In Vitro Vascular Permeability Imaging Assay (Green)

32 assays (4 x 8-Well Chamber Slides)

Catalog No. 17-10398

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures.

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Introduction

A fundamental requirement for the physiological performance of organs is the formation of diffusion barriers that separate and maintain compartments of different structure. The endothelial cell lining of the internal vasculature defines a semi-permeable barrier between the blood and the interstitial spaces of the body. This barrier is composed of intercellular adherens, tight, and gap junction complexes, as well as desmosomes [1]. Junction substructure components such as connexins, integrins, cadherins, catenins, occludins, desmoplakins, selectins, and platelet endothelial cell adhesion molecule-1 (PECAM-1) all act as interface regulators for paracellular permeability of ions, nutrients, therapeutic agents, and macromolecules [2, 3]. Endothelial cell adhesive characteristics provide strength and stability for neighboring cells and the cellular cytoskeleton by interacting with actin and myosin contractile filaments [4, 5]. Junctional molecules also influence cell signaling and trigger responses that are translated into cell morphology changes and physiological angiogenesis [6, 7].

A multitude of vasoactive cytokines, growth factors, and signal modulators react with endothelial cell substructural components to control permeability. Vascular endothelial growth factor (VEGF), interleukin-1 alpha and beta (IL-1α and IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) have been shown to increase endothelial monolayer permeability [8, 9, 10, 11]. Thrombin stimulation of cytoskeletal signaling pathways has been shown to increase cell permeability [12]. In contrast, junctional adhesion molecule (JAM) decreases permeability by initiating cell adhesion [14] and angiopoietin-1 (Ang-1) can protect endothelial barrier function through regulation of junctional complexes [15, 16].

Disruptions of the barrier integrity are manifested as microvascular hyperpermeability, which is associated with many systemic disease states. Pathological angiogenic disease states include heart disease, diabetes, cancer, stroke, hypertension, arthritis, and Alzheimer's [1, 17, 18]. Increases in tissue permeability may be caused by weak, hemorrhaging vessels that become oedematous, and intensifies with irregular fluid flow through the vessels [17]. Expanding the knowledge of endothelial junction behavior and the agents that influence that behavior will lead to new therapies for controlling endothelial permeability.

EMD Millipore's *In Vitro* Vascular Permeability Imaging Assay provides optimized materials and protocols to enable detailed spatial analysis of intercellular permeability in endothelial monolayers. All of the components necessary for affixing a thin film of biotinylated matrix to glass culture surfaces and staining sites of intercellular permeability are provided. In addition, compatible reagents are provided for correlating tight junctions (VE-cadherin) and nuclei with sites of permeability. This assay may be used for assessing activity of inhibitors and promoters of vascular permeability, and correlating permeability with ultrastructural features and signaling events.

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Assay Principle

The EMD Millipore *In Vitro* Vascular Permeability Imaging Assay provides the reagents necessary for affixing a thin, uniform layer of biotinylated gelatin to a glass culture substrate which, in the presence of an endothelial monolayer, binds to a fluorescently labeled streptavidin only at sites of intercellular permeability. A poly-L-lysine coating is first adsorbed to the glass substratum. The substrate is then treated with a dilute glutaraldehyde solution to bi-functionally "activate" the surface for further protein binding. Subsequent incubation of the surface with biotinylated gelatin allows covalent coupling between the poly-L-lysine and gelatin via reactive aldehyde (-CHO) groups. The biotin-coated glass is now prepared for cell culture by disinfection with 70% ethanol, followed by quenching of free aldehydes with amino acid-containing growth medium.

The endothelial cell of interest is seeded onto the gelatin surface and allowed to form a confluent monolayer. Treatment compounds of interest may be introduced at desired time points during the culture period. Sites of intercellular permeability are then stained with fluorescent streptavidin [19], which are microscopically visualized and may be quantified using image analysis software algorithms. The assay also provides anti-VE-cadherin and DAPI, for visualization of adherens junctions and nuclei, respectively, to allow colocalization of sites of adherens junction remodeling and increased permeability. The basic method allows potential activators or inhibitors to be investigated for their influence on the degree and sites of vascular permeability. The assay may be further combined with immunocytochemical staining for other molecules of interest to colocalize sites of permeability with signaling events.

