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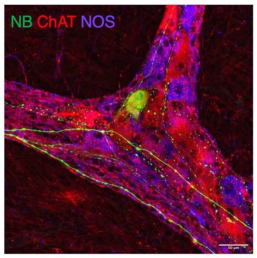
Studying viscerofugal neurons in human colon



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Bao Nan Chen[1], Adam G Humenick[1], Simon Brookes[1]

[1]Human Physiology, College of Medicine and Public Health, Flinders University, Bedford Park, South Australia 5042

SPARC
Tech. support email: **info@neuinfo.org**

Manmeet Bains
UCLA

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Humenick AG, Johnson ME, Chen BN, Wee M, Wattchow DA, Costa M, Dinning PG and Brookes SJH (2024). Antibody elution with 2-ME/SDS solution: uses for multi-layer immunohistochemical analysis of wholemount preparations of human colonic myenteric plexus. Heliyon 10(5):e26522, 2024 Mar 15. DOI: 10.1016/j.heliyon.2024.e26522

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. DOI: 10.3389/fnins.2022.1072002).

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We use this protocol and it's working

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Abstract

This protocol explains how viscerofugal neurons can be studied in ex vivo specimens of human colon. Viscerofugal neurons are characterized by having cell bodies in myenteric ganglia and axons that project out of the gut via colonic or mesenteric nerves. The protocol covers obtaining and handling live human colonic tissue specimens, biotinamide tracing of viscerofugal neuron cell bodies from axons in colonic nerves, tissue culture, fixation and immunohistochemical processing. This protocol was used in: Chen BN, Humenick A, Hibberd TJ, Yew WP, Wattchow DA, Dinning PG, Costa M, Spencer NJ, Brookes SJH (2024) Characterisation of viscerofugal neurons in human colon by retrograde tracing and multi-layer immunohistochemistry. Frontiers in Neuroscience 17:1313057. doi: 10.3389/fnins.2023.1313057

Guidelines

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.

Materials

- Specimen of live human colon
- Krebs solution for preparation containing: 118mM NaCl, 4.8mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 25mM $NaHCO_3$, 1.0mM NaH_2PO_4, 11mM glucose, bubbled with 95% O_2/5% CO_2, pH 7.4. Note: to avoid contaminated aerosols, Krebs solution that has been in contact with human tissue should not be bubbled.
- Sylgard 184 Elastomer (Dow Corning) and glass petri dishes
- Sharpened entomological pins without heads (~ 200uM diameter ~ 8-10mm long eg: (Australian Entomological Supplies, E184) – about 50 pins.
- Sharpened tungsten needles and fine forceps for micro-dissection.
- 100mls of sterile DME/F12 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich)
- Tissue culture incubator (37˚C, 5% CO2) with low voltage orbital mixer
- 4% paraformaldehyde (Sigma-Aldrich) as 4% formaldehyde in 0.1M phosphate buffer, pH 7.2
- Phosphate-buffered saline (PBS) pH 7.4 (137mM NaCl, 10mM phosphate buffer, pH7.4)
- Triton X-100 (Sigma-Aldrich)
- Carbonate-buffered glycerol; (Sigma-Aldrich) at pH 8.6
- Epifluorescence microscope; Olympus IX71 or equivalent with appropriate fluorescence filters and camera for 3-4 independent fluorophores and/or Laser-scanning confocal microscope (Zeiss LSM880 or equivalent) with 4 channel detection.
- Primary and secondary antisera for immunofluorescence labelling. Streptavidin-conjugated fluorophore(s) for use with biotinylated secondary antisera
- Artificial Intracellular Medium for biotinamide tracing: (150 mM monopotassium L-glutamic acid, 7 mM $MgCl_2$, 5mM glucose, 1 mM ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 20 mM Hepes buffer, 5 mM disodium adenosine triphosphate, 0.02% saponin, 1% dimethyl sulfoxide, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 20 µg/mL gentamycin) dissolved in water. These can be stored as frozen (-20˚C) aliquots in Eppendorf vials
- Biotinamide solution (5% N-[2-aminoethyl] biotinamide hydrobromide, (Molecular Probes, Invitrogen USA) dissolved in Artificial Intracellular Medium).
- Elution Solution containing 2% w/v sodium dodecyl sulphate (SDS), 62.5mM Tris-HCl (pH6.8) and 0.8% mercaptoethanol
- Water bath with agitation (to reach temperature of 60˚C)

Collecting tissue

1 All handling of un-fixed (live) 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 (opaque) and transported back to the laboratory.

