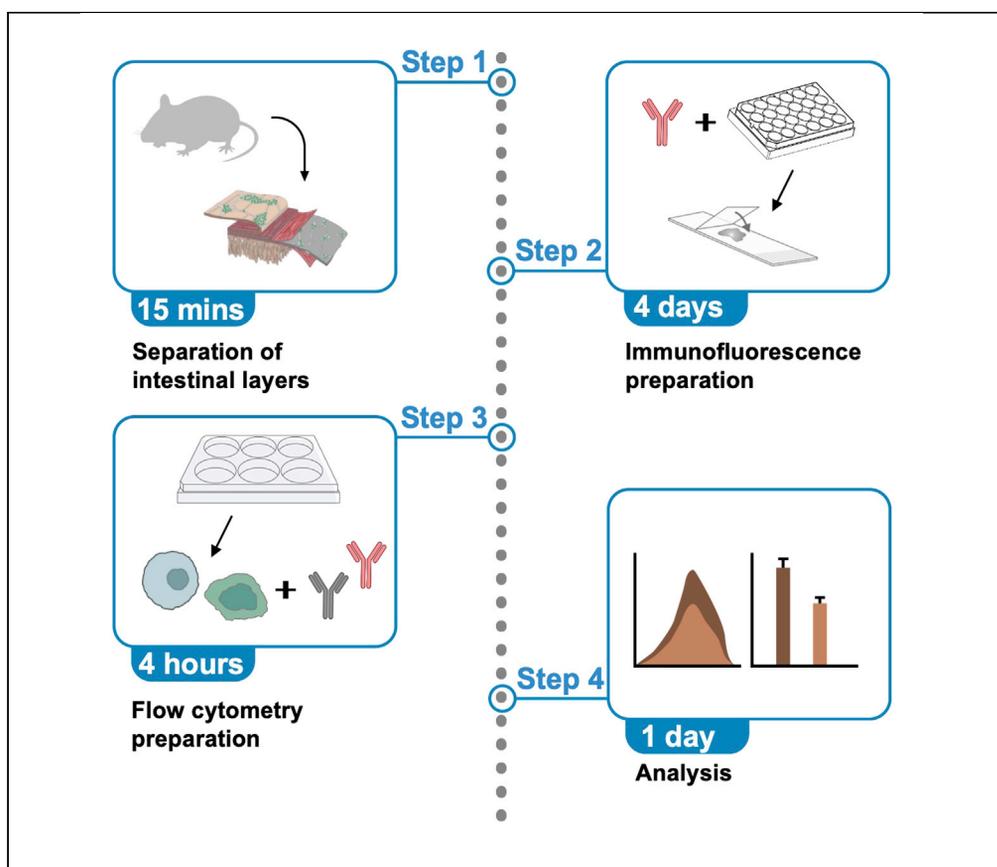


## Protocol

# Isolation of myenteric and submucosal plexus from mouse gastrointestinal tract and subsequent flow cytometry and immunofluorescence



The myenteric plexus is located between the longitudinal and circular layers of muscularis externa in the gastrointestinal tract. It contains a large network of enteric neurons that form the enteric nervous system (ENS) and control intestinal functions, such as motility and nutrient sensing. This protocol describes the method for physical separation (peeling) of muscularis and submucosal layers of the mouse intestine. Subsequently, the intestinal layers are then processed for flow cytometry and/or immunofluorescence analysis.

Tomasz Ahrends,  
Molly Weiner, Daniel  
Mucida

tahrends@rockefeller.edu  
(T.A.)  
mucida@rockefeller.edu  
(D.M.)

