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Intracellular recording and dye filling of human myenteric neurons

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We use this protocol and it's working

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Abstract

This protocol explains how to make intracellular recordings and dye fills with glass micropipettes filled with potassium chloride and 5,6 carboxyfluorescein. It also includes immunohistochemical processing of dye fills. This protocol was used for the study: WP Yew, A Humenick, BN Chen, DA Wattchow, M Costa, PG Dinning, SJH Brookes (2023). Electrophysiological and morphological features of myenteric neurons of human colon revealed by intracellular recording and dye fills. Neurogastroenterology & Motility. e14538, 2023, **https://doi.org/10.1111/nmo.14538)**

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

List of materials:

- Specimen of live human colon
- **Modified Krebs solution containing:**

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

- Sylgard 184 Elastomer (Dow Corning) and glass petri dishes
- Entomological pins without heads (~ 200uM diameter ~ 8-10mm long eg: (eg: Australian Entomological Supplies, E184)
- Tungsten wire **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**GF64187657
- Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P6148 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 X-100)
- Activated charcoal **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**242276
- 5(6)-Carboxyfluorescein **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**21877
- Sucrose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S0389
- Von Frey hair (50-100mg force)
- Carbonate-buffered glycerol; 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
- Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8900

Collecting tissue

1 Handling all 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 small section of live human colonic tissue (at least 20mm x 20mm) 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 tissue 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 Place the sealed container in an opaque second, larger watertight container and transport back to the laboratory.

Handling tissue for recording

1d 17h 23m

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, preparation is rinsed repeatedly in fresh oxygenated Krebs solution to flush away contents.

8 The preparation is then pinned out as a flat sheet with serosal side uppermost, in a 15cm petri dish lined with 3mm of Sylgard 184 Elastomer (Dow Corning). Up to thirty 10mm headless stainless steel insect pins are used to stretch the preparation and pin it flat.

10m

- The preparations is kept immersed in fresh carbogenated Krebs solution at Room temperature, which is replaced at 00:10:00 intervals throughout the dissection.
- The oral end of the preparation is marked with a series of 5 x 1mm cuts for subsequent orientation.

9 The preparation is illuminated via a single directional fibre-optic source orientated parallel to the surface of the Sylgard to maximise contrast.

10 The serosa is cleaned of fat and major vessels using micro- spring scissors and fine forceps (#55), then the preparation is turned over and repinned, mucosa uppermost.

11 The mucosa and as much submucosa as possible are then removed by sharp dissection and discarded.

12 The circular muscle is then peeled from the edge of the preparation to the centre in 0.5 – 2mm wide strips, using a shearing movement to remove as much circular muscle as possible. This is done from both sides. Remaining loose strands of smooth muscle are peeled off taking care not to touch the underlying tissue with forceps.

13 The orientation of the fibre optic illumination is then systematically moved to try to identify lipofuscins in nerve cell bodies, in order to localize myenteric ganglia.

- Lipofuscins are concentrated in the peri-nuclear cell bodies of myenteric neurons, are pale cream in colour and resemble a bunch of grapes.
- The myenteric plexus may also be identified by the oblique orientation of internodal strands which distinguishes them from circular and longitudinal smooth muscle strands with their characteristic orthogonal orientations.

14 When a myenteric ganglion is identified, its position is marked on the tissue. To do this, a light (eg: 50 mg - 100 mg) von Frey hair is dipped into sucrose syrup, then used to pick up graphite particles. When pressed on the tissue, the sucrose dissolves, leaving the graphite particles attached to the tissue. Several spots around the ganglion are marked in this way.

15 The preparation is then re-pinned in a small recording chamber (35 x 55mm) whose base is a coverslip, with a 1mm thick coating of Sylgard 184, using 50µm tungsten wire pins approximately 2mm long.

- Additional pins are placed around the marked myenteric ganglion to hold the tissue firmly against the Sylgard base of the chamber.

