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Characterisation of parasympathetic ascending nerves in human colon

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We use this protocol and it's working

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Abstract

This protocol explains how ascending nerves (also called “shunt fascicles”) in the plane of the myenteric plexus of human distal colon can be identified using fluorescence immunohistochemical labelling of wholemount preparations. It includes obtaining and handling live human tissue specimens, fixation, initial labelling to identification with a fluorescence microscope. This protocol was used in: Johnson ME, Humenick A, Peterson RA, Costa M, Wattchow DA, Sia TC, Dinning PG and Brookes SJH (2022). Characterisation of parasympathetic ascending nerves in human colon. Frontiers in Neuroscience: 16:1072002. https://doi.org/10.3389/fnins.2022.1072002).

Materials

• Specimen of live human colon
• Krebs solution for preparation containing:

	A	B
	NaCl	118mM
	KCl	4.8mM
	CaCl2	2.5mM
	MgSO4	1.2mM
	NaHCO3	25mM
	NaH2PO4	1.0mM
	glucose	11mM
	bubbled with	95% O2/5% CO2
	pH	7.4

• Sylgard 184 Elastomer (Dow Corning) and glass petri dishes
SYLGARD™ 184 Silicone Elastomer Kit **Dow Corning** **Catalog #**04019862
• Entomological pins without heads (~ 200µM diameter ~ 8-10mm long eg: (Australian Entomological Supplies, E184)
• 4% paraformaldehyde (Sigma-Aldrich) as 4% formaldehyde in 0.1M phosphate buffer, pH 7.2)
• Phosphate-buffered saline (PBS) pH 7.4 ([M] 137 millimolar (mM) NaCl, [M] 10 millimolar (mM) phosphate buffer pH7.4)
• Triton X-100 (Sigma-Aldrich) Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T8787
• Carbonate-buffered glycerol; (Sigma-Aldrich) at pH 8.6
• Epifluorescence microscope; Olympus IX71 or equivalent with appropriate fluorescence filters and camera and/or Laser-scanning confocal microscope (Zeiss LSM880 or equivalent) with 4 channel detection
• Primary and secondary antisera for chosen immunofluorescence labelling
• Anti-Glucose Transporter GLUT1 antibody [EPR3915] **Abcam** **Catalog #**ab115730
•
Monoclonal Anti-Neurofilament 200 (Phos. and Non-Phos.) antibody produced in mouse **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**N0142

Safety warnings

Special consideration should be given to biosafety concerns regarding the handling of live human tissue containing blood and potential pathogens, up to the point where tissue is fixed in formaldehyde. Care is taken to avoid splashes and aerosols. Users should consult their Institution Biosafety Committee for advice.

Collecting tissue

1 All handling of un-fixed human tissue must be exclusively done by staff trained in occupational health and safety requirements for handling hazardous material, wearing appropriate PPE (personal protective equipment) (gloves, gowns and masks) and working in areas designated for human tissue, with availability of a Microbiological Safety Cabinet. Users of this protocol should check local requirements with their Institutional Biosafety Committee before starting experimental work.

2 Before surgery, written informed consent must be obtained from patients, by surgical staff not involved in the project. A patient information form is supplied to the patient and the patient's signature is witnessed.

3 A 2 cm ring of live human colonic tissue is 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). The oral end of the ring is marked with a suture for future reference.

4 The specimen is immersed immediately in Room temperature carbogenated Krebs solution in a sterile container with a watertight screw top.

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

Handling tissue for fixation

10m

6 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. This is a condition of our Ethics permit.

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

8 The ring preparation is then opened into a flat sheet by cutting along the edge of a taenia coli, then pinned out, mucosal side uppermost, in a 15cm petri dish lined with 3mm depth of Sylgard 184 Elastomer (Dow Corning). 10mm headless stainless steel insect pins are used to stretch the preparation and pin it flat. The preparations is kept immersed in fresh carbogenated Krebs solution, which is replaced at 00:10:00 intervals throughout the dissection.

10m

9 The mucosa and submucosa are then removed by sharp dissection and discarded. The preparation is then turned over and re-pinned. The serosa is then cleared of fat, blood vessels

and adhering tissue. The oral end of the specimen (marked with a suture) is marked on the tissue with a series of small (1mm) parallel cuts.

10 The preparation is re-pinned in a clean dish and covered in PBS (pH 7.4) under maximal longitudinal and circular tension. PBS is poured off and replaced with cold 4% paraformaldehyde and the dish is covered and placed in a refrigerator Overnight . 10m

11 The next day, the preparation is unpinned and placed in fresh 4% paraformaldehyde in [M] 0.1 Molarity (M) phosphate buffer, pH 7.2 in a sealed container and placed on an orbital mixer at Room temperature for a further 24:00:00 , to ensure complete penetration by the fixative. 1d

12 All tools and containers that have been exposed to unfixed human tissue or contaminating solutions are immersed in 0.1% bleach solution for at least 00:10:00 prior to normal cleaning and washing in water. Surfaces are wiped with 0.1% bleach solution, followed by 70% ethanol to remove any residue. Tools and containers (Sylgard-lined petri dishes) are then sealed in Wipak Steriking autoclave bags and subjected to steam autoclaving at 121 °C for 00:30:00 to decontaminate before the next experiment. 40m

Processing for immunohistochemistry 30m

13 The specimen is then repeatedly rinsed in PBS for at least 3 x 10 minutes to remove paraformaldehyde.

13.1 The specimen is then repeatedly rinsed in PBS for at least 00:10:00 to remove paraformaldehyde (1/3). 10m

13.2 The specimen is then repeatedly rinsed in PBS for at least 00:10:00 to remove paraformaldehyde (2/3). 10m

13.3 The specimen is then repeatedly rinsed in PBS for at least 00:10:00 to remove paraformaldehyde (3/3). 10m

14 The tissue is pinned in PBS and the circular muscle is removed by sharp dissection, exposing the myenteric plexus. The taenia are also dissected to remove most of their bulk from the serosal surface. This process can take many hours.

