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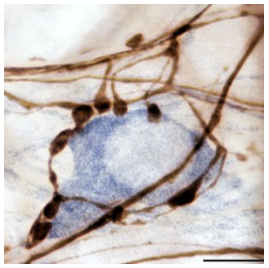
High resolution labeling of vagal efferent fibers using Dextran-Biotin with counterstaining V.1



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We use this protocol and it's working

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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. 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 were removed and processed as whole mounts. ABC-DAB was used to create a permanent gold-brown stain of all labeled efferent neurites. Subgroups of samples were also counterstained with either the panneuronal chromogen cuprolinic blue or with nNOS antibodies and steel gray chromogen to label nitrergic cells.

Materials

STEP MATERIALS

- Sprague-Dawley **Envigo**
- 2018 Teklad global 18% protein rodent diet **Envigo**
- Dextran-Biotin 10k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D1956
- Buprenorphine (Buprenex) **Midwest Veterinary SUpply** **Catalog #** 191.26890.3
- Rimadyl (Carprofen) **Zoetis**
- Ketamine **Patterson Veterinary** **Catalog #**07-803-6637
- Xylazine **Akorn Inc** **Catalog #**NDC: 59399-110-20
- Heparin **Henry Schein Animal Health** **Catalog #**049130
- Cytoseal XYL **Fisher Scientific** **Catalog #**22-050-262
- Vectastain Elite ABC HRP kit **Vector Laboratories** **Catalog #**PK-6100
- 3,3-DIAMINOBENZIDINE.4HCl.xH2O Pure 98% * 5 g **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**32750-5G
- Cuprolinic Blue (quinolinic phthalocyanine) **American Elements**
- Goat serum **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G9023
- Avidin/Biotin blocking kit **Vector Laboratories** **Catalog #**SP-2001
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- nNOS polyclonal antibody **Thermo Fisher Scientific** **Catalog ##** 61-7000
- Biotin-SP (long spacer) AffiniPure Goat Anti-Rabbit IgG (H L) **Jackson ImmunoResearch Laboratories, Inc.** **Catalog #**111-065-144
- Vectastain Elite ABC HRP kit **Vector Laboratories** **Catalog #**PK-6100
- VECTOR® SG Peroxidase (HRP) Substrate Kit **Vector Laboratories** **Catalog #**SK-4700
- Glycopyrrolate **Akorn Inc**
- Isoflurane **Akorn Inc** **Catalog #**NDC: 59399-106-01

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Troubleshooting

Animals

1 Two to four month old male

Sprague-Dawley **Envigo**

rats in the weight range of 180g to 360g at the time of tracer injection were 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 was maintained on a 12-hour light–dark schedule. Pelleted chow

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

and filtered tap water were available ad libitum, except for the night before tracer injection, when food but not water was removed. All procedures were conducted in compliance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 80-23, revised 1996), and were approved by the Purdue University Animal Care and Use Committee.

Neural tracer injections

2 Rats were anesthetized with isoflurane

Isoflurane **Akorn Inc** **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

Glycopyrrolate **Akorn Inc**

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

3 The medulla was exposed and the obex was used as the reference point for coordinates for injection sites into the dorsal motor nucleus of the vagus nerve (dmnX). Each animal received a single injection into left and right sides of the dmnX, but the injection location within the dmnX was varied across the population of rats studied. 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

was 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 were made into the same location and the pipette was 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 the muscle and skin incisions were closed with interrupted sutures. The animal was transferred first to a circulating-water heating pad until its righting reflexes had returned and then to its home cage.

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

(0.01 mg/kg) was given s.c. prior to suturing as analgesia. Further analgesia was provided the day following surgery:

Rimadyl (Carprofen) **Zoetis**

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

Tissue Fixation and GI dissection

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

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

and

Xylazine **Akorn Inc Catalog #**NDC: 59399-110-20

(275 mg/kg ketamine and 27.5 mg/kg xylazine). The animals had food available ad libitum until they were anesthetized, to facilitate the stomach being full and relaxed in accommodation. Once unresponsive to paw pinch, the abdomen and chest cavity were opened and

Heparin **Henry Schein Animal Health Catalog #**049130
(0.5 ml; 1,000 units/ml)was injected into the heart, followed by transcardial perfusion with 200 ml of 0.01 M sodium phosphate-buffered saline (PBS; pH 7.4; 38°C). The stomach was distended with approximately 10 to 15 ml of PBS to provide uniformity in organ size. Tissue fixation was then achieved by transcardiac perfusion of 500 ml of 4% paraformaldehyde (PF) in 0.1 M PBS (pH 7.4; 4°C).

