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Editorial summary: This protocol describes how to perform whole-mount immunostaining and imaging on adult mouse small intestinal villi. All gut cell types can be seen at high resolution and in 3D without the need for image reconstruction.

Proposed Tweet: #NewNProt: High resolution 3D imaging of immunostained whole-mount #mouse #intestine [LINK]

Suggested Ontology terms

Biological sciences / Biological techniques / Immunological techniques / Immunohistochemistry

Health sciences / Gastroenterology / Gastrointestinal system / Small intestine

Biological sciences/Immunology/Imaging the immune system

Biological sciences/Physiology/Cardiovascular biology/Angiogenesis

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High-resolution 3D analysis of mouse small intestinal stroma

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ABSTRACT

Here we detail a protocol for whole-mount immunostaining of mouse small intestinal villi that can be used to generate high-resolution, 3D images of all gut cell types including: blood and lymphatic vessels, neurons, smooth muscle cells, fibroblasts and immune cells. The Procedure describes perfusion, fixation, dissection, immunostaining, mounting, clearing, imaging and quantification, using intestinal vasculature as an example. Since intestinal epithelial cells prevent visualization with some antibodies, we also provide an optional protocol to remove these cells prior to fixation. In contrast to alternative current techniques, our protocol enables the entire villus to be visualized with increased spatial resolution of cell location, morphology and cell-cell interactions allowing for easy quantification of phenotypes. The technique, which takes 7 days from mouse dissection to microscopic examination, will be useful for researchers interested in most aspects of intestinal biology, including mucosal immunology, infection, nutrition, cancer biology and intestinal microbiota.

INTRODUCTION

Vascular biology research relies heavily on analyzing vessel patterning and fine detailed vessel structures by whole-mount immunostaining. Common mouse models for studying blood and lymphatic vessel patterning are the postnatal pup retina and embryonic mesentery or skin, respectively[1-4]. These tissues can be flat-mounted for easy imaging and analyzed for vessel density, number of endothelial cells, and filopodia, among other parameters. These tissue models, used in combination with genetic tools for gene inactivation in blood or lymphatic endothelial cells, have led to significant advances in our understanding of sprouting (lymph)angiogenesis, vascular patterning and maturation[5,6]. The current challenge is to now understand the molecular mechanisms and principles that govern tissue-specific organization and function of adult vascular beds, which can be critical for our ability to treat a range of human diseases. Here we present a step-by-step protocol for 3D visualization of all stromal cells and structures, including blood and lymphatic vessels, in adult mouse small intestine.

The adult mouse small intestine is a highly specialized organ, which harbors billions of bacteria and whose epithelial cells are constantly regenerating, so that the entire intestinal lining is replaced every few days. Its critical role in absorbing all dietary nutrients, as well as performing immunosurveillance of the intestinal microbiota, create a remarkable tissue microenvironment[7]. In particular, adult small intestinal blood capillaries and lymphatic vessels perform a distinct role, namely absorption and systemic distribution of all dietary nutrients, and display properties different from most adult vessels. Therefore, understanding molecular mechanisms underpinning the patterning and function of these vessels may lead to novel treatments for gut-related

diseases such as inflammatory bowel disease or colorectal cancer. Indeed, intestinal villus blood vessels are one of a handful of VEGF-dependent adult vascular beds[8,9] and their density is regulated by intestinal microbiota[10,11] Dietary fat absorption is carried out by intestinal lymphatic vessels, also called lacteals, and is uniquely promoted by lacteal contractions controlled by the autonomic nervous system[12]. In addition, our own and other recent work show that both intestinal villus blood and lymphatic vessels display hallmarks of (lymph)angiogenesis, such as filopodia and increased proliferation, and they slowly, but steadily regenerate, paralleling renewal of epithelial cells[13,14]. Furthermore, we found that lymphatic expression of the Notch ligand Dll4 is critical for maintaining such continuous lacteal regeneration. Therefore, by combining cell-specific genetic inactivation with advanced imaging techniques we were able to study the molecular mechanisms underlying lymphatic vessel specialization in the small intestine. Here we present in detail the protocol for intestinal whole-mount immunostaining.

Development of the protocol

One of the areas of interest in our laboratory has been the study of lymphatic collecting vessels with a focus on lymphatic valves[3,15-17]. The primary tool for analyzing phenotypes in transgenic mouse models for us and others is whole-mount immunostaining of the mouse mesentery, whose flat structure, similar to retina, allows high-resolution 3D analysis of blood and lymphatic vessels[3,15-19]. However, as we began to analyze the intestine, we realized that an equivalent detailed protocol was not available for small intestinal villi. Thin sectioning of paraffin or cryoblocks is often used for intestinal imaging and works very well to analyze the epithelium, however the complex structure of villi make it difficult to correctly image the 3D

stroma organization, especially blood and lymphatic vessels (Figure 1a). For example, accurately measuring lacteal length or blood vessel density can be difficult as villi sectioning prevents unambiguous identification of the lacteal tip and visualization of the cage-like blood vascular network. Imaging of thick cryosections is feasible and allows a more complete image of the villus stroma[8,9] (Figure 1b), however, overall 3D structure and fine, but important, details of cell morphology, such as filopodia, are difficult to visualize and quantify. Furthermore, it is challenging to reliably analyze co-localization or interaction of different cell types in tissue sections. Therefore, we wanted to apply whole-mount immunostaining to allow high-resolution visualization of all cell types in small intestinal villi.

Whole mount imaging of intestine by confocal microscopy has been previously used to study the submucosal[20,21] and villus stroma[10,22,23], however a detailed step-by-step protocol has not been reported. Stappenbeck et al. examined blood vessels in small intestinal villi in whole-mount preparations[10]. Fu et al. incorporated perfusion fixation into whole-mount immunostaining of neuronal components of the small intestine and colon with visualization of blood vessel by dye intracardiac perfusion, focusing on the submucosal layer[22]. Furthermore, both Fu et al. and Appleton et al. performed tissue clearing of intestinal whole-mounts to increase fluorescent signal strength[22,23]. Recently, we used a new whole-mount immunostaining protocol to examine in detail both intestinal submucosal and villus stroma at high resolution[13]. Our protocol combines the tissue fixation procedure of Stappenbeck et al.[10] and perfusion fixation, confocal microscopy and solvent-based clearing of Fu et al.[22]. However, we have also incorporated several major improvements into our approach (Table 1). For example, while solvent-based clearing allows many antibodies to be used effectively for

immunostaining of intestinal villi, some immunostainings are not easily visible in the presence of epithelial cells (even with solvent clearing); this can be due to a weak signal that is indistinguishable from epithelial cell background noise or due to specific high expression of the antigen to be stained in the epithelium (Supplementary Figure 1). To overcome this limitation, we improved the protocol by developing a procedure for physically removing the epithelial cells so the stroma is rendered visible. A second key improvement was the development of a novel mounting strategy that allows villi to be imaged laterally ("side-view") rather than transversally ("top down"; imaging with the tip of the villus pointing directly towards the microscope), which facilitates visualization of 3D structures without the need for image reconstruction. Importantly, likely due to the combination of cardiac perfusion fixation and specific tissue fixation conditions, we were able to visualize important structural features of angiogenic endothelial cells, such as filopodia, at high resolution[13]. Thus, the optimized protocol described here enables intestinal villi stroma to be visualized in more detail than previously possible using alternative approaches (Figure 2).

Overview of the Procedure

The Procedure begins with intracardiac paraformaldehyde (PFA) perfusion of mice. This is a critical step in the protocol as, in our hands, poor perfusion results in the rapid degradation of the tissue (presumably due to intestinal mucous, bacteria and/or digestion-related enzymes present in the gut) and an inability to stain and mount the intestine. After perfusion-fixation, the intestine is dissected and cleaned. Samples are then pinned to silicone plates before being re-fixed, extensively washed and immunostained using standard whole-mount immunostaining methods. After staining, strips of villi are carefully cut; strips should be 1-2 villi thick to ensure that villi will

be relatively transparent and easy to image. In order to obtain high-resolution images, the epithelial cells (which can prevent efficient stroma visualization) must be cleared before mounting onto slides for 3D imaging (Figure 3). We provide two different strategies for epithelial clearing. The first approach leaves epithelial cells in place (WM/EP(+)), which are rendered clear using a commercially available clearing solution; they can be either imaged by immunostaining or ignored if immunostaining is performed for stromal cells only. However, in our experience several antibodies cannot be used for whole-mount intestinal stroma immunostaining when epithelial cells are present, due to either high epithelial background staining or high specific epithelial staining that renders the stromal staining undetectable (Supplementary Table 1). Therefore we have also included an optional protocol to remove epithelial cells (WM/EP(-)), allowing imaging with a wider range of antibodies than in the presence of epithelial cells (Figure 3).

We also provide an optional protocol for paraffin embedding tissue from the same samples used for whole-mount. In our hands, simultaneous harvest of samples for both whole-mount and paraffin embedding gives flexibility in analyzing e.g. endothelial cell proliferation as well as epithelial cell parameters. A common technique for preparing intestinal tissue is the “Swiss roll” where the intestine is cut open and flattened and then rolled and embedded in paraffin[24]. Although the Swiss roll technique for paraffin embedding of intestinal tissue is well known and established in many laboratories, we have included it in context of also performing intestinal whole-mount immunostaining (Supplementary Method 1). Note that removal of epithelial cells for WM/EP(-) is not efficient in the duodenum, therefore

the upper duodenum half should be used for paraffin embedding and the lower part for WM/EP(+).

Applications, advantages and limitations

Here we show the utility of the protocol for immunostaining blood and lymphatic vessels to measure relative lacteal length and number of lacteal and villus blood vessel filopodia. However we have used the same method to visualize other cell types including neurons, smooth muscle cells, immune cells and fibroblasts (Figure 2a-d). Therefore, our protocol can be used to discover novel cell-cell interactions in small intestinal villi. In addition to cell immunostaining, the whole-mount technique also works well to stain extracellular matrix (ECM) proteins such as fibronectin, tenascin C and periostin (Figure 2f, g, Supplementary Table 1 and ref. 13).

