Apr 12, 2021

Protocol for "Characterization of projections of long interneurons in human colon" - Brookes Lab

Simon Brookes[1], Bao Nan Chen[2], Adam Humenick[2]
[1]Flinders University of South Australia; [2]Flinders University

1 Works for me dx.doi.org/10.17504/protocols.io.btwinpce

Simon Brookes

ABSTRACT

This protocol outlines the basic methods for retrograde tracing of enteric neuronal pathways in live surgical specimens of human colon.The methods use the carbocyanine tracer, DiI and combine this with multiple-labelling immunohistochemistry to analyse pathways to specific targets within the gut wall.The methods outline basic sterile handling of tissue, health and safety issues and the sequence of manipulations to produce output on an epifluorescence microscope.

DOI

dx.doi.org/10.17504/protocols.io.btwinpce

PROTOCOL CITATION

Simon Brookes, Bao Nan Chen, Adam Humenick 2021. Protocol for "Characterization of projections of long interneurons in human colon" - Brookes Lab. **protocols.io** https://dx.doi.org/10.17504/protocols.io.btwinpce

CREATED

Apr 02, 2021

LAST MODIFIED

Apr 12, 2021

OWNERSHIP HISTORY

Apr 02, 2021 Gabrielle Pine

Apr 02, 2021 Simon Brookes

PROTOCOL INTEGER ID

48810

Preparation

1 In weeks prior to the experiment, coat a batch of small glass beads (500 µm diameter - Sigma) with 1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI - Invitrogen) dissolved in ethanol.

1.1 Let the ethanol evaporate until a viscous sticky paste is present and ensure glass beads are evenly coated.

1.2 Dry in oven at 50˚C overnight to harden DiI.

2 Make up sterile culture medium (Dulbecco's Modified Eagle's Medium/Nutrient Mix F-12 Ham (Sigma D8900), 10% v/v Fetal Bovine Serum (Gibco 10100147), 100 iu/mL penicillin, 100 µg/mL streptomycin (Sigma P4333), 2.5 µg/mL amphotericin (Sigma A9528).

3 Equilibrate in 200ml batches in incubator with 5% CO2 overnight

4 Sterilise dissection tools and petri dishes in an autoclave at 121°C for 20 minutes.

5 Sterilise modified Krebs solution (NaCl; 118 mmol/L, KCl; 4.8 mmol/L, MgSO4; 11.2 mmol/L, NaHCO3; 25 mmol/L, NaH2PO4; 1.0 mmol/L).

6 Make up several litres of phosphate buffered saline and filter (0.22µm pores) ready for use (NaCl: 8.5g/l, Na2HPO4.2H2O: 1.341 g/l, NaH2PO4.2H2O: 0.39g/l).

Main Protocol

7 Bubble sterile Krebs solution with 95% O2, 5% CO2, to reach pH; 7.4.

7.1 Add final concentration of 1 µmol/L nicardipine (Sigma N7510) and 1 µmol/L hyoscine (Hyoscine hydrobromide Sigma S1875).

7.2 Using a syringe filter (0.22µm pores) add 10mls of 100x CaCl2/glucose solution (250mM CaCl2; 1100mM glucose) to bubbled Krebs solution

8 Collect human colonic specimen from operating theatres and transport to laboratory in oxygenated Krebs solution.

8.1 Ensure that informed consent has been obtained prior to accepting the tissue.

8.2 When dealing with fresh human specimens, wear appropriate PPE (gloves, lab coat).

9 Open the specimen (complete tube) into a flat sheet by cutting down the longitudinal axis of the bowel wall.

Citation: Simon Brookes, Bao Nan Chen, Adam Humenick (04/12/2021). Protocol for "Characterization of projections of long interneurons in human colon" - Brookes Lab. https://dx.doi.org/10.17504/protocols.io.btwinpce

10 Under a dissection microscope, pin the specimen as a flat sheet, mucosal side up, in a Sylgard-lined petri dish (Sylgard from Dow Corning 4019862) using 5mm headless sharpened stainless steel entomology pins in dish (E185, Australian Entomological Supplies, South Murwillumbah, NSW 2484, Australia)

11 Remove the mucosa and submucosa using sharp dissection, with sterile forceps and spring scissors.

12 Turn the specimen over and pin serosal side up. Remove any mesentery, fat cells and loose connective tissue to reveal the strands of longitudinal muscle and taenia coli.

