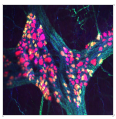

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Human colon tissue clearing and Immunohistochemistry

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

Optical Clearing of Tissue

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ABSTRACT

INTRODUCTION: Bowel pathology is routinely evaluated by sectioning tissue, staining, and light microscopy. Tissue sectioning does not provide robust information about three-dimensional structures. Defining bowel anatomy in three dimensions is especially valuable for understanding human bowel motility disorders. Intrinsic mechanisms controlling bowel motility include the enteric nervous system (ENS), pacemaker cells called interstitial cells of Cajal, smooth muscle cells and PDGFRalpha+ cells. One problem is that the cells that control motility are distributed throughout the bowel wall and the bowel wall is too thick and opaque to permit direct visualization without sectioning.

OBJECTIVES: To develop an efficient and reproducible method to make human colon translucent, stain with antibodies, and visualize cells that control bowel motility in three dimensions.

METHODS: Tissue is cleaned, trimmed to remove fat, pinned flat, gently stretched and then fixed in 4% paraformaldehyde before storage. To begin clearing tissue is treated with 100% methanol, then permeabilized with Dent's bleach (Methanol, DMSO and hydrogen peroxide) and rinsed with PBS. Blocking is performed for 3 days at room temperature. Incubation with primary antibodies occurs for 14 days at 37 ^{o}C. Unbound primary antibody is washed out over the course of one day. Secondary antibody staining is performed for three days at 37 ^{o}C. After washing in PBS, tissue is dehydrated using a graded methanol series and then cleared using Murray's Clear (Benzyl Benzoate: Benzyl Alcohol). For confocal imaging, tissue is mounted in Murray's Clear.

RESULTS: This approach provides efficient tissue clearing and reproducible staining with a subset of tested antibodies. We routinely obtain images using two or three antibodies and can visualize stained cells all the way through the bowel wall thickness. Confocal imaging permits excellent visualization and three dimensional reconstruction. We routinely stain 1 cm x 1 cm human colon pieces, but the same methods can be used to stain and image colon tissue many centimeters in length.

CONCLUSION: We established an efficient and reproducible method for clearing, staining with antibodies and imaging colon tissue. This approach works well to generate three dimensional images of stained cells. We can visualize many cell types that control bowel motility, but the approach will also probably work to see other cell types with appropriate antibodies. Only a subset of antibodies work with this organic solvent based fixation and clearing method.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Kahleb D. Graham*, Silvia Huerta López*, Rajarshi Sengupta, Archana Shenoy, Sabine Schneider, Christina M. Wright, Michael Feldman, Emma Furth, Federico Valdivieso, Amanda Lemke, Benjamin J. Wilkins, Ali Naji, Edward Doolin, Marthe J. Howard, Robert O. Heuckeroth (2020) Robust, 3-Dimensional Visualization of Human Colon Enteric Nervous System Without Tissue Sectioning, Gastroenterology, in press

GUIDELINES

- This method works best with fresh unfixed tissue.
- Cleaning colon and removing fat or debris is important before fixation.
- Tissue is gently stretched and pinned before fixation. During the process try to get the tissue as flat and uniform as possible.

- Use stainless steel pins when pinning tissue as these will not rust.
- Fixed tissue can be stored in 50% 1X PBS, 50% Glycerol, 0.05% Sodium Azide) at 4 ^{O}C for at least a month and retains good staining characteristics.
- Use sealed containers for prolonged incubations at 37 ^{O}C to avoid evaporation, which would change reagent concentrations and can dry tissues.
- Dilute primary antibodies in **Blocking Solution** (see below) when ready to use.
- Dilute secondary antibodies in 1X Phosphate Buffered Saline.
- 500 microliters antibody solution is enough to cover a 1 cm x 1 cm fixed colon sample.
- Staining is performed on a rocker. For 1 cm x 1 cm tissue, we use 24-well plates for blocking, washing and antibody staining steps.