### Highlights

We describe the method for physical separation of mouse intestinal layers

Flow cytometry analysis of immune cell subsets in myenteric and submucosal plexus

Immunofluorescence analysis of enteric nervous system in myenteric and submucosal plexus

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## Protocol

## Isolation of myenteric and submucosal plexus from mouse gastrointestinal tract and subsequent flow cytometry and immunofluorescence

Tomasz Ahrends,<sup>1,3,\*</sup> Molly Weiner,<sup>1</sup> and Daniel Mucida<sup>1,2,4,\*</sup><sup>1</sup>Laboratory of Mucosal Immunology, The Rockefeller University, New York, NY, USA<sup>2</sup>Howard Hughes Medical Institute, The Rockefeller University, New York, NY, USA<sup>3</sup>Technical contact<sup>4</sup>Lead contact\*Correspondence: [tahrends@rockefeller.edu](mailto:tahrends@rockefeller.edu) (T.A.), [mucida@rockefeller.edu](mailto:mucida@rockefeller.edu) (D.M.)  
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## SUMMARY

The myenteric plexus is located between the longitudinal and circular layers of muscularis externa in the gastrointestinal tract. It contains a large network of enteric neurons that form the enteric nervous system (ENS) and control intestinal functions, such as motility and nutrient sensing. This protocol describes the method for physical separation (peeling) of muscularis and submucosal layers of the mouse intestine. Subsequently, the intestinal layers are then processed for flow cytometry and/or immunofluorescence analysis.

For complete details on the use and execution of this profile, please refer to Ahrends et al. (2021).

## BEFORE YOU BEGIN

The protocol below describes the specific steps for the isolation and analysis of mouse distal small intestine (ileum). However, we have also used this exact protocol for proximal small intestine (duodenum) and large intestine (colon). The protocol focuses on the analysis of steady-state myenteric plexus, but we have also used it to describe changes in enteric neuron numbers and muscularis macrophage phenotype following enteric infection and microbiome manipulation.

Animal studies must be approved by an Institutional Animal Care and Use Committee (IACUC) and performed in accordance with IACUC guidelines.

## Mice

Mouse strain selection will depend on the experiment. We have extensively analyzed 7–12 week old C57BL/6J mice, as well as various transgenic strains.

## Prepare Sylgard plates

⌚ Timing: 48 h

Sylgard-coated plates are used to pin the intestinal layers for fixation following their separation.

1. Mix 10 parts Sylgard Base with 1 part Sylgard Curing Agent.
2. Add 2 g of Charcoal Powder and mix thoroughly with a tongue depressor.
3. Pour into Single Well plate, fill 1/3 of the well depth and set in a level, dust-free location to solidify for 48 h at 20°C–25°C.



## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
CD64-APC (Clone X54-5/7.1)	BioLegend	cat. # 13906
CD11b-FITC (Clone M1/70)	BD	cat. # 553310
CD206-AF700 (Clone MMR)	BioLegend	cat. # 141732
Arg1-PE-Cy7 (Clone A1exF5)	Thermo Fisher Scientific	cat. # 25-36-97-82
CD45.2-PerCP-Cy5.5 (Clone 104)	BD	cat. # 552950
Human polyclonal anti-Hu (ANNA-1)	Gift of V. Lennon	N/A
Goat anti-human IgG (H+L)-AF568	Thermo Fisher Scientific	cat. # A-21090
<b>Chemicals, peptides, and recombinant proteins</b>		
LIVE/DEAD Fixable Near-IR Cell Stain Kit	Thermo Fisher Scientific	cat. # L34975
HBSS, calcium, magnesium	GIBCO	cat. # 24020117
Dulbecco's Phosphate Buffered Saline (PBS)	Corning	cat. # 21040CV
Fetal Bovine Serum (FBS)	Sigma-Aldrich	cat. # F0926-500ML
Collagenase D	Thermo Fisher Scientific	cat. # 50-100-3282
Dispase	Corning	cat. # CB40235
HEPES 1M	Invitrogen	cat. # 15630-080
Triton X-100	Alfa Aesar	cat. # A16046
Normal Goat Serum	Jackson ImmunoResearch	cat. # 005-000-121
Fluoromount-G	SouthernBiotech	cat. # 0100-01
16% Paraformaldehyde (PFA)	Electron Microscopy Sciences	Cat. # 15714-S
Sodium Pyruvate (NaPyr) (100mM)	Invitrogen	cat. # 11360070
Bovine Serum Albumin (BSA)	Jackson ImmunoResearch	Cat. # 001-000-162
Sylgard 184 Silicone Elastomer	Electron Microscopy Sciences	Cat. # 24236-10
Activated charcoal powder	Sigma-Aldrich	Cat. # 05105-250G
DNase I	Roche	cat. # 04536282001
<b>Critical commercial assays</b>		
eBioscience Foxp3 / Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	cat. # 00-5523-00
<b>Experimental models: Organisms/strains</b>		
Mouse: C57BL6/J	The Jackson Laboratory	#000664
<b>Software and algorithms</b>		
Fiji	ImageJ	<a href="https://imagej.net">https://imagej.net</a>
FlowJo	Tree Star	RRID:SCR_008520
<b>Other</b>		
Microscope Slides Fisherbrand Superfrost Plus White	Fisher Scientific	Cat. # 12-550-15
Rectangular Coverslips 40 × 22mm	Thermo Fisher Scientific	Cat. # 2980-224
Tongue Depressors	Thermo Fisher Scientific	cat. # 23-400-121
Minutien Pins, 0.22mm diameter	Roboz Surgical Instruments	cat. # RS-6083-20
Nunc OmniTray Single-Well Plates with Lids	Thermo Scientific	Cat. # 242811