This protocol is best suited for analysis of the intestinal villus stroma. Epithelial cells are easily imaged using this protocol and stromal/epithelial interactions can be clearly visualized. Key advantages of the protocol include: perfusion fixation to ensure tissue quality for the duration of the procedure; epithelial clearing to enable the use of all antibodies validated for whole-mount immunostaining; and lateral mounting to avoid the need for image reconstruction. This mounting approach also facilitates analysis, as slides can be quickly examined by epifluorescencent microscopy to determine the quality of the immunostaining and detection of a phenotype. As a result of these improvements, this whole-mount protocol is superior to either paraffin or frozen sections for detecting fine structures and for analyzing stromal cell protein expression, patterning, location or co-localization.

However, if the goal of an experiment is to solely analyze epithelial cells, performing the traditional “Swiss roll” paraffin embedding technique should be sufficient, as well as less time consuming, for dissection, immunostaining and analysis. Furthermore, as PFA perfusion is a critical step in this protocol, combining whole-mount intestinal immunostaining with cell sorting is not feasible. Similarly, it is not possible to perform RNA or protein analysis from mice where PFA perfusion has been performed. Finally, since proper cleaning of the intestine requires vigorous flushing, our protocol will not be useful to visualize the intestinal mucus layer.

Level of expertise needed to implement the protocol

The techniques described in this protocol can be performed by anyone comfortable with handling mice, mouse dissection and confocal microscopy imaging. PFA perfusion and pinning of the intestine onto silicone plates are skills that are relatively straightforward after some practice. If preparing samples for paraffin embedding and whole-mount simultaneously, in our experience it is better to have two people: one experimenter handling the intestinal whole-mount, the other performing PFA perfusion and tissue preparation for paraffin embedding. In our hands, even with good PFA perfusion it is best to start the overnight intestine fixation as quickly as possible after dissection to avoid tissue degradation. If other tissues will also be collected at the same time it is better to add experimenters as necessary, so that all tissues are collected in a timely manner.

Experimental design

Application to younger mice: The protocol described here is for adult mice, however we have also successfully used this protocol to analyze the intestine from P0 to 3-week-old mouse pups. PFA perfusion fixation is essential for proper adult sample preparation and is well described by Gage et al.[25] However, we do not perform PFA perfusion on pups, as it is technically challenging, and rather work rapidly to fix the intestine as quickly as possible.

Conditional mutants: In our previous study[13] we used *Prox1-CreERT2*[18] mice for conditional deletion in lymphatic endothelial cells and *Pdgfβ-iCreERT2* mice[26] for recombination in blood intestinal endothelial cells. Recombination in lymphatic endothelial cells has been reported for *Pdgfβ-iCreERT2* mice in some tissues[27], but we did not observe it in small intestinal lymphatic vessels. Tamoxifen was administered by a single or multiple intraperitoneal injection(s) to 8- to 12-week-old mice which were analyzed after 3 or 10 weeks[13], however any treatment, mutant mouse line or other time points can be used.

Choice of mice: The gut microbiota plays an important role in intestinal physiology, and studies of microbiota composition show large variations in cage effects between mice of different strains, sex and housing unit[28]. Therefore, if possible, when analyzing mutant mouse models, co-housed littermates of the same sex should be used for experiments.

MATERIALS

REAGENTS

- Adult mice (8- to 12-weeks-old)

Caution: All procedures involving animal experiments should follow approved institutional and governmental animal protocols and comply with the relevant guidelines and regulations of the local animal ethics committee.

- Xylosol (Graeub, cat. no. 763.12)
- Ketasol (Graeub, cat. no. 668.51)
- 10X PBS (see Reagent Setup)
- Picric acid (Sigma, cat. no. 197378)

Caution: Picric acid is a hazardous reagent; use a chemical fume hood and wear protective gloves and mask when you are preparing the fixative. Protect from light.

- paraformaldehyde (PFA, Sigma, cat. no. 158127)

Caution: PFA is a hazardous reagent; use a chemical fume hood and wear protective gloves and mask when you are preparing the fixative.

- Ethylenediaminetetraacetic acid (EDTA, Applichem, cat. no. A3234)
- NaH_2PO_4 (Sodium phosphate monobasic, Sigma, cat. no. S3139)
- Na_2H2PO_4 (Sodium phosphate dibasic, Applichem, cat. no. A2943)
- Sylgard® 184 Silicone elastomer kit (Dow Corning)
- 87% Glycerol (Applichem, cat. no. A0970)
- D(+)-sucrose (Applichem, cat. no. A2211)
- NaN_3 (Sodium azide, Applichem, cat. no. A1430)

Caution: Sodium azide is a hazardous reagent; use a chemical fume hood and wear protective gloves and mask when you are preparing the fixative.

- Donkey serum (Bio-Rad, cat. no. C065B)
- Bovine serum albumin (Albumin fraction V, Applichem, cat. no. A1391)
- Triton X-100 (Applichem, cat. no. A1388)
- Primary Antibodies (rat anti-Pecam1, BD Pharmigen, cat. no. 557355; goat anti-Vegfr2, R&D, cat. no. AF644; rabbit anti-Lyve1, AngioBio, cat. no. 11-034, rat anti-Ki67, BD Pharmigen, cat. no. 556003; rabbit anti-Erg, Abcam, cat. no. ab92513; goat anti-Prox1, R&D, cat. no. AF2727; goat anti-Vegfr3, R&D, cat. no. AF743, also see Supplementary Table 1)
- Secondary Abs (donkey anti-rat IgG Alexa Fluor 488, cat. no. A-21208; donkey anti-goat IgG Alexa Fluor 555, cat. no. A-21432; donkey anti-rabbit IgG Alexa Fluor 647, cat. no. A-31573; donkey anti-rabbit IgG Alexa Fluor 555, cat. no. A-31572; donkey anti-goat IgG Alexa Fluor 647, cat. no. A-21447)

Caution: Protect from light.

- ProLong Gold antifade reagent with DAPI (Life Technologies, cat. no. P36931)
- KCl (Potassium chloride, Applichem, cat. no. A1362)
- NaCl (Sodium chloride, Sigma, cat. no. 71382)
- KH_2PO_4 (Potassium hydrogen phosphate, Applichem cat. no. A1042)
- 70% EtOH
- FocusClear (CelExplorer Labs CO., cat. no. FC-101)
- Xylene (ReactoLab, cat. no. 99814)
- 100% EtOH
- 90% (vol/vol) EtOH
- High pH antigen retrieval buffer (Dako, EnVision FLEX Target Retrieval Solution, High pH, cat. no. K8004)
- M.O.M. blocking reagent (Vector Laboratories, cat. no. BMK-2202)

EQUIPMENT

- Dissection microscope (e.g. Leica MZ16)
- Perfusion apparatus (2 Infu-Surg Pressure Infusion bags, 1000 ml, Moog, cat. no. 4010)
- Small Animal Butterfly Catheter Infusion Set (Harvard Apparatus, cat. no. 725965)

- 3-way stopcock (e.g. CardioMed cat. no. 17-369C)
- 20 ml syringe (Braun, cat. no. 4616200V)
- 25G syringe needles (Braun, cat. no. 4657853)
- Dissecting forceps (Aesculap, cat. no. BD210R)
- Small forceps, 0.2 mm (Aesculap, cat. no. BD330R)
- Very small forceps, 0.115 mm (Aesculap, cat. no. BD331R)
- Artery scissors (Fine Science Tools, cat. no. 14080-11)
- Very small scissors (Fine Science Tools, cat. no. 15000-00)
- Scissors (Aesculap, cat. no. BC064R)
- Microscope slides, 76x25 mm (Menzel-Gläser)
- Secure-Seal spacer 20mm diameter, 0.12mm deep (Molecular Probes, cat. no. S24736)
- Confocal microscope (e.g. Leica SP5)
- Insect pins (Fine Science Tools, cat. no. 26002-20)
- Coverslips, 24x24 mm (Menzel-Gläser)
- Aluminum foil (Co-op Supermarket, Alustar)
- 0.22 micron filter sterilizers (TPP, cat. no. 99500)
- Orbital shaker (Labnet, Orbit 1000)
- Parafilm (Pechiney Plastic Packaging, cat. no. PM-996)
- Marked 15 cm silicon plate (see Equipment Setup)
- 50 ml syringe (Braun, cat. no. 4610083F)
- 18 G syringe needles (Milian, cat. no. 921030)
- Whatman paper (VWR, cat. no. 10426892)
- Benchkote paper (Whatman, cat. no. 2300731)
- 15 cm cell culture dish (TPP, cat. no. 93150)
- 10 cm cell culture dish (TPP, cat. no. 93100)
- 6-well cell culture dish (TPP, cat. no. 92406)
- 12-well cell culture dish (TPP, cat. no. 93412)
- 50 ml tube (TPP, cat. no. 91050)
- Paraffin embedding cassette (Biosystems, 81-0035-00)
- Fluorescent stereomicroscope (e.g. Leica M205FA)
- Heated block (e.g. Desaga, Thermoplate S)
- Microwave oven (e.g. Sharp)
- FIJI software http://fiji.sc/Fiji
- Lymphatic vessel analysis protocol (LVAP) FIJI plugin[29] (http://www.ludwig.edu.au/archive/RaminShayan2008/index.html)

REAGENT SETUP

10X PBS 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH2PO4. Dissolve 2 g KCl, 80 g NaCl, 6.1 g Na_2HPO_4 and 2 g KH_2PO4 in 1 L water. Adjust the pH to 7.4 with NaOH and autoclave. Store at RT (24°C). 1X PBS can be made by diluting 10X PBS 1:10 and stored at RT for mouse perfusion and at 4°C for all other uses and stored for months.

1M Phosphate buffer, pH 7 To make 500 ml, mix 195 ml 1M NaH_2PO_4 with 305 ml 1M Na_2H2PO_4 and filter sterilize. Can be stored for months at RT.

4% (wt/vol) paraformaldehyde solution Weigh 40g of PFA and mix with 1L of 1X PBS at 60ºC until dissolved. Filter sterilize, make 50ml aliquots and store at -20ºC for up to 6 months. Once thawed, store at 4ºC for up to 7 days then discard. Use at RT for perfusion, all other uses at 4°C.
CAUTION- Paraformaldehyde is a hazardous reagent; use a chemical fume hood and wear protective gloves and mask when you are preparing the fixative. Protect from light.
CRITICAL- Plan 3 hours for dissolving, filtering and aliquoting the 4% PFA.