Application

EMD Millipore's *In Vitro* Vascular Permeability Imaging Assay kit is ideal for interrogating compounds that may disrupt or protect an endothelial monolayer. Each assay kit contains sufficient reagents for the coating of four 8-well glass chamber slides, although alternate substrate formats may also be utilized. The EMD Millipore *In Vitro* Vascular Permeability Imaging Assay is intended for research use only, not for diagnostic or therapeutic applications.

Kit Components

17-10398-1 (2-8℃ Storage):

1. Poly-L-Lysine, 2X: (Part No. CS207800) One vial containing 5 mL

2. DAPI: (Part No. 90229) One vial containing 100 µL at 100 µg/mL in water.

17-10398-2 (-20℃ Storage):

3. Glutaraldehyde, 16X: (Part No. CS207801) One vial containing 1 mL.

4. Biotin-Gelatin: (Part No. CS210598) One vial containing 225 µL.

5. Fluorescein-Streptavidin: (Part No. CS210499) One vial containing 10 µL

6. Anti-VE Cadherin: (Part No. MABT129) One vial containing 100 µL

7. Donkey anti-Mouse IgG, Cy3 Conjugate: (Part No. CS210500) One vial containing 50 µL

Materials Not Supplied

1. Sterile cell culture hood
2. Pipettors, liquid aspirators, etc. for handling of cells and liquid reagents
3. Sterile plasticware (cell culture flasks, centrifuge tubes, pipettes, pipette tips, etc. for handling of cells and liquid reagents)
4. Sterile glass substrate (e.g., chamber slide, coverslip, glass-bottom dish/multi-well plate)
5. Sterile deionized water
6. Sterile Dulbecco's phosphate-buffered saline (DPBS), without calcium or magnesium
7. 70% ethanol in sterile water
8. Human umbilical vein endothelial cells (HUVEC) such as EndoGRO™ Human Umbilical Vein Endothelial Cells (Cat. No. SCCE001) or endothelial cell type of interest
9. Endothelial cell Basal Medium
10. Endothelial cell Growth Medium
11. Vascular permeability factor (e.g., IL-1β, TNF-α, VEGF, Ang-1, thrombin etc.)
12. Hemocytometer (e.g. Scepter™ Handheld Automated Cell Counter)
13. Trypan blue or equivalent viability stain
14. Low speed centrifuge for cell harvesting
15. CO_2 tissue culture incubator
16. 3.7% formaldehyde in DPBS (or equivalent) for cell fixation
17. Blocking/permeabilization buffer for antibody/DAPI staining (e.g., 2% blocking serum/0.25% Triton X-100 in DPBS)
18. Slide mounting media (with anti-fade reagent) and cover glasses, if appropriate
19. Microscope/image acquisition system (for phase contrast and fluorescence)
20. Fluorescence filters for fluorescein, Cy3 and DAPI imaging (see **Table 2** for specific excitation/emission wavelengths)
21. Image analysis software (e.g., NIH ImageJ)

Related Products Available from EMD Millipore

PHCC20040	Scepter™ 2.0 Handheld Automated Cell Counter, w/ 40 μM Sensors
PHCC20060	Scepter™ 2.0 Handheld Automated Cell Counter, w/ 60 μM Sensors
PEZGS0816	Millicell EZ Slide Chamber slide
ECM642	*In Vitro* Vascular Permeability Assay (96-Well)
ECM644	*In Vitro* Vascular Permeability Assay (24-Well)
SCCE001	EndoGRO Human Umbilical Vein Endothelial Cells (HUVEC)
SCCME001-004	EndoGRO Media Products for Human Endothelial Cell Culture
IL038	Recombinant Human Interleukin-1β
GF023	Recombinant Human Tumor Necrosis Factor-α
SM-2002-C	0.05% Trypsin-EDTA in Hank's Balanced Salt Solution
BSS-1006-B	EmbryoMax® 1X Dulbecco's Phosphate Buffered Saline

Precautions

Refer to MSDS for further information.

Component	Hazards
Glutaraldehyde	Toxic, corrosive, sensitizer (handle in fume hood or biosafety cabinet)
DAPI	Potential mutagen

Storage

Store Poly-L-Lysine and DAPI at 2-8℃.
Store Biotin-Gelatin, Glutaraldehyde, fluorescein-streptavidin, anti-VE cadherin, and Donkey anti-Mouse IgG, Cy3 conjugate at -20℃.

Use all reagents within 4 months from date of receipt.