## MATERIALS AND EQUIPMENT

<b>Digestion Buffer</b>		
Reagent	Final concentration	Amount
HBSS Ma2+/Ca2+ + 5% FCS		7.6 mL
Collagenase D	400 U/mL	1 mL
Dispase	2.5 U/mL	500 μL
HEPES	25 mM	250 μL
NaPyr	1 mM	100 μL
DNase I	50 μg/mL	50 μL
<b>Total</b>		<b>10 mL</b>

Store at 4°C for on ice and use on the same day.

### FACS Buffer

Reagent	Final concentration	Amount
FCS	2%	1 mL
PBS		49 mL
<b>Total</b>		<b>50 mL</b>

Store at 4°C for up to 1 week.

### 4% Paraformaldehyde (PFA) solution

Reagent	Final concentration	Amount
16% PFA solution	4%	12.5 mL
PBS		37.5 mL
<b>Total</b>		<b>50 mL</b>

Store at 4°C up to 1 month.

### Permeabilization Buffer

Reagent	Final concentration	Amount
Triton-X	0.2%	2 mL
PBS		998 mL
<b>Total</b>		<b>1000 mL</b>

Store at 20°C–25°C for up to 1 year.

### Blocking Buffer

Reagent	Final concentration	Amount
Permeabilization Buffer		45.2 mL
BSA (Bovine Serum Albumin)	5%	2.4 g
Normal Goat Serum	5%	2.4 mL
<b>Total</b>		<b>50 mL</b>

Store at 4°C for up to 2 weeks.

**Note:** Depending on the antibodies used for immunofluorescent staining, the source of serum should be adjusted – e.g., if secondary donkey antibodies are used, prepare blocking buffer with normal donkey serum.

## STEP-BY-STEP METHOD DETAILS

### Separation of mouse intestinal layers

⌚ Timing: 15 min

1. Separation of myenteric and submucosal plexus.
  - a. Euthanize mouse by cervical dislocation or other approved method.
  - b. Lay euthanized mouse on its back on a cold surface (e.g., metal dissection block placed on ice); spray abdominal area with 70% ethanol. Lift the skin of the lower abdomen with tweezers and make a V-shaped incision with scissors; cut away skin to expose abdominal cavity.
  - c. Locate the beginning of the small intestine at the base of the stomach; cut the beginning of the small intestine from the stomach and carefully pull out from abdominal cavity, allowing mesenteric fat to peel away from intestine as it is pulled, and gently removing as much remaining fat as possible with tweezers.