Fixation solution 0.5% (wt/vol) paraformaldehyde, 15% (vol/vol) picric acid, 100 mM phosphate buffer, pH 7. To make 250 ml mix 31.25 ml 4% paraformaldehyde, 37.5 ml picric acid, 25 ml 1M phosphate buffer, pH 7 and 156.25 ml water.
CRITICAL: Prepare fresh before beginning dissection and store at 4ºC. Discard after finishing dissection.
CAUTION-Picric acid is a hazardous reagent; use a chemical fume hood and wear protective gloves and mask when you are preparing the fixative.

Xylosol/Ketasol mix For 5 ml mix 500ul Ketasol with 400ul Xylosol and bring up to 5 ml with 1X PBS. Discard after finishing dissection.
CRITICAL- Protect from light.

PBS+ 1 mM EDTA To make 500 ml, add 1 ml of 500mM EDTA to 499 ml of 1xPBS. Can be stored for months at 4ºC.

10% (vol/vol) sucrose solution Add 50 g sucrose to 450 ml of PBS. Once the sucrose is dissolved, add 1X PBS to 500 ml, filter sterilize. Can be stored for months at 4ºC.

20% (vol/vol) sucrose solution+10% glycerol Add 100 g sucrose and 57.5 ml 87% glycerol to 450 ml of PBS. Once the sucrose and glycerol are dissolved, add 1X PBS to 500 ml, filter sterilize. Can be stored for months at 4ºC.

Donkey serum Heat inactivate donkey serum at 65°C for 30 min., let cool, filter sterilize and make 10 ml aliquots. Can be stored for months at -20°C.

Blocking buffer 5% (vol/vol) donkey serum, 0.5% (wt/vol) bovine serum albumin (BSA), 0.3% (vol/vol) Triton X-100, 0.1% (wt/vol) NaN_3. n 300 ml of 1X PBS dissolve 2.5 g BSA, 0.5 g NaN_3, 25 ml of donkey serum, and 1.5 ml Triton X-100. Add 1X PBS to 500 ml and filter sterilize. Can be stored for months at 4°C.

Wash buffer 1X PBS, 0.3% (vol/vol) Triton X-100. Add 3 ml Triton X-100 to 1 L 1X PBS, shake vigorously. Can be stored for months at 4°C.

PBS+NaN_3 1X PBS, 0.1% (wt/vol) NaN_3. Add 1 g of NaN_3 to 1 to 1 L 1X PBS, shake vigorously. Can be stored for months at 4°C.

M.O.M. Blocking reagent in 1X PBS Add one drop of blocking reagent to 2.5 ml of 1X PBS. CRITICAL: Make fresh and discard any extra after blocking.

EQUIPMENT SETUP

Perfusion apparatus Fill one infusion bag with room temp. 1X PBS and one bag with room temp. 4% PFA. Arrange tubing so that each bag drains to a 3-way stopcock. At the third position of the 3-way stopcock attach the Small Animal Butterfly Catheter. Run 1X PBS and 4% PFA until all bubbles are removed from tubing. If a perfusion apparatus is not readily available a 20 ml syringe filled with 4% PFA and fitted with a 25 G needle can be substituted to perfuse the mice.

Silicone plates Mix the curing agent well with the silicone according to manufacturer's directions in a fume hood. Immediately, pipette approximately 25 ml of silicone into each of either 10 cm, 6-well or 12 well culture dish. Pipette approximately 35 ml of silicone into 15 cm culture dish. Distribute all silicone onto culture dishes of various sizes. Leave in hood to cure for 4 days before use. After 4 days wash with soap and water and sterilize with 70% (vol/vol) ethanol. Plates can be recycled after use by removing any remaining tissue and washing as above.

CRITICAL- Using the silicone plates before the end of the 4 day cure will result in detachment of the silicone from the culture dish.

Marked 15 cm silicone plate Using a ruler, make 1 cm markings on 15 cm silicone plate with an indelible lab pen. For ease of use, invert numbers labeling the marks such that the numbers are readable when looking from the top down.

Whatman paper disc Draw a circle with a pencil on Whatman paper using a 10 cm cell culture dish as a model. Cut out circle with scissors. Use scissors to further cut the Whatman paper to reduce the circle diameter so it easily fits inside a 10 cm cell culture dish.

Benchkote paper-coated dish Draw a circle with a pencil on Benchkote paper using a 15 cm cell culture dish as a model. Cut out circle with scissors. Use lab tape to attach Benchkote paper, plastic side up, to the bottom of a 15 cm cell culture dish.

PROCEDURE

Day 1: Mouse perfusion, tissue dissection, preparation and fixation. Timing: 6-8h

CRITICAL: Good perfusion fixation is necessary to prevent intestine degradation and allow long-term storage. Poorly fixed whole-mount intestinal samples tend to degrade very quickly during storage. A detailed video and protocol is available from Gage et al.[25]

1. Anaesthetize the animal as approved in the animal experimentation protocol (e.g. by injecting 15µl/ g mouse of Xylosol/Ketasol mix). Wait 5-10 minutes until there is no response to the foot pinch test.
2. Transfer the mouse to the fume hood containing the perfusion apparatus and place the animal in a supine position. Spray the mouse with 70% EtOH to prevent hair contaminating the intestine sample. Make an incision in the skin from the gonadal region to just under the neck and remove the skin to expose the thorax.
3. Make an incision between the ribs and continue cutting in a dorsal direction following the contours of the ribcage until the other side of the animal is reached. Cut enough of the ribcage to be able to easily lift and expose the heart.
4. Start pumping 1X PBS through the perfusion apparatus. Cut the right atrium with small scissors and insert the perfusion needle into the left ventricle.
5. Allow 1X PBS perfusion to continue for 30-60 seconds then switch to 4% PFA solution. Allow 4% PFA solution to perfuse for 2-3 minutes.

Troubleshooting

CRITICAL STEP: Good perfusion fixation is necessary to prevent intestine degradation and to allow long-term storage. Poorly fixed whole-mount intestinal samples tend to degrade very quickly during storage. Furthermore, proper perfusion fixation is critical for successful implementation of the WM/EP(-) protocol by allowing efficient removal of intestinal epithelial cells without villus destruction.

CAUTION PFA is toxic, only perform PFA perfusion in a well-ventilated fume hood.

6. After completion of perfusion make an incision in the abdominal cavity to expose the intestine. Cut the colon as near to the rectum as possible and cut the duodenum as near to the stomach as possible.
7. Slowly pull out the duodenum to remove the pancreas and mesentery. Periodic pulling of the mesentery with the forceps while pulling the duodenum may be necessary to completely remove the mesentery. Place the entire small intestine, cecum and colon into the marked 15 cm silicon plate with ice-cold 1X PBS on ice.
8. Measure and record the length of the small intestine from the pyloric sphincter to the cecum. Cut the small intestine at the junction with the cecum. Using the ruler on the 15 cm silicone plate cut 5 cm down from the pyloric sphincter and label as duodenum. The rest of the length is considered the jejunum and ileum. Cut this piece in half and use the upper part as jejunum and lower part as ileum (Figure 3). Cut the colon from the cecum. Secure tissue with one pin on the upper end of each dissected piece of intestine to keep the pieces organized.

Critical step: During the tissue dissection procedure it is important to keep track of the upper end of each of the pieces. This is critical for downstream analysis to be able to compare similar regions of the intestine among different animals.

9. Using the 50 ml syringe fitted with an 18G needle, flush the gut contents from each of the small intestine pieces by fully flushing from both ends. Because the colon muscles tightly hold feces, the colon cannot be flushed in the same manner; instead, cut the colon lengthwise using artery scissors and remove feces manually or by gentle shaking in 1X PBS.

   Troubleshooting

10. Cut the duodenum, jejunum and ileum open lengthwise using artery scissors. Pin the upper end of each part to the 15 cm marked silicone dish.
11. (OPTIONAL) If intending to perform simultaneous analysis on whole-mount and paraffin-embedded samples (see Supplementary Method 1), divide the jejunum and ileum into the appropriate number of equal pieces and designate one piece for each different treatment (i.e. WM/EP(+), WM/EP(-) or paraffin). Mark the upper ends of the pieces designated for WM/EP(-) with a small cut with the scissors.

    CRITICAL STEP: removal of epithelial cells for WM/EP(-) is not efficient in the duodenum, therefore the upper duodenum half should be used for paraffin embedding and the lower part for WM/EP(+).
12. Perform post-fixation of the intestinal pieces according to Option A for WM/EP(+), Option B for WM/EP(-), or Supplementary Method 1 for paraffin embedding.

    A. WM/EP(+) Timing: 10 minutes

i. Pin the piece of intestine for WM/EP(+)onto a 10 cm silicone plate containing 1X PBS using insect pins (Figure 4a). First pin the top of the intestine at 1 cm intervals, then pin the bottom, making sure the intestine is flush with the silicone plate.

ii. Rinse the pinned intestine in the 10 cm silicone plate once with ice-cold 1X PBS.

iii. Add fixation buffer until intestine pieces are fully submerged. Incubate the samples on an orbital shaker overnight (O/N) at 4°C.

B. WM/EP(-) Timing: 20 minutes

i. Place pieces on intestine for WM/EP(-) into 25 ml of 1mM EDTA PBS solution in a 50 ml tube and gently shake to submerge. Leave on ice for 5 min.

ii. Gently shake again and then leave on ice for 5 min.

iii. Transfer the pieces of intestine back to the 15 cm silicone plate in ice-cold 1X PBS.

iv. Pin the piece of intestine for WM/EP(-) onto a 10 cm silicone plate containing 1X PBS using insect pins (Figure 4a). First pin the top of the intestine at 0.5 cm intervals, then pin the bottom, making sure the intestine is flush with the silicone plate.

Critical step: WM/EP(-) pieces should be pinned tightly, flush with the plate, with no “loose” intestine. Otherwise, the following epithelial cell removal step is more difficult.

v. Use the end of closed large forceps to gently “brush” off villus epithelial cells (Figure 4b, c). Do not press down on the tissue while brushing or intestinal villus stroma will also be brushed away.

