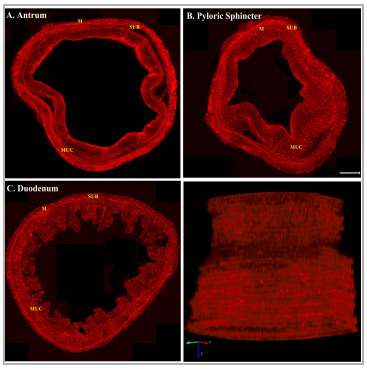

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We use this protocol and it's working

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Topographical distribution and morphology of SP-IR axons in the antrum, pylorus, and duodenum of mice

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ABSTRACT

This protocol describes the process of identifying the distribution and morphology of substance P (SP) immuno-reactive axons and terminals in the antrum, pylorus, and duodenum of mice. The antrum-pylorus-duodenum (APD) region was sectioned and immunohistochemically labeled for SP. To determine the distribution and morphology of SP-IR axons and calculate their density in different layers of APD, a Zeiss M2 Imager was used to scan each serial section. Each section was presented as a montage of approximately 50 all-in-focus maximum projection images. To determine the detailed structures of SP-IR axons and terminals, the confocal microscope was used to scan the regions of interest.

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Keywords: substance P, nociceptive afferent, myenteric plexus, longitudinal and circular muscles, submucosa, mucosa, sections, confocal microscope

Animals

1 12 to 13-week old male Mouse cohorts for feeding **The Jackson Laboratory Catalog #C57BL/6J** were used. The animals were housed in a room in which light/dark cycles were set to 12 h/12 h (6:00 AM to 6:00 PM light cycle) and supplied with food and water ad libitum. Animals were fasted overnight, and food was provided 2h before perfusion. A catheter was introduced during perfusion to deliver saline to ensure uniform extension of the stomach across all animals. All procedures were approved by the University of Central Florida Animal Care and Use Committee and strictly followed the guidelines established by NIH. All experiments conformed to the University of Central Florida guidelines on the ethical use of animals.

Tissue preparation

2 Animals were deeply anesthetized with 5% Isoflurane **Covetrus Catalog #029404** and the absence of the hind paw pinch withdrawal reflex was used as an indicator of sufficient anesthesia.

A cut was made in the inferior vena cava to drain the blood and perfusate.

The mice were perfused through the left ventricle with 38◦C phosphate-buffered saline (0.1 M PBS, pH = 7.4) for 5 min, then fixed by perfusion with 4% paraformaldehyde at 4◦C (PFA 4 % in PBS, pH = 7.4).

The whole stomach with the proximal portion of the duodenum was removed. The APD was cut and separated from the whole stomach, then the contents within the APD were removed, and rinsed with distilled water, followed by a 24 h post-fixation in the same fixative.

After post-fixation, the APD was washed 3 times in PBS (10 min each) and then cryoprotected in graded sucrose (10%, 20%, and 30%, sucrose in PBS) at 4 ◦C until the samples sank in the sucrose (10% and 20%) and overnight for 30%.

The samples were then dried and embedded in

Tissue-Tek® O.C.T. Compound, Sakura® Finetek **VWR International Catalog #25608-93(**
and frozen using dry ice and then stored at - 20 ◦C for the next day's cryosection.

Using

Equipment	
CM1900	NAME
Cryostat	TYPE
Leica	BRAND
Cryostat	SKU
https://www.leicabiosystems.com/us/histology-equipment/cryostats/leica-cm1900/	LINK

, the sections (100 µm) were cut and mounted on
SuperFrost Plus Glass Slides **Electron Microscopy Sciences Catalog #71869-10** .
Each APD sample was sectioned transversely or longitudinally, ensuring that the sections were complete (complete uninterrupted whole circular shape) and continuous (each sample was sectioned into 28–30 consecutive sections). Slides were air-dried under the fume hood for 2h and then processed for SP-labeling.