- d. Divide the small intestine into four equal pieces; collect the ileum, which is the most distal fourth, approximately 6–7 cm of small intestinal tissue proximal to the beginning of the cecum).

**Note:** To account for variation in neuronal density in different intestinal segments, analysis should always be performed with the same part of the small intestine (e.g., last 3 cm of the ileum).

- e. Insert scissors into intestinal opening; carefully pull intestine over scissor blade; make a straight cut to the right of the mesenteric attachment over the length of the tissue until intestine is fully opened (pulling the entire length of tissue onto the scissor blade allows fewer cuts and enables a straighter cut, which prevents tearing of the tissue during the layer separation).
- f. Locate the outside layer of the intestine (the smooth side with mesenteric attachment, as seen in [Methods video S1](#) at 00:30 min and [Figure 1](#)); grip with tweezers and vigorously shake in a petri dish filled with PBS on ice to wash.
- g. Place the intestine on a cold surface (e.g., metal dissection block) with the muscularis (outer) layer facing up (luminal side facing down). Use small, damp paint brushes to completely flatten the tissue, making sure the sides don't fold under (See [Methods video S1](#) at 00:06 min).
- h. With one pair of tweezers, grip the intestine at the beginning of the tissue segment, and use a second pair to gently make a dent in the tissue directly below the pair of gripping tweezers, making sure not to pierce through the entire thickness of the tissue (as seen in [Methods video S1](#) at 00:45 min); carefully stroke downwards on the tissue from the indentation with second pair of tweezers until you see the layers separating, and the muscularis rolling away. Gradually adjusting both tweezers down the length of the tissue, keep scraping the muscularis layer away until you reach the end of the length tissue. See [Methods video S1](#).

**Note:** Depending on the experimental question, submucosal layer (tissue remaining after muscularis isolation) of the intestine can be similarly analyzed by flow cytometry or microscopy.

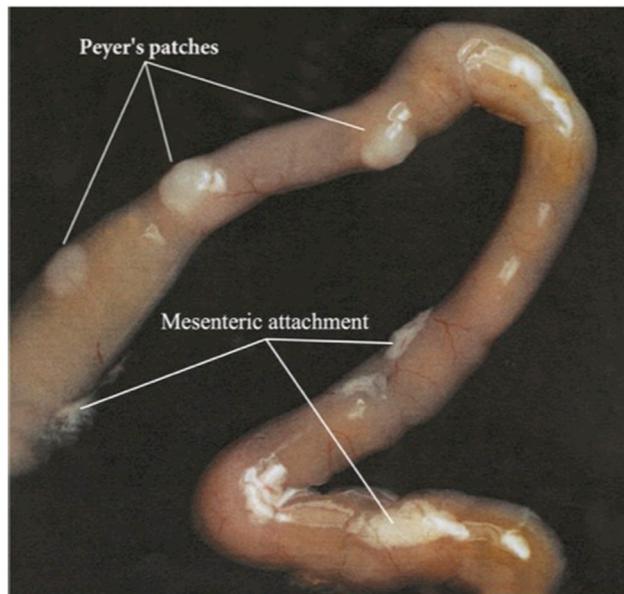
- i. Store tissue in PBS on ice until all animals are processed.
2. Depending on the desired analysis, immediately continue with “[flow cytometry preparation](#)” or “[immunofluorescence preparation](#)”.

**Note:** Flow cytometry and immunofluorescent analysis can be performed together for each sample. Use 1/3 of the tissue for imaging and 2/3 for flow cytometry. Make sure to use the same part of the tissue for each type of analysis (e.g., lower ileum for imaging and upper ileum for flow cytometry).

