mL of 2 mg/mL per rat) to counterstain neurons in the peripheral ganglia. The animals were perfused 4 days after the FG injection.

Tissue fixation and heart dissection

4 16 days following post-op, the rats were weighed and deeply anesthetized with Isoflurane **Covetrus Catalog #**029404 (5%). Upon the absence of response to hind-paw pinching, 0.3 mL of
Heparin **Henry Schein Animal Health Catalog #**049130 was injected into the left ventricle to prevent blood coagulation followed by a cut to the inferior vena cava.

The animal was perfused through the left ventricle of the heart with 500 mL of physiological saline at 37°C and then with 500 mL of 4% paraformaldehyde in phosphate-buffered saline (0.1 M PBS, pH 7.4) at 4°C.

The heart was pinned to a dissecting dish containing PBS (pH 7.4), and the specimen was further dissected under a Leica surgical microscope. In brief, the lungs and trachea were removed with fine forceps, the atria were separated from the ventricles, and the aortic arch was removed from the atria. The right and left atria were separated along the interatrial septum. The right atrium tissue also contained the great vessels, including the IVC, superior vena cava (SVC), and left precaval vein (LPCV), whereas the junctions of all pulmonary veins (PVs) remained attached to the left atrium.

In this study, we have identified structures corresponding to the right cranial vein (RCV), left cranial vein (LCV), and caudal vein (CV), and we consistently referred to them here as the SVC, LPCV, and IVC in both our current study and in prior research.

DAB and Cuprolinic Blue staining

5 Similar to the protocol that was previously used for the rat stomach (**Walter et al., 2016**), all steps of tracer processing and neuronal counterstaining of whole mount tissue were done in room temperature (~22°C), on a shaker, and with the tissue free floating.

The samples were washed 6 times x 5 minutes each in phosphate-buffered saline (PBS; 0.1 M, pH 7.4), followed by a 30-minute soaking in methanol:hydrogen peroxide block (4:1 ratio) to inactivate endogenous peroxidase.

Following additional PBS washes, the tissues were blocked with a PBS solution containing 0.5% Triton X-100 and 0.08% sodium azide NaN3 for 5 days to facilitate penetration of the reagent.

The samples were rinsed in PBS 6 times x 5 minutes each, followed by a one-hour incubation in

VECTASTAIN Elite ABC HRP Kit (Peroxidase, Standard) **Vector Laboratories Catalog #**PK-6100

. The DB-filled spinal afferent axons were visualized using

3,3′-Diaminobenzidine tetrahydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D5905

, a traditional dye that produces a golden-brown color on labeled axons.

Samples were then rinsed in PBS 6 times x 5 minutes each, soaked in DAB solution for 5 minutes, and rinsed in distilled water 6 times x 5 minutes. The pan-neuronal marker

Cuprolinic Blue (quinolinic phthalocyanine) **American Elements**

which binds to RNA in the cytoplasm of neurons (**Phillips et al., 2004**) was used to counterstain the enteric neurons in another group of samples (n = 6).

Briefly, the samples were rinsed in distilled water and then incubated in 0.5% Cuprolinic Blue solution which dissolved in 0.05 M sodium acetate buffer containing 1.0 M $MgCl_2$ (pH 4.9) for 2 hours in a humidified slide warmer (38°C).
Next, the samples were rinsed in distilled water and incubated in 0.05 M sodium acetate buffer containing 1.0 M $MgCl_2$ (pH 4.9) for 2 minutes, followed by a repeating rinse in distilled water.
After the staining process, samples were mounted on slides, flattened under lead blocks for 6 hours, air-dried overnight in a fume hood, dehydrated in an ascending

concentration of alcohol (75%, 95%, 100%, and 100%), and cleared in xylene. Coverslips were used to cover the tissue after applying DPX **Merck Millipore (EMD Millipore) Catalog #**1.00579.0500 mounting medium.

Spinal afferent axon screening and tracing

6

Equipment

Eclipse 80i	NAME
Fluorescent Microscope	TYPE
Nikon	BRAND
M318E	SKU
https://www.microscopyu.com/museum/eclipse-80i	LINK

(Lens: 20X, NA 0.5) with brightfield optics was first used to systematically examine all flat-mount stomachs. Once a DB-labeled spinal axon was identified, three criteria were used to inspect the quality of the axon: (a) adequate labeling, (b) completeness of the axon, and (c) minimal tissue artifacts such as folds and tears.

