Journal of Visualized Experiments

En face immunofluorescence staining – A method to observe vascular endothelial cells directly

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59325R2
Full Title:	En face immunofluorescence staining – A method to observe vascular endothelial cells directly
Keywords:	En face; Immunofluorescence; aorta endothelial; morphology; in situ; Atherosclerosis
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TITLE:

Using En Face Immunofluorescence Staining to Observe Vascular Endothelial Cells Directly

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KEYWORDS:

En face, immunofluorescence, aorta endothelial, morphology, in situ, atherosclerosis

SUMMARY:

Here, we present a protocol for immunofluorescence staining to observe the endothelial cells of the mouse aorta directly. This technique is useful when studying the cellular and molecular phenotype of endothelial cells in different flow patterns and in the development of atherosclerosis.

ABSTRACT:

Aberrant changes in endothelial phenotype and morphology are considered to be initial events in the pathogenesis of atherosclerosis. Direct observation of the intact endothelium will provide valuable information for understanding the cellular and molecular events in the dysfunctional endothelial cells. Here, we describe a modified en face immunofluorescence staining technique which enables scientists to obtain clear images of the intact endothelial surface and analyze the molecule expression patterns in situ. The method is simple and reliable for observing the entire endothelial monolayer at different sites of the aorta. This technique may be a promising tool for understanding the pathophysiology of atherosclerosis, especially at an early stage.

INTRODUCTION:

The early changes in the vasculature primarily initiate in the endothelium, which functions as a selective barrier between the blood and the vessel wall with its intercellular tight junctional complexes[1]. Substantial evidence points to a critical role for the mechanical effects of blood flow in modulating endothelial function[2]. Fluid shear stress, a frictional force generated by blood flow, differentially shapes endothelial cell morphology and function, depending on the specific flow paradigms at different vascular sites[2,3]. Atherosclerotic lesions preferentially occur at sites of disturbed blood flow (d-flow), such as vessel curvatures, flow dividers, and branch points, as compared to regions of steady flow (s-flow), such as the straight segment of the artery.

Therefore, direct observation of endothelial morphology and molecule expression patterns should provide important insights into the structural and functional phenotypes of endothelial cells under varying flow paradigms.

Cultured endothelial cells may not express the actual phenotype as they do in vivo partly due to the loss of impact of fluid shear stress, surrounding cytokines, and cell-cell or cell-extracellular matrix interactions. To aid this, the intact endothelial cell monolayer can be studied on transverse sections using classical immunohistochemistry. However, the endothelial monolayer is so thin and fragile that it usually cannot be observed clearly. En face immunohistochemistry has been used to observe the inner surface of the endothelium but is either complicated or erratic in its results because the endothelium is easily stripped from the underlying tissue, or just part of the arterial wall of rats or rabbits, whose walls are thick, is mounted[4,5].

Mouse models have considerable advantages over other animals in many respects. Here, we employ a modified en face immunofluorescence technique to analyze endothelial cells of the aortic arch and thoracic aorta in C57BL/6 mouse. Such a technique has been widely used to study the endothelial pathophysiology in different flow patterns and in the development of atherosclerosis[6–10]. This method allows scientists to observe the entire surface of the endothelium clearly and to compare the expression patterns of a given protein in regions under different fluid shear stress.

PROTOCOL:

All animal experiments were conducted in accordance with experimental protocols approved by the Committee on Animal Resources of Shanghai Jiao Tong University.

1. Perfusion of the mouse aorta

1.1. Briefly, anesthetize 12-week-old C57BL/6 mice with intraperitoneal injections of sodium pentobarbital (50 mg/kg body weight). Confirm proper anesthetization by gently pinching the tail.

NOTE: If no movement is observed, the animal is sufficiently anesthetized to start the experiments.

1.2. Tape the mouse's paws to a stack of paper towels with adhesive tapes.

1.3. Hold up the skin of the mouse with forceps and cut the skin with a pair of scissors from the abdomen to the top of the thorax.

