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Imaging the gut with “CLARITY”

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TITLE:

Imaging the gut with "CLARITY"

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KEYWORDS:

CLARITY staining, mouse gut, neurons, EEC, glia, hydrogel embedding

SUMMARY:

We describe the protocol for passive CLARITY (PACT) staining of mouse intestine to enable visualization of subepithelial tissues, including neurons, glia and enteroendocrine cells (EEC) without tissue sectioning. The protocol involves hydrogel embedding of formaldehyde-fixed tissue, and subsequent delipidation using an anionic detergent to "clear" the tissue.

ABSTRACT:

CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging compatible Tissue hYdrogel) has recently evolved as a valuable technique involving acrylamide embedding to delipidate tissue (without sectioning) and to preserve the 3-D tissue structure for immunostaining. The technique is highly relevant in imaging the dynamic gut environment where different cell types interact during homeostasis and disease states. This method optimized for the mouse gut is described here, which helps to trace cell types like epithelia, enteroendocrine, neurons, glia, and the neuronal projections into the epithelia or enteroendocrine cells that mediate microbial sensing or nutrient chemo sensing respectively. The gut tissue (1-1.5 cm) is fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4 °C overnight on day 1. On day 2, PFA is discarded, and the tissue is washed thrice with PBS. The tissue is hydrogel embedded to preserve its integrity by incubation in 4% hydrogel (acrylamide) solution in PBS (diluted from 30% ProtoGel) overnight at 4 °C. On day 3, the tissue-hydrogel solution is incubated at 37 °C for 1 h to allow hydrogel polymerization. Tissue is then washed thrice gently with PBS to remove excess hydrogel. The subsequent step of delipidation (clearing) involves tissue incubation in sodium dodecyl sulfate (8% SDS in PBS) at 37 °C for 2 days (days 4 & 5) on a shaker at room temperature (RT). On day 6, the cleared tissue is thoroughly washed with PBS to remove SDS. Tissue can be immunostained by incubation in primary antibodies (diluted in 0.5% normal donkey serum in PBS containing 0.3% Triton X-100), overnight at 4 °C, and subsequent incubation in appropriate secondary Alexa Fluor antibodies for 1.5 h at RT, and nuclear staining with DAPI (1: 10000). The tissue is transferred onto a clean glass slide and mounted using VectaShield for confocal imaging.

INTRODUCTION:

CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging compatible Tissue hYdrogel) has recently evolved as a valuable technique involving acrylamide (hydrogel) embedding and tissue delipidation to preserve the 3-D tissue structure for immunostaining (without sectioning)[1,2]. The hydrogel-embedded tissue is optically transparent and macromolecule-permeable, with proteins and nucleic acids being preserved after removal of lipids by detergent. CLARITY was recognized as one among ten notable breakthroughs in 2013 by *Science*[3]. Although developed initially to image lipid-rich brain tissues where lipids affect light scattering and imaging quality, the technique is currently widely used for imaging other tissues like intestine, liver, kidney and heart. The technique offers the ability to stain and image a tissue by eliminating the need for sectioning, which otherwise might result in a biased evaluation of cell-cell interactions due to irregular distribution of cell types. The technique also provides the opportunity to perform confocal z-stacks and recreate 3-dimensional image of the tissue, which can help determine the densities, microarchitecture, and cell-cell interaction among various cell types more realistically than from a tissue section. Moreover, the technique has been modified to allow immunostaining of dense tissues like bone, as well as in situ hybridization studies. The hydrogel embedded 3D tissue offers an attractive platform for elucidating the interactions between epithelial, enteroendocrine, glial and neuronal cells, which have been recently cited to be crucial to the understanding of pathophysiology of diseases like Parkinson's Disease, Alzheimer's, Autism Spectrum Disorders etc[4].

PROTOCOL:

All animal experiments described were approved by the Emory University Committee on the Use and Care of Animals. C57BL/6 mice (both male and female can be used) at 8-12 weeks of age were allowed free access to the food and water prior to euthanasia.

1. Removal of intestine (Day 1)

1.1. Euthanize the mouse by carbon dioxide asphyxiation method, at a flowrate of 1.6 mL of carbon dioxide gas until cessation of breathing is observed for 2 min).

1.2. Place the mouse supine on a dissecting board with limbs pinned. Sterilize the abdomen with 70% ethanol. Using forceps, cut open the skin with scissors.

1.3. Lift the liver gently and identify the stomach. Then holding the stomach with forceps, cut off the esophagus just above the stomach. Holding the stomach with forceps, gently snip away the mesenteries and detach the intestine from the abdominal cavity up to the rectum. Cut off the detached intestine at the rectum.

1.4. Transfer the detached intestine into an ice-cold PBS solution in a Petri dish. Now gently snip off the mesentery between the ileal segments and straighten out the intestine so that starting from the stomach all the mesenteries are snipped off, up to the colon.

1.5. Cut 1-1.5 cm of the desired intestinal region for CLARITY staining into a new Petri dish containing ice-cold PBS.

1.6. Using a 5 mL syringe attached to a blunt end of a 20 G needle, flush out the fecal contents with ice cold PBS.

NOTE: Any region of the gut can be used for CLARITY staining using the same technique. The intestinal segment may be cut open along the mesentery or used intact for the subsequent steps.