MATERIALS

NAME	CATALOG #	VENDOR
Paraformaldehyde	P6148	Sigma Aldrich
Benzyl Alcohol	305197	Sigma Aldrich
Methanol	A452-4	Fisher Scientific
DMSO	472301	Sigma Aldrich
Benzyl Benzoate	B6630	Sigma Aldrich
Hydrogen Peroxide	H1009-500ML	Sigma Aldrich
Triton X-100	9002-93-1	VWR Scientific
Normal Donkey Serum	017-000-121	Jackson Immunoresearch
Minutien Pins Stainless Steel 0.1 mm diameter	26002-10	Fine Science Tools
Minutien Pins Stainless Steel 0.2 mm diameter	26002-20	Fine Science Tools
Sodium Azide ReagentPlus > 99.5%	S2002-25G	Sigma Aldrich

SAFETY WARNINGS

Toxic reagents include:
Benzyl alcohol
Benzyl Benzoate
Methanol
Paraformaldehyde

Note that Murray's clear dissolves many types of plastic.

Use personal protective equipment (PPI) when working with human tissues because of the risk of blood borne infections. PPI should also be used when handling toxic chemicals. This includes eye protection, gloves, and mask to prevent mucus membrane exposures.

BEFORE STARTING

Prepare the following materials and solutions:

1. Sylgard^{TM} 184 Elastomer (Dow Corning, Midland MI, USA) coated plates for pinning tissue were prepared according to manufacturer instructions.

2. Dent's Bleach (Permeabilization Solution) (50mL)

- *20mL* 100% MeOH
- *5g/5mL* DMSO
- *5mL* 30% Hydrogen peroxide
- This solution must be made fresh.

3. Murrays Clear

- 2:1 Benzyl Benzoate: Benzyl Alcohol
- Note that this destroys plastic
- This should be a clear colorless solution

4. Blocking Solution

- 0.5% Triton X-100 (5 mL Triton x-100 diluted in 1X PBS)
- 5% Normal Donkey Serum (NDS) diluted in 0.5% Triton X-100
- 0.05% Sodium Azide
- This solution must be made fresh and can be stored at 4C for up to 2 weeks.

5. Washing Solution

- 1X phosphate buffered saline (PBS)
- 0.05% Sodium Azide

Fresh tissue processing and fixation

1 Transport fresh tissue from human colon in cold sterile 1X phosphate buffered saline (PBS) on ice. Tissues placed in cold PBS within 1-2 hours of resection were used for imaging. 2h

2 Transfer tissue using forceps and fresh PBS into Sylgard™ coated dish. Lay tissue flat with the luminal side down and the serosa side up. 1m

3 Using dissecting scissors and forceps remove any fat tissue that is present and discard according to Biosafety instructions. 20m

4 Pin one side of the tissue and begin to stretch and pin along the edge of the tissue until the tissue looks uniform and as flat as possible. Pins are then repositioned several times, with each round of repositioning stretching the tissue further as colon relaxes. On average, colon area stretched to about 2.6x more than the original unstretched dimensions. 20m

5 Remove the PBS and add 4% Paraformaldehyde (PFA). Let issue sit at 4C overnight. 16h

6 Replace the 4% PFA with fresh PBS, and begin to carefully remove the pins. Trim the edges of the tissue. 20m

7 Using small dissecting scissors cut out a full thickness strip of tissue (ex. 1cm x 1 cm) to be used for staining. The remaining tissue can be transferred to a solution of (50% 1X PBS, 50% Glycerol, 0.05% Sodium Azide) and stored at 4C for future use. 5m

Immunostaining

8 *The following incubations are done in 24-well plates (2 cm diameter/well) with approximately 500ul of solution. Additional solution is added if needed to cover tissue. Samples are placed on a rocker (moderate speed) at room temperature for these steps.*

For tissue taken out of storage, wash three times in 1X PBS for 5 minutes each wash. 15m

