High resolution labeling of vagal efferent fibers using Dextran-Biotin with counterstaining V.2

COMMENTS 0

DOI
dx.doi.org/10.17504/protocols.io.kxygxmqqwl8j/v2

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VERSION 2
DEC 12, 2022

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ABSTRACT

This protocol describes the methods used to trace and enable morphometric quantification of preganglionic efferent neurites in the rat stomach and duodenum. A mixture of dextran conjugates was injected into the dorsal motor nucleus of the vagal nerve (dmnX) of young adult Sprague-Dawley rats and after a survival period of 19 days for optimal tracer transport, stomachs and sections of proximal duodenum were removed and processed as whole mounts. ABC-DAB was used to create a permanent gold-brown stain of all labeled efferent neurites. Protocols are included for labeling the efferent-labeled whole mounts with either NADPH-diaphorase (NADPHd) histochemistry or antibodies to nNOS to visualize the nitrergic subpopulation of myenteric neurons, or with Cuprolinic Blue to visualize the entire population of myenteric neurons.

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PROTOCOL CITATION

Deborah Jaffey, Terry Powley, Jennifer Mcadams, Robert Phillips 2022. High resolution labeling of vag efferent fibers using Dextran-Biotin with counterstaining. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygxmqqwl8j/v2
Version created by Deborah Jaffey

FUNDERS ACKNOWLEDGEMENT

NIH
Grant ID: OT2OD023847

KEYWORDS

Efferent, vagal, tracing, preganglionic, ABC-DAB, cuprolinic blue, nNOS, dmnX, rat

CREATED

Aug 16, 2022

LAST MODIFIED

Dec 12, 2022

PROTOCOL INTEGER ID

68744

MATERIALS TEXT

STEP MATERIALS

Sprague-Dawley **Envigo** Step 1

2018 Teklad global 18% protein rodent diet **Envigo** Step 1

Dextran-Biotin 10k, Lysine fixable **Thermo Fisher Scientific Catalog #D1956** Step 3

Buprenorphine (Buprenex) **Midwest Veterinary SUpply Catalog # 191.26890.3** Step 4

Rimadyl (Carprofen) **Zoetis** Step 4

Ketamine **Patterson Veterinary Catalog #07-803-6637** Step 5

Xylazine **Akorn Animal Health Catalog #NDC: 59399-110-20** Step 5

Heparin **Henry Schein Animal Health Catalog #049130** Step 5

Cytoseal XYL **Fisher Scientific Catalog #22-050-262** Step 14

Vectastain Elite ABC HRP kit **Vector Laboratories Catalog #PK-6100** In 2 steps

3,3-DIAMINOBENZIDINE.4HCl.xH2O Pure 98% * 5 g **Sigma-aldrich Catalog #32750-5G** Step 9

Cuprolinic Blue (quinolinic phthalocyanine) **American Elements** Step 10

Goat serum **Sigma Aldrich Catalog #G9023** Step 11

Avidin/Biotin blocking kit **Vector Laboratories Catalog #SP-2001** Step 11

Avidin/Biotin blocking kit **Vector Laboratories Catalog #SP-2001** Step 11

nNOS polyclonal antibody **Thermo Fisher Scientific Catalog ## 61-7000** Step 11

Biotin-SP (long spacer) AffiniPure Goat Anti-Rabbit IgG (H L) **Jackson Immunoresearch Catalog #111-065-144** Step 11

Vectastain Elite ABC HRP kit **Vector Laboratories Catalog #PK-6100** In 2 steps

VECTOR® SG Peroxidase (HRP) Substrate Kit **Vector Laboratories Catalog #SK-4700** Step 11

Glycopyrrolate **Akorn Animal Health** Step 2

Isoflurane **Akorn Animal Health Catalog #NDC: 59399-106-01** Step 2

Animals

1 Sprague-Dawley **Envigo**

rats (males: two to four months, mean weight 247g [sd 42g]; females: two to five months, mean weight: 189g [sd 15g] at the time of tracer injection) are housed individually in wire hanging cages or in vented rack plastic cages in an Association for Assessment and Accreditation of Laboratory Animal Care-approved temperature (22–24 °C) and humidity (40–60%)-controlled colony room. The room is maintained on a 12-hour light–dark schedule. Pelleted chow

2018 Teklad global 18% protein rodent diet **Envigo**

and filtered tap water are available ad libitum, except for the night before tracer injection, when food but not water is removed. All procedures are conducted in compliance with the National Institute of Health *Guide for*

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the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), and are approved by the Purdue University Animal Care and Use Committee.