2. Fixation of intestinal tissue (Day 1)

2.1. Fix the tissue in 4% paraformaldehyde (PFA) solution in PBS.

2.2. Transfer 1-1.5 cm piece of intestinal segment of interest into a 15 mL conical tube filled with 4% PFA at 4 °C overnight for fixation.

3. Hydrogel embedding (Day 2)

3.1. Transfer the intestinal segment from 4% PFA solution with forceps into a new 15 mL conical tube with PBS solution and wash three times (5 min each, on a shaker at 150-200 rpm) to remove any residual PFA.

3.2. Preparation of 4% hydrogel

3.2.1. Use the 30% gel solution (e.g., ProtoGel) to prepare the hydrogel. For preparing 4% hydrogel solution, dilute the 30% stock solution is diluted in PBS. To prepare 12 mL of 4% hydrogel, take 1.6 mL of 30% gel solution and add 10.4 mL of PBS.

3.3. Transfer the fixed tissue from step 3.1. to the 4% hydrogel solution in PBS in a 15 ml conical tube and incubated at 4 °C overnight.

NOTE: It is highly recommended that the tissue is washed free of PFA using PBS after overnight incubation. Tissue stored longer in PFA does not give good results with subsequent steps and staining. The fixed tissue after PBS washes may be stored at 4 °C for a week.

4. Hydrogel polymerization and delipidation (clearing) step (Day 3)

4.1. Take the conical tube containing the tissue in hydrogel solution out from 4 °C. Transfer to a 37 °C water bath and incubate for 1 h. This step allows for hydrogel polymerization.

4.2. After 1 h, take out the conical tube containing the hydrogel embedded tissue of the water bath, and pour off the acrylamide solution. Now gently rinse the tissue with a single PBS wash at room temperature to remove the excess hydrogel.

4.3. Delipidation step

4.3.1. Delipidate by incubating the tissue in sodium dodecyl sulfate (8% SDS) in PBS. Transfer the hydrogel embedded tissue from step 4.2 to an 8% SDS solution in a 50 mL conical tube. Make sure that the tissue is completely immersed in the SDS solution, and that the tube is capped.

4.3.2. Transfer the capped 50 mL tube containing the tissue in SDS onto a 37 °C shaker (200 rpm) at room temperature for 2 days (Days 4 & 5) to allow delipidation.

NOTE: Ensure that SDS solution is filled up to at least 20-25 mL in the 50 mL conical tube, to compensate for any evaporation that might occur at 37C during the 2 days of incubation.

4.4. On days 4 & 5, incubate the tissue in SDS at 37 °C shaker (200 rpm) at room temperature.

5. Washing off the detergent from the cleared tissue (Day 6)

5.1. Take the tissue from the SDS solution. The tissue will appear 'clear' or transparent.

5.2. Transfer the tissue to a new 50 mL comical tube with PBS solution and wash over the course of the day on a shaker (150-200 rpm) at room temperature with several changes of PBS, to ensure that all traces of SDS are removed. At least 10 changes of PBS solution will ensure that tissue is free off the SDS, and a good rule of thumb is to check if the final PBS wash has no traces of froth/ foam from the SDS. **This is a critical step as SDS can interfere with the subsequent staining steps.**

NOTE: At the end of the day, the cleared tissue after several washes in PBS is now ready for immunostaining or maybe stored at 4 °C for up to 3 weeks.

5.3. Storing cleared intestinal tissues for more than a month in PBS solution will affect the tissue integrity and quality of staining. Use the cleared intestinal tissue immediately or within 1-2 weeks of clearing.

6. Immunofluorescent staining for neurons, glia and enteroendocrine cells

6.1. Transfer the cleared tissue to a 1.5 or 2 mL tube and incubate in 500 µL volume of respective primary antibodies diluted in 0.5% normal donkey serum in PBS containing 0.3% Triton X-100) overnight at 4 °C. The antibodies (Ab) used: Tuj1 or β-3-tubulin (Pan neuronal marker, 1: 1000), Glial Fibrillary Acid Protein (GFAP, 1: 500), Chromogranin A (1: 250).

6.2. After overnight incubation, subject the tissue to 3 PBS washes (5 min each using 1-1.5 mL of PBS) to remove unbound primary Ab.

6.3. Transfer the tissue to a new tube, and subsequently incubate with 500 µL of appropriate

secondary Alexa Fluor Ab (AF) for 1.5 h at room temperature in the dark. The secondary Ab used are AF 488 for Tuj1 (1: 1000), AF-555 for GFAP (1: 500) and AF 555 (1 :250) for Chromogranin A.

6.4. After incubation, wash the tissue thrice with PBS as in Step 6.2.

6.5. For staining the nuclei, incubate the tissue in DAPI (1: 10000 dilution or 0.2 µL in 2 mL PBS) for 5 min at room temperature.

6.6. Finally, transfer the stained tissue onto a clean glass slide, add coverslip on top of the tissue and mount using 100 µL of VectaShield for confocal imaging.

6.7. Place the slides in the dark at room temperature in a slide holder for 30 min for drying, after which they can be imaged or stored at -20 °C. Mouse gut wall is fairly thin and once opened along the mesentery, it can be mounted on glass slides directly with coverslip. If working with larger tissues like spleen, kidneys, chamber (cavity) slides or spacers may be used. For bigger tissues like, a cavity slide may be used for mounting.

