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

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SPARC
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This protocol describes the methods used to trace and enable morphometric quantification of vagal afferent neurites in mucosal layers of the rat GI system. 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 and intestines were removed and selected pieces embedded in gelatin, sectioned and processed. ABC-DAB was used to create a permanent gold-brown stain of all labeled afferent neurites. Some samples were also counterstained for ghrelin and gastrin.

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Mucosal afferent, vagal, tracing, ABC-DAB, gastrin, ghrelin, nodose, rat

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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 Animal**

Health 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 Animal Health**

(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 **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. For the evening meal following surgery the animals are given a bowl of "chow mash", chow mixed with water to form a porridge. The next day they are returned to solid food.

Perfusion, GI Dissection and Fixation

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 Animal Health 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.

☒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). Immediately after the beginning of the perfusion, the GI tract was flushed with 4% PF solution to preserve the integrity of the mucosa.

6 After the perfusion, the distal esophagus and the proximal colon were transected, and the entire GI tract was freed and removed.

4mm blocks of tissue from desired areas of the GI tract were cut out with scissors and placed in 4%

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paraformaldehyde solution overnight.

Gelatin Embedding

7 Prepare 750 ml each of 5%, 10% and 15% gelatin by weight solutions in ultrapure water, as follows:

Add 0.75mg of thymol to 750 ml of ultrapure water and stir on a hot plate at 45°C until dissolved (about 30 min)
☒ Thymol **Fisher**
Scientific Catalog #AC150331000
Remove from the heat and add the gelatin and stir until dissolved.
☒ Gelatin Type 8 (Bloom strength 100, narrow range) **Great Lakes Gelatin**
Then place the container of gelatin in a water bath at 45°C for one hour with agitation of the bath and occasional stirring of the gelatin.

8 Place tissue into tissue baskets and into the tissue processor in an initial container of 4% PF, and run a program overnight with the following parameters.
Ultrapure Water @ 25°C - 2hrs
Ultrapure Water @ 25°C - 4hrs
5% Gelatin @ 45°C - 2hrs
10% Gelatin @ 45°C - 2hrs
15% Gelatin @ 45°C - 2hrs

Leica TP1020 Semi-enclosed Benchtop Tissue Processor
Leica Biosystems **TP1020**

Time the program so that it ends at an appropriate time the following morning.

9 Next morning make another 750 ml of 15% gelatin with 0.1% thymol as above. Fill peel-away molds half-way with 15% gelatin 30min before the end of the tissue processing and allow to harden in the refrigerator for 30 min.

Peel Away Disposable Embedding Molds
Electron Microscopy Sciences **70182**

10 Fill the molds with more 15% gelatin and position the tissue samples with the desired orientation for sectioning, and allow to harden in the refrigerator for 30min. Carefully cut away mold and place hardened gelatin block in 4% paraformaldehyde solution overnight.

11 Move gelatin blocks into a water solution 24 hours after placing in 4% paraformaldehyde. Section tissue blocks on a vibratome with a section thickness of 100µm. Place sections into PBS.

Leica VT1200 S Fully automated vibrating blade microtome
Leica Biosystems **VT1200S**

DAB labeling

12 Rinse for 3x10min in PBS, followed by a 3% hydrogen peroxide – methanol block (1:4) for 30 min to quench endogenous peroxidase activity.

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Then rinse again for 3x10min in PBS, and then soak for 3–5 days in PBS containing 0.5% Triton X-100 and 0.08% Na azide at 4°C to facilitate penetration of reagents.

13 Rinse 3x10min in 0.3% PBST, and the incubate for 48 hours 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 0.5% PBST, mixed 30 min before using), at 4°C

14 Rinse in PBS (3x10min) and then react with DAB
☒3,3-DIAMINOBENZIDINE.4HCl.xH2O Pure 98% * 5 g **Sigma-aldrich Catalog #32750-5G**
(0.7 mg/ml DAB, 5.6 ug/ml 3% H_2O_2 in Tris buffered saline) for 90 minutes at 4°C. Then rinse for 3x10min in cold dH_2O.

Gastrin Counterstaining

15 If no counterstaining is wanted - proceed directly to the slide mounting section. For gastrin counterstaining use this section and then proceed to slide mounting; for ghrelin counterstaining use the next section and then proceed to slide mounting.