### Immunofluorescence preparation

⌚ **Timing: 4 days**

3. Place tissue on a Sylgard-coated plate; stretch and flatten using damp paint brushes, avoiding any wrinkles or folds, and pin down with Minutien pins. See [Figure 2](#).
4. Cover the pinned tissue with 4% PFA solution and keep at 4°C for 16 h.
5. Discard PFA solution and transfer tissue to a 24-well plate filled with PBS. Wash 4 times for 15 min per wash at 20°C–25°C on an orbital shaker, transferring tissue to a fresh well of PBS after each wash.
6. Transfer tissue to 24-well plate filled with Permeabilization Buffer. Incubate at 20°C–25°C on an orbital shaker for 2 h, moving the tissue to a second PBS-filled well after 1 h.



**Figure 1. Peyer's patches and mesenteric attachment visible on the muscularis layer of the mouse small intestine**

**▮▮ Pause Point:** Tissue can be kept in Permeabilization Buffer at 4°C for up to 1 week. For longer storage (2 months) secure the plate with parafilm and keep tissue in Permeabilization Buffer supplemented with 0.05% sodium azide.

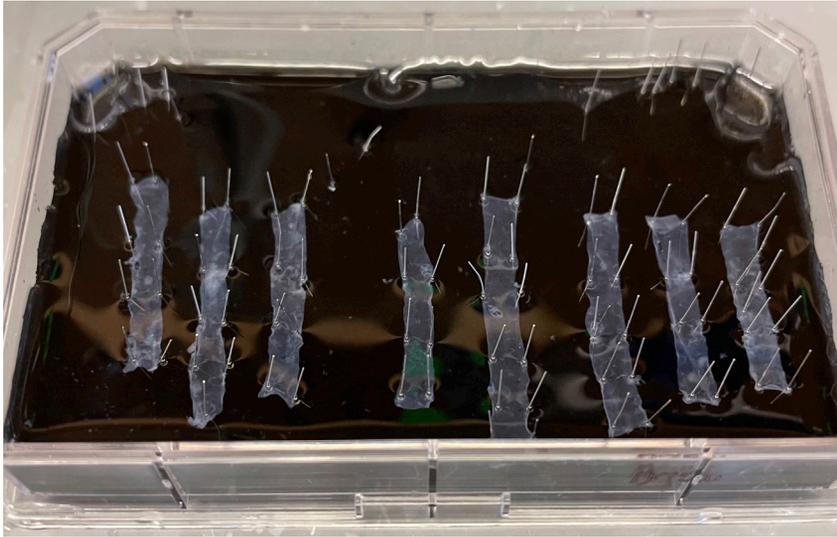
7. Transfer tissue to 24-well plate filled with Blocking Buffer. Incubate at 20°C–25°C on an orbital shaker for 1 h.
8. Prepare 600  $\mu$ L of primary antibody solution per 4 cm piece of muscularis, diluted in Blocking Buffer. Place tissue in antibody solution in 24-well plate; seal with parafilm and incubate for 48 h at 4°C on a platform rocker.
9. Transfer tissue to a 24-well plate filled with PBS. Wash 4 times for 15 min per wash at 20°C–25°C on an orbital shaker, transferring tissue to a new PBS-filled well after each wash.
10. Prepare 600  $\mu$ L of secondary antibody solution per 4 cm piece of muscularis, diluted in Permeabilization Buffer. Incubate tissue in solution in a 24-well plate for 2–3 h at 20°C–25°C, covered from light on an orbital shaker.
11. Transfer tissue to a 24-well plate filled with PBS. Wash 4 times for 15 min per wash at 20°C–25°C on an orbital shaker, transferring tissue to a new PBS-filled well after each wash.
12. Transfer tissue to a microscope slide, completely flatten with damp paint brushes, cover with a few drops of Fluoromount-G mounting medium and carefully place a coverslip on the tissue sample. Avoid introducing air bubbles by lowering the coverslip gradually from one side to the other.
13. Allow slides to dry for 16 h in dark at 20°C–25°C, or until the media solidifies.