NOTE: It is to be noted that if the intestinal segment was not cut open at the beginning step (refer to Note in Step 1), it is advisable to cut open the intestinal tube with micro scissors that will facilitate the steps involving mounting.

7. Confocal Imaging

NOTE: The cleared, stained and mounted tissue can be imaged immediately or can be stored in slide boxes at -20 °C.

7.1. Image using a confocal microscope (e.g., Olympus FV1000).

7.2. Take z-stacks across 50 µm thickness of the gut tissue to visualize the regions from the gut lumen toward the serosal layer.

7.3. Store the z stack images as image files, .avi movie files or convert into 3D image with the help of the software[5].

REPRESENTATIVE RESULTS:

The images from the CLARITY-cleared mouse gut tissue are represented in **Figure 1**. A successful completion of the protocol yields high quality, crisp images where all cellular details can be visualized clearly. The DAPI staining for nuclei is a very good index to assess the quality of the CLARITY protocol and the subsequent immunostaining as it can depict the tissue integrity. Further, the cell shape also provides a clue as to how the protocol has been successful especially in case of neuronal, glial and enteroendocrine cells with a distinct cellular morphology. A distorted cell with scanty/ aberrant staining shows that tissue preparation has been compromised.

The mouse ileum stained with pan neuronal marker Tuj1 (β-3-tubulin, red) and co-stained with glial marker Glial Fibrillary Acid Protein (GFAP, green) is shown in **Figure 1A**. A 3D image of mouse colon stained with neuronal marker Tuj1 (red) generated from the z-stack using the imaging software is shown in **Figure 1B**. The staining for enteroendocrine cells producing Chromogranin A in mouse colon is shown in **Figure 1C**. In all images, blue represents the nuclear stain with DAPI. **Figure 1A** and **Figure 1B** have been captured at 40x magnification and, **Figure 1C** at 60x. Co-staining as represented in **Figure 1A** can be used to assess co-localization of various markers of interest like receptors on specific cell types, immune cells, etc. or nuclear co-localization with DAPI. 3D images can be made use of to compare cell densities (for example myenteric neuronal density) between two experimental conditions or a wildtype and knockout mouse strain etc. The density of neuronal projections can also be assessed in 3D images. Changes in enteroendocrine cell populations with receptors of interest in normal and pathophysiological conditions can be evaluated by staining for various markers like Chromogranin as shown in **Figure 1C**. In addition, changes in intensity and/or localization of various tight junction proteins that maintain epithelial barrier integrity can be assessed via CLARITY staining under normal or inflammatory conditions. Cleared splenic tissues maybe stained for specific immune cell types based on the research interests involving inflammatory mouse models.

Thus, without the time-consuming sectioning steps, and antigen retrieval processes, the tissue can be visualized in much higher quality and details across its entire thickness via z stacks, and can provide the details in 3D. The technique also has the advantage that it can be also used for staining tissues from saline-perfused animals.

Figure 1: Representative immunostaining of cleared intestinal tissues. Confocal images (enface) of **(A)** Cleared mouse ileum stained with neuronal marker Tuj1 (β-3-tubulin) and glial marker Glial Fibrillary Acid Protein (GFAP), with nuclear stain DAPI and **(B)** 3D image generated from z stacks of mouse colon stained with Tuj1 **(C)** Cleared mouse colon stained with Chromogranin A and DAPI.

DISCUSSION:

The CLARITY method is highly useful for staining mouse gut to visualize various cell types including epithelia, neurons, and glial cells in 3D, especially the network of neuronal projections that extend across the gut wall to the lumen[5] and their innervation to glial and EEC cells. The method presented here was modified according to the original study by Yang et al. 2014[1].

The critical steps in the protocol include immediate rinsing off the fixative after overnight fixation of the gut tissue, subsequent hydrogel embedding and delipidation. The PBS washes following delipidation are crucial to remove the residual detergent used in delipidation step.

The CLARITY staining is superior in that it allows for tissue imaging preserving the tissue structure, proteins and nucleic acids, without sectioning, provides excellent quality images even in perfused tissues, and 3D evaluation of tissue micro architecture and cellular interactions like neuron-glia, EEC-glia etc. It is also observed that CLARITY stained slides retain good fluorescence even after storage at -20 °C, and hence can be imaged later if needed.

Furthermore, several modifications of the CLARITY technique have enabled it suitable for in situ hybridization studies. For example, modification of the process using carbodiimide based fixation can help preserve integrity of nucleic acids (RNA) and subsequent quantification[6]. A new method called 3D imaging of solvent-cleared organs (3DISCO) have been specifically developed in 2012 for clearing mouse brain and spinal cord tissues, and whole body of *Drosophila melanogaster* (fruit fly). 3DISCO involves para formaldehyde fixation of tissues, followed by serial dehydration steps using 50-100% of tetrahydrofuran in water, followed by lipid extraction with dichloromethane, and final immersion in dibenzyl ether for refractive index matching. The 3DISCO protocol is best suited for fixed tissues with strong fluorophores like green fluorescent protein in transgenic models. Several modifications of the 3DISCO protocol like iDISCO (immunolabeling-enabled imaging of solvent-cleared organs) and uDISCO (ultimate labelling of solvent cleared organs) have been perfected to allow for immunostaining before clearing (iDISCO) and to preserve fluorescence (uDISCO). CUBIC is another clearing method developed in 2014 where in addition to lipids, iron-based light absorbing chromophores are also removed from tissues. A comparison of various tissues and the recommended clearing process is detailed in an elegant review by Muntifering et al. (2018)[7].