1.4. Open the abdominal cavity below the ribcage with a sharp pair of scissors.

1.5. Lift the sternum with forceps and cut the diaphragm; then, cut away the ribcage to expose the thoracic cavity.

1.6. Cut off the vena cava just above the liver, with scissors.

1.7. Pressure perfuse (100 mmHg) the arterial tree for 5 min with prechilled normal saline containing 40 units/mL heparin through the left ventricle until the lungs and liver become pale. Then, perfuse with prechilled 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 3 min.

1.8. Remove all the muscles, organs, and fat until the aorta is exposed.

1.9. Place the mouse under a dissecting microscope.

2. Dissection and longitudinally opening of the aorta

2.1. Expose the aorta clearly under a dissecting microscope and remove the connective tissues along the aorta as clean as possible, with delicate forceps and a pair of delicate scissors (**Figure 1**).

2.2. Dissect the thoracic aorta from the heart to the celiac trunk with a pair of delicate scissors and put the aorta into a 6 cm cell culture dish with PBS. Cut open the aorta longitudinally, along the lesser curve, and along the greater curve until the straight segment is met. Cut open the three branches of the aortic arch, including the innominate, left common carotid and the left subclavian artery, with microscissors, as shown in **Figure 2**.

3. Pretreatment and immunostaining of the aorta

3.1. Permeabilize the aorta with 0.1% polyoxyethylene octyl phenyl ether in PBS for 10 min and block it with 10% normal goat serum in Tris-buffered saline (TBS) containing 2.5% polysorbate 20 for 1 h at room temperature in a 12-well cell culture plate.

3.2. Next, incubate the aorta with 5 g/mL rabbit anti-VCAM-1 and 5 g/mL rat anti-VE-cadherin in the blocking buffer, overnight at 4 °C.

3.3. After rinsing the sample 3x with washing solution (TBS containing 2.5% polysorbate 20), apply the fluorescence-conjugated secondary antibodies (1:1,000 dilution, Alexa Fluor 555-labeled anti-rabbit IgG and Alexa Fluor 488-labeled anti-rat IgG) for 1 h at room temperature. Rinse 3x in the washing solution.

3.4. Counterstain the aorta with 4’,6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 10 min and rinse it 3x in the washing solution.

4. Mounting of the aorta on the glass slide

4.1. Place the aorta on a coverslip with the luminal surface downward and move it slowly to the antifade mounting solution previously dropped on the coverslip. Gently stretch the aorta to keep

the specimen flat.

4.2. Inverse the coverslip and put it on the slide glass. Care must be taken not to allow any air bubbles to remain between the specimen and the glass.

5. Observation of the aorta

5.1. Examine the aorta with a laser-scanning confocal microscope.

5.2. Analyze color intensities of different channels from the desired region in the en face images with Image-Pro Plus software.

REPRESENTATIVE RESULTS:

A 12-week-old C57BL/6 mouse was euthanized and perfused with normal saline containing 40 units/mL heparin and, then, prechilled 4% paraformaldehyde. The mouse aorta was exposed under a dissecting microscope (**Figure 1**), dissected, and cut open longitudinally (**Figure 2**). En face immunofluorescence staining of the vascular endothelial cells was performed as illustrated in **Figure 3** and **Table 1**. En face immunofluorescence of the vascular cell adhesion protein-1 (VCAM-1) expression with VE-cadherin as endothelial marker was shown under varying flow patterns from different regions of the mouse aorta. DAPI was also counterstained to show the cell nuclei for better visualization. The endothelial and smooth muscle cells can be easily distinguished from the morphology of the cell nuclei when looking through the *z*-stacks under the microscope since the endothelial cell nuclei are oval shaped and bigger than the spindle-shaped smooth muscle cell nuclei. The representative en face images are shown in **Figure 4**. The aorta was examined by the LSM 710 Laser Scanning Microscope (**Table of Materials**) with a FLUAR 40x/1,3 oil lens. From the en face immunofluorescence staining, we can clearly and directly observe that the expression of VCAM-1 was more abundant in regions under disturbed flow (lesser curvature of the aorta arch) than in those under steady flow (greater curvature of the aorta arch and the thoracic aorta).