**Optional:** For long-term storage (> 4 weeks) the slides can be sealed with nail polish.

**▮▮ Pause Point:** Stained slides may be stored in the dark at 4°C for at least 2 weeks prior to microscopy analysis.

14. Acquire 10 randomly selected, evenly spaced, complete z-stacks for each slide.

For our studies, we used a Zeiss LSM 780 confocal microscope with 25 $\times$  glycerol objective, 0.6 $\times$  zoom, 1 airy unit pinhole and 512  $\times$  512 pixels xy resolution.



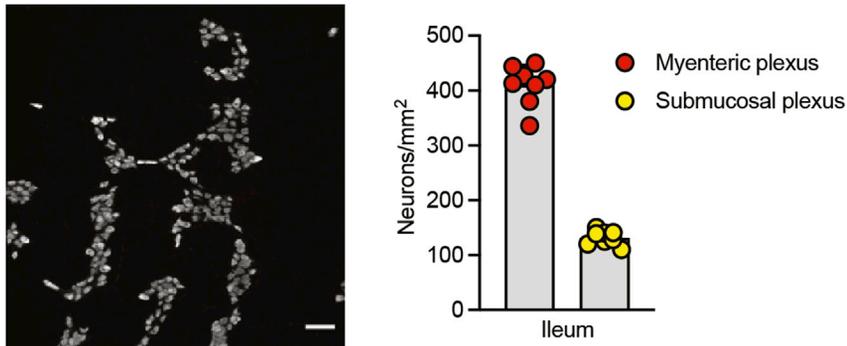
**Figure 2.** Sylgard plate with multiple pinned fragments of ileum muscularis

### Flow cytometry preparation

⌚ Timing: 3–4 h

15. If analyzing the submucosa, remove Peyer's patches with scissors or tweezers (there are 6–10 visible Peyer's patches in the entire small intestine). See [Figure 1](#).
16. Place tissue into a 6-well plate without liquid and use scissors to cut thoroughly into very fine (less than 0.5 mm) pieces, for approximately 15 s.
17. Add Digestion Buffer to the plate and place it on a shaker at 37°C. Use following times and Digestion Buffer volumes (longer digestion time is required for muscularis to ensure proper dissociation):
  - a. Muscularis: 40 min in 4 mL.
  - b. Mucosae: 20 min in 6 mL.
18. Following digestion, homogenize tissue with 18G needle/10 mL syringe by aspirating up and down twice.
19. Add 5 mL HBSS+5% FCS to each well.
20. With a 10 mL serological pipet, filter the cell suspension through a 70  $\mu\text{m}$  cell strainer directly into a 15 mL falcon tube. If 5–6 cm of tissue is analyzed, approximately  $6 \times 10^6$  cells are recovered from muscularis and  $10 \times 10^6$  from the submucosal layer.
21. Centrifuge at 400 g for 5 min at 4°C, aspirate supernatant, resuspend the pellet in 100  $\mu\text{L}$  of FACS buffer and pipette to transfer the cells to a round-bottom 96-well plate.
22. Centrifuge plate at 400 g for 5 min at 4°C and remove supernatant by quickly inverting the plate over the sink.
23. Resuspend cells in 50  $\mu\text{L}$  of surface antibody mix, diluted in FACS buffer. Incubate for 30 min on ice, covered from light.
24. Wash cells by adding 100  $\mu\text{L}$  of FACS buffer to each well. Centrifuge plate at 400 g for 5 min at 4°C and remove supernatant by quickly inverting the plate over the sink.

**Optional:** For intracellular staining, make Fix/Perm Buffer by diluting 1 part Fix/Perm Concentrate in 3 parts Fix/Perm Diluent from the Fixation/Permeabilization Solution Kit (See [key resources table](#)). Resuspend cells in 50  $\mu\text{L}$  of Fix/Perm Buffer and incubate for 30 min on ice, and covered from light. Wash cells by adding 100  $\mu\text{L}$  of Perm buffer to each well. Centrifuge at 400 g for 5 min at 4°C and remove supernatant by quickly inverting the plate over the sink.