9 Incubate tissue in 100% Methanol for 1 hour on ice. 1h

10 Transfer to permeabilization solution (Dent's Bleach: 5mL 30% hydrogen peroxide, 5mL DMSO, 20mL 100% Methanol) for 2 hours at room temperature. 2h

11 Wash tissue three times in 1X PBS for 5 minutes each wash. 15m

12 *The following incubations are done in 2.0mL Eppendorf tubes with ~500uL- 1mL of solution on a shaker at 40 rpm-100 rpm at 37C. Tissue should be completely covered with incubation solution.* 3d

Transfer tissue into Blocking Solution (4% Normal Donkey Serum in 0.5% PBS-T (0.5%TritonX100 in 1X Phosphate Buffered Saline with 0.05% Sodium Azide)) for 3 days at room temperature.

13 Incubate tissue in primary antibody diluted in Blocking Solution (4% Normal Donkey Serum in 0.5% PBS-T (0.5%Triton X-100 in Phosphate Buffered Saline with 0.05% Sodium Azide)) for 14 days at 37C. Be sure to place samples in air-tight sealed tubes to prevent contamination. Tubes are additionally sealed with Parafilm™. 2w

14 Wash tissue three times in 1X PBS with 0.05% Sodium Azide for 2 hours per wash and leave tissue in 1X PBS with 0.05% Sodium Azide overnight at room temperature on the rocker. 22h

15 Incubate in Secondary Antibody diluted in 1X PBS with 0.05% Sodium Azide for 3 days at 37C. 3d

16 Wash tissue three times in 1X PBS with 0.05% Sodium Azide for 2 hours per wash and leave tissue in 1X PBS with 0.05% Sodium Azide overnight at room temperature on the rocker. 22h

Dehydration and clearing

17 *The following incubations are done in 24-well plates with approximately 500ul of solution (with added liquid if needed to cover tissue. Incubations occur on a rocker (moderate speed) at room temperature.* 4h

Dehydrate tissue following methanol series: 50% methanol for 30 minutes, 70% methanol for 30 minutes, 80% methanol for 30 minutes, 95% methanol for 30 minutes, 100% methanol for 30 minutes, 100% methanol for 30 minutes, 100% methanol for 30 minutes.

18 Transfer tissue directly out of 100% methanol into Murray's Clear (2:1 Benzyl Benzoate: Benzyl Alcohol) before imaging. Tissue clearing will depend on the thickness of your specimen. 30m

19 Once the tissue has become completely transparent, mount on glass slides using Murray's Clear as your mounting media (if imagining on inverted Confocal microscope a coverslip can be taped at the edges of the slide to prevent it from falling off with only a minimal amount of Murray's Clear). 10m

Image aquisition

20 Images can be acquired using confocal or light sheet microscopy. We acquired images using an LSM 710 Confocal microscope. Details about our microscope inclue: 5h

-10x and 20x Plan-Apochromat objectives
-Zeiss Zen software (version 2.3 14.0.14.201))
-Z-axis increments were 4µm (10x objective) or 1µm (20x objective)
-Each image slice was 900x900 (10x) or 1200x1200 pixels (20x).
-10x Z-stacks were stitched to cover large regions.
-Laser-scanning operated under multi-track to sequentially acquire multi-channel images.
-Each channel used 100% laser power.
-Excitation/long-pass emission filters: Alexa Fluor® 647 (excitation: 633 nm, emission: 656-755-nm filter), Alexa Fluor® 594 (excitation: 561 nm, emission: 588-656-nm filter), Alexa Fluor® 488 (excitation: 488 nm, emission: 493-584-nm filter)

21 Z-stacks through the myenteric and submucosal plexus can be acquired using the 10X objective and the stitching feature on Zen software.

22 Detailed volumetric and quantification of neuronal subtypes can be obtained from high-resolution images using the 20X Objective. Due to the limited working distance allowed with this objective, the myenteric and submucosal plexus must be imaged independently by turning the tissue over.

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