16 The whole preparation is then treated with enzymatic Liberase TH (Cat# LIBTH-RO; Sigma-Aldrich) diluted from frozen stock 1:50 (stock: 50 undetermined working solution) in DME/F12 medium (Cat# 8900; Sigma-Aldrich), incubated for 00:08:00 – 00:18:00, in 5 mL of solution at 36 °C. 18m

- This softened the connective tissue around the ganglion, making micropipette penetration easier and reducing the chance of the micropipette tip breaking.

17 A glass micropipette (1.0 outer diameter, 0.58mm inner diameter) is then tip-filled with 5%w/v 5(6)-carboxyfluorescein (Cat# 21877; Sigma-Aldrich) buffered in [M] 20 millimolar (mM) Tris in [M] 1 Molarity (M) KCl (pH 7.0), then backfilled with [M] 1 Molarity (M) KCl solution and mounted on a micromanipulator.

18 Hyperpolarising current pulses are passed through the electrode (-200 pA, 33Hz, 10ms) to monitor membrane potential and electrode characteristics as the pipette tip is advanced through the myenteric ganglion.

- Impalement of a cell is indicated by a negative deflection reflecting membrane potential and an increase in pulse time constant, reflecting cell membrane capacitance. In some cases, lipofuscin autofluorescence was used to locate nerve cell bodies.

19 At the end of the recording period, large hyperpolarizing current pulses (200ms duration at 2Hz, 100 – 500pA) are applied for 00:01:00 – 00:05:00 to electrophorese carboxyfluorescein into the cell. 5m

20 At the end of this process, the preparation is drawn to identify ganglion position, then fixed in situ in 4% paraformaldehyde in [M] 0.1 Molarity (M) phosphate buffer, pH 7.2 for 16:00:00 at 4 °C, then unpinned and immersed in 4% paraformaldehyde in [M] 0.1 Molarity (M) phosphate buffer, pH 7.2 for a further 24:00:00 at 4 °C. 1d 16h

21 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. 50m

- 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.
- Excess paraformaldehyde is removed from the recording chamber by filling with 3% hydrogen peroxide for 00:10:00 .

Processing for immunohistochemistry

5d 3h

22 After fixation, the specimen is then repeatedly rinsed in PBS for at least 3 x 10 minutes to remove paraformaldehyde.

- It is then mounted in carbonate-buffered glycerol (mounting medium) and viewed with epifluroscence on an Olympus IX71 microscope, using an FITC filter set to determine whether the recorded cell had been successfully labelled with carboxyfluorescein.

23 If successful, the preparation is then unmounted, rinsed in PBS 3 x 10 minutes to remove mounting medium then 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

24 It is then rinsed in PBS (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.

25 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.

3d

26 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

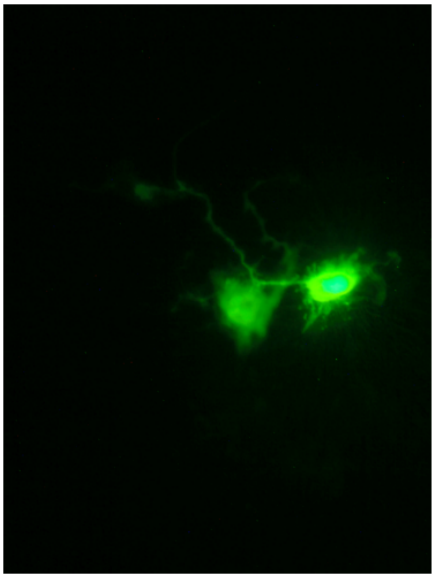
26.1 Secondary antisera are applied overnight followed by repeated washing in PBS for 3 x 10 minutes.

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

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

Banner image

29



Protocol references

Manuscript References:

1. Yew WP, Humenick AG, Chen BN, Wattchow DA, Costa M, Dinning PG, Brookes SJH (2023). Electrophysiological and morphological features of myenteric neurons of human colon revealed by intracellular recording and dye fills. Neurogastroenterology & Motility. e14538, 2023, **https://doi.org/10.1111/nmo.14538)**