CLARITY is also an excellent tool to measure and compare cell densities (neurons, immune cells, etc.) during normal and pathological conditions. For example, host-pathogen interactions or inflammation characterized by immune cell infiltration into gut tissues and ganglia can be quantified by CLARITY staining and is superior to enumerating cell numbers from images obtained from tissue sections[8]. Similarly, quantifying neuronal and glial cell densities in sub mucosal and myenteric plexi is possible by CLARITY staining as we do not have specific markers to differentiate between sub mucosal versus myenteric neuronal or glia populations[5].

The limitations involve a comparatively longer time that is required to delipidate or clear the tissue, which requires 5 days for the intestinal tissue. In addition to neurons, glia and EEC, cleared tissue can also stained for various immune cell types in the intestine, and other tissues like liver, spleen and kidney. The delipidation step would have to modified accordingly to clear these tissues, as larger tissues like liver would need more delipidation time to clear (3-5 days).

CLARITY staining is a valuable tool to image the gut environment focusing on epithelial, enteroendocrine, neuronal, glial and immune cells, measuring cell densities (neuronal, immune or glial cell densities) and cell-cell interaction under homeostasis and pathological conditions. As the interactions between immune-neuron, immune-glia, neuron-glia and glia-EEC have been highly implicated in the pathophysiology of brain-gut disorders like Parkinson's disease, Alzheimer's and autism spectrum disorders[4], CLARITY would be an excellent tool to image the dynamic gut environment and correlate the gut characteristics to progressing brain pathology. In addition, CLARITY staining for specific cell types could also be used as a marker or index of disease pathology. For example, the accumulation of aberrant proteins like synuclein in the gut and changes in gut motility maybe correlated to the development of brain pathology and changes in cognitive behavior.

Thus, CLARITY offers unlimited imaging possibilities in intact tissue in 3D without the bias of interpretation that may result from tissue sectioning in the context of uneven distribution of cell types. Furthermore, numerous variations of tissue clearing method like three-dimensional imaging of solvent-cleared organs (3DISCO) have evolved utilizing organic solvents[9,10]. More over CLARITY method has been extensively modified to incorporate carbodiimide-based chemistry to stably retain RNAs in clarified tissue so as to enhance the tissue integrity and RNA stability to offer more possibilities for in situ hybridization in the cleared tissue, which could be exploited in analysis of microRNAs relevant to different disease pathologies[6]. The method can also be suitably adapted for staining other mouse tissues like brain[1], skeletal muscle[11], ovaries[12],and other tissues of research interest like human biopsy tissue[13], zebra fish[8] and *Drosophila*, that are also key research models in addition to the murine model.

ACKNOWLEDGMENTS:

The authors wish to acknowledge support from American Gastroenterology Association (AGA) AGA-Rome Functional GI motility Disorders Pilot Research Award (to BC), U.S. National Institutes of Health grant AI64462 (ASN) and the Emory University Integrated Cellular Imaging Microscopy Core.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Yang, B. et al. Single-cell phenotyping within transparent intact tissue through whole-body clearing. *Cell*. **158**, 945-958, doi:10.1016/j.cell.2014.07.017 (2014).

2 Chung, K. et al. Structural and molecular interrogation of intact biological systems. *Nature*. **497**, 332-337, doi:10.1038/nature12107 (2013).

3 2013 Runners-Up. CLARITY makes it perfectly clear. *Science*. **342**, 1434-1435, doi:10.1126/science.342.6165.1434-b (2013).

4 Fung, T. C. The microbiota-immune axis as a central mediator of gut-brain communication. Neurobiology of Disease. **136**, 104714, doi:10.1016/j.nbd.2019.104714 (2020).

5 Chandrasekharan, B. et al. Interactions Between Commensal Bacteria and Enteric Neurons, via FPR1 Induction of ROS, Increase Gastrointestinal Motility in Mice. *Gastroenterology*. doi:10.1053/j.gastro.2019.03.045 (2019).

6 Sylwestrak, E. L., Rajasethupathy, P., Wright, M. A., Jaffe, A. & Deisseroth, K. Multiplexed Intact-Tissue Transcriptional Analysis at Cellular Resolution. *Cell*. **164**, 792-804, doi:10.1016/j.cell.2016.01.038 (2016).

7 Muntifering, M. et al. Clearing for Deep Tissue Imaging. *Current Protocols in Cytometry*. **86**, e38, doi:10.1002/cpcy.38 (2018).

8 Cronan, M. R. et al. CLARITY and PACT-based imaging of adult zebrafish and mouse for whole-animal analysis of infections. *Disease Models & Mechanisms*. **8**, 1643-1650, doi:10.1242/dmm.021394 (2015).

9 Erturk, A. et al. Three-dimensional imaging of solvent-cleared organs using 3DISCO. *Nature Protocols*. **7**, 1983-1995, doi:10.1038/nprot.2012.119 (2012).