Rinse 3x10min in PBS, then soak for 48 hours in 2% PBST, 0.08% Na Azide, 5% NDS, 2% BSA at 4°C.
☒Normal Donkey Serum **Sigma Aldrich Catalog #D9663-10ml**

☒Bovine Serum Albumin **Jackson Immunoresearch Catalog #001-000-173**

16 Rinse 3x10min in PBS, followed by 2 hours in Avidin blocking solution
☒Avidin/Biotin blocking kit **Vector Laboratories Catalog #SP-2001**
Rinse 3x10min in PBS followed by 2 hours in Biotin blocking solution
☒Avidin/Biotin blocking kit **Vector Laboratories Catalog #SP-2001**
Rinse 3x10min in PBS, followed by 3 days in primary at 4°C (Gastrin antibody diluted 1:7,500 in 0.5% PBST, 0.08% Na Azide, 5% NDS, 2% BSA)
☒Gastrin (GAST) Guinea Pig Polyclonal Antibody **OriGene Catalog #BP5046**

17 Rinse 3x10min in 0.3% PBST, followed by 48 hours in secondary at 4°C (Biotin Anti-Guinea Pig diluted 1:500 in 0.5% PBST, 0.08% Na Azide, 5% NDS, 2% BSA)
☒Biotin Anti-Guinea Pig IgG (H L) made in donkey **Jackson Immunoresearch Catalog #706-065-148**

18 Rinse 3x10min in 0.3% PBST, followed by 48 hrs @ 4°C in
☒Vectastain Elite ABC HRP kit **Vector Laboratories Catalog #PK-6100**
(bottle A and bottle B each diluted 1:50 in 0.5% PBST, mixed 30 min before using)

19 Rinse 3x10min in PBS followed by 20 min in
☒VECTOR® SG Peroxidase (HRP) Substrate Kit **Vector Laboratories Catalog #SK-4700**
Rinse 3x10min in cold dH_2O

Ghrelin Counterstaining

20 Rinse 3x10min in PBS, then soak for 48 hours in 2% PBST, 0.08% Na Azide, 5% NGS, 2% BSA at 4°C.

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Goat serum **Sigma Aldrich Catalog #G9023**

Bovine Serum Albumin **Jackson Immunoresearch Catalog #001-000-173**

21 Rinse 3x10min in PBS, followed by 2 hours in Avidin blocking solution

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

Rinse 3x10min in PBS followed by 2 hours in Biotin blocking solution

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

Rinse 3x10min in PBS, followed by 3 days in primary at 4°C (Ghrelin antibody diluted 1:800 in 0.5% PBST, 0.08% Na Azide, 5% NGS, 2% BSA)

Ghrelin (Rat, Mouse) - Antibody **Phoenix Pharmaceuticals Catalog #H-031-31**

22 Rinse 3x10min in 0.3% PBST, followed by 48 hours in secondary at 4°C (Biotin Anti-Rabbit diluted 1:500 in 0.5% PBST, 0.08% Na Azide, 5% NGS, 2% BSA)

Biotin-SP (long spacer) AffiniPure Goat Anti-Rabbit IgG (H L) (min X Hu, Ms, Rat Sr Prot) **Jackson Immunoresearch Catalog #111-065-144**

23 Rinse 3x10min in 0.3% PBST, followed by 48 hours at 4°C in

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

(bottle A and bottle B each diluted 1:50 in 0.5% PBST, mixed 30 min before using)

24 Rinse 3x10min in PBS followed by 20 min in

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

Rinse 3x10min in cold dH_2O

Slide mounting

25 Transfer each section to a gelatin coated glass slide, 3-4 sections per slide, maintaining a consistent orientation and section order and label slide with pencil, and allow the slides to dry overnight. Then soak in 4% PF for 1 hour, rinse 3 times with dH_2O, and allow the slides to dry overnight. Finally dehydrate 4x4min in ethanol followed by 2x6min xylene, and coverslip with

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

Neurite Tracing and Morphometry

26 Scan all sections systematically under a Leica DMRE or DM5500 microscope to identify afferents associated with mucosal layers, villi or crypts/glands and suitable for tracing.

Suitable afferent sections can then be traced using Neurolucida 360 software

Neurolucida
by MBF BioScience

(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. Once tracing of an arbor is complete, morphometric analysis can be performed using the Neurolucida Explorer software.

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