Neural tracer injections

2 Anesthetize overnight-fasted rats with isoflurane

Isoflurane **Akorn Animal Health Catalog #NDC: 59399-106-01**

(starting at 5% and then dropping to 2% or less as needed) and then mounted in a stereotaxic frame. After anesthesia, inject

Glycopyrrolate **Akorn Animal Health**

(0.2 mg/ml, s.c.) to minimize secretions.

3 Expose the medulla and use the obex as the reference point for injection sites into the dorsal motor nucleus of the vagus nerve (dmnX). In order to obtain some isolated efferents, each animal should receive a single injection into left and right sides of the dmnX, varying the injection location within the dmnX across the population of rats studied. Using more injections per side (up to six) may result in heavy labeling with many interlocking neurites. A 7.5% solution of lysine fixable, 10000 MW dextran-biotin conjugate in ultrapure DI water or PBS.

Dextran-Biotin 10k, Lysine fixable **Thermo Fisher Scientific Catalog #D1956**

is pressure injected through a glass micropipette (ID 25 µm) with a Picospritzer III

Equipment

Picospritzer III Intracellular Microinjection Dispense System	NAME
Injection system	TYPE
Picospritzer	BRAND
052-0500-900	SKU
https://ph.parker.com/us/12051/en/picospritzer-iii-intracellular-microinjection-dispense-systems-picospritzer-micro-dispense-system/052-0500-900	LINK
100 psi, 2 channel	SPECIFICATIONS

into the dmnX at 40 psi. Two 4-6 msec applications of the conjugate are made into the same location and the pipette is left in each site for 2–3 min to allow the solution to disperse, and prevent excess leakage from the site of penetration.

4 Following removal of the glass pipette, close the muscle and skin incisions with interrupted sutures. Transfer the animal first to a circulating-water heating pad until its righting reflexes have returned and then to its home cage. Give

Buprenorphine (Buprenex) **Midwest Veterinary SUpply Catalog # 191.26890.3**

(0.01 mg/kg) s.c. prior to suturing as analgesia. Provide further analgesia the day following surgery:

Rimadyl (Carprofen) **Zoetis**

(5 mg/kg, s.c.).

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Tissue Fixation and GI dissection

5 Following dmnX injections, allow a time course of 19 days for the dextrans to transport to the GI tract. Rats are then weighed and euthanized with a lethal dose of a combination of

Ketamine **Patterson Veterinary Catalog #07-803-6637**

and

Xylazine **Akorn Animal Health Catalog #NDC: 59399-110-20**

(275 mg/kg ketamine and 27.5 mg/kg xylazine). The animals should have food available ad libitum until they are anesthetized, to facilitate the stomach being full and relaxed in accommodation.

Once unresponsive to paw pinch, open the abdomen and chest cavity and inject

Heparin **Henry Schein Animal Health Catalog #049130**

(0.5 ml; 1,000 units/ml) into the heart, followed by transcardial perfusion with 200 ml of 0.01 M sodium phosphate-buffered saline (PBS; pH 7.4; 38°C). Distend the stomach with approximately 10 to 15 ml of PBS to provide uniformity in organ size. Tissue fixation is then achieved by transcardiac perfusion of 500 ml of 4% paraformaldehyde (PF) in 0.1 M PBS (pH 7.4; 4°C).

6 After perfusion, transect the distal esophagus and the duodenum, and free and remove the stomach and portion of the duodenum. If collecting duodenum samples, separate the duodenum from the stomach by cutting at or slightly distal to the pylorus, as preferred. Open the stomach with a longitudinal cut along the greater curvature, and gently rinse away the material in the stomach with tap water. If collected, open the duodenum along the line of the mesenteric attachment and also rinse with tap water.

For analysis focused on the stomach:
To ensure that the entire stomach is preserved and sampled, trim the specimen to include the distal lower esophageal sphincter and the proximal pylorus. Including too much of either sphincter can make flattening the whole mount difficult. Separate the ventral and dorsal stomach walls by an incision along the lesser curvature, thus yielding two whole mounts per animal.

For analysis focused on the pylorus:
Ensure that the stomach includes the pylorus. Cut from a point about 2 cm from the pylorus on the ventral side across the lesser curvature at about 1.5cm from the pylorus and to a point on the dorsal side equivalent to the starting point to generate a whole mount including pylorus and partial antrum. If wanted, the remaining portion of the stomach can then be separated into separate ventral and dorsal sides as above.