Troubleshooting

vi. Rinse the pinned intestine in the 10 cm silicone plate once with ice-cold 1X PBS.

vii. Add fixation buffer until intestine pieces are fully submerged. Incubate the samples on an orbital shaker O/N at 4°C.

Day 2: Sample washing. Timing: 1-2h

13. Remove fixation buffer from 10 cm silicone dishes containing pinned intestine (from step 12A(iii) or 12B(vii)) and replace with ice-cold 1X PBS.
14. Wash on an orbital shaker, 4°C for 5 min. Repeat two more times.

Caution: Removal of fixation buffer must be done in fume hood.

15. After last wash with 1X PBS, remove 1X PBS and replace with 10% sucrose solution.
16. Incubate the samples on an orbital shaker for 3 hours at 4°C.
17. Remove 10% sucrose solution and replace with 20% surcrose+10% glycerol solution.
18. Incubate the samples on an orbital shaker O/N at 4°C.

Day 3: Primary antibody incubation. Timing: 4-5h

19. Remove 20% surcrose+10% glycerol solution, rinse once with ice-cold 1X PBS, and submerge intestine pieces in PBS+NaN_3.

Pause point: Seal plates with parafilm. Samples can be stored at 4ºC for up to several months.

20. Using a dissection microscope to visualize the intestine, cut it into 0.5-1cm long pieces and transfer them to a 6- or 12- well silicone plate and re-pin with 4 pins/ piece. Up to two pieces can fit in one well of a 12-well plate; up to 5 pieces can fit in one well of a 6-well plate (Figure 4d).

Critical step: When comparing the same immunostaining among several mice (e.g. wild-type vs mutant mice), make sure to take a 1 cm long piece from the corresponding place on the intestine from each mouse.

21. Add blocking buffer to samples; use 1 mL/well for a 12-well plate or 2 mL/well for a 6-well plate. Incubate the samples on an orbital shaker for 1-2h at 4°C.

22. Prepare an appropriate primary antibody mix in blocking buffer. Prepare 1 mL/well for a 12-well plate or 2 mL/well for a 6-well plate. Table 2 outlines combinations of primary and secondary antibodies we have used successfully. For example, staining for Pecam1 and Lyve1 will allow analysis of lacteal length (see Step 45A), staining for Vegfr3 will allow visualization of lacteal filopodia (step 45C), and staining with Vegfr2 will allow analysis of blood vessel length and branching and blood vessel filopodia (Step 45B and D)

CRITICAL STEP- Primary antibodies can be omitted or replaced with antibodies from the same species and of the same isotype as a negative control.

23. Remove blocking buffer from samples and add primary antibody mix. Incubate the samples on an orbital shaker O/N at 4°C.

Day 4: Primary antibody washing and secondary antibody incubation. Timing: 6h

24. Remove primary antibody mix and add ice-cold PBS+0.3% Triton-X100. Wash on an orbital shaker, 4°C for 1h. Repeat 4 more times.
25. Prepare an appropriate secondary antibody mix in blocking buffer. Prepare 1 mL/well for a 12-well plate or 2 mL/well for a 6-well plate.
26. Replace PBS+0.3% Triton-X100 with secondary antibody mix. Incubate the samples on an orbital shaker O/N at 4°C.

Critical step: Although primary antibodies can be left on samples for several days, secondary antibodies cannot. Let secondary antibodies incubate for a maximum of 16 hours at 4°C.

Day 5: Secondary antibody washing and post-immunostaining fixation. Timing: 6h

27. Remove secondary antibody mix and add ice-cold PBS+0.3% Triton-X100. Wash on an orbital shaker, 4°C, 10 times for 30 min each. At this point, samples can be analyzed using a fluorescent stereomicroscope to determine that primary and secondary antibody immunostaining worked.
28. Replace PBS+0.3% Triton-X100 with ice-cold 1X PBS and wash twice for 10 min. each at 4°C on an orbital shaker.
29. Replace 1X PBS with ice-cold 4% PFA. Incubate the samples on an orbital shaker for 2 days at 4°C.

CAUTION PFA is toxic, only work with PFA in a well-ventilated fume hood.

Day 6: Mounting. Timing: 6h

30. Remove 4% PFA and add ice-cold PBS. Wash on an orbital shaker, 4°C, 3 times for 30 min each.

Caution: Remove 4% PFA in fume hood.

31. Label microscope slides with sample ID and antibodies used in immunostaining
32. Remove the protective cover of one sticky side of the spacers and stick onto microscope slide. Leave the other sticky side untouched.
33. Transfer plate to dissection microscope. Work in as little light as possible and use the bottom lamp of the dissection microscope to look at the samples.
34. Move pins away from two corners using small forceps to leave a strip 2-3 mm wide that remains flush with the silicone. Focus the microscope with the highest magnification on the bottom corner.
35. Use very fine scissors to cut a strip of intestine, trying to make it one or two villi wide. Cutting into the silicone gel under the intestinal sample facilitates obtaining a good strip. When it is cut, you can gently move the strip out of the way and begin to cut the next strip (Figure 4e).

Troubleshooting

36. Repeat step 35 to obtain a total of 10-15 strips.
37. Use very fine forceps to place the strips on the glass slide inside the spacer area.

38. Using a paper towel or tissue paper absorb excess PBS by placing it near intestinal strips.
39. For WM/EP(+) samples only, place 2 drops of FocusClear on intestine strips, use very small forceps to spread out the strips as much as possible and incubate for at least 20 minutes (maximum 25 minutes) at room temperature. Use P200 micropipettor to remove as much FocusClear as possible.
40. Add 2-3 drops of Prolong Gold mounting medium to intestine strips. Tilt slide around to help spread out the mounting medium.
41. Use very fine forceps to arrange the strips horizontally and vertically so they all fit inside the area of the spacer. Intestine strips should also be placed so that villi are clearly visible and submucosa does not fold over on top of villi (Figure 4f).
42. Remove upper spacer cover, leaving the upward surface of the spacer sticky, and firmly affix the coverslip.
43. Smear excess mounting medium around the edges of the coverslip to seal the coverslip to the slide.
44. Place in slide storage box at 4°C.

Pause point: If samples are kept at 4°C and mounting medium remains, slides can be kept for up to a year.

Day 7: Confocal microscopy. Timing: 6-8h

45. Acquire images for the vessel parameter to be analyzed. If WM staining 1 (see Table 2) was performed, lacteal length (Option A) and lacteal filopodia (Option C) can be analyzed. If WM staining 2 (see Table 2) was performed,

blood vessel length and branching (Option B) and blood vessel filopodia (Option D) can be analyzed.

A. Lacteal length

i. Image mounted samples with WM staining 1 using the 488 and 633 nm lasers with the 20X objective of a confocal microscope, so that Pecam1 and Lyve1 staining on several villi can be imaged simultaneously on a single image (Figure 5a). Adjust the z-stack so that whole villi can be imaged (usually 80-100 μm) and set z-stack step size as recommended by image acquisition software. Take images from 10-15 different regions in each sample.

B. Blood vessel length and branching

i. Image mounted samples with WM staining 2 using the 555 nm laser with the 20X objective of a confocal microscope, so that Vegfr2 staining on several villi can be imaged simultaneously on a single image (Figure 5a). Adjust the z-stack so that whole villi can be imaged (usually 80-100 μm) and set z-stack step size as recommended by image acquisition software. Take images from 10-15 different regions in each sample.

C. Lacteal filopodia

i. Examine mounted samples with WM staining 1 using the orange filter of a fluorescent microscope, to analyze Vegfr3, with the 63X objective. Count the number of filopodia/ lacteal blinded and record on spreadsheet for appropriate analysis.

D. Blood vessel filopodia

i. Examine mounted samples with WM staining 2 using the orange filter of a fluorescent microscope, to analyze Vegfr2, with the 63X objective. Count the number of blood vessel filopodia/villus blinded and record on spreadsheet for appropriate analysis.

Troubleshooting

Day 8: Image analysis. Timing: 6-8h

46. Analyze confocal images obtained at step 45A as outlined in Option A. Use Option B to analyze images obtained as described in step 45B.

A. Lacteal length

i. Import images from step 45A into FIJI, convert into maximum intensity projections to view the complete z-stack and import into the LVAP FIJI plugin. Using the "Total Width" counter, measure the lacteal length from the lacteal tip to the bottom (Figure 5a).

ii. Using the "Intervessel Distance" counter, measure the blood vessel network length from the top of the villus to the bottom (same bottom as for lacteal measurement (Figure 5a).

iii. Click "measure" and cut and paste data to a spreadsheet for appropriate analysis.

iv. Divide the lacteal length by villus length, multiply by 100 to obtain relative lacteal length.

B. Blood vessel length and branching

i. Import images from step 45B (Figure 5b) into FIJI, convert into maximum intensity projections to view the complete z-stack and import into the LVAP FIJI plugin. Make images binary (Process>Binary>Make Binary)

and adjust settings (dilate, open, close) until blood vessel network is clear (Figure 5c).

Critical step: Use the same settings for all analyses from one experiment.

ii. Click “Skeletonize” (Process>Binary>Skeletonize) to draw vessel network outline (Figure 5d).

iii. Click “Analyze Skeleton” (Plugins>Skeleton>Analyze Skeleton) for network quantification.

iv. Cut and paste data for branchpoints and length into a spreadsheet for analysis.

v. Calculate vessel length and branching/vessel length for each sample.

Troubleshooting

TROUBLESHOOTING

Troubleshooting advice is provided in Table 3.