Immunohistochemistry

3 APD area from 6 animals were used for SP single labeling, and another 2 were used for SP double labeling with vesicular acetylcholine transporter (VAChT) or tyrosine hydroxylase (TH). All animals were injected (intraperitoneally) with Fluorogold **Fluorochrome** (0.3 ml of 3 mg/ml per animal) to counterstain neurons in the myenteric and submucosal ganglia. All IHC steps were performed on slides to preserve the original anatomical outline and avoid shape distortion which may result during tissue handling. All procedures were performed in a wet chamber and at room temperature (except for VAChT which was processed at 4 ◦C).

3.1 The sections were washed 3 times in PBS (10 min each) and blocked for 24h in a blocking mixture containing 2% bovine serum albumin, 10% normal donkey serum, 2% Triton X-100, and 0.08% sodium azide in 0.1 M PBS, pH = 7.4. The tissue samples were incubated for 2 days with a mixture of primary antibodies diluted in a solution comprising of 2% bovine serum albumin, 4% normal goat and donkey serum, 0.5% Triton X-100, and 0.08% sodium azide in 0.01 M PBS,

pH = 7.4).

The primary antibodies that were used:
Polyclonal
Rabbit anti-substance P antibody; AB_572266 **Immunostar Catalog #20064** was used for single labeling (specificity was determined by the vendor using preadsorption).
Either polyclonal
Goat anti-Vesicular Acetylcholine Transporter (VAChT) antibody **Merck MilliporeSigma (Sigma-Aldrich) Catalog #ABN100**
, polyclonal
Sheep anti-tyrosine hydroxylase antibody **Merck Millipore (EMD Millipore) Catalog #AB1542**
, or monoclonal Monoclonal Rabbit anti-PGP 9.5 **Abcam Catalog #ab108986** were used for double labeling.

The sections were carefully washed 3 times (10 min each) in PBS-T (0.5 % Triton X-100 in 0.01 M PBS) to remove unbound primary antibodies and then incubated for 24 h with secondary antibodies in PBS-T.

The secondary antibodies that were used:
Alexa Fluor 594 donkey anti rabbit **Thermo Fisher Scientific Catalog #A-21207**,
Donkey anti-Sheep IgG (H L) Cross-Adsorbed Secondary Antibody Alexa Fluor 488 **Therm Fisher Scientific Catalog #A-11015**
Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 **Thermo Fisher Scientific Catalog #A11055**
RRID: AB_2534102),
Donkey Anti-rabbit IgG Affinipure Fab Fragment Alexa Fluor 488 **Jackson ImmunoResearch Laboratories, Inc. Catalog #711-547-003**
.
Unbound secondary antibodies were removed from tissues by 6 × 5 min washes in PBS.

3.2 For double-labeled tissues with SP and PGP 9.5, the antibodies were administered in the following sequence: blocking, SP primary antibody, PBS-T wash, Fab fragment secondary antibody, PBS wash, blocking, PGP 9.5 primary antibody, PBS-T wash, secondary antibody, and a final wash with PBS.

The use of fragmented antibodies instead of fully intact secondary antibody was to reduce cross-reactivity (Gramer et al., 2013; Kholodenko et al., 2019; Yanakieva et al., 2022). Sections were coverslipped with
VECTASHIELD® Vibrance™ Antifade Mounting Medium H-1700 **Vector Laboratories Catalog #H-1700-10**
. The slides were air-dried overnight in the fume hood.

Negative controls were performed by omitting primary or secondary antibodies. Positive controls were performed by double labeling the tissue with SP and PGP 9.5. The listing of antibodies used in this study is summarized in the table below.