For analysis focused on the LES and pylorus:
Separate the stomach from the duodenal bulb preserving the pylorus with the stomach. Start cutting at the greater curvature near the corpus-antrum border, and extend the cut rostrally with an arcing incision that roughly parallels the greater curvature before wrapping medially to the angle of His and then extend/replicate the same cut on the opposite wall of the stomach.

At this point the whole mount still contains the intact LES. Two methods of opening the LES to enable flattening of the whole mount are used. In one (see D below) make a slit in the ventral or dorsal wall spanning from the edge of the whole mount to the lumen of the esophagus. In the other (see C below), cut from the forestomach to the lumen of the esophagus.

This reference may be helpful:

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CITATION

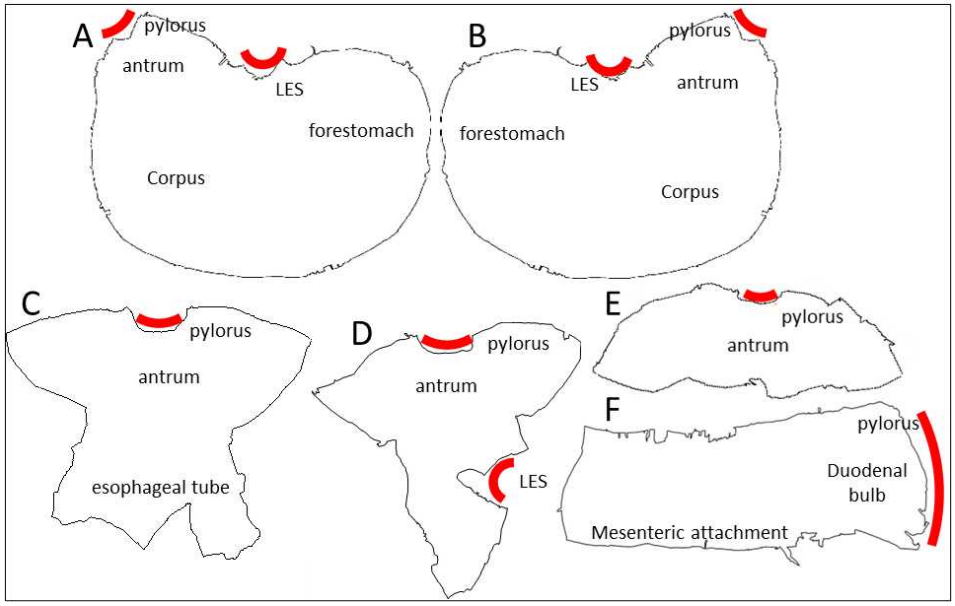
Powley TL, Baronowsky EA, Gilbert JM, Hudson CN, Martin FN, Mason JK, McAdams JL, Phillips RJ (2013). Vagal afferent innervation of the lower esophageal sphincter.. Autonomic neuroscience : basic & clinical.

LINK

https://doi.org/10.1016/j.autneu.2013.03.008

For analysis focused on the proximal duodenum:
Open the duodenum by cutting along the mesenteric attachment and wash gently with tap water and trim at the distal end to about 1.5 in.

The figure below illustrates some types of whole mounts used.



A and B: dorsal and ventral stomach whole mounts (internal view); C and D: antral/LES whole mounts; E: pylorus whole mount; F: duodenal whole mount.

7 Place the whole mounts in fix (4% paraformaldehyde in PBS) for at least 18 hours or up to about six months. After this time, peeling (see below) becomes difficult.

Isolate the external muscle wall of the stomach as a whole mount by removing the gastric mucosa and submucosa with forceps (referred to as peeling) while holding the wholemount under PBS. If counterstaining for nNOS+ is planned, then peeling should be completed within 24 hours of perfusion and processing immediately begun. For Dextran labeling alone and for other counterstaining discussed below, the delay time to processing is not critical. Following peeling, return the whole mounts to fix solution.

Staining

8 Whole mounts are processed free floating for all tracer processing, immunohistochemistry, and neuronal counterstaining.

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The following staining / counterstaining options are described below: (1) Dextran biotin alone; (2) Dextran biotin with Cuprolinic Blue; (3) Dextran biotin with nNOS; (4) Dextran biotin with NADPHd; (5) Dextran biotin with NADPHd and Cuprolinic Blue.

For all groups independent of planned counterstaining remove from fix solution, rinse for 3x5min in PBS and treat with a 3% hydrogen peroxide – methanol block (1:4) for 30 min to quench endogenous peroxidase activity.

9 **Dextran biotin labeling alone.**

Following methanol/peroxide block, rinse tissue 6x5min in PBS, and then soak 3–5 days in PBS containing 0.5% Triton X-100 and 0.08% Na azide to facilitate penetration of all reagents through the muscle sheets.