6 After the perfusion, the distal esophagus and the proximal duodenum were transected, and the stomach was freed and removed. The organ was then opened with a longitudinal cut along the greater curvature, and the material in the stomach was gently rinsed away with tap water. To ensure that the entire stomach was preserved and sampled, the specimen was then trimmed to include the distal lower esophageal sphincter and the proximal pylorus. Next, the ventral and dorsal stomach walls were separated by an incision along the lesser curvature, thus yielding two whole mounts per animal. The external muscle wall of the stomach was then isolated as a whole mount by removing the gastric mucosa and submucosa with forceps.The whole mounts were then placed in fix (4% paraformaldehyde in PBS) for at least 18 hours or up to about six months. After this time, peeling (see below) became difficult.

The external muscle wall of the stomach was then isolated as a whole mount by removing the gastric mucosa and submucosa with forceps (referred to as peeling). If counterstaining for nNOS+ was planned, then peeling was completed within 24 hours of perfusion and processing immediately begun. For Dextran labeling alone and for cuprolinic blue counterstaining, the delay time to processing was not critical.

Staining

7 Whole mounts were processed free floating for all tracer processing, immunohistochemistry, and neuronal counterstaining.

All groups independent of planned counterstaining were removed from fix solution and rinsed for 3×5min in PBS and treated with a 3% hydrogen peroxide – methanol block (1:4) for 30 min to quench endogenous peroxidase activity.

One group was processed for Dextran labeling alone. Following methanol/peroxide block, tissue was rinsed for 6×5min in PBS, and then soaked 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. Whole mounts were then rinsed 6×5min in PBS, and then incubated 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 was established, the specimens were rinsed in PBS (6×5min) and then reacted with DAB

3,3-DIAMINOBENZIDINE.4HCl.xH2O Pure 98% * 5 g **Merck MilliporeSigma (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.

A second group was processed for counterstaining with the panneuronal chromogen cuprolinic blue (quinolinic phthalocyanine). The following solutions were prepared:

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**

In this group, following the methanol-hydogen peroxide soak and PBS rinse, whole mounts were rinsed 3×5min in ultrapure DI water, and then soaked in the cuprolinic blue solution for 2 hours, placed on a slide warmer at 37°C. Following the soak, samples were rinsed with magnesium chloride solution for 2 min, rinsed 3×2min in ultrapure DI water and 3×5min in PBS. Following completion of the counterstaining, processing continued as above for Dextran labeling with the 3-5 day blocking buffer step.

A third group was processed for counterstaining of nNOS+ cells. In this group, processing for Dextran was initiated within 24 hours of perfusion. After conclusion of the Dextran labeling above, the tissue was washed for 3×5min in cold ultrapure DI water, followed by 3×5min rinses in PBS, and then incubated overnight at room temperature in serum block (PBS, 2% Triton X-100, 0.08% Na Azide,

Goat serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G9023

(5%), 2% BSA). The following day, this tissue was washed for 3×5min in PBS, incubated for 15 min in an avidin blocking solution

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

then washed for 3×5min in PBS, and then incubated for 15min in a biotin blocking solution

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

washed for 6×5 min in PBS, and then incubated 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. Whole mounts were flipped after 24 hours. Then, the tissue was washed for 6×5 min in PBST (0.3% Triton X-100, PBS), incubated for 2hr in

Biotin-SP (long spacer) AffiniPure Goat Anti-Rabbit IgG (H L) **Jackson ImmunoResearch Laboratories, Inc. Catalog #**111-065-144

(1:500 diluted with buffer (2% normal goat serum, 2% BSA, 0.3% Triton X-100 in PBS), washed for 6×5 min in PBS, incubated 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), washed for 6×5min in PBS, and stained for 5 min with steel gray chromogen

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

8 Finally, the tissue from all groups was washed for 6×5 min in cold ultrapure H_2O and mounted circular muscle side up on gelatin coated slides. The following process was used to flatten the tissue: it was covered with a non-gelatin coated slide, followed by a Saran wrap layer on which was placed a 4-6 lb weight and left overnight. The following day the weight and covers were removed, the samplesleft to air dry overnight, and then dehydrated in an ascending series of alcohols and xylene (4 min in 70% EtOH, 95% EtOH, 2 × 100% EtOH, and 2×6 min xylene), and coverslipped with

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

Neurite Tracing and Morphometry

9 All whole mounts were scanned systematically under a Leica DMRE or DM5500 microscope 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 was also taken into account.

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 was used to trace neurites. All branches of an arbor were digitized in three dimensions as the parent neurite repeatedly branched, arborized, and

finally terminated. At this morphometry step, however, a percentage of the neurites had to be dropped from analysis because comprehensive Neurolucida digitization encountered 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 were retained and used for analysis of neurite distribution. Before morphometric analysis the parent branch of the neurite was removed so analysis information would reflect the branch length distribution and area of innervation specific to the arbor itself.