Handling tissue for preparation

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 Krebs solution equilibrated with carbogen (95% O_2/5% CO_2), 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). It is cut down to approximately 25 x 25mm and pinned flat with 10mm headless stainless steel insect pins.

9 The preparation is kept immersed in fresh carbogenated Krebs solution, which is replaced at 10 minute intervals throughout the dissection. The oral end of the specimen is marked by a series of 5 parallel short cuts, approximately 1mm long in one corner.

10 The mucosa and submucosa are then removed by sharp dissection and discarded. The preparation is then turned over and re-pinned under tension.

11 The fat and connective tissue on the serosal surface is then closely examined for large blood vessels that enter the gut wall. When a large vessel is located, the nearby tissue is carefully searched for paravascular nerve trunks which are identifiable by their distinctive smooth and/or glassy appearance and lack of lumen.

12 The nerve trunk is then traced to confirm that it runs into the wall of the colon (and has not been cut during surgery) and it is mobilized over a length of at least 5mm by dissecting surrounding connective tissue. Identifying and dissecting the extrinsic nerve trunk is demanding and may take several hours. Some preparations do not contain a suitable nerve trunk.

13 The perineurial sheath surrounding the nerve trunk is then removed using fine forceps and sharp tungsten needles.

14 The preparation is then transferred to a clean, sterile 12cm Sylgard-lined petri dish, fitted with a Perspex block (30mm x 20mm x 3mm); the latter with an eccentric circular well (12mm diameter; 1 mL volume) that just breaks through one edge of the block.

15 The tissue is pinned with its edge close to the well so that the extrinsic nerve trunk can be pulled into the 1mL well and sealed in place with a cut-down coverslip and silicon grease (Sigma-Aldrich-Merck)

16 Krebs solution is aspirated from the 1mL well using a 3ml syringe, to check for leaks. If Krebs flows in then a leak is identified: the coverslip is lowered and extra silicon grease added until the barrier between the chamber and the well is water-tight. The 1mL well is then filled with paraffin oil (Sigma-Aldrich-Merck).

17 The nerve within the 1mL well is then rinsed under the paraffin several times with Artifical Intracellular Medium (see list of materials) to remove adhering Krebs solution. Then a small drop (~10µl) of 5% biotinamide, dissolved in Artifical Intracellular Medium, is applied to the nerve trunk away from the coverslip under the paraffin. In this way, the paraffin oil and silicon grease act as a barrier preventing any leakage of biotinamide outside the 1ml well.

18 The Krebs solution in the petri dish is then aspirated and replaced with DME/F12 culture medium, supplemented with 10% fetal bovine serum

19 The dish is then transferred into an incubator held at 37˚C with 5% carbon dioxide on an orbital mixer for 12 – 24 hours, giving time for the biotinamide to enter and diffuse down the axons in the colonic nerve trunk (Tassicker 1999).

20 If it is intended to study neuropeptide or 5-HT immunoreactivity in viscerofugal nerve cell bodies, then special steps are taken at the end of incubation, before fixation.

a. Colchicine (Sigma-Aldrich), at a final concentration of 83µM is added to the DME/F12 culture medium at the time of set-up.

b. In the last 90 minutes before fixation, cell bodies are loaded with 5-HT by adding the monoamine oxidase B inhibitor, pargyline (Sigma-Aldrich; 50 µM) to DME/F12 medium in the dish, followed 30 min later by addition of 5-HT mesylate (Sigma-Aldrich) 2µM for 1 h.

21 At the end of the period of incubation, the nerve trunk is then cut close to the main preparation. The main preparation can then be unpinned without any leakage of biotinamide from the 1ml chamber. The main preparation is then transferred to another Sylgard-lined petri dish, repinned under maximal stretch and rinsed in PBS (pH 7.4) to remove culture medium, colchicine and 5-HT. The PBS is then poured off and replaced with cold 4% paraformaldehyde and the dish is covered and placed in a refrigerator overnight.

22 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 on an orbital mixer at room temperature for a further 24 hours, to ensure complete penetration by the fixative.