FIGURE AND TABLE LEGENDS:

Figure 1: Exposing the mouse aorta under a dissecting microscope. Remove the connective tissues along the aorta as cleanly as possible. a = innominate artery; b = left common carotid artery; c = left subclavian artery.

Figure 2: Dissection of the mouse thoracic aorta and cutting it open longitudinally. (**A**) The thoracic aorta from the heart to the celiac trunk was cut open along the lesser curve longitudinally, and along the greater curve, until the straight segment was met. The three branches of the aortic arch, including the innominate, left common carotid, and left subclavian artery, were also cut open with microscissors. Red dashed lines indicate the cutting line. (**B**) The aorta was opened and spread flat on the glass slides.

Figure 3: Schema for en face immunofluorescence staining of vascular endothelial cells. First,

permeabilize the dissected aorta with 0.1% polyoxyethylene octyl phenyl ether in PBS for 10 min and block it with 10% normal goat serum in TBS containing 2.5% polysorbate 20 for 1 h at room temperature in a 12-well cell culture plate. Next, incubate the aorta with primary antibody in the blocking buffer overnight at 4 °C, and rinse it three times with washing solution (TBS containing 2.5% polysorbate 20). Then, apply fluorescence-conjugated secondary antibodies for 1 h at room temperature and rinse three times. Counterstain the aorta with DAPI for 10 min and rinse it three times in the washing solution. At last, mount the aorta on a glass slide and observe the aorta by a laser-scanning confocal microscope.

Figure 4: Representative en face staining results. (**A**) En face immunofluorescence analysis of the lesser curvature of a mouse aorta (d-flow areas) and the greater curvature or thoracic aorta (s-flow areas) to compare the endothelial VCAM-1 expression under different flow paradigms. Endothelial cell morphology is shown by VE-cadherin staining. (**B**) Representative d-flow areas (lesser curvature) and s-flow areas (greater curvature and thoracic aorta) are indicated by arrows.

Table 1: Detailed information about the en face immunofluorescence staining procedure.

DISCUSSION:

The endothelium is exposed to numerous proatherogenic factors, including lipids, inflammatory mediators, and fluid shear stress[1,11,12]. Direct observation of endothelial cells in situ provides the special advantages to analyze changes in cell morphology, intercellular junctions, and molecule expression patterns in response to the injury stimuli.

Previous studies have provided two different en face immunohistochemical techniques to observe the endothelium of the arterial wall[4,5]. One is to obtain an endothelial monolayer by stripping the endothelium from the vessel wall by a special technique, which is difficult to master and could result in the loss of much endothelium at the branching sites of the artery[4]. The other is mounting the whole arterial wall as we described. Whole-mount preparation is easy to perform and can maintain the whole endothelial monolayer in rabbit and rats[5,13]. However, spreading the whole mouse aortic specimen flat is not easy, and failure to do so may largely affect the quality of the observation. The preparation of the aortic specimen as described here, with the curvatures cut open and the luminal surface faced downward on the coverslip, makes it easier to spread the aortic specimen flat on the slide glass. Moreover, a laser-scanning confocal microscope is required to better focus on the thin endothelial cell monolayer lining the blood vessel.

A key point of this technique is to stretch the aortic specimen as flat as possible to get a better observation of the aorta. In addition, heparin should be added to normal saline before perfusion, and the aortic specimen should be treated gently throughout the process. Paraformaldehyde should be freshly prepared and kept at 4 °C until usage.

There are limitations to this technique. First, the capacity of en face immunofluorescence to detect subendothelial substance, such as deposited low-density lipoprotein, is limited when compared to regular immunohistochemistry of tissue sections. Second, the fluorescent signals are readily bleached after a scan by the confocal microscope, especially when performing *z*-axis

analysis. Moreover, the expression of the target molecule can only be semiquantified, according to its fluorescent intensity.