TIMING

Day 1: Steps 1-12, mouse perfusion, tissue dissection, preparation and fixation: 6-8h

Day 2: Steps 13-18, sample washing: 1-2h

Day 3: Steps 19-23, primary antibody immunostaining: 4-5h

Day 4: Steps 24-26, primary antibody washing and secondary antibody immunostaining: 6h

Day 5: Steps 27-29, secondary antibody washing and post-immunostaining fixation: 6h

Day 6: Steps 30-44, WM mounting: 6h

Day 7: Step 45, confocal microscopy: 6-8h

Day 8: Step 46, image analysis: 6-8h

ANTICIPATED RESULTS

We have used this intestine whole-mount protocol to obtain high-resolution 3D images of intestinal villus stroma by immunostaining with a variety of antibodies[13]. The resolution obtained allows many different analyses to be performed on the same samples. Proper preparation, clearing, immunostaining and mounting will allow full visualization of both lymphatic and blood vessels in the villus and submucosal intestine (Figure 5a) and detection of lymphatic and blood filopodia (Figure 5e, f). Loss of 3D villus structure, incomplete immunostaining in the submucosal vessels and inability to detect vessel filopodia are all indicators of reduced quality (see Table 3). Co-immunostaining with Pecam1 and Lyve1 allows accurate determination of lacteal length and blood vascular density (Figure 5a). Furthermore, immunostaining with Vegfr2 allows precise measurement of villus blood capillary length and branching (Figure 5b). In addition, this protocol allows the visualization of blood capillary and lacteal filopodia and organization of intercellular junctions (Figure 5e, f and ref. 13). The protocol can be combined with the conventional “Swiss roll” protocol for paraffin embedding, allowing measurement of endothelial cell proliferation by staining for Ki67, lymphatic endothelial transcription factor Prox1 (Figure 5g) and pan-endothelial transcription factor Erg (not shown). By using this procedure we are also able to visualize most stromal cell types and components in the small intestine, such as neurons, immune cells, pericytes, smooth muscle cells, fibroblasts and extracellular matrix proteins (Figure 2 and ref. 13). We anticipate this protocol will be used to investigate cell morphology, position and cell-cell interactions in the intestine for researchers from a plethora of fields, as well as serve a standard tool in the vascular biology toolbox for the study of both vessel patterning and specialized function.

AUTHOR CONTRIBUTIONS

JBL performed the experiments; JBL and TVP designed the protocol and wrote the manuscript.

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Competing Financial Interests

The authors declare that they have no competing financial interests.

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FIGURE LEGENDS

Figure 1: Whole-mount immunostaining allows high-resolution imaging of intestinal stroma. (a) Image comparison of intestinal villus blood capillaries (red) and lymphatic lacteals (green) obtained using either paraffin section (left) or whole-mount immunostaining (right). Blood capillaries stained with either Meca32 (left) or Pecam1 (right); lacteals stained with Lyve1; nuclei stained with DAPI (blue, left). **(b)** Image comparison of intestinal villus blood capillaries (red, Vegfr2) obtained using either thick cryosections (200 μm, top) or whole-mount staining (bottom). Blood endothelial cell nuclei are stained for Erg (green). Scale bars: 50 μm: a; 20 μm: b. Animal experiments were approved by the Animal Ethics Committee of Vaud, Switzerland.

Figure 2: All intestinal stromal cell types can be visualized with intestinal whole-mount immunostaining. (a) Whole-mount immunostaining of villus blood capillaries (red, Vegfr2) and neurons (green, Pgp9.5). Note close association of

neurons and blood capillaries. **(b)** Whole-mount immunostaining of intestinal villus lacteal (red, Lyve1), smooth muscle cells (SMCs; cyan, αSMA) and macrophages (green, F4/80). **(c)** Cell-cell interactions can be easily observed as a subset of villus SMCs (cyan, αSMA) are also in contact with lacteals (red, Lyve1). **(d)** $CD11b^+$ (red) and $CD3^+$ (violet) immune cells are readily visualized in close proximity to villus SMCs (cyan, αSMA) using intestinal whole-mount staining. (**e)** $Vimentin^+$ fibroblasts (green) and villus SMCs (red, αSMA) imaged using intestinal whole-mount immunostaining. **(f)** The extracellular matrix protein tenascin C (green) can be visualized around blood capillaries (red, Vegfr2) and throughout the entire villus by whole-mount immunostaining. **(g)** Fibronectin (green) can be observed highly expressed in villi, especially on villus SMCs (red, αSMA). Images with epithelial cells (WM/EP(+)): a, b, c, d. Images without epithelial cells (WM/EP(-)): e, f, g. Scale bars: 50 μm: a, b, d; 20 μm: c, e, f, g. Animal experiments were approved by the Animal Ethics Committee of Vaud, Switzerland.

Figure 3: Experimental outline for preparing intestine for simultaneous imaging by paraffin sectioning, whole-mount with epithelial cells (WM/EP(+)) and whole-mount without epithelial cells (WM/EP(-)). The small intestine is dissected, cleaned and divided into the duodenum (duo), jejunum and ileum. These pieces can then be further partitioned for use in paraffin sections, WM/EP(+) or WM/EP(-) and fixed. The piece for paraffin sectioning is prepared in a Swiss roll and further processed as described in Supplementary Method 1, while the pieces for WM are pinned to silicone plates and epithelial cells are removed from the piece dedicated to WM/EP(-). One cm pieces are then cut and transferred to 6- or 12-well plates for immunostaining. After staining, fine scissors are used to cut strips of the intestine 2-3 villi thick and samples dedicated to WM/EP(+) are cleared using a commercial clearing agent. These

villi strips are then carefully positioned to be flat against the slides inside the spacer and coverslips are affixed prior to confocal microscopy.

Figure 4: Experimental details. (a) After dissection, but before addition of fixation buffer, the whole intestine is pinned on 10 cm silicone-coated dishes. An example is shown on how to pin the whole intestine including both pieces for WM/EP(+) and WM/EP(-). **(b, c)** Whole-mount pinned intestine for WM/EP(-) before (**b**) and after (**c**) scraping off epithelial cells. **(d)** 1 cm intestinal pieces pinned into a silicone-coated 6-well dish for whole-mount immunostaining. **(e)** An example of cutting villi strips for whole-mount immunostaining. **(f)** An example of mounted villi strips, stained for Lyve1 (white), placed correctly on a microscope slide. Scale bars: 1 mm: b, c, f; 500 μm: e. Animal experiments were approved by the Animal Ethics Committee of Vaud, Switzerland.

Figure 5: Expected results. (**a)** Intestinal whole-mount image for measuring lacteal length as a percentage of the blood capillary network length. Lacteals (red, Lyve1) and blood capillaries (green, Pecam1) are imaged using a 20X objective of a confocal microscope. Lacteal length (white bars) and blood capillary network length (yellow bars) are measured and lacteal length is calculated as lacteal length/ blood capillary network length. **(b, c, d)** Intestinal whole-mount imaging for measuring blood capillary vessel length and branching. (**b**) Blood capillaries (red, Vegfr2) are imaged using the 20X objective of a confocal microscope. (**c**) The images are imported into FIJI and converted into binary images. (**d**) Using FIJI the binary images are skeletonized and the resulting network analyzed for length and branching. (**e**) Blood capillary filopodia (green, Vegfr2; arrowheads) can be easily visualized and counted

after whole-mount immunostaining. (**f**) Lacteal filopodia (red, Lyve1; arrowheads) can be easily visualized and counted after whole-mount immunostaining. Immunostaining for Vegfr3 can also be effectively used for counting lacteal filopodia. (**g**) Example paraffin section image of lymphatic endothelial cells positive (white, $Prox1^{+}Ki67^{+}$, arrowhead) or negative (red, $Prox1^{+}$, arrow) for Ki67 (green). DAPI stained nuclei are blue. Scale bars: 100 μm: a; 50 μm: b; 20 μm: e, f, g. Animal experiments were approved by the Animal Ethics Committee of Vaud, Switzerland.

TABLES

Table 1: Comparison of different methods for intestinal imaging

Method	Sample thickness	3D imaging?	Lateral imaging?	Clearing necessary?	Filopodia detection?
Paraffin/frozen thin sections	1-5µm	no	yes	no	no
Frozen thick sections	5-200µm	no	yes	no	no
Stappenbeck et al. whole-mount[10]	whole villus	partial	no	no	no
Fu et al. and Appleton et al. whole-mount[22,23]	whole villus	yes	no	yes	no
Small intestinal whole-mount[13]	whole villus	yes	yes	yes	yes

Table 2: Whole-mount immunostaining antibody mixes

	Primary antibodies	Dilution (final conc.)	Secondary antibodies	Dilution (final conc.)	Antibody against:
Staining 1*	Pecam1	1:400 (1.25 µg/ml)	donkey anti-rat Alexa 488	1:500 (4 µg/ml)	Blood and lymphatic vessels
	Vegfr3	1:100 (1 µg/ml)	donkey anti-goat Alexa 555	1:500 (4 µg/ml)	Lymphatic vessels and filopodia
	Lyve1	1:500 (2 µg/ml)	donkey anti-rabbit Alexa 647	1:500 (4 µg/ml)	Lymphatic vessels and filopodia
Staining 2	Vegfr2	1:100 (1 µg/ml)	donkey anti-goat Alexa 555	1:500 (4 µg/ml)	Blood vessels and filopodia

*****Primary antibodies can be omitted or replaced with antibodies from the same species and of the same isotype as a negative control.

Table 3: TROUBLESHOOTING

Step	Problem	Possible reason	Solution
5	Poor perfusion	Puncture of the ventricle septum or outer heart wall	Gently insert needle into left ventricle to start perfusion. Once the needle is inserted, do not move it, rather let the needle rest on the mouse. If there are multiple punctures try to squeeze heart together with forceps and perfusion needle while perfusing.
9	Inefficient intestine flushing	Holes in intestine from 18 G needle	The 18 G needle can be substituted by a rounded gavage needle
12B(v)	Epithelial cells do not come off easily	Poor fixation	Good perfusion fixation is critical for epithelial cells to be easily scraped off with the forceps. Poor perfusion makes it very difficult to remove the epithelial cells without tissue damage.
12B(v)	Epithelial cells do not come off easily	Not enough pins	Place one pin every 0.5 cm on both the top and bottom of the opened intestine to avoid "loose" intestine. Loose intestine makes easily scraping of epithelial cells difficult.
35	Unable to cut intestine strips for mounting	Using wrong/dull scissors	Sharp, very small scissors are necessary to properly cut the tissue. Also, cutting into the silicone gel also helps make better intestine strips.
45A-D	Villus size is significantly different among samples	Not keeping intestine in right order during dissection	While organizing intestinal tissue during processing for whole-mount and paraffin immunostaining always keep one pin in the upper part of the piece to keep of directionality (steps 10-11).