Assay Protocol

The given protocol volumes assume preparation of 4 x 8-well glass chamber slides.
For alternate per sample volumes, see **Table 1** (or scale volumes as appropriate for culture surface area). **NOTE: All reagents should be diluted to 1X only immediately before use.**

Substrate Preparation and Cell Seeding:

Perform the following steps in a sterile cell culture hood with sterile plasticware.
Mix all reagents gently before use.

1. Dilute Poly-L-Lysine, 2X solution to 1X with sterile, deionized water by mixing 4 mL Poly-L-Lysine, 2X + 4 mL sterile, deionized water = 8 mL total 1X solution.

2. Add 250 µL of 1X Poly-L-Lysine solution to each well; incubate at room temperature for 20 minutes.

3. Remove poly-L-lysine solution; rinse twice with sterile Dulbecco's PBS (DPBS, without calcium or magnesium) using 500 µL/well.

4. Dilute Glutaraldehyde, 16X solution to 1X with sterile DPBS:
    a. Thaw Glutaraldehyde, 16X to room temperature. Remaining glutaraldehyde may be re-frozen for subsequent use.
    b. Mix 0.5 mL Glutaraldehyde, 16X + 7.5 mL sterile DPBS = 8 mL total 1X solution.

5. Add 250 µL of 1X Glutaraldehyde solution to each well; incubate at room temperature for 15 minutes.

6. Remove glutaraldehyde solution (dispose of as hazardous waste); rinse twice with sterile DPBS using 500 µL/well.

7. Dilute Biotinylated-Gelatin in 1X sterile DPBS:
    a. Thaw Biotinylated-Gelatin to room temperature. Remaining Biotinylated-gelatin may be re-frozen for subsequent use.
    b. Heat biotinylated-gelatin stock at 60℃ for 5 mi nutes.
    c. Dilute 10mg/mL biotinylated-gelatin stock to a 0.25mg/mL solution in 1X DPBS.
    d. Mix 200 µL 10mg/mL stock + 7.8 mL sterile DPBS = 8 mL total 0.25mg/mL solution.

8. Add 250 µL of 0.25mg/mL biotinylated- gelatin mixture to each well; incubate at room temperature for 10 minutes.

9. Remove gelatin mixture; rinse twice with sterile DPBS using 500 µL/well.

10. Disinfect substrates with 70% ethanol in sterile water using 500 µL/well; incubate at room temperature for 30 minutes.

11. Remove ethanol; rinse twice times with sterile DPBS using 500 µL/well.

12. To quench residual free aldehydes, add 500 µL of growth media per well and incubate at room temperature for 30 minutes, protected from light.

13. For cell seeding, use cells that are ~70-80% confluent.
    a. Detach cells as appropriate for cell type of interest.
    b. Remove sample for cell counting (e.g., via hemocytometer, with viability stain).
    c. Centrifuge and resuspend cells in growth media at desired concentration.

14. Remove growth media used for quenching; seed cells in fresh growth media at 500 µL/well. Seeding densities should be empirically determined for each cell type over desired culture period. Typically, 28,000 HUVEC per well are plated and incubated for 2 days to achieve a confluent monolayer with well-developed cell-cell contacts. Allow chamber slides to sit undisturbed on a horizontal surface at room temperature for ~15-30 minutes to encourage even cell distribution.

15. Place chamber slides into a 5% CO_2 tissue culture incubator at 37℃ for the remainder of the desired culture duration.
    a. Cell treatments may also be performed at the time of seeding, following an initial adhesion phase (e.g., 2-4 hours), or after formation of a confluent monolayer.

**__For coverslip preparation/seeding:__

- Individual glass coverslips may also be utilized for gelatin coating.
- A 12 mm-diameter round coverslip may be coated/cultured in a standard 24-well plate.
- Before coating, tracking of coverslip "sides" may be facilitated by labeling the "non-coated side" of each coverslip with a solvent-resistant marker (or other marking strategy).
- Handle coverslips with 70% ethanol-disinfected forceps (serrated tips).
- The poly-L-lysine, glutaraldehyde and gelatin coating steps (with associated DPBS rinses) may be performed by filling the 24-well plate with the recommended reagent volume, then inverting the coverslip onto the liquid ("coated side" down). For alternate coverslip sizes, appropriately-sized dishes or multi-well plates may be utilized, or reagent volumes may be dropped onto Parafilm surfaces, followed by coverslip inversion (volumes may need to be adjusted for size).
- The ethanol, media quench, cell culture, fixation and staining steps (with associated rinses) may be performed by sinking the coverslip in the well ("coated side" up), followed by filling the 24-well plate with the recommended reagent volume. For alternate coverslip sizes, appropriately-sized dishes or multi-well plates should be utilized.