15 The preparation is then cut down to a size that can be accommodated by available slides and coverslips, noting how the pieces fit together. The oral edge of each piece is marked.

16 The specimens are now immersed in 0.5% Triton X100 dissolved in PBS to permeabilise the tissue for 24:00:00 on an orbital mixer at Room temperature . 1d

17 It is then repeatedly rinsed in PBS (at least 3 x 10 minutes) before immunohistochemical labelling (for details see: "Immunohistochemical labelling of the innervation of dissected human colon wholemounts" **dx.doi.org/10.17504/protocols.io.n92ldpb47l5b/v1** .

17.1 It is then repeatedly rinsed in PBS for 00:10:00 before immunohistochemical labelling (for details see: "Immunohistochemical labelling of the innervation of dissected human colon wholemounts" **dx.doi.org/10.17504/protocols.io.n92ldpb47l5b/v1** (1/3). 10m

17.2 It is then repeatedly rinsed in PBS for 00:10:00 before immunohistochemical labelling (for details see: "Immunohistochemical labelling of the innervation of dissected human colon wholemounts" **dx.doi.org/10.17504/protocols.io.n92ldpb47l5b/v1** (2/3). 10m

17.3 It is then repeatedly rinsed in PBS for 00:10:00 before immunohistochemical labelling (for details see: "Immunohistochemical labelling of the innervation of dissected human colon wholemounts" **dx.doi.org/10.17504/protocols.io.n92ldpb47l5b/v1** (3/3). 10m

18 Tissue is then immersed in chosen primary antibodies diluted in hypertonic PBS (containing [M] 0.3 Molarity (M) NaCl), for 24:00:00 - 72:00:00 , then repeatedly rinsed in PBS for at least 3 x 10 minutes. 4d

19 Tissue is immersed in secondary antibodies chosen to match primaries. All secondary antisera are diluted in hypertonic PBS for 12:00:00 - 24:00:00 , then repeatedly rinsed in PBS for at least 3 x 10 minutes. 1d 12h

20 To reveal ascending nerves an antiserum raised against Glucose Transporter 1 (GluT1) is effective (Primary raised in rabbit, AbCam, Ab115730 used at 1:500). This labels the perineurium of extrinsic nerves whose path can then be traced in the wall of the colon, in the plane of the myenteric plexus. Alternatively, the distinctive long, braided appearance of axons in ascending nerves is revealed by antisera to 200kDa neurofilament subunit (NF200, Mouse Sigma N0142, applied at 1:1000).

21 Secondary antisera are applied Overnight followed by repeated washing in PBS 3 x 10 minutes. 10m

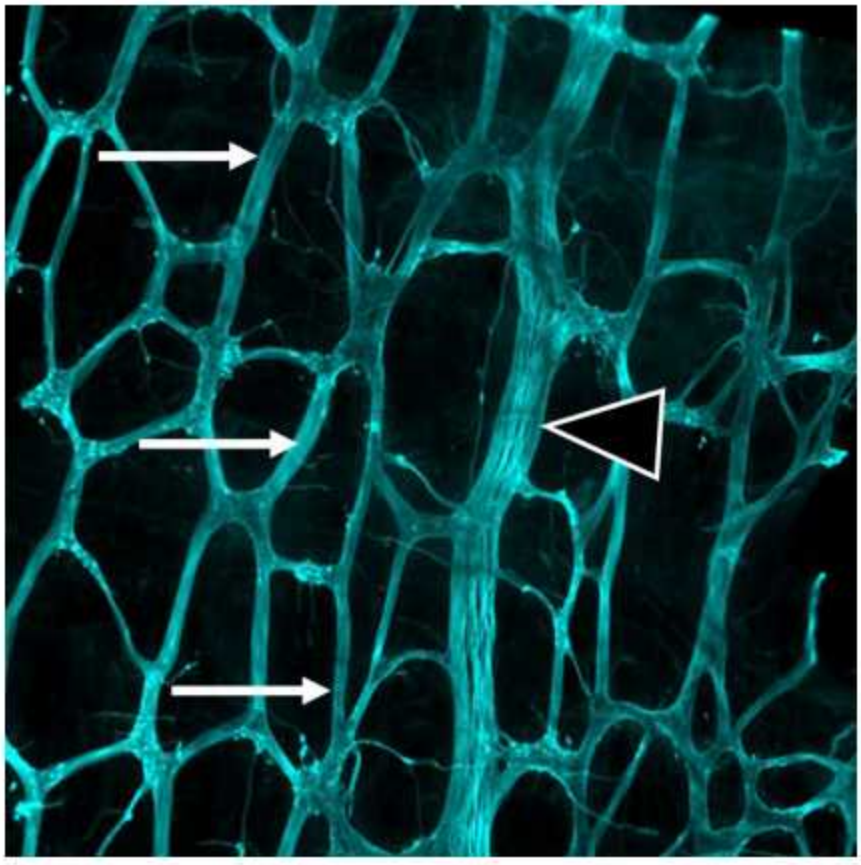
21.1 Wash in PBS for 00:10:00 (1/3). 10m

21.2 Wash in PBS for 00:10:00 (2/3). 10m

21.3 Wash in PBS for 00:10:00 (3/3). 10m

22 Tissue is soaked in carbonate-buffered glycerol (pH 8.6) and mounted on a slide in the same solution, coverslipped and viewed and photographed on an epifluorescence microscope (Olympus IX71) with appropriate filter sets.

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



Banner Image

Protocol references

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