In summary, this modified en face immunofluorescence staining technique provides an easy way to analyze the morphology and protein expression pattern of vascular endothelial cells in regions under different blood flow paradigms[6,7] and in the pathogenesis of atherosclerosis.

ACKNOWLEDGMENTS:

This study was supported by the National Natural Science Foundation of China (Grant No. 81670451, 81770430), the Shanghai Rising-Star Program (Grant No. 17QA1403000), and the Science Technology Committee of the Shanghai Municipal Government (Grant No. 14441903002, 15411963700).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Gimbrone Jr., M. A., Garcia-Cardena, G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circulation Research.* **118** (4), 620-636 (2016).
2. Zhou, J., Li, Y. S., Chien, S. Shear stress-initiated signaling and its regulation of endothelial function. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **34** (10), 2191-2198 (2014).
3. Tarbell, J. M. Shear stress and the endothelial transport barrier. *Cardiovascular Research.* **87** (2), 320-330 (2010).
4. Warren, B. A. A method for the production of "en face" preparations one cell in thickness. *Journal of Microscopy.* **85** (4), 407-413 (1965).
5. Azuma, K. et al. A new En face method is useful to quantitate endothelial damage in vivo. *Biochemical and Biophysical Research Communications.* **309** (2), 384-390 (2003).
6. Son, D. J. et al. The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis. *Nature Communications.* **4**, 3000 (2013).
7. Go, Y. M. et al. Disturbed flow enhances inflammatory signaling and atherogenesis by increasing thioredoxin-1 level in endothelial cell nuclei. *PLOS ONE.* **9** (9), e108346 (2014).
8. Kundumani-Sridharan, V., Dyukova, E., Hansen 3rd., D. E., Rao, G. N. 12/15-Lipoxygenase mediates high-fat diet-induced endothelial tight junction disruption and monocyte transmigration: a new role for 15(S)-hydroxyeicosatetraenoic acid in endothelial cell dysfunction. *The Journal of Biological Chemistry.* **288** (22), 15830-15842 (2013).
9. Liu, Z. H. et al. C1q/TNF-related protein 1 promotes endothelial barrier dysfunction under disturbed flow. *Biochemical and Biophysical Research Communications.* **490** (2), 580-586 (2017).
10. Wang, X. Q. et al. Thioredoxin interacting protein promotes endothelial cell inflammation in response to disturbed flow by increasing leukocyte adhesion and repressing Kruppel-like factor 2. *Circulation Research.* **110** (4), 560-568 (2012).
11. Mitra, S., Deshmukh, A., Sachdeva, R., Lu, J., Mehta, J. L. Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy. *The American Journal of the Medical Sciences.* **342** (2), 135-142 (2011).
12. Stancel, N. et al. Interplay between CRP, Atherogenic LDL, and LOX-1 and Its Potential Role in

the Pathogenesis of Atherosclerosis. *Clinical Chemistry.* **62** (2), 320-327 (2016).
13. Nerem, R. M., Levesque, M. J., Cornhill, J. F. Vascular Endothelial Morphology as an Indicator of the Pattern of Blood Flow. *Journal of Biomechanical Engineering.* **103** (3), 172-176 (1981).