	Glass Substrate (Surface Area per Well or per Sample)		
Per Well or Per Sample 1X Coating/Staining Volumes	8-Well Chamber Slide (0.7 cm^2)	96-Well Plate (0.2 cm^2)	12 mm-Diameter Round Coverslip (1.1 cm^2) in 24-Well Plate (*see **Assay Protocol** for further details)
Poly-L-Lysine	250 µL	70 µL	250 µL (*invert)
DPBS Rinses	500 µL	140 µL	500 µL (*invert/sink)
Glutaraldehyde	250 µL	70 µL	250 µL (*invert)
Biotin-Gelatin	250 µL	70 µL	250 µL (*invert)
70% Ethanol	500 µL	140 µL	500 µL (*sink)
Growth Media (quench/ cell seeding)	500 µL	140 µL	500 µL (*sink)
Fixative (e.g., 3.7% Formaldehyde)	250 µL	70 µL	250 µL (*sink)
Fluorescent Staining Buffer Rinses (user-provided)	500 µL	140 µL	500 µL (*sink)
Antibody/DAPI Staining Solution	200 µL	60 µL	200 µL (*sink)

__Table 1.__ Recommended coating/staining volumes for example glass substrates.

Sample Fixation and Staining:

The following steps do not need to be performed under sterile conditions.

1. At the desired time-point after plating and treatment, remove culture media and add fluorescein-strepavidin diluted in DPBS at a 1:2000 dilution, 250 μL/well and incubate at room temperature for 5 min.

2. Remove fluorescein-strepavidin solution, and fix cells with 3.7% formaldehyde in DPBS (or preferred fixative) using 250 μL/well. Incubate for 30 minutes at room temperature, protected from light.

3. Remove formaldehyde solution (dispose of as hazardous waste) and rinse twice with DPBS using 500 μL/well. Samples may be stored at this point in DPBS at 2-8°C, protected from light.

4. Prepare desired blocking/permeabilization buffer for fluorescent staining, e.g., DPBS with 2% blocking serum + 0.25% Triton X-100.

5. Immediately before staining, remove DPBS and rinse samples twice with fluorescent staining buffer using 500 μL/well.

6. Dilute anti-VE cadherin antibody in fluorescent staining buffer. Dilute anti-VE cadherin antibody 1:100.
   a. Mix 64 μL of anti-VE cadherin antibody + 6336 μL fluorescent staining buffer = 6400 μL total antibody solution.

7. Add 200 μL of antibody solution to each well; incubate 1 hour at room temperature, protected from light.

8. After staining, remove DPBS and rinse samples twice with fluorescent staining buffer using 500 μL/well.

9. Dilute Donkey anti-Mouse IgG, Cy3 conjugate and DAPI in fluorescent staining buffer. Use Donkey anti-Mouse IgG, Cy3 conjugate at a 1:200 dilution and DAPI at a 1:100 dilution.
   a. Mix 32 μL of Donkey anti-Mouse IgG, Cy3 conjugate + 64 μL of DAPI + 6304 μL fluorescent staining buffer = 6400 μL total staining solution.

10. Add 200 μL of staining solution to each well; incubate 1 hour at room temperature, protected from light.

11. Remove staining solution (dispose of as hazardous waste); rinse twice with DPBS using 500 μL/well.

12. For chamber slides, remove chambers and cover with slide mounting media (preferably containing anti-fade reagent) and appropriate-thickness cover glasses for magnification/imaging modality of choice. Allow mounted cover glasses to hard-set, or seal wet mounting media, as appropriate.
    a. For multi-well plates, leave in the last DPBS rinse for imaging.

b. For coverslips, use slide mounting media to invert coverslips ("coated side" down) onto a glass slide or cover glass base for imaging. Allow mounted coverslips to hard-set, or seal wet mounting media, as appropriate.
c. Stained samples may be stored at 2-8℃, protected from light.