**Figure 3. Neuronal quantification in the ileum myenteric and submucosal plexus assessed by IF staining of ANNA-1**  
Left: representative image; scale bar 50  $\mu$ m

Resuspend cells in 50  $\mu$ L of intracellular antibody mix, diluted in Perm buffer (diluted 1:10 in water). Wash cells by adding 100  $\mu$ L of Perm buffer to each well. Centrifuge at 400 g for 5 min at 4°C and remove supernatant by quickly inverting the plate over the sink.

25. Resuspend cells in 300  $\mu$ L of FACS buffer and transfer to FACS tubes.
26. Acquire samples on FACS machine.
  - a. For our studies, we used LSR-II flow cytometer (Becton Dickinson, USA) and analyzed the data using FlowJo software package (Tri-Star, USA).

### EXPECTED OUTCOMES

For a sample image of immunofluorescence analysis see [Figure 3](#). For sample plots of flow cytometry analysis see [Figure 4](#).

### QUANTIFICATION AND STATISTICAL ANALYSIS

Immunofluorescence image analysis was performed in Fiji. The “Cell counter” plugin was used to enumerate neuronal cell bodies in each image. For each sample, 10 randomly selected images were acquired and average number of neurons per sample was calculated. To calculate the density of neurons per mm<sup>2</sup>, the average number of neurons in each sample was multiplied by 3.125. This number was calculated based on the dimensions of acquired images and microscope magnification settings.

For representative gating strategy of flow cytometry data see [Figure 4](#).

### LIMITATIONS

Depending on mouse strain, age, treatment conditions, and intestinal segments, we observed variability of the thickness of each layer. Thin muscularis layer (e.g., in Balb/c mice, weanlings, or in the duodenum) is prone to tearing during the separation.