10 Qi, Y. et al. FDISCO: Advanced solvent-based clearing method for imaging whole organs.

Science Advances. **5**, eaau8355, doi:10.1126/sciadv.aau8355 (2019).
11 Milgroom, A. & Ralston, E. Clearing skeletal muscle with CLARITY for light microscopy imaging. *Cell Biology International* **40**, 478-483, doi:10.1002/cbin.10578 (2016).
12 Hu, W., Tamadon, A., Hsueh, A. J. W. & Feng, Y. Three-dimensional Reconstruction of the Vascular Architecture of the Passive CLARITY-cleared Mouse Ovary. *Journal of Visualized Experiments*. doi:10.3791/56141 (2017).
13 Chen, Y. et al. Three-dimensional imaging and quantitative analysis in CLARITY processed breast cancer tissues. *Scientific Reports*. **9**, 5624, doi:10.1038/s41598-019-41957-w (2019).

Figure 1A

Click here to access/download;Figure;Figure 1A.tif

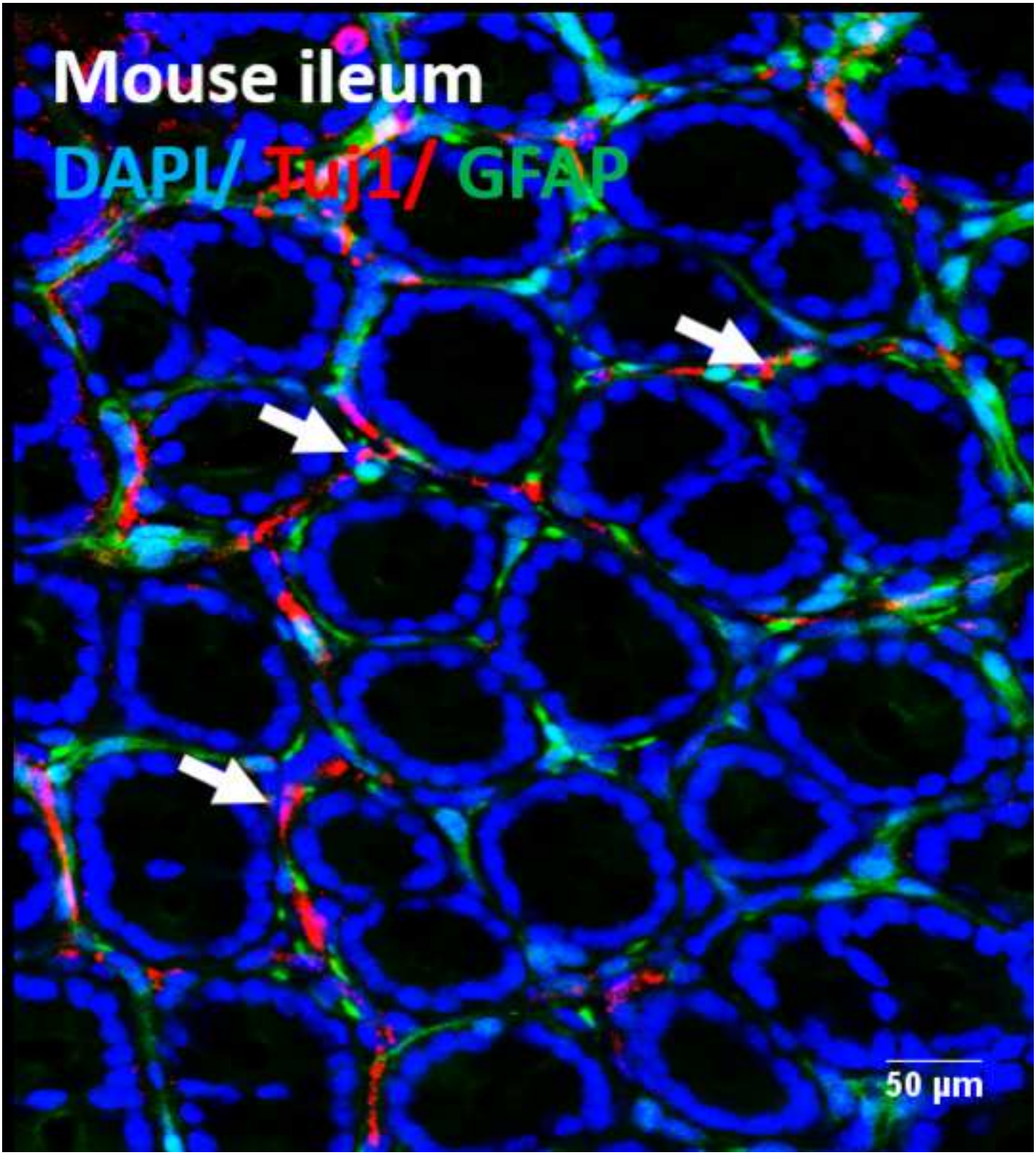

Figure 1B

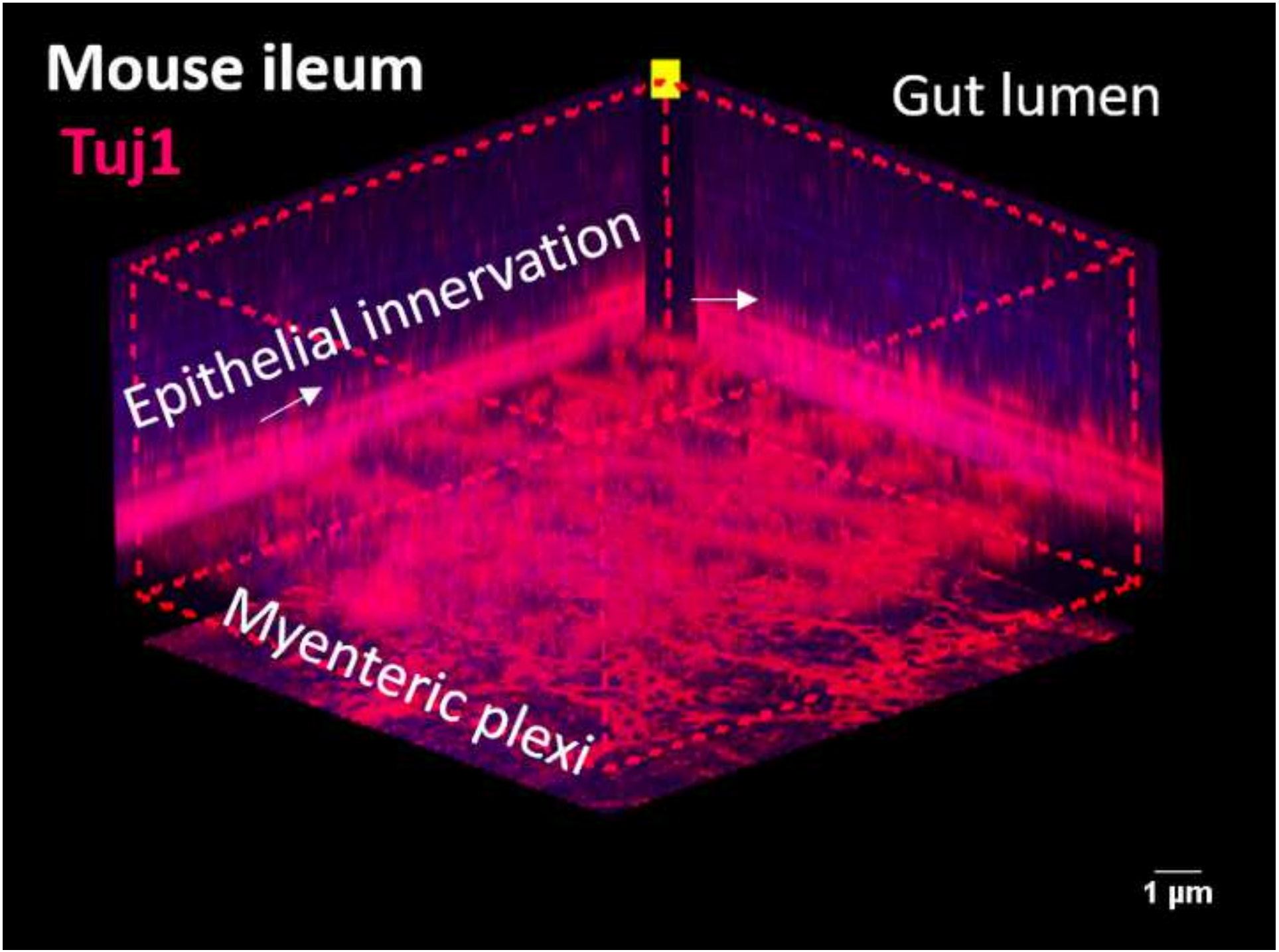

Figure 1C

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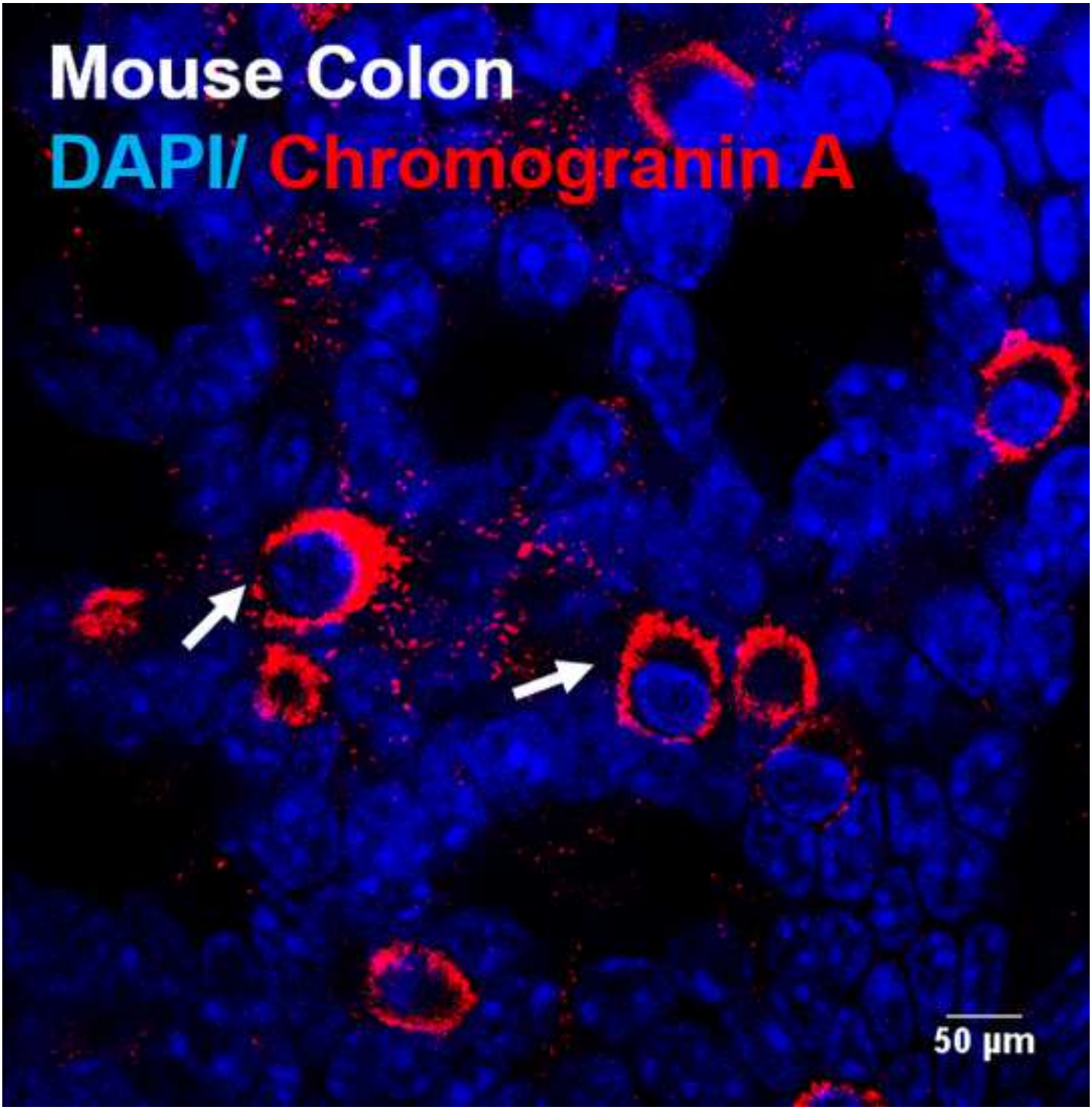