Rinse whole mounts 6x5min in PBS, and then incubate for 60 minutes in avidin– biotin–horseradish complex

Vectastain Elite ABC HRP kit **Vector Laboratories Catalog #PK-6100**

(bottle A and bottle B each diluted 1:50 in PBS, mixed 30 min before using).

After the avidin–biotin complex is established, rinse in PBS (6x5min) and then react with DAB

3,3-DIAMINOBENZIDINE.4HCl.xH2O Pure 98% * 5 g **Sigma-aldrich Catalog #32750-5G**

and H_2O_2 (0.7 mg/ml DAB, 5.6 ug/ml 3% H_2O_2 in Tris buffered saline) for 3 minutes to yield a permanent gold-brown stain of all labeled afferent neurites.

Complete process with step 14.

10 **Counterstaining with the panneuronal chromogen cuprolinic blue (quinolinic phthalocyanine) alone**

Prepare the following solutions:

A. Sodium acetate buffer: 11ml of acetic acid stock solution [6 ml glacial acetic acid in 500 ml ultrapure DI water], 89 ml sodium acetate stock solution [13.6g sodium acetate in 500 ml ulltrapure DI water], 300 ml ultrapure DI water
B. Magnesium chloride solution: 100 ml sodium acetate buffer, 20.3g magnesium chloride
C Cuprolinic Blue solution: 10 ml magnesium chloride solution, 50mg cuprolinic blue

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

Remove whole mounts from fix solution and rinse for 3x5min in PBS and treat with a 3% hydrogen peroxide – methanol block (1:4) for 30 min to quench endogenous peroxidase activity.

Rinse whole mounts 3x5min in ultrapure DI water, and then soak in the cuprolinic blue solution for 2 hours, placed on a slide warmer at 37°C. Following the soak, rinse samples in ultrapure DI water (4x2min), place in magnesium chloride solution for 2 min, rinse 3x2min in ultrapure DI water and 3x5min in PBS.

Following completion of the counterstaining, continue processing per step 8 for Dextran labeling with the 3-5 day soak in Triton X-100 buffer.

11 **nNOS Counterstaining**

In this group, processing for Dextran (step 8) must be initiated within 24 hours of perfusion.

After conclusion of the Dextran labeling above, wash the tissue 3x5min in cold ultrapure DI water, followed by 3x5min rinses in PBS, and then incubate overnight at room temperature in serum block (PBS, 2% Triton X-100, 0.08% Na Azide,

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Goat serum **Sigma Aldrich Catalog #G9023**
(5%), 2% BSA).

The following day, wash this tissue 3x5min in PBS, incubate for 15 min in an avidin blocking solution
Avidin/Biotin blocking kit **Vector Laboratories Catalog #SP-2001**
wash for 3x5min in PBS, and then incubate for 15min in a biotin blocking solution
Avidin/Biotin blocking kit **Vector Laboratories Catalog #SP-2001**
wash for 6x5 min in PBS, and then incubate for 48 hours in
nNOS polyclonal antibody **Thermo Fisher Scientific Catalog ## 61-7000**
(1:2000 diluted with buffer (2% normal goat serum, 2% BSA, 0.08% Na Azide, 0.3% Triton X-100 in PBS)) at room temperature. Flip whole mounts after 24 hours.

Wash tissue 6x5 min in PBST (0.3% Triton X-100, PBS), incubate for 2hr in
Biotin-SP (long spacer) AffiniPure Goat Anti-Rabbit IgG (H L) **Jackson Immunoresearch Catalog #111-065-144**
(1:500 diluted with buffer (2% normal goat serum, 2% BSA, 0.3% Triton X-100 in PBS), wash for 6x5 min in PBS, incubate for 60 min in
Vectastain Elite ABC HRP kit **Vector Laboratories Catalog #PK-6100**
(bottle A and bottle B each diluted 1:50 in PBS, mixed 30 min before using), wash for 6x5min in PBS, and stain for 5 min with steel gray chromogen
VECTOR® SG Peroxidase (HRP) Substrate Kit **Vector Laboratories Catalog #SK-4700**

Complete process with step 14.

12 NADPH-diaphorase Counterstaining

Following perfusion, post-fix whole mounts in 4% PF for 2-3 hours, and then peel.

Make a solution of Trizma Pre-set Crystals pH 7.6: 1.44g dissolved in 100ml ultrapure water and rinse the whole mounts in Trizma solution (5x5min).