Figure 1

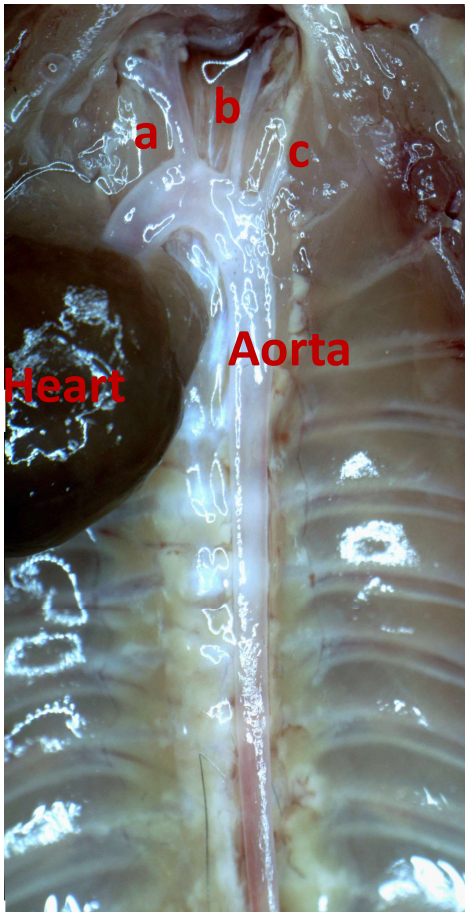

Figure 2

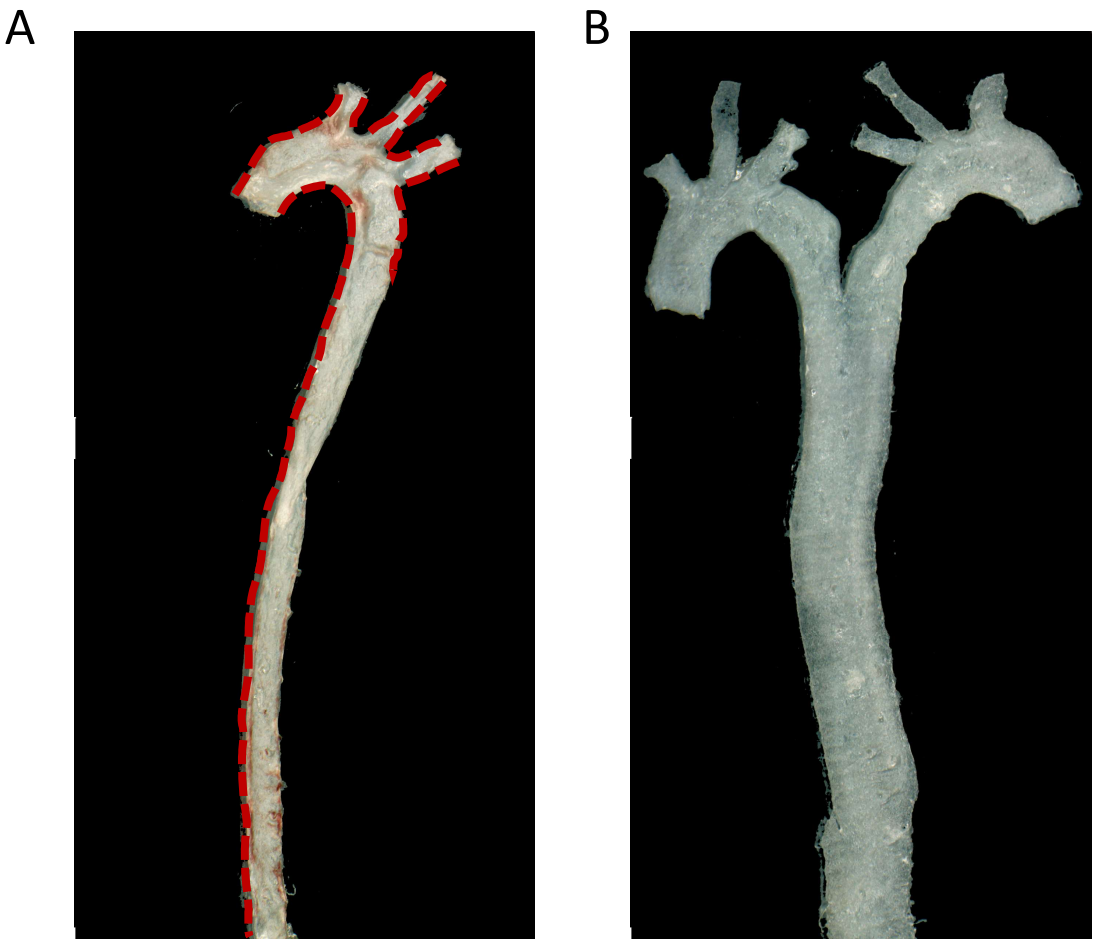

Figure 3

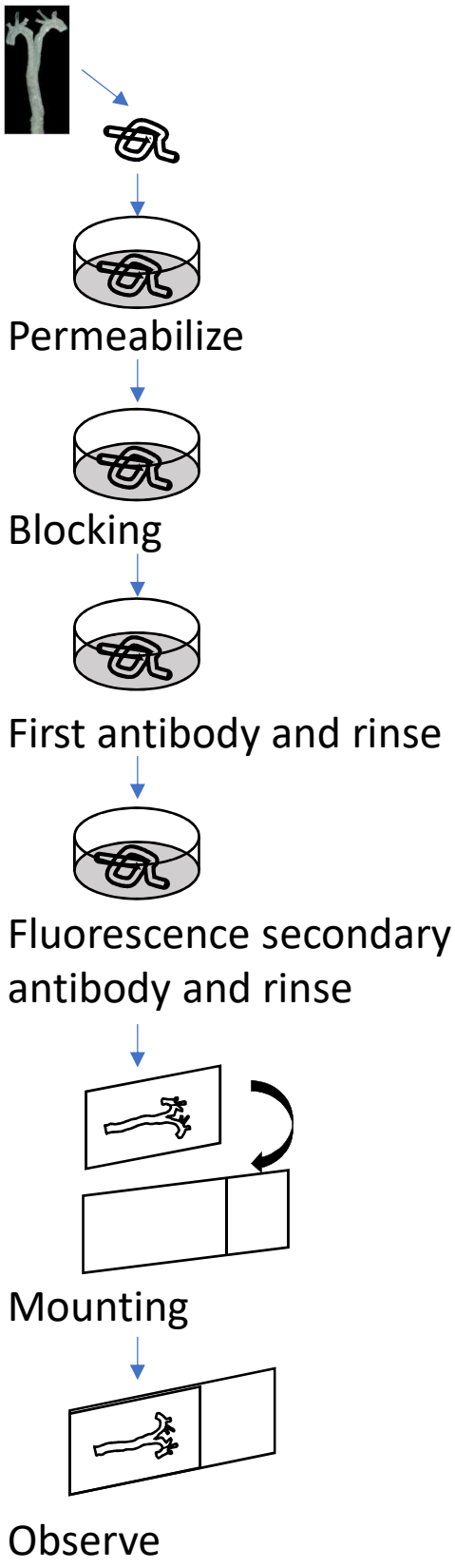

Figure 4

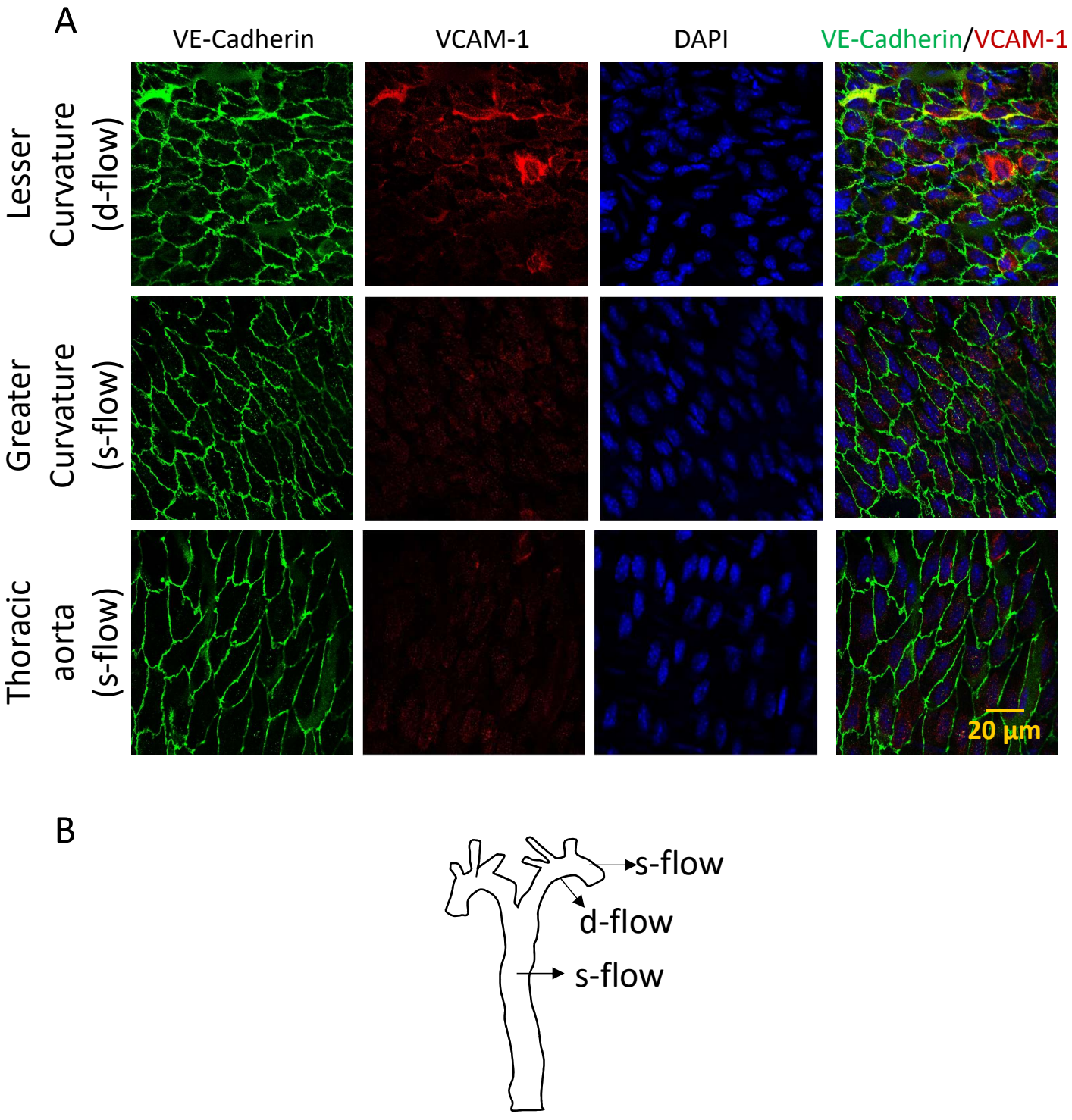

Table 1. Detailed information for the e*n face* immunofluorescence staining procedure.

Step	Process	Buffer	Temperature	Time
1	Permeabilize	0.1% polyoxyethylene octyl phenyl ether in PBS	Room temperature	10 min
2	Blocking	10% normal goat serum in Tris-buffered saline (TBS) containing 2.5% polysorbate 20	Room temperature	1 h
3	First antibody (5 g/mL)	Blocking buffer	4 ℃	Overnight
4	Rinse	TBS containing 2.5% polysorbate 20	Room temperature	5 min, 3 times
5	Fluorescence secondary antibody (1:1000)	Blocking buffer	Room temperature	1 h
6	Rinse	TBS containing 2.5% polysorbate 20	Room temperature	5 min, 3 times

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Antifade mountant	Servicebio	G1401	
Delicate Forceps	RWD Life Science	F11001-11	
Delicate Scissors	RWD Life Science	S12003-09	
Dissecting Forceps	RWD Life Science	F12005-10	
Mciro Spring Scissors	RWD Life Science	S11001-08	
Polyoxyethylene octyl phenyl ether (Triton X-100）	Amresco	M143	
Polysorbate 20 (Tween 20)	Amresco	0777	
VCAM-1 antibody	Abcam	ab134047	
VE-Cadherin antibody	BD Biosciences	555289	
Alexa Fluor 555 labeled anti-rabbit IgG	invitrogen	A-31572	
Alexa Fluor 488 labeled anti-rat IgG	invitrogen	A-21208	
Laser Scanning Microscope	Carl Zeiss		

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