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number
4% paraformaldehyde (PFA) in PBS	ThermoFischer Scientific	J19943
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	ThermoFischer Scientific	D1306
Alexa Fluor-488	ThermoFischer Scientific	A-11034
Alexa Fluor-555	ThermoFischer Scientific	A-21428
Anti- GFAP (Glial Fibrillary Acid Protein)	Abcam	ab7260
Anti-beta III Tubulin (or Tuj1)	Abcam	ab18207
Chromogranin A	Abcam	ab15160
ProtoGel 30%	National Diagnostics	EC-890
Sodium dodecyl sulfate	ThermoFischer Scientific	28364
Vectashield mounting medium	Vector Laboratories	H-1000

Comments/Description
Used for tissue fixation
1/10,000 dilution
1/1000 dilution
1/500 dilution
1/500 dilution
1/1000 dilution
1/250 dilution
Toxic, hazardous, handle with care- make 4% solution in PBS for use
8% in PBS

We thank the Reviewers for their thoughtful critiques, comments and suggestions that we believe has helped in significantly improving and revising the manuscript.

The rebuttal to reviewers comments is marked in red, and changes have been made in the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe the use of Clarity, an optical tissue clearing method, to clear the mouse gut, to perform immunohistochemistry and to acquire 3D image with confocal microscopy.

Minor Concerns:

There are other articles describing Clarity's use for clearing mouse intestine (BMC Dev Biol. 2014 Dec 21;14:48. doi: 10.1186/s12861-014-0048-3).

In the discussion, other methods used for clearing the gut may be discussed, like CUBIC.- We have now discussed other methods like CUBIC, 3DISCO etc (Lines 231-246**).**

Some technical details are missing:

Step 5: The temperature of the washes with PBS is not mentioned- room temperature.

Step 6: It's not specified if the antibodies and DAPI incubation are performed in a shaker or resting conditions. resting

In Figure 1, the magnification bar is missing and the magnification objective used for acquiring the images is not mentioned in the text. Added scale bar. 40x

Some pictures of the uncleared and clear gut after clarity is missing. Now added

Some tips and troubleshooting may be introduced and it would help to the clearing process success.

Reviewer #2:

Manuscript Summary:

This manuscript nicely describes the technique of processing intestinal tissue using CLARITY for immunostaining. The benefit of this technique is that, unlike standard tissue processing, sectioning is not necessary with CLARITY, therefore preserving 3D tissue architecture. In addition, the technique is easy to perform and requires standard lab equipment. The major limitation is the extended time needed for delipidation, but is lessened when one considers the time normally involved with sectioning.

Major Concerns:

None

Minor Concerns:

Some clarifications to the protocol include:

To clarify step 4.2, is the tissue removed from the hydrogel with forceps and placed into a new conical tube containing PBS to remove the excess hydrogel? Is it washed one time or

like in step 3.1 three times with shaking? – rinsing off excess hydrogel gently
For step 5.2 can the authors provide a range for the number of PBS changes needed since this is critical? At least 3-5 washes, to remove SDS completely, PBS is clear and not frothy
No blocking step is needed between steps 5.3 and 6? Works fine without a separate blocking step as primary antibody is diluted in blocking reagent (1.5 % NDS/ overnight)
In step 6.1, the primary antibody incubation occurs in a conical tube? In steps 6.2 and 6.4, the PBS washes are with shaking and again in fresh conical tubes? In step 6.3, the secondary antibody incubation is in a conical tube? Corrected to Eppendorf tube (1.5.mL) for antibody incubations.

