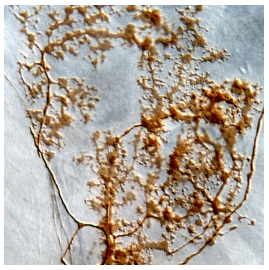
Dec 19, 2019

High resolution labeling of vagal afferent fibers using Dextran-Biotin with counterstaining



DOI
dx.doi.org/10.17504/protocols.io.2ipgcdn

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.2ipgcdn

Protocol Citation: Terry Powley, Jennifer Mcadams, Robert Phillips 2019. High resolution labeling of vagal afferent fibers using Dextran-Biotin with counterstaining. **protocols.io https://dx.doi.org/10.17504/protocols.io.2ipgcdn**

Manuscript citation:
Powley TL, Hudson CN, McAdams JL, Baronowsky EA, Phillips RJ. Vagal Intramuscular Arrays: The Specialized Mechanoreceptor Arbors That Innervate the Smooth Muscle Layers of the Stomach Examined in the Rat. J Comp Neurol. 2016 Mar 1;524(4):713-37. doi: 10.1002/cne.23892. Epub 2015 Oct 13.

Protocol status: Working
We use this protocol and it's working

Created: May 01, 2019

Last Modified: December 19, 2019

Protocol Integer ID: 22831

Keywords: Afferent, vagal, tracing, IGLE, IMA, ABC-DAB, cuprolinic blue, nNOS, nodose, rat

Abstract

This protocol describes the methods used to trace and enable morphometric quantification of vagal afferent neurites in the rat stomach. A mixture of dextran conjugates was injected into the nodose ganglia of young adult Sprague-Dawley rats and after a survival period of 14 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 afferent 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 3k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D7135
- Dextran-Biotin 10k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D1956
- 35-gauge NANOFIL needle **World Precision Instruments** **Catalog #**NF35BV-2
- 10 microliter Nanofil syringe **World Precision Instruments** **Catalog #**Nanofil
- Fast Green FCF **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**F7252
- Buprenorphine (Buprenex) **Midwest Veterinary SUpply** **Catalog #** 191.26890.3
- 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
- Isoflurane **Akorn Inc** **Catalog #**NDC: 59399-106-01
- Glycopyrrolate **Akorn Inc**

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- Vectastain Elite ABC HRP kit **Vector Laboratories** **Catalog #**PK-6100
- Cytoseal XYL **Fisher Scientific** **Catalog #**22-050-262

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 provided ad libitum, except for the night before tracer injection, when food but not water was removed. All husbandry practices conformed to the NIH *Guide for the Care and Use of Laboratory Animals* (8th edition) and were reviewed and approved by the Purdue University Animal Care and Use Committee. All efforts were made to minimize any suffering as well as the number of animals used.

Neural tracer injections

2 Overnight-fasted animals were anesthetized with
Isoflurane **Akorn Inc** **Catalog #**NDC: 59399-106-01
starting at an initial concentration of 5%, dropping to 2% or less as required to maintain anesthesia. After anesthesia,
Glycopyrrolate **Akorn Inc**
(0.2 mg/ml, s.c.) was injected to minimize secretions.

3 For dextran injections, each anesthetized animal was placed in a supine position, and a midline incision of the skin of the ventral neck was made. Both nodose ganglia were then exposed by blunt dissection of the overlying muscles. Each animal received bilateral injections (1 µl per ganglion) of lysine-fixable dextran biotin solution consisting of a 1:1 mixture of 3K and 10K MW dextrans in ultrapure DI water or PBS - final concentration 15% dextran biotin consisting of 7.5% each of
Dextran-Biotin 3k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D7135
and
Dextran-Biotin 10k, Lysine fixable **Thermo Fisher Scientific** **Catalog #**D1956
Injections were performed with a
35-gauge NANOFIL needle **World Precision Instruments** **Catalog #**NF35BV-2
and
10 microliter Nanofil syringe **World Precision Instruments** **Catalog #**Nanofil

To control the injection placement and to check the distribution of the tracer within the ganglia under direct visual control,

Fast Green FCF **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F7252

was added to the dextran tracer (0.01 mg per 100 µl solution).

4 After both ganglia had been injected, 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 and again the following morning for analgesia.

Tissue Fixation and GI Dissection

5 After a survival period of 14 days for optimal tracer transport, each rat was given a lethal dose (180 mg/kg^{-1}, i.p.) of sodium pentobarbital or 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. When each animal was completely unresponsive to nociceptive pinching and prodding, the GI tract was exposed with a midline abdominal incision. To ensure that the stomach was normally distended at the time of fixation, the organ was inspected for normal distension or accommodation, and, as required, physiological saline that had been warmed to body temperature was slowly infused into the stomach by gavage catheter. With the stomach normally dilated

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

(0.5ml, 1000 units/ml) was then injected into the heart and the animal was transcardially perfused through the vasculature, first with physiological saline and then with 4% paraformaldehyde in 0.1 mol $liter^{-1}$ phosphate-buffered saline (PBS; pH 7.4).

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 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 H2O 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. Once tracing of an arbor was complete, morphometric analysis was performed using Neurolucida software.