The spinal axons that satisfied all criteria were re-evaluated using a higher magnification lens (40X, NA 0.75) to assess the morphology of any intertwined neighboring individuals that could potentially lead to the failure of distinction and digitization of the entire tracing of a single spinal afferent axon. Such neighboring spinal axons were removed from the inventory, and the axons that satisfied all qualifications were marked and ready to be digitized.

Axon tracing, digitization, and analysis were performed using

Software	
Neurolucida	NAME
MBF BioScience	DEVELOPER
https://www.mbfbioscience.com/neurolucida	SOURCE LINK

(RRID:SCR_001775). The software controlled the motorized stage of

Equipment	
Axio Imager.M2	NAME
Upright microscope	TYPE
Zeiss	BRAND
Zeiss	SKU
https://www.zeiss.com/microscopy/en/products/light-microscopes/widefield-microscopes/axio-imager-2-for-life-science-research.html	LINK

(RRID:SCR_018876, Oberkochen, Germany) equipped with brightfield optics and a long-working-distance 40X objective lens (NA 0.75). All spinal afferent axons were traced in real time and in their original three dimensional space. The tracing file was then saved as .xml.

Image acquisition

7 Single-field and multiple-field (or mosaic) photomicrographs were acquired using Zeiss Axio Imager M2 microscope with a 20X (NA 0.8) objective lens and a 63X oil immersion (NA 1.4) objective lens. The AxioCam 208 color camera was mounted on the microscope with BrightField and Differential Interference Contrast (DIC) optics. Mosaic photomicrographs consisted of the whole innervating field of a spinal afferent axon and

were assembled via Photoshop. To capture the varying depths of a spinal afferent axon within a smooth muscle whole mount, each image consisted of multiple focal z-planes that were stacked using Photoshop to generate a partial projection image. Modifications, including brightness and contrast adjustments, and scale bar additions were conducted utilizing Photoshop or

Software

ImageJ/Fiji	NAME
Windows 7	OS
National Institutes of Health	DEVELOPER
http://wsr.imagej.net/distros/win/ij152-win-java8.zip	REPOSITORY
https://imagej.nih.gov/ij/	SOURCE LINK

(RRID: SCR_003070).

Equipment

TCS SP5 Laser Scanning	NAME
Confocal Microscope	TYPE
Leica	BRAND
TCSSP5	SKU
https://www.leica-microsystems.com/products/confocal-microscopes/p/leica-tcs-sp5/	LINK

with 40x oil immersed lens (NA. 1.25) and a 405 nm laser (excitation) was used to detect FG-labeled neurons in the flat-mounts of the atria. Simultaneously, DIC optics were used

to detect DB-labeled spinal afferents. Single optical section, partial, and maximum projection images were used to show the innervation on cardiac targets.

The distribution map of spinal afferent axons in the atria

8 Each left and right atria were contoured using Neurolucida. One left and one right atria image were selected to serve as reference images. Then all contours were averaged using the interactive landmark-based deformable image alignment BigWarp tool provided by the open source ImageJ Fiji plugin.

To align the landmarks with the reference images, similar fiducial points were selected for each image. Following image alignments, the tracing data were added using the calculator tool of ImageJ to show the total traced axons of the aligned images. The first bifurcation of each parent axon was resembled by a dot in the reference images.

Quantification

9 Morphometric analysis was performed automatically using

Software

NAME	DEVELOPER	SOURCE LINK
Neurolucida Explorer	MicroBrightField Bioscience	https://www.mbfbioscience.com/neurolucida-explorer

on traced axons, and information, including axon diameter, receptive fields, terminals, and node (bifurcation point) numbers, were obtained. Values are expressed in the table as mean values ± standard error of mean (SEM). In categorizing the size of small and large axons, the distribution of axon diameters was considered. Given that the majority of axons fell either below 0.7 or above 0.9 µm in diameter with few intermediary values, a threshold of 0.9 was selected for categorization purposes.

Receptive field was calculated using convex hull spatial analysis. A convex polygon is generated by connecting distal branches of each axon, and the surface area is reported as the receptive field parameter for each axon. An independent t-test was performed using

Software	
Prism	NAME
GraphPad	DEVELOPER
https://www.graphpad.com/scientific-software/prism/	SOURCE LINK

. Significance was considered at $p < 0.05$.