Imaging and Analysis:

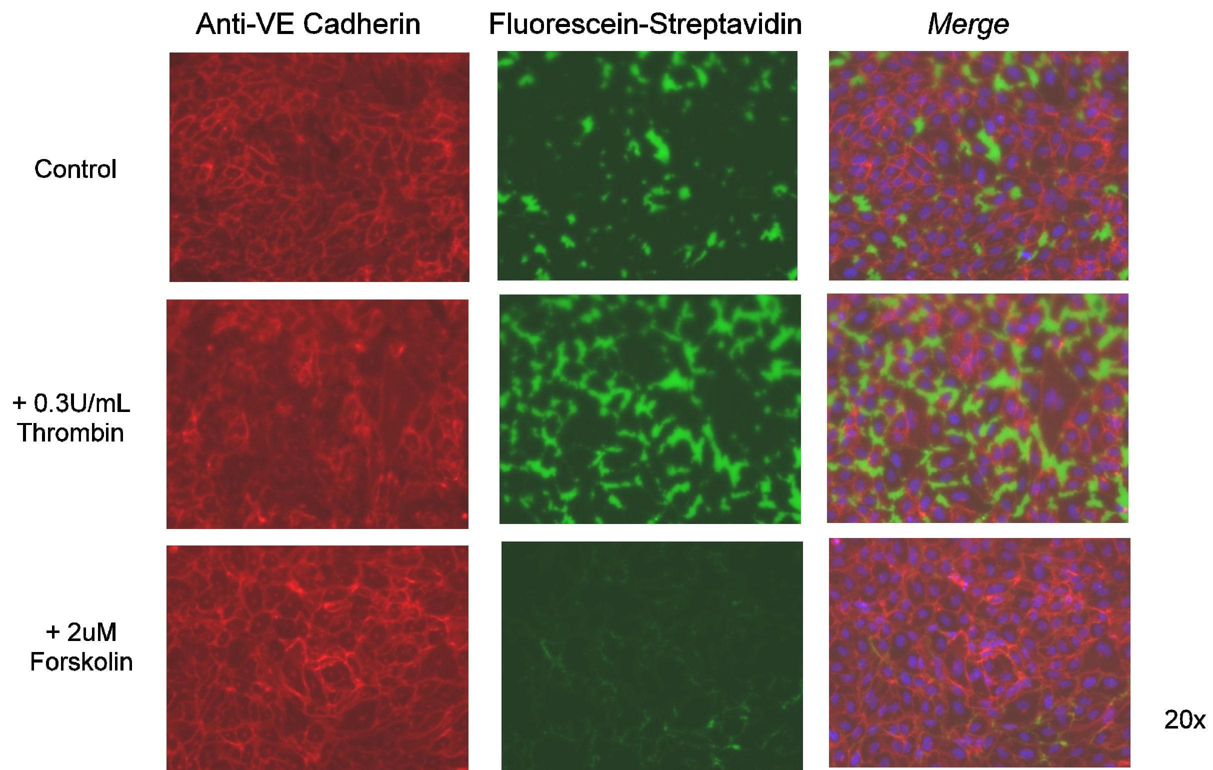
1. Perform fluorescent microscopic imaging of samples using appropriate fluorescence filters as listed in Table 2 below.

Fluorescent Label	Excitation Wavelength	Emission Wavelength
Fluorescein-strepavidin	492 nm	518 nm
Cy3	550 nm	570 nm
DAPI	358 nm	461 nm

Table 2. Fluorescence filter specifications.

2. Selection of imaging magnification and/or modality (e.g., widefield or confocal fluorescence) will depend upon application of interest. Lower magnifications (e.g., 20X objective) may be sufficient for quantification of large scale changes in permeability, while co-localization studies may require higher magnification/resolution visualization (e.g., 63X oil objective). Optimal imaging settings/techniques must be determined by the end user.