Trizma Pre-set Crystals pH 7.6 **Sigma Aldrich Catalog #T7943**

Make NADPH solution: 0.1M Trizma; 1.0 mg/ml β-NADPH; 0.33 mg/ml NBT; 5ul/ml Triton X-100, mixing in this order: add β-NADPH, then NBT, then Trizma and while mixing add Triton X-100. When solution turns purple, it is ready to add the tissue.
β-NADPH **Sigma Aldrich Catalog #N1630**
Nitrotetrazolium Blue chloride (NBT) **Sigma Aldrich Catalog #N6876**
Triton X-100 **Sigma Aldrich Catalog #T9284**
Place whole mounts in the NADPH solution and soak at 37°C for 1hr. (Stomach and colon whole mounts require 3 ml solution each; intestinal whole mounts 1 ml each; colon sub-mucosal whole mounts 2.5ml each.)

Rinse with Trizma solution (5x5min).

Continue processing per step 9 for Dextran labeling.

13 NADPH-diaphorase and Cuprolinic Blue Counterstaining

Follow step 12 for NADPHd counterstaining EXCEPT: soak whole mounts in NADPH solution for 2 hours.

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Follow step 10 for Cuprolinic Blue counterstaining.

Then continue processing per step 9 for Dextran labeling.

14 Wash the tissue from all groups for 6x5 min in cold ultrapure H2O and mount circular muscle side up (serosal side up is recommended for antral-LES samples for best flattening) on gelatin coated slides.

The following process is used to flatten the tissue: cover with a non-gelatin coated slide, followed by a Saran wrap layer on which is placed a 4-6 lb weight and left overnight.

The following day remove the weight and covers , leave the samples to air dry overnight, and then dehydrate in an ascending series of alcohols and xylene (4 min in 70% EtOH, 95% EtOH, 2 x 100% EtOH, and 2x6 min xylene), and coverslip with

Cytoseal XYL **Fisher Scientific Catalog #22-050-262**

Neurite Tracing and Morphometry

15 **Tracing Directly from Tissue:**

To identify vagal afferents (IMA, IGLE) suitable for tracing [i.e. well-labeled, complete, sufficiently isolated from other neurites to enable unequivocal identification of the complete neurite, and with relatively few artifacts such as folds, tears, debris etc. obscuring the neurite; in the case of counterstained whole mounts the quality of the counterstaining is also taken into account] all whole mounts are scanned systematically under a Leica DMRE or DM5500 microscope.

Once identified, neurites are traced/digitized using a Neurolucida system

Software	
Neurolucida	NAME
MBF BioScience	DEVELOPER

(RRID:SCR_001775) controlling the motorized stage of a Zeiss (Oberkochen, Germany) Axio Imager Z2 microscope equipped with DIC optics and long-working-distance (×40 and ×63 oil) objectives. All branches of an arbor are digitized in three dimensions as the parent neurite repeatedly branches, arborizes, and finally terminates. At this morphometry step a percentage of the neurites will have to be dropped from analysis because comprehensive Neurolucida digitization encounters a flaw, artifact, discontinuity, or intermingled branch of a neighboring arbor not seen in the earlier scanning that made identification of the target arbor problematic. Where appropriate for more qualitative observations, the arbors dropped from the morphometry analyses are retained and used for analysis of neurite spatial distribution. Once tracing of an arbor is complete, morphometric analysis is performed using Neurolucida Explorer software.

16 **Tracing from Virtual Tissue Stacks**

As an alternative to tracing directly from tissue, it is also possible to generate image stacks (virtual tissue stacks) and trace neurites through the stacks using Neurolucida 360 software as above but via an offline Wacom tablet or similar. This has a number of advantages: (1) a second microscope can be used to generate the tissue images; (2) “permanent” virtual tissue is generated which makes it easier to share with colleagues

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and other analyses (such as characterization of interactions with neighboring cells) can be completed without returning to the original tissue; (3) reconstructing large, broken, or very tangled fibers can be easier with virtual tissue, since the operator can easily zoom in and out to assess completeness/continuity; by contrast, changing magnification on the live-tracing microscope requires changing lenses and immersion medium. There are disadvantages to this method, however: (1) tracing complete efferents sometimes requires many large tissue stacks, potentially up to ten images, each up to 4GB in size even after heavy compression to JPEG 2000 format; (2) iteration between tracing and virtual stack generation is needed as the full extent of the fiber is only revealed through tracing; (3) piecing together tracings from multiple stacks can be difficult and can introduce errors; (4) vertical discrimination is more difficult when tracing in a virtual stack because of the “steps” between image planes in the virtual stack.

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