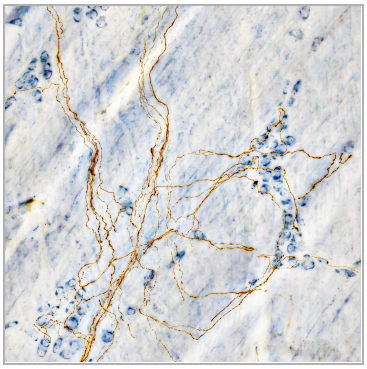

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Anterograde tracing of spinal afferent innervation in rat stomach flat-mounts

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ABSTRACT

This protocol describes the methods used to trace and digitize spinal afferent axons in the rat stomach. A mixture of dextran conjugates was injected into the dorsal root ganglia T7- T11 of adult Sprague-Dawley rats. 16 days after the tracer injection, the animals were sacrificed, ventral and dorsal stomachs 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.

Keywords: stomach, myenteric plexus, smooth muscles, blood vessels, spinal afferent, anterograde labeling, axonal tracing

Animals

1 Four to five-month-old male Sprague-Dawley **Envigo** rats (n = 20) 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). Whilst 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 transverse process by blunt dissection to expose the lateral aspect of the T7-T11 vertebrae. T7-T11 DRG were then exposed by drilling holes at T7-T11 intervertebral foramina.

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 µm.

The micropipette was inserted at a 45-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 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 righting reflexes prior to being transferred to their home cages.

Tissue fixation and GI dissection

3 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.

In order to shape the stomach in normal distension at the time of fixation, a catheter was slowly

intubated into the stomach and 10 mL of physiological saline at 37°C was infused into the stomach.

After the perfusion, the whole stomach was removed from the abdominal cavity by introducing cuts to the distal esophagus and the proximal duodenum. Next, a midline cut along the lesser and greater curvature was made to yield two equal halves. The chyme was rinsed off to expose the mucosa.

8 hours into post-fixation with paraformaldehyde, the whole mount muscle wall of the stomach was separated from the gastric mucosa and submucosa using forceps. The muscle wall includes the longitudinal muscle, myenteric plexus, and circular muscle.

DAB and Cuprolinic Blue staining

4 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) Catalo #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

5

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.

All spinal axons were categorized based on their relative locations (e.g., fundus, corpus, antrum) and gastric targets (e.g., myenteric ganglia, longitudinal muscle sheet, circular muscle sheet, blood vessels).

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

(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.

The Neurolucida system was also used to locate and outline the myenteric ganglia on which the spinal afferent axons formed terminals or where the axon processes coursed in close proximity with and on the same layer without making direct innervation. The criteria for identifying myenteric ganglia and myenteric plexus connectives were established (Walter et al., 2016; Bar-Shai et al., 2004; Berthoud et al., 1997), where at least two myenteric neurons clustered together were considered a ganglion. Two clusters of neurons were considered two ganglia if they were separated by a distance of at least three average neuron long-axis diameters, with or without the presence of a string of consecutive single-file neurons.

The digital reconstruction of spinal afferent axons in the stomach was performed on a 2D montage image instead of the live tissues like we mentioned above. In order to do this, we scanned the whole stomach in bright field using a 20X (NA 0.8) objective lens on the Zeiss Axio Imager M2 and performed a maximum projection for all image stacks using

Software

Zen Digital Imaging for Light Microscopy NAME

Zeiss DEVELOPER

(RRID:SCR_013672). The image tiles were stitched together using

Software

Adobe Photoshop NAME

Adobe DEVELOPER

(RRID: SCR_014199) to create a montage of the whole stomach. The montage was then loaded into

Software

Neurolucida 360 NAME

MBF Bioscience DEVELOPER

(RRID:SCR_016788) for axon tracing, digitization, and analysis.

Image acquisition

6 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	SOURCE LINK

(RRID: SCR_003070).