23 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 10 minutes 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 30 minutes to decontaminate before the next experiment.

Processing for immunohistochemistry

24 The specimen is then repeatedly rinsed in PBS (3 x 10 minutes) to remove fixative.

25 The circular muscle is removed by sharp dissection, exposing the myenteric plexus. The taenia (if present) are also dissected to remove most of their bulk from the serosal surface. Thinning the taenia can take several hours but is necessary for wholemount preparations.

26 The specimens are immersed in 0.5% Triton X100 dissolved in PBS to permeabilise the tissue for 24 hours on an orbital mixer at room temperature.

27 After further washes in PBS to remove Triton X100 (3 x 10 minutes), tissue is immersed in fluorophore-linked streptavidin (eg: streptavidin-AF488 (1:2000) or streptavidin-AF555 (1:2000) overnight to visualize the extent of biotinamide filling.

28 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. The biotinamide fill is then judged on the number of viscerofugal nerve cell bodies and intensity of their labelling in myenteric ganglia. A decision whether to proceed with immunohistochemical analysis is made on this basis.

29 If the decision is to proceed, the location of each labelled viscerofugal nerve cell body is recorded using two Mitutoyo linear scales connected to a Mitutoyo 2D-ALC Decoder (ALC-3701, Mitutoyo Corporation, JPN) connected to the stage of an Olympus IX71 epifluorescence microscope. XY coordinates of objects and outlines are recorded, together with an identifying keypress, in Microsoft Excel, via Bill Redirect Software. (**www.billproduction.com**). From this data, a map of the outline of the specimen and location of each labelled viscerofugal neuron is created in Microsoft Excel

30 The preparation is then unmounted, rinsed in PBS (3 x 10 minutes) before immunohistochemical labelling (for details see protocol: "Immunohistochemical labelling of the innervation of dissected human colon wholemounts" dx.doi.org/10.17504/protocols.io.n92ldpb47l5b/v1)

31 Tissue is immersed in up to 3 chosen primary antibodies diluted in hypertonic PBS (containing 0.3M NaCl and 0.05% sodium azide), for 24 - 72 hours at room temperature, then rinsed in PBS (3 x 10 minutes or more)

32 Tissue is immersed in secondary antibodies chosen to match the primaries. All secondary antisera are diluted in hypertonic PBS and applied for 12-24 hours, then repeatedly rinsed in PBS for at least 3 x 10 minutes.

33 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. Confocal microscopy, a Zeiss LSM880 was used to collect hyperstacks of images in 1µm steps with a 20x objective.

Relabelling the preparation with other immunohistochemical markers

34 If more than 3 markers are to be assessed in each viscerofugal nerve cell body, a protocol for elution of antisera can be used prior to restaining with a new combination of antisera.

35 After photography and analysis, the preparation is then unmounted and rinsed in PBS (3 x 10 minutes) to remove mounting medium.

36 50mls of Elution Solution is then made up (Humenick et al 2024) - mixing 10 mL 10% SDS, 6.25 mL 0.5 M Tris-HCl (pH 6.8), 33.75 mL distilled water and 0.4 mL 2-mercaptoethanol) and pre-warmed in an agitating water bath to 56˚C. The tissue is then immersed in this solution and incubated for 1 hour.

37 After rinsing (3 x 10 minutes of PBS), the preparation is then mounted in carbonate-buffered glycerol and viewed on an Olympus IX71 epifluorescence microscope to check in each filter combination that elution has been complete. Over-exposed images are taken in each channel to ensure that low levels of labelling (detectable by the camera but not by human observers) have not persisted. Note that labelling with biotin/streptavidin complexes is very resistant to elution and viscerofugal neurons and axons are still visible after the elution process.

38 The preparation is then unmounted, rinsed in PBS (3 x 10 minutes), then immersed in a new set of primary antisera followed by secondary antisera as described above.

39 The re-stained preparation is then mounted and each viscerofugal neuron is located using the coordinates mapped earlier. Each cell is then scored as immunoreactive/non-immunoreactive for each marker.

40 The process of elution, checking, and restaining can be repeated at least 7 times (the most used in the present study). Care must be taken to avoid physical damage to the preparation by repeated handling with fine forceps and accumulation of debris during repeated solution changes.

Protocol references

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