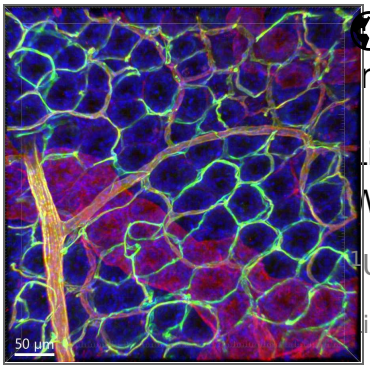

Mouse colon vasculature labeling combined with immunofluorescence

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ABSTRACT

To study the distribution, morphology and innervation of vasculature in different mouse colonic segments and layers, as well as spatial relationships of the vasculature with the enteric plexuses, glia and macrophages

MATERIALS

Animals

Mice, C57BL/6J (000664, Jackson Laboratories, Sacramento, CA), male and female 8-12 weeks old

Reagents and supplies

1. Isoflurane
2. 0.9% saline
3. wheat germ agglutinin-Alexa Fluor (WGA-AF) 448 (W11261, ThermoFisher Scientific)
4. 0.01 M phosphate-buffer saline (PBS), stock solution 10X
5. paraformaldehyde (P6148, Millipore-Sigma)
6. Sylgard™ 184 silicone elastomer (Electron Microscopy Science, Hatfield, PA)
7. Acrylamide
8. VA-044 Initiator
9. A4F0 hydrogel (Yang et al. 2014): 4% acrylamide and 0.25% VA-044 Initiator in 0.01M PBS. (A4F0: 4% acrylamide without paraformaldehyde)
10. Sucrose
11. Primary antibodies (Table 1)
12. Secondary antibodies: donkey anti-rabbit IgG 555 or 647, donkey anti-rat IgG 594 (all purchased from ThermoFisher)

13. Normal donkey serum
14. Triton-X 100
15. OCT Compound (Tissue-Plus™, 23-730-571, Fisher Scientific)
16. DAPI (4', 6-diamidino-2-phenylindole; 62247, ThermoFisher Scientific)
17. RIMS (refractive index matching solution) (Yang et al. 2014)
18. Vectashield anti-fade mounting medium (Vector Laboratories, Burlingame, CA)
19. iSpacer (Sunjin Lab, Hsinchu City, Taiwan, R.O.C.) (Yuan et al. 2021)

Table

1. Primary antibodies

A	B	C	D	E	F
Antibody	**RRID***	Species	Source	Catalog No.	**Dilution**
CD31	AB_393571	Rat	BD Biosciences	550274	1:50
PGP9.5	AB_10891773	Rabbit	Abcam	ab108986	1:1,000
αCGRP	AB_518147	Rabbit	Peninsula	T-4032	1:2,000
VIP	AB_2890602	Rabbit	CURE/UCLA	ab7913	1:1,000
TH	AB_390204	Rabbit	Millipore	AB152	1:1,000
GFAP	AB_305808	Rabbit	Abcam	ab7260	1:2,000
S100B	AB_882426	Rabbit	Abcam	AB56242	1:1,000
Iba1	AB_839504	Rabbit	WAKO	019-19741	1:1,000

*: RRID: Research Resource Identifiers

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We use this protocol and it's working

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Keywords: colon, fluorescent vessel painting, immunohistochemistry, microvessel, mouse, vasculature, wheat germ agglutinin

Tissue collection and preparation

1

1.1 Remove the whole colon is removed from the ileocecal junction to the end of distal colon at the level of pelvic brim where the iliac artery runs, after the cardiovascular perfusion of WGA.

1.2 Open the colon along the mesenteric margin, and pin the colon flat on a dish coated with Sylgard™ 184 silicone elastomer.

1.3 Fix the colon in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) overnight at 4°C.

1.4 Divide the colon samples to the proximal, mid and distal colon (Wang et al. 2022; Wang et al. 2023).

1.5 To improve penetration of antibodies for immunofluorescence, or for visualization of vasculature in different layers, the colonic samples could be prepared as the following approaches:
(1) Clear the samples using A4P0 hydrogel. The methodological details as described in previous report (Yang et al. 2014) and the protocol on protocols.io (Yuan et al. https://dx.doi.org/10.17504/protocols.io.bqi2muge).
(2) Treat the samples with 2% Triton-X 100 and 10% normal donkey serum in 0.01M PBS for 1 h at room temperature (RT) and then overnight at 4°C.
(3) Because the layers of the proximal colon with mucosal folds are thick and poorly immunolabeled, the proximal colon samples are prepared as: all layers without the mucosa and separating the wall in two parts at the submucosal layer.
(4) Frozen thick sections of colonic walls. The colon of mice perfused with the paraformaldehyde immersed in 20% sucrose for 2 days for cryoprotection, and embedded in Tissue-PlusTM O.C.T. Compound, snap-frozen and sectioned at 200 μm in a cryostat.

Vasculature painting

2

2.1 Dilute WGA AF-488 in 0.1M phosphate-buffer saline (PBS) at 30 μg/ml.

2.2 Anesthetized mice deeply with 5% isoflurane.

2.3 Flush the blood system with 0.9% saline via a cardiac cannula

2.4 Perfuse WGA-AF 488 (30 µg/g of body weight) at 1 ml/g of mouse body weight and a rate of 3 ml/min.

For immunostaining in thick colonic wall sections, some mice were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) following WGA perfusion.

Immunohistochemistry

3 Process samples of mice perfused with WGA-AF 488 by the following steps:

3.1 2% Triton-X 100 and 10% normal donkey serum in 0.01M PBS for 1 h at room temperature (RT) and then overnight at 4°C (the same step of 5(2) in Tissue Preparation)

3.2 Primary antibodies (Table 1) in 0.3% Triton-X 100-PBS for 2 h RT, at 4°C 2-5 days

3.3 Secondary antibodies for 3 h at RT, then overnight at 4°C.

3.4 Counterstaining: DAPI 1:2,000, 30 min at RT.

3.5 Mount the colonic tissues onto glass slides in a frame (iSpacer), and seal by glass coverslip in Vectashield anti-fade mounting medium or RIMS

Imaging and quantitative analysis

4

4.1 Acquire microscopic images were acquired with Z-stacks in Zeiss confocal microscopes (LSM 710 and 880, Carl Zeiss Microscopy GmbH, Germany), using objectives of 10X, 20X and 63X.

4.2 The optical sections were scanned at intervals of 2 or 2.5 µm in 10X, 1 µm in 20X and 0.5 µm in 63X objectives.

4.3 The image segmentation, quantitation and visualization using Imaris 9.8 and 9.9 for neuroscientists (Bitplane Inc., Concord, MA).

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