13 Cut specimen to size

14 Wash preparations in sterile Krebs for 15 minutes, changing solution 3 times and agitating solution frequently to remove faecal matter, cell debris etc. Use suction device that empties into a bleach-filled container (10% bleach)

15 Re-pin each preparation in a fresh sterile Sylgard-lined petri dish filled with sterile Krebs, longitudinal muscle/tenia coli side up.

16 Remove circular muscle strips by sharp dissection until it is possible to identify a myenteric ganglion near the end of specimen (oral or anal end depending on which polarity of interneuron is being targeted).

17 With fine forceps, apply a DiI-coated glass bead to an identified ganglion or internodal strand in the area cleared of circular muscle and press lightly in place. Leave for 10 minutes for DiI to stick to tissue.

18 Rinse carefully with sterile Krebs solution three times without dislodging the bead

19 Replace sterile Krebs solution with culture medium (Dulbecco's Modified Eagle's Medium/Nutrient Mix F-12 Ham, 10% v/v Fetal Bovine Serum, 100 iu/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin) and transfer to an orbital mixer in an incubator (37°C, 5% CO_2).

20 Incubate preparations for 4 nights in 5% CO_2 incubator with orbital mixer, replacing culture medium every 24 hours.

21 If neuropeptides in nerve cell bodies are to be studied, add via syringe with 0.22µm filter to a final concentration of 83 µmol/L colchicine (Sigma C9754) for the final 24 hours of incubation.

22 After 4 nights in culture, remove petri dish from incubator and replace culture medium with 1X PBS (phosphate buffered saline, pH7.4).

Citation: Simon Brookes, Bao Nan Chen, Adam Humenick (04/12/2021). Protocol for "Characterization of projections of long interneurons in human colon" - Brookes Lab. https://dx.doi.org/10.17504/protocols.io.btwinpce

23 Under a dissection microscope, using forceps, carefully remove the DiI-coated glass bead and any residual DiI deposits, regularly wiping forceps tips with 100% ethanol to prevent unintentional DiI spread.

24 Pin preparation in a fresh Sylgard-lined petri dish, stretching the muscle maximally in both circular and longitudinal directions.

25 Replace 1X PBS with modified Zamboni's fixative (2% formaldehyde (Paraformaldehyde Sigma 441244) and 15% saturated picric acid in water (Saturated Picric Acid ChemSupply PA154) in 0.1 mol/L phosphate buffer, pH 7.0) to immersion-fix the preparation.

26 Place the petri dish containing the fixative and preparation in a fridge at 4°C for 24 hours.

27 After 24 hours, un-pin preparation from petri dish and place in a specimen pot in fresh Zamboni's fixative for 24 hours at room temperature on an orbital mixer to complete fixation. Note: preparations should hold their shape after 24 hours in refrigerator; this additional fixation is to ensure proper penetration of fixative through the preparation.

28 Wash for 30 minutes on an orbital shaker at room temperature, replacing 1X PBS every 10 minutes.

29 Pin preparation in a fixative-designated petri dish, serosal side up, and remove any remaining connective tissue bundles via sharp dissection with forceps and spring scissors. Thin down the taenia coli.

30 Turn the preparation over and dissect away any remaining submucosa and circular muscle, leaving a preparation of myenteric plexus and thin longitudinal muscle/tenia.

31 Un-pin the preparation and place in a specimen pot containing 50% carbonate/bicarbonate buffered glycerol (pH 8.6) for 10 minutes (Glycerol; Sigma G7893) on an orbital mixer at room temperature.

32 Transfer preparation to a specimen pot containing 70% carbonate/bicarbonate buffered glycerol, then 100% carbonate/bicarbonate buffered glycerol for 10 minutes each on an orbital mixer at room temperature.