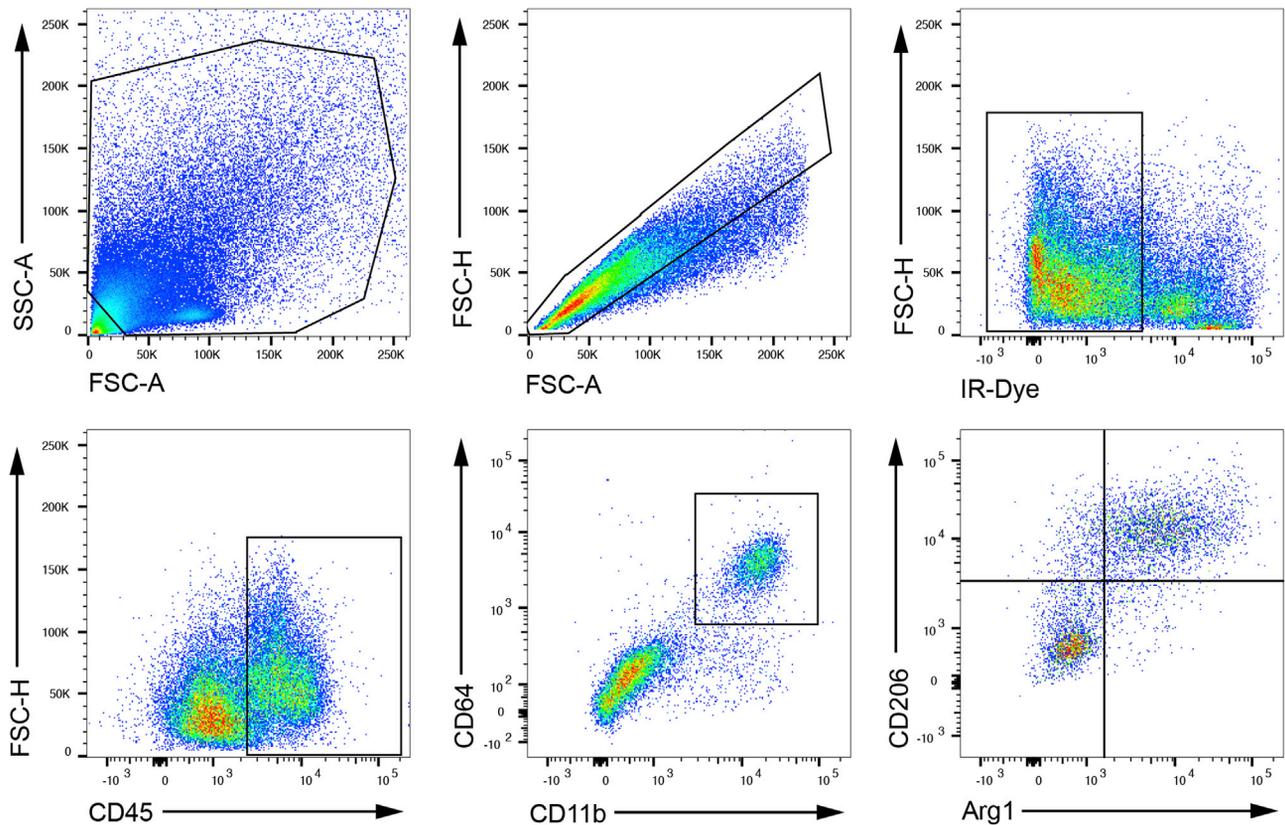
### TROUBLESHOOTING

#### Problem 1

Thin muscularis layer (See step 1).

#### Potential solution

For immunofluorescence analysis, cut open the intestine and fix the tissue without separating the layers, then separate after fixation. This makes the muscularis layer stiffer and therefore easier to isolate. If this method is used, all samples should be processed in this way to avoid technical



**Figure 4.** Flow cytometry analysis of macrophages isolated from ileum muscularis

variability. Flow cytometry will not be possible if samples are fixed prior to single-cell suspension preparation.

#### Problem 2

Weak staining/signal in immunofluorescence analysis. (See step 14)

#### Potential solution

Samples can be re-stained. Submerge each slide in PBS for 5 h (or 16 h) to dissolve the mounting media and release tissue samples. Continue with a regular staining protocol as outlined above (see step 6).

#### Problem 3

Thin muscularis layer in weanlings. (See step 1)

#### Potential solution

Immunofluorescence analysis of enteric neurons in weanlings (up to 3 weeks old) can be performed without the separation of the layers. Due to low thickness of the tissue, both layers can be imaged by confocal microscopy.

#### Problem 4

Low cell viability during flow cytometry analysis. (See step 26)

### Potential solution

Limit the time between the separation of intestinal layers and enzymatic digestion, ideally to less than 1 h. If one person is performing the experiment, up to 10 mice should be analyzed at a time. Try staggering experimental groups in time or dividing the workload between two people.

### Problem 5

Inconsistent enteric neuron densities after image analysis (see step 14). This can occur when small intestine stretches due to experimental manipulations e.g., long-term antibiotic treatment.

### Potential solution

Instead of density per mm<sup>2</sup>, the quantification of enteric neurons can be performed per ganglion. A myenteric ganglion can be defined as a continuous group of ANNA1+ cells (neurons) that are separated by less than 15 mm in distance. Only complete ganglia should be counted per field of view. The following ganglia should be excluded: 1. Ganglia that were truncated; 2. No clear separation (> 15 mm) was noted between the last ANNA1+ cell and the edge of the field of view. In the case of single ANNA1+ cells that are separated by 15 mm on all sides, this was counted as extraganglionic. The number of quantifiable ganglia was averaged across a minimum of 10 images per gut segment per animal.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniel Mucida ([mucida@rockefeller.edu](mailto:mucida@rockefeller.edu)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate/analyze datasets/code.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101157>.

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## AUTHOR CONTRIBUTIONS

Methodology, T.A. and M.W.; Writing, T.A.; Editing, M.W. and D.M.; Funding and supervision, D.M. All authors critically reviewed, edited, and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

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