45A-D	Immunostaining background	Intestine not cleaned enough	Fully clean intestine by flushing in both directions and shaking in 1X PBS. If intestine is not properly cleaned the tissue degrades more quickly and has more background after immunostaining (Step 9).
45A-D	Villus size is significantly different among samples	Not keeping intestine in right order during dissection	To note the upper part of the intestine for the WM/EP(-) preparation, make a small notch in the upper part of the intestinal pieces with very small scissors (Step 11).
45A-D	Immunostaining only on villus tips	Primary antibody incubation time is too short	Increase primary antibody incubation time. Primary antibodies can be left on for several days without an increase in background (Step 23).
46B	Skeletonized outline of blood vessels doesn't match image	Low quality images	Blood vessel networks can be drawn by hand using photo processing software (e.g. Photoshop) and imported into FIJI for network analysis

SUPPLEMENTARY INFORMATION

Supplementary Figure 1: Example of necessity for WM/EP(-) protocol where strong epithelial staining prevents stroma visualization in most villi. Whole-mount immunostaining of intestinal villi for Dll4 (cyan). Dll4 is highly expressed in intestinal epithelial cells preventing visualization of stromal Dll4 (outlined). Scale bar: 50 μm.

Supplementary Method 1: Preparation, immunostaining, imaging and quantification of intestinal paraffin sections

Supplementary Table 1: Antibodies tested for whole-mount and paraffin section immunostaining.

Supplementary Table 2: Paraffin immunostaining antibody mix

Supplementary Table 3: Blood and lymphatic endothelial cell proliferation analysis strategy