33 Mount the preparation, in 100% carbonate/bicarbonate buffered glycerol and coverslip.

34 Using a fluorescent microscope with appropriate filters (eg:CY3 filter for DiI), view the preparation through the circular muscle side to confirm that neurons in the myenteric plexus have been filled with DiI.

35 Un-mount the preparation, and place back in a specimen pot containing 100% carbonate/bicarbonate buffered glycerol for 48 hours at room temperature on an orbital mixer to p ermeabilise.

Citation: Simon Brookes, Bao Nan Chen, Adam Humenick (04/12/2021). Protocol for "Characterization of projections of long interneurons in human colon" - Brookes Lab. https://dx.doi.org/10.17504/protocols.io.btwinpce

36 Wash 5 times in 1X PBS on an orbital mixer at room temperature, changing 1X PBS every 30 minutes to thoroughly remove the glycerol

37 Immerse in primary antisera in a 20ml scintillation vile, dissolved in hypertonic PBS, and incubate at room temperature for 72 hours on an orbital mixer.

38 Wash 5 times in 1X PBS on an orbital mixer at room temperature, changing 1X PBS every 10 minutes

39 Immerse in secondary antisera, dissolved in hypertonic PBS, and incubate at room temperature for 24 hours on an orbital mixer.

40 Wash 5 times in 1X PBS on an orbital mixer at room temperature, changing 1X PBS every 10 minutes

41 Pass preparation through 50%, 70% and 100% carbonate/bicarbonate buffered glycerol diluted with PBS, 10 minutes in each, on an orbital mixer at room temperature.

42 Re-mount in 100% carbonate/bicarbonate buffered glycerol and coverslip

43 View preparation, through the circular muscle, on a fluorescent microscope with a cross-hair eyepiece, fitted with stage measurement system (eg: Mitutoyo linear scales (1 µm resolution), connected to a Mitutoyo 2D-ALC Decoder (ALC-3701; Mitutoyo Corporation). Connection via an RS232/USB connector to a personal computer allows coordinates to be recorded in Microsoft Excel via Bill Redirect Software (http://www.billproduction.com).

44 Set the DiI application site to co-ordinate (0,0) then record co-ordinates of the outline of the preparation at regular intervals.

45 Increase magnification to 20X. Start at one end of the preparation and move across the tissue at 0.5 mm intervals. When a neuron enters the field of view, record its co-ordinates and immunohistochemical code (i.e. NOS +ve, ChAT -ve coded a “N”).

46 Once all co-ordinates have been recorded, preparation can be recreated as a “map” in Microsoft Excel, showing the preparation outline, DiI application site, location of axons and neurons with their chemical coding.

47 Clean up

48 Staff Occupational Health and Safety is of paramount importance throughout – it should be assumed that all tissue is infectious. During all handling of unfixed tissue and solutions that contact unfixed tissue, appropriate PPE must be

Citation: Simon Brookes, Bao Nan Chen, Adam Humenick (04/12/2021). Protocol for "Characterization of projections of long interneurons in human colon" - Brookes Lab. https://dx.doi.org/10.17504/protocols.io.btwinpce

worn (gloves, gown)

49 Care must be taken that contaminated solutions (which have been in contact with unfixed human tissue) are never be bubbled, allowed to splash or allowed to form aerosols. Spills must be cleaned up immediately with bleach.

50 All microscopes, surfaces, benches, biosafety hood benchtop, must be wiped down with 1% bleach, followed by 70% ethanol in water, and allowed to dry.

51 All tools (forceps, scissors), containers and petri dishes that came in contact with unfixed specimens must be immersed in 10% bleach for 10 minutes to sterilise. They should then be autoclaved before subsequent use.

52 Discard any excess tissue and disposable materials (e.g. specimen pots, paper towels) into an appropriate “Contaminated-Waste” bin.

Citation: Simon Brookes, Bao Nan Chen, Adam Humenick (04/12/2021). Protocol for "Characterization of projections of long interneurons in human colon" - Brookes Lab. https://dx.doi.org/10.17504/protocols.io.btwinpce