	A	B	C	D	E
	Antibody	Host	Concentration	Excitation wavelength	Company
	Anti-SP (1)	Rabbit	1:400	n/a	Immunostar
	Anti-VAChT (1)	Goat	1:400	n/a	Millipore Sigma
	Anti-TH (1)	Sheep	1:400	n/a	Millipore Sigma
	Anti-PGP 9.5 (1)	Rabbit	1:400	n/a	Abcam
	Alexa Fluor Anti-rabbit (2)	Donkey	1:200	594	Thermo Fisher Scientific
	Alexa Fluor Anti-goat (2)	Donkey	1:200	488	Thermo Fisher Scientific
	Alexa Fluor Anti-sheep (2)	Donkey	1:200	488	Thermo Fisher Scientific
	Alexa Fluor 488-AffiniPure Fab Fragment	Donkey	1:200	488	Jackson ImmunoResearch

(1) primary antibody, (2) secondary antibody

Data acquisition and analysis

4 Image stacks of the serial sections were collected using

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

(20× objective lens, z-step: 1.5 µm, 594 nm filter) lens. Maximum projection images were acquired from the image stacks and organized into a montage to generate a complete image of each section.

To show the details of the SP, TH, and VAChT axon and varicosity distributions and projections to different targets in various layers and regions of the APD,

Equipment

TCS SP5	NAME
Laser scanning confocal microscope	TYPE
Leica	BRAND
Leica	SKU
https://www.leica-microsystems.com/products/confocal-microscopes/p/leica-tcs-sp5/	LINK

was used to acquire optical section images at high magnification (40× oil immersion objective lens, zoom X 1.5, Z-step: 1 µm).

Maximum projection images were used to show the overall innervation across the whole tissue thickness, while single optical sections revealed the details at a single cell/axon/varicosity level.

The helium-neon (HeNe) laser (excitation 543 nm) was used to detect SP-IR axons, the argon–krypton (ArKr) laser (excitation 488 nm) was used to detect TH, and VAChT, and the background autofluorescence of the tissues (e.g. smooth muscles, blood vessels, mucosal glands), and the 405 nm laser (excitation) was used for the detection and verification of FG-labeled myenteric and submucosal ganglionic neurons.

Software	
ImageJ/Fiji	NAME
Windows 7	OS
National Institutes of Health	DEVELOPER
http://wsr.imagej.net/distros/win/ij152-win-java8.zip	SOURCE LINK

(https://imagej.net/, RRID: SCR_003070) and

Software	
Adobe Photoshop	NAME
Adobe	DEVELOPER

(https://www.adobe.com/products/photoshop.html, RRID: SCR_014199) were used for the image processing and enhancement including brightness, contrast adjustments, and scale bar addition. All serial sections in each APD were well preserved without damage during sectioning, processing, and mounting.

Density analysis

5 The density of the SP-IR axons was the amount of nerve signals in a defined area of tissue.

The density analysis of SP-IR axons in the sections started by importing the montages acquired with the Zeiss M2 imager into Photoshop where the muscular layer was separated from the submucosa and mucosa.

Each subsection was exported as a .png file into

Software	
Icy	NAME
Windows/MacOS/Linux	OS
BioImage Analysis Lab (Institut Pasteur)	DEVELOPER
http://icy.bioimageanalysis.org	SOURCE LINK

(RRID: SCR_010587) where two-pixel densities (all signals and axon signals) were calculated and exported into an Excel file.

The total surface area (all signals) was calculated by applying a low threshold to keep all the signals produced by muscle, ganglia, axons, or any background from the tissue. The axon signals were calculated by applying a high threshold to exclude all signals coming from the tissue and keep only the signals that were produced by SP-IR axons. The plugin Skeletonize was applied on the binarized image to ensure that axons are expressed by a centerline and the thickness of the diameter will not affect the density calculations.

To calculate the axon density in each region in Excel, the number of axon signal pixels was divided by the number of surface area pixels. Data are expressed as means of all samples' densities in percentages ± standard error of the mean (SEM).

Percentage data were normalized and passed the Shapiro-Wilk test before statistical analysis. One-way analysis of variance followed by all-pairwise multiple comparisons (Tukey's HSD) was used for statistical comparisons. The statistical analyses were performed using

Software	
Prism	NAME
GraphPad	DEVELOPER

(version 8; GraphPad Software, Inc., RRID:SCR_002798). $P < 0.05$ was considered statistically significant.