Supplementary Table 4: Troubleshooting for Supplementary Method 1

Figure 1 Petrova

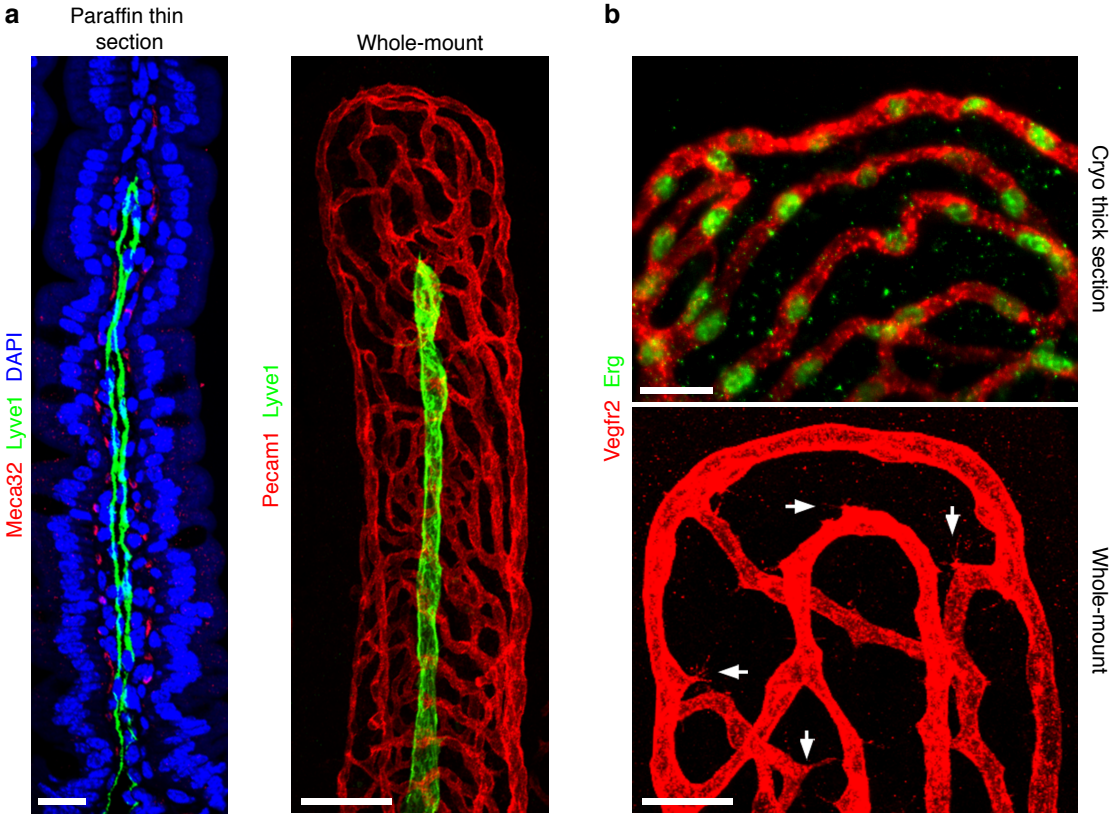


Figure 2 Petrova

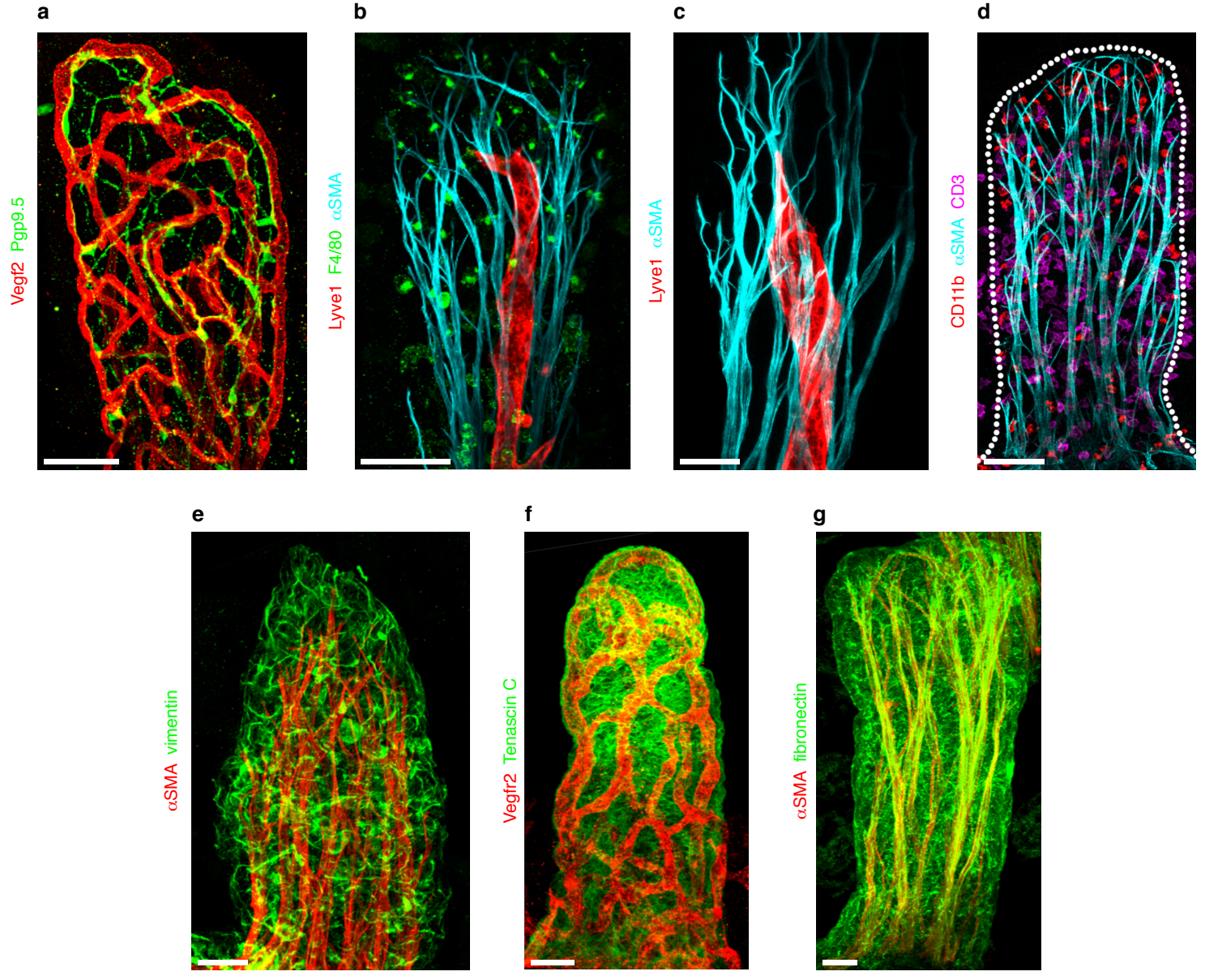


Figure 3 Petrova

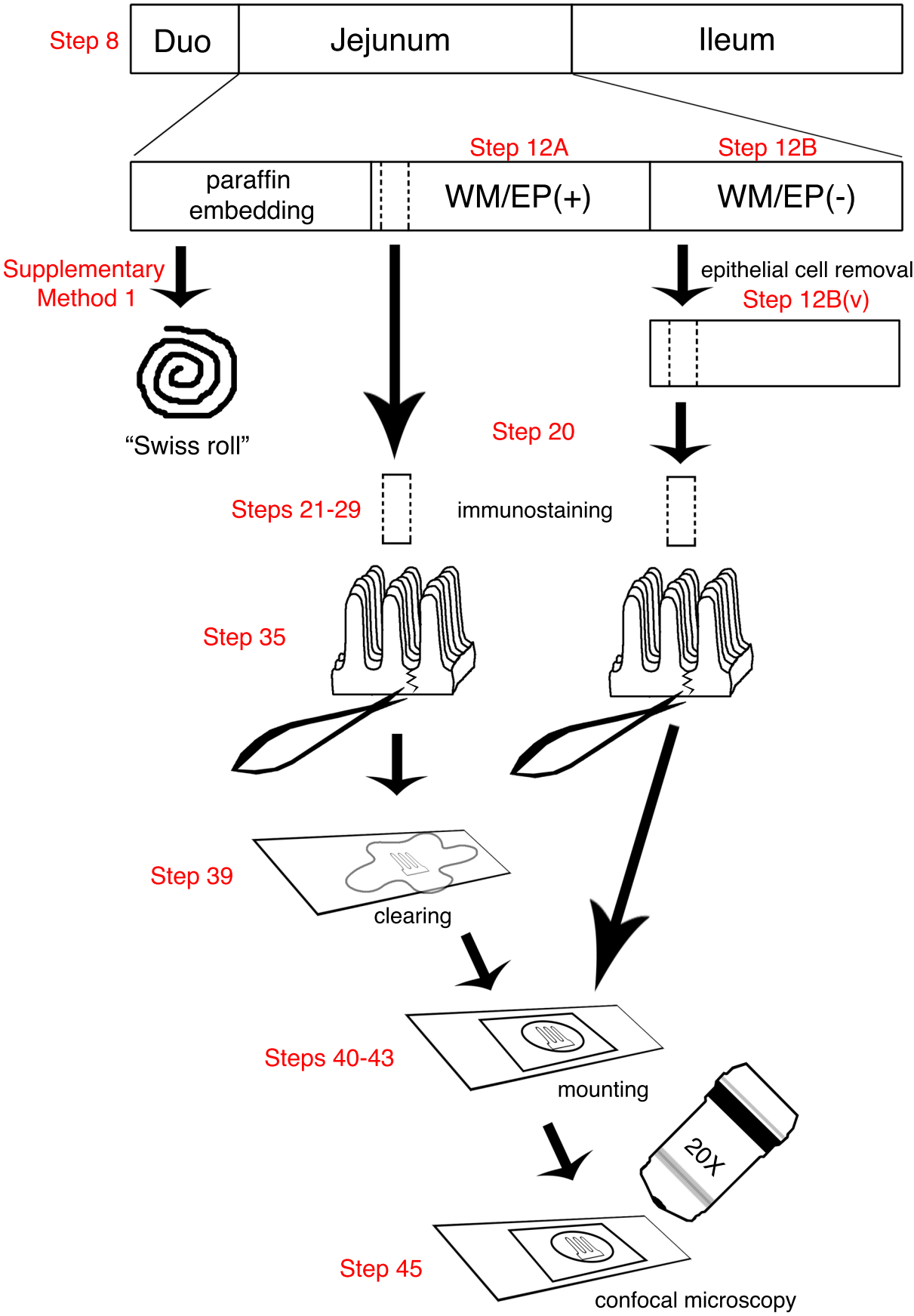
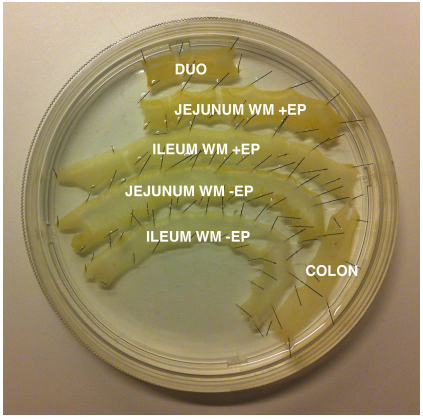


Figure 4 Petrova

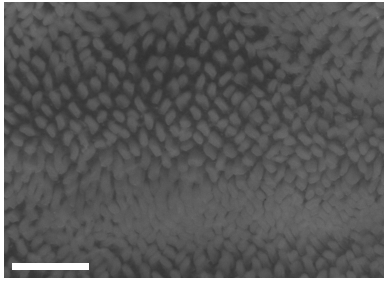
a

Pinned intestine in 10 cm silcone dish



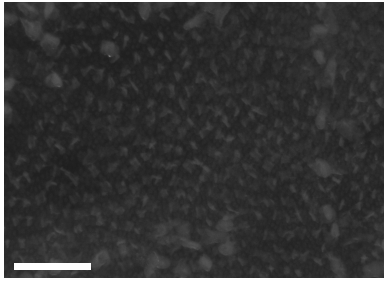
b

Whole-mount +EPTH



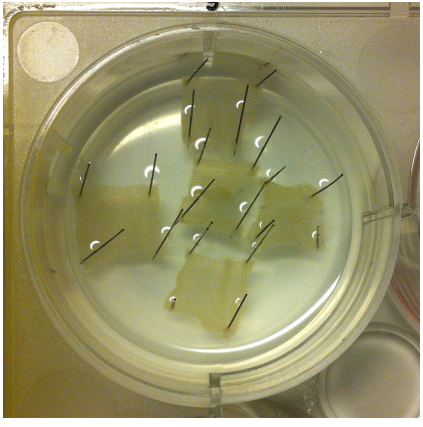
c

Whole-mount -EPTH



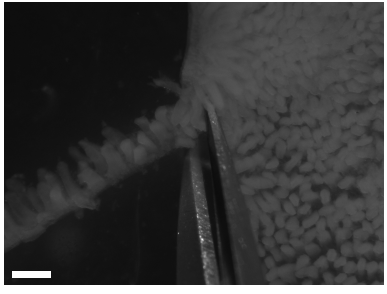
d

1 cm pieces in 6-well dish for immunostaining



e

Cutting intestine strips



f

Stained, cleared, mounted strips on slide

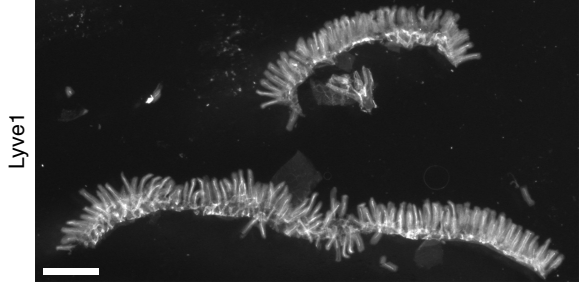
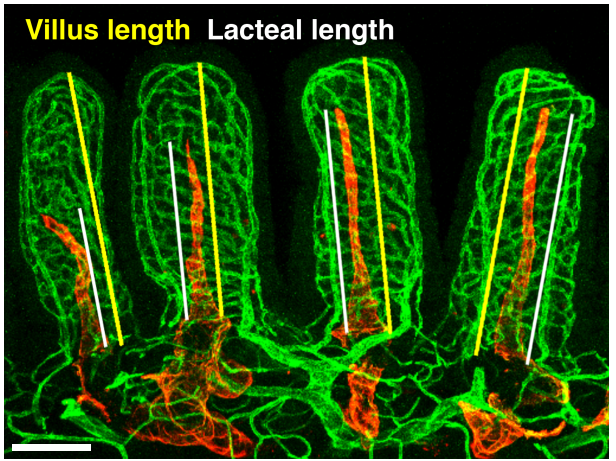
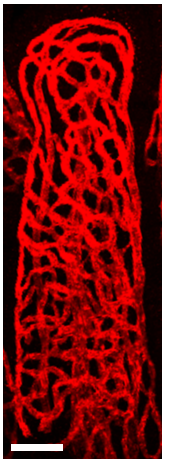
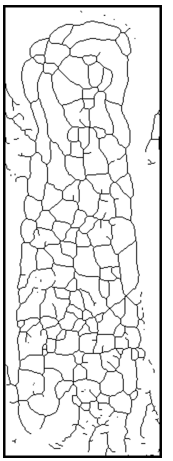
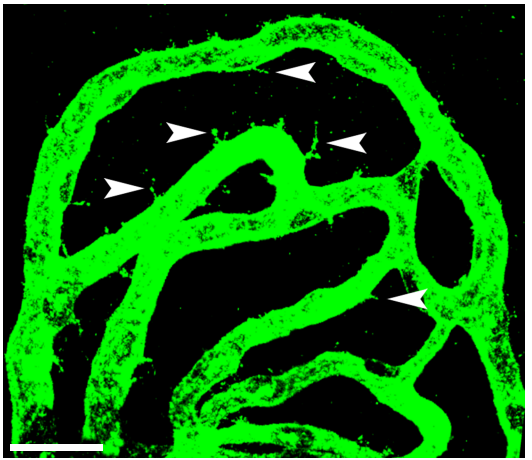


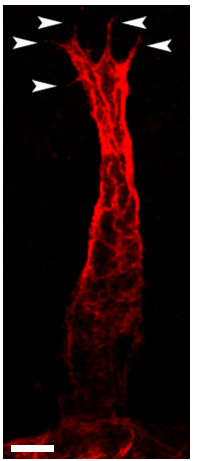
Figure 5 Petrova

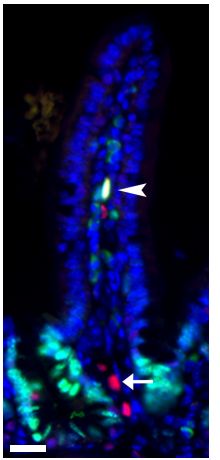


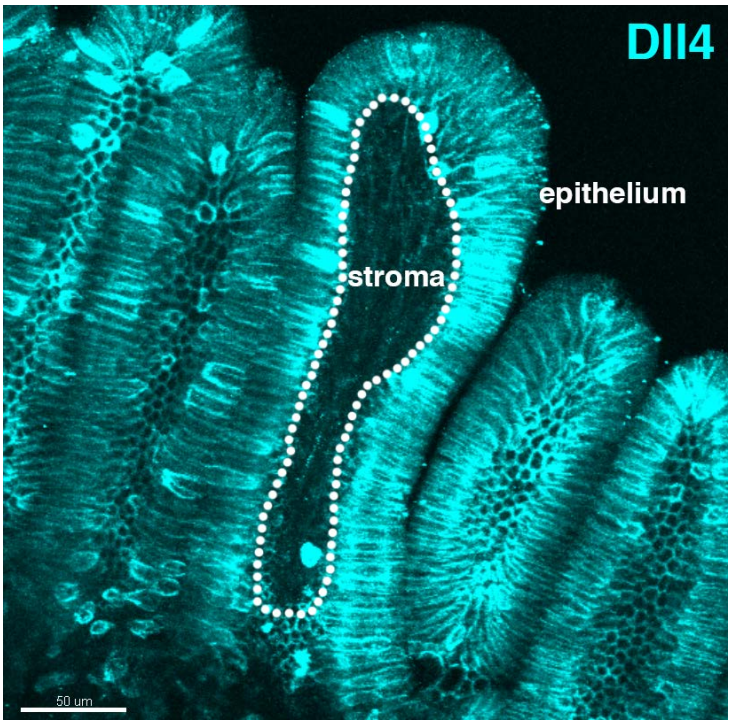












Supplementary Figure 1

Example of necessity for WM –EP protocol where strong epithelial staining prevents stroma visualization in most villi.

