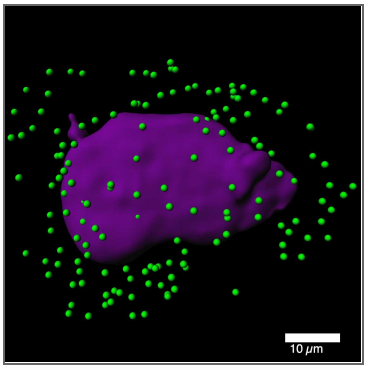

Targets of sympathetic nerves in myenteric plexus of human colon

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ABSTRACT

This protocol outlines basic methods to measure distributions of axonal varicosities (comparing TH and ENK-immunoreactive varicosities) relative to the somatic border of human colonic myenteric nerve cell bodies which have been characterized for NOS and ChAT immunoreactivities. It uses an antibody elution method.

MATERIALS

- Specimen of live human colon (or other gut) tissue
- Krebs solution for preparation containing in (mM): (NaCl; 118mM, KCl; 4.8mM, $CaCl_2$; 2.5mM, $MgSO_4$; 1.2mM, $NaHCO_3$; 25mM, NaH_2PO_4; 1.0mM, glucose; 11mM, bubbled with 95% O_2, 5% CO_2, pH; 7.4)
- Sylgard 184 Elastomer (Dow Corning, Midland MI)
- 4% paraformaldehyde; (Sigma-Aldrich)
- phosphate-buffered saline (PBS)
  pH 7.4 (137mM NaCl, 10mM phosphate buffer pH7.4)
- Triton X-100; (Sigma-Aldrich)
- Glycerol; (Sigma-Aldrich)
- Epifluorescence microscope;
  Olympus IX71 or equivalent with appropriate filters and camera
- Laser-scanning confocal
  microscope (Zeiss LSM880 or equivalent) with 4 channel detection
- Primary and secondary antisera

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

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Keywords: colon, myenteric, sympathetic, cholinergic, enteric nervous system

SAFETY WARNINGS

Safety warnings relating to use of human tissue are included in the protocol.

1 All handling of un-fixed human tissue is exclusively done by staff trained in occupational health and safety requirements for handling hazardous material, wearing appropriate PPE (gloves, gowns and masks) and working in areas designated for human tissue. Users of this protocol should check local requirements with their Institutional Biosafety Committee.

2 Prior written informed consent is obtained from patients by surgical staff not involved in the project, prior to surgery - a patient information form is supplied and the patient's signature is witnessed.

3 Specimens of live human colonic tissue are cut from un-involved ends of excised specimens in the operating theatres, under the supervision of surgical staff (to avoid interference with needs of pathologists). They are placed into room-temperature carbogenated Krebs solution

4 The container is sealed then placed in a second sealed, watertight container and transported back to the laboratory

5 The specimen is then anonymised by replacing patient name with a code number. 6 pieces of data are recorded for each de-identified specimen: patient sex, age, region of bowel, reason for

operation, date of surgery and surgeon's name. All other patient data is then deleted

6 In the laboratory, in a Microbiological Safety Cabinet, preparations are rinsed repeatedly in fresh oxygenated Krebs solution to flush away contents

7 Preparations are then pinned out mucosal side uppermost in a petri dish lined with 3mm depth of Sylgard 184 Elastomer (Dow Corning, Midland MI) using headless stainless steel insect pins (Australian Entomological Supplies, E184). Preparations are immersed in fresh carbogenated Krebs solution which is replaced at 10 minute intervals.

8 The mucosa and submucosa are removed by sharp dissection and discarded. The preparation is then turned over and re-pinned to allow the serosa to be cleared of fat, blood vessels and adhering tissue.

9 The preparation is re-pinned in a clean dish and covered in Phosphate-buffered saline (PBS pH7.4) under maximal longitudinal and circular tension. PBS is poured off and replaced with cold 4% paraformaldehyde (4% formaldehyde in 0.1M phosphate buffer, pH 7.2) and the dish is covered and placed in a refrigerator overnight

10 The next day, the preparation is unpinned and placed in fresh 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2 in a sealed container and placed on an orbital mixer at room temperature for 24 hours, to ensure complete penetration by the fixative.

11 All tools, containers and surfaces that have been exposed to unfixed human tissue or contaminating solutions are immersed in 0.1% bleach solution for at least 10 minutes prior to normal cleaning and washing, followed, where possible, by wiping with 70% ethanol to remove any bleach residues.

12 The specimen is then repeatedly rinsed in PBS for at least 3 x 30 minutes to remove paraformaldehyde

13 The tissue is pinned in PBS and the circular muscle is removed by sharp dissection, exposing the myenteric plexus.

14 A sample of tissue with little adhering circular muscle, approximately 10mm x 15mm is then isolated.

15 The tissue is then transferred to a sealed container with 0.5% Triton X100 dissolved in PBS to permeabilise the tissue for 24 hours on an orbital mixer at room temperature

16 It is then repeatedly rinsed in PBS for at least 3 x 10 minutes

17 Tissue is then immersed in HuC/D, Tyrosine Hydroxylase (TH) and leu-Enkephalin (ENK) primary antibodies diluted in hypertonic PBS with 3M NaCl, for 24 - 72 hours, then repeatedly rinsed in PBS for at least 3 x 10 minutes

18 Tissue is immersed in secondary antibodies. A biotinylated secondary for HuCD is followed by streptavidin-AMCA. An AF488-coupled secondary antiserum is used for TH and a CY5-coupled antiserum is used for ENK. All antisera are diluted in hypertonic PBS for 12-24 hours, then repeatedly rinsed in PBS for at least 3 x 10 minutes

19 Tissue is soaked in carbonate/bicarbonate buffered glycerol (pH8.6) and mounted on a slide in the same solution, coverslipped and viewed and photographed on an epifluorescence microscope (Olympus IX71) with appropriate filter sets

20 For confocal microscopy, a Zeiss LSM880 was used to collect stacks of images in 1µm steps with a 20x objective

21 After viewing and photography, the TH and ENK labelling was eluted by treating preparations in 50 mls of 2-mercaptoethanol/SDS buffer (2-ME/SDS) in a pre-heated waterbath (at 56˚C) with agitation at 60 rpm for one hour. The tissue was then washed in PBS-Tween (Sigma Aldrich, North Ryde, NSW, Australia) three times for 10 min, before washing in PBS. This process eluted the primary and secondary antisera for TH and ENK but did not affect the labelling for HuCD (due to the biotin-streptavidin complexes which protected AMCA labelling from elution)

22 Tissue was then immersed in ChAT and NOS1 primary antibodies diluted in hypertonic PBS with 0.3M NaCl, for 24 - 72 hours, then repeatedly rinsed in PBS for at least 3 x 10 minutes

23 ChAT was visualised with a CY3-coupled secondary antiserum and NOS with a CY5-coupled antiserum

24 The preparation was then imaged as decribed above and each HuC/D-labelled cell body was scored for ChAT and NOS immunoreactivity, giving 4 possible combinations.

25 Image stacks were analysed using Imaris software (Bitplane AG, Imaris x64, version 8.4.1). HuCD cell bodies were reconstructed as "surfaces" and the centre of each TH and ENK varicosity was identified using the "spots" function. This data was then analysed to calculate the density (per volume) of "spots" at incremental 2 µm shells measured from each cell's "surface".