Reviewer #3:
Manuscript Summary:
Nicely written, clear, straightforward protocol by Authors who describe a state of the art method - Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging compatible Tissue hYdrogel or CLARITY - to preserve the 3D tissue structure for immunostaining (without sectioning); a technique that is highly relevant imaging the dynamic gut environment where different cell types interact during homeostasis and disease states. A few minor suggestions.

Major Concerns:
none

Minor Concerns:
Abstract:
- Enteroendocrine cells mentioned twice "We have optimized …. " is this because they have two roles - microbial sensing and nutrient sensing? This sensing may benefit from some revision - also are you looking at epithelial cells or sub-epithelial cells or both? Summary statement sates ",…visualization of subepithelial tissues".- we can visualize epithelia, subepithelial cells, EEC , Enteric neurons, glia
- 4 0 C overnight needs formatting in this version of the abstract- formatted

Title: nice title
Summary: are you looking at epithelium or subepithelial tissues or both- we can visualize epithelia, subepithelial cells, EEC and enteric glia , enteric neurons
Abstract: see comments above; 4 degree formatting not necessary here
Introduction:
- Line 53: " … the technique currently widely used …" the word IS is missing-corrected.

- Line 64: " … Alzheimer's, Autism Spectrum Disorders etc. (ref) " the reference is missing
Protocol:
- Line 86: Does the note "NOTE: Any region of the gut can be used for clarity staining using the same technique" apply only to mouse gut? Or can it be used for human tissue e.g. biopsies or surgical specimens as well? You mention human biopsy tissue in Discussion line 233. If only mouse, please revise "any region of the mouse gut …." any region of mouse gut.
- Figure 1 - can you label A, B, C? Would it be worthwhile to show how method is an

improvement to current immunostaining techniques - e.g. how does Figure 1 A and 1 C compare to traditional immunostaining/confocal imaging; you describe improvements in Lines 191-196 of discussion. With same antibody dilutions we use for regular immunostaining, we can get better clarity of image, z stacks and 3D reconstruction possible, no need to section tissues/ antigen retrieval steps etc compared to immunostaining.
Discussion:
- I realize focus is on immune-neuron interactions; but are there any information on myofibroblasts lining the crypts? Has immunostaining for myofibroblasts been done using this technique? No references so far.
- Does "Clarity" need to be capitalized - would be consistent either way e.g. line 224 vs line 228 – have uniformly used the capitalized version in "CLARITY".

Reviewer #4:
Manuscript Summary:
The manuscript by Chandrasekharan and Neish describes a modified CLARITY protocol for optical clearing and immunostaining of mouse intestinal tissue. The modifications appear to be based on the original CLARITY protocol by Chung et al. (2013) and the PACT variant by Yang et al. (2014). The manuscript provides a clear protocol. The subject matter is timely and significant.

Major Concerns:
Abstract: The authors state: "The subsequent step of delipidation (clearing) involves tissue incubation in sodium dodecyl sulfate (8% in PBS) at 37C for 2 days (days 4 & 5) on a shaker at room temperature (RT)." There seems to be conflicting instruction as to the appropriate temperature for incubation. Is it 37C or is it room temperature? 37C

Sacrifice is performed by carbon dioxide asphyxiation. Did the authors attempt transcardial perfusion, and, if so, does perfusion improve the quality of tissue preservation and immunostaining? It would be helpful if the authors could comment on this. Have cleared mouse gut after PBS perfusion, works fine, similar results compared to non-perfused tissue.

In the original CLARITY protocol by Chung et al. (2013), hydrogel polymerization was facilitated by replacing the air in the conical tube with nitrogen (as oxygen may impede hydrogel formation). The protocol by Chandrasekharan and Neish does not call for this nitrogen replacement step. Can the authors comment on this difference? Here we are using acrylamide solution that has already been degassed.

Step 4.3 Delipidation step. The authors state: "Transfer the capped 50ml tube containing the tissue in SDS on a 37C shaker (200 rpm) at room temperature for 2 days (Days 4 & 5) to allow delipidation." Perhaps I am mis-reading the instruction, but I am still confused as to the appropriate temperature. 37°C

Step 4.4 Same comment as above regarding appropriate temperature.
Step 6.6 Most optical clearing protocols require the construction or use of a small chamber

assembled on the slide (to accommodate the relatively large tissue sample). I would be very interested if the authors could expand on their procedure for mounting the stained tissue. If there is no chamber and no use of spacers, does the tissue not become deformed or "squished" by the cover slip? More explanation on mounting would definitely be appreciated. Mouse gut wall is fairly thin and once opened along the mesentery, it can be mounted on glass slides with coverslip directly, if working with larger tissues like stomach/ cecum, chamber slides/ spacers may be used. Have now added this in the mounting section.

CLARITY is known to produce tissue expansion of the hydrogel-embedded sample, especially during delipidation at higher temperatures like 37C. Did the authors observe such expansion and can they comment on it? With mouse gut tissue there is negligible/ no expansion, but there is some degree of swelling/ expansion when tissues like mouse brain and liver are cleared.

Minor Concerns:
Introduction: Last sentence requires a reference. Reference is added (Line 64)
Discussion line 196 requires references. References added (Lines 282-284),