Whole-mount immunostaining of intestinal villi for Dll4 (cyan). Dll4 is highly expressed in intestinal epithelial cells preventing visualization of stromal Dll4 (outlined). Scale bar: 50 µm.

Supplementary Information

High-resolution 3D analysis of mouse small intestinal stroma

Jeremiah Bernier-Latmani and Tatiana V. Petrova

Supplementary Method 1- Preparation, immunostaining, imaging and quantification of intestinal paraffin sections

Day 1: Dissection and fixation. Timing: 6-8h

1. Place pieces of intestine designated for paraffin embedding (from Main Protocol, step 11) on a Benchkote paper 15 cm dish. Use a dissection microscope position to ensure the intestinal pieces are placed luminal side down. Use small forceps to spread intestinal pieces flat.
2. Once intestinal pieces are well positioned, place the 10 cm Whatman disc on top of them and press gently, yet firmly, down to stick the pieces to the Whatman disc. Gently pull the Whatman disc up; the intestinal pieces should be stuck villi-up onto the Whatman paper. Use small forceps to spread intestinal pieces flat.
3. Place the intestinal pieces on the Whatman paper into a regular 10 cm cell culture dish and fill with 4% paraformaldehyde until the intestinal pieces are fully submerged. Incubate the samples on an orbital shaker O/N at 4°C.

Troubleshooting

Day 2-3: Washing and paraffin embedding. Timing: 2 days

4. Remove 4% PFA and replace with ice-cold 1X PBS. Wash on orbital shaker, 4°C for 10 min. Repeat three more times.

Caution: Removal of 4% PFA must be done in fume hood.

5. After last 1X PBS wash prepare intestine pieces for Swiss roll. Roll pieces of intestine (villi inside) and fix with two pins/ roll.
6. Place all pieces from one mouse in marked cassette and close cassette. Submerge cassette in ice-cold 70% EtOH.

Pause point: Incubate the samples 4°C until paraffin embedding.

7. Remove insect pins from samples, embed in paraffin and cut 5 µm thick sections.

Day 4: Primary antibody immunostaining. Timing: 3h

8. Place slides with paraffin sections on a heated block at 60°C for 10 min to melt wax.
9. Immediately transfer slides to 100% xylene and agitate by hand for 3 minutes to deparaffinize the slides.

Troubleshooting

Critical step: It is important to transfer the warm slides as quickly as possible to the xylene bath to ensure efficient deparaffinization.

10. Repeat step 2 twice more in new xylene baths agitating for 2 minutes each.
11. Transfer slides to 100% EtOH bath and agitate for 2 minutes.
12. Repeat step 4 twice more in new 100% EtOH baths agitating for 2 minutes each.
13. Transfer slides to 90% EtOH bath and agitate for 2 minutes.
14. Transfer slides to 70% EtOH bath and agitate for 2 minutes.
15. Transfer slides to water bath, briefly agitate and replace water.
16. Pour enough 1X high pH retrieval buffer to cover slides.
17. Cook slides in microwave at high heat for 5 min.
18. Open microwave and examine levels of retrieval buffers. If the slides are exposed add enough 1X high pH retrieval buffer to cover them again.
19. Cook slides again in microwave at medium heat for 10 min.
20. Let slides cool for 30 min. at RT.
21. Wash slides 3 times with 1X PBS.
22. Add M.O.M. blocking reagent in 1X PBS and incubate for 30 min. at RT.

23. Replace blocking reagent with blocking buffer and primary antibody mix with antibodies against Ki67, Erg and Prox1 (Supplementary Table 2).

Pause point: Incubate the slides O/N at 4°C.

Day 5: Primary antibody washing, secondary antibody immunostaining, secondary antibody washing and mounting. Timing: 3h

24. Wash slides 4 times with PBS+0.3% Triton-X100.
25. Incubate slides with secondary antibody mix for 1 hour at RT.
26. Wash slides 7 times with PBS+0.3% Triton-X100.
27. Rinse slides once with 1X PBS.
28. Dry slides and mount coverslips with ProLong Gold antifade reagent with DAPI.
29. Place in slide storage box at 4°C

Pause point: If samples are kept at 4°C and mounting medium remains, slides can be kept for up to a year.

Day 6: Confocal microscopy. Timing: 6-8h

30. Image stained paraffin sections from step 29 using the 405, 488, 543, and 647 lasers (Supplementary Table 2) with the 40X objective of a confocal microscope, to analyze DAPI, Ki67, Prox1 and Erg, so that several villi can be imaged at once. Set pseudocolors as follows: white, DAPI; green, Ki67; red, Prox1; blue, Erg. Take images from 10-15 different regions in each sample.

Day 7: Image analysis. Timing: 6-8h

31. Import images from step 52 into FIJI. Open Cell Counter (Plugins> Analyze>Cell Counter).
32. Turn off the DAPI channel (white). When counting proliferating blood and lymphatic endothelial cells switch off Prox1 (red) and Erg (blue), respectively,

to avoid confusion. Once a $Ki67^{+}Erg^{+}$ is observed, switch back on Prox1 to verify if the cell is a lymphatic endothelial cell. Count cells according to Supplementary Table 3 using Cell Counter.

33. Copy and paste results into a spreadsheet for analysis.

TROUBLESHOOTING

Troubleshooting advice is provided in Supplementary Table 4.

TIMING

Day 1: Steps 1-3, tissue dissection, preparation and fixation: 6-8h

Day 2-3: Steps 4-7, sample washing and paraffin embedding: 2-3h

Day 4: Steps 8-23, primary antibody immunostaining: 3h

Day 5: Steps 24-29, primary antibody washing and secondary antibody immunostaining, secondary antibody washing and mounting: 3h

Day 6: Step 30, confocal microscopy: 6-8h

Day 7: Steps 31-33, image analysis: 6-8h

Supplementary Table 1: Antibodies tested for whole-mount and paraffin section immunostaining.

	Antibody	Dilution (final conc.)	Supplier	Catalog number	Clonality/ clone name	WM/EP(-) needed?
Wholemount	α-SMA-Cy3 (mouse)	1:1000 (1µg/ml)	Sigma	C6198	monoclonal/ 1A4	no
	β-catenin (rabbit)	1:200 (from original stock)	Millipore	06-734	Polyclonal	yes (for stroma)
	CD3 (armenian hamster)	1:1000 (0.5µg/ml)	Biolegend	100301	monoclonal/ 145-2C11	no
	CD11b (rat)	1:200 (1µg/ml)	Biolegend	101201	monoclonal/ M1-70	no
	Dll4 (goat)	1:300 (0.66µg/ml)	R&D	AF1389	Polyclonal	yes (for stroma)
	Endomucin (rat)	1:250 (from original stock)	eBioscience	14-5851-82	monoclonal/ V.7C7	yes
	Erg (rabbit)	1:300 (from original stock)	Abcam	ab92513	monoclonal/ EPR3864	no
	F4/80 (rat)	1:200 (from original stock)	Invitrogen	MF48000	monoclonal/ BM8	no
	Fibronectin (rabbit)	1:200 (5µg/ml)	Millipore	AB2033	Polyclonal	yes
	Lyve-1 (rabbit)	1:400 (2.5µg/ml)	AngioBio	11-034	Polyclonal	no
	Lyve-1 (rat)	1:100 (5µg/ml)	R&D	MAB2125	monoclonal/ 223322	no
	NG2 (rabbit)	1:1000 (1µg/ml)	Millipore	AB5320	Polyclonal	yes
	Pecam-1 (rat)	1:400 (1.25µg/ml)	BD Pharmingen	557355	monoclonal/ MEC 13.3	no
	Periostin (goat)	1:1000 (0.2µg/ml)	R&D	AF2955	Polyclonal	yes
	Pgp9.5 (rabbit)	1:1000 (from original stock)	Abcam	ab10404	Polyclonal	no
	Tenascin C (rat)	1:1000 (0.5µg/ml)	R&D	MAB 2138	monoclonal/ 578	yes
	Vegfr-2 (goat)	1:100 (2µg/ml)	R&D	AF644	Polyclonal	no
	Vegfr-3 (goat)	1:100 (2µg/ml)	R&D	MAB3491	Polyclonal	no
	Vimentin (chicken)	1:500 (from original stock)	Millipore	AB5733	Polyclonal	yes
Paraffin	Ki67 (mouse)	1:300 (1.66µg/ml)	BD Pharmingen	556003	monoclonal/ B56	
	Meca32 (rat)	1:300 (0.1µg/ml)	BD Pharmingen	550563	monoclonal/ MECA32	
	Prox1 (goat)	1:150 (1.33µg/ml)	R&D	AF2727	Polyclonal	
Secondary Abs	Alexa Fluor 488	1:500 (WM) 1:300 (paraffin)	Invitrogen			
	Alexa Fluor 555		Invitrogen			
	Alexa Fluor 647		Invitrogen			

Supplementary Table 2: Paraffin immunostaining antibody mix

Primary antibodies	Dilution (final concentration)	Secondary antibodies	Dilution (final concentration)
Ki67	1:300 (1.66 µg/ml)	donkey anti-mouse Alexa 488	1:300 (6.6 µg/ml)
Prox1	1:300 (0.33 µg/ml)	donkey anti-goat Alexa 555	1:300 (6.6 µg/ml)
ERG	1:200 (4 µg/ml)	donkey anti-rabbit Alexa 647	1:300 (6.6 µg/ml)

Supplementary Table 3: Blood and lymphatic endothelial cell proliferation analysis strategy

Cell-type	Ki67 status	Color
LEC	Neg	magenta
LEC	+	white
BEC	Neg	blue
BEC	+	cyan

Assumes pseudocolors as the following: green, Ki67; red, Prox1; blue, Erg. LEC, lymphatic endothelial cell; BEC, blood endothelial cell.

Supplementary Table 4: Troubleshooting for Supplementary Method 1

Step	Problem	Possible reason	Solution
3	Intestinal pieces for paraffin embedding do not stick to Whatman paper	Well-perfused intestine sticks less well to Whatman paper.	Use insect pins to pin intestinal pieces to Whatman paper.
30	Immunostaining does not work on paraffin sections	Incomplete deparaffinization.	Transfer slides from the heated block to 100% xylene solution as quickly as possible. Also, ensure that 100% xylene is clean and does not already contain paraffin wax (step 9).