Example Data



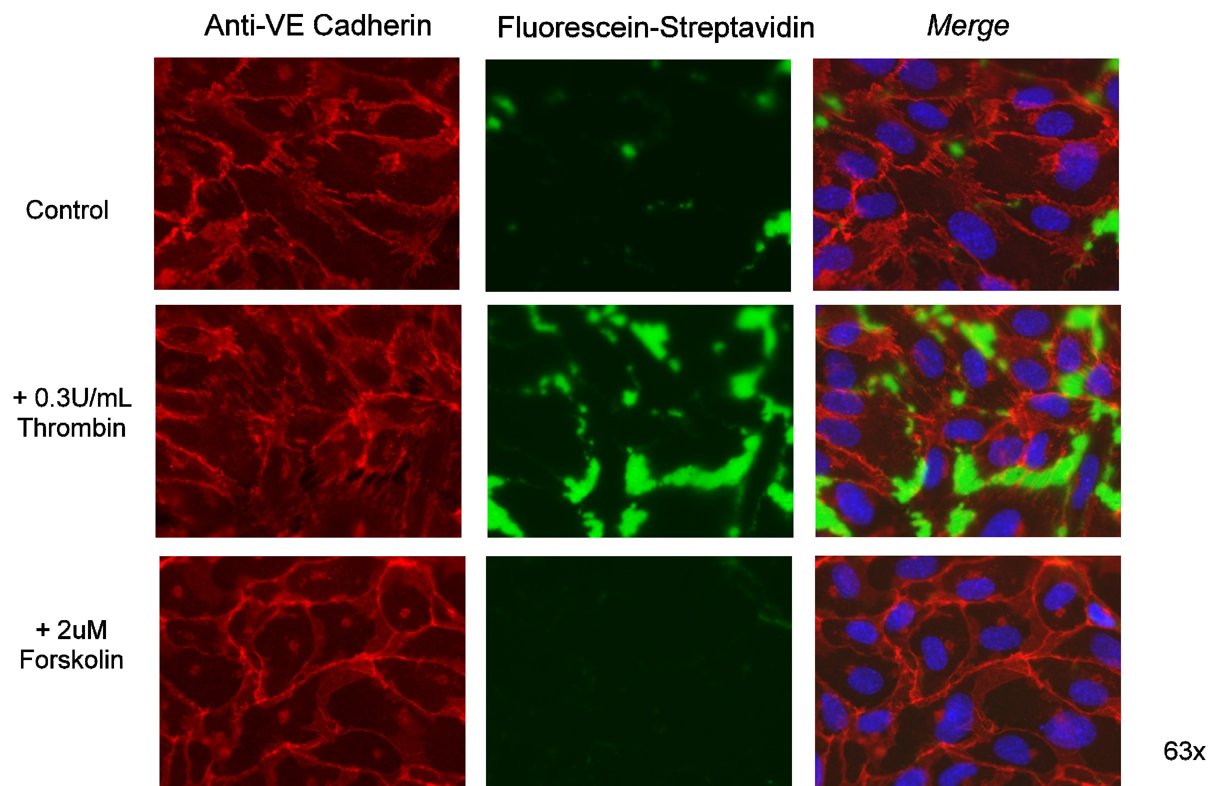


Figure 1. Imaging of the effects of thrombin and forskolin on permeability of HUVEC monolayers.

Biotinylated gelatin matrices were coated onto 8-well glass chamber slides as described in the **Assay Protocol**. HUVEC were seeded onto the gelatin substrates at 20,000 cells/cm^2 in EndoGRO media for a culture duration of 48 h. Cells were left untreated or treated with thrombin or forskolin for 15 minutes and 30 minutes respectively, then Fluorescein-streptavidin was added as indicated in the protocol. Fixation and staining with anti-VE-cadherin (red) and DAPI (blue) were performed according to the **Assay Protocol**. Cells were imaged on a widefield fluorescent microscope, with a 20x dry lens or 63X oil immersion objective lens.

Thrombin disrupts VE-cadherin-containing tight junctions and increases permeability of the monolayer to fluorescein-streptavidin. In contrast, forskolin increases tight junctions containing VE-cadherin and reduces penetration of fluorescein-streptavidin through the monolayer.

Troubleshooting

Problem	Possible Solutions
Incomplete/non-confluent endothelial cell monolayer	• Use endothelial cells at lower passage number or from new thaw/stock of cells. • Allow a longer culture duration for monolayer formation. • Plate cells at a higher initial seeding density. • Growth media or supplemental components may be old/degraded – use freshly-made growth media. • Avoid mechanical disruption or excessive fluid shear that may compromise insert membrane attachment or monolayer integrity. • During monolayer formation, treat cells with known endothelial survival factors, e.g., Ang-1, VEGF, etc.
Low fluorescent signal with known activator (e.g., TNF-α or IL-1β)	• Increase fluorescein-streptavidin permeation time. • Use a higher concentration (lower dilution) of fluorescein-streptavidin for permeability testing. • Decrease initial endothelial cell seeding density. • Ensure that fluorescein-streptavidin stock or plate well solutions are protected from light.

Problem	**Possible Solutions**
High fluorescent signal in control wells	• Check monolayer and insert integrity (see Possible Solutions for "incomplete/non-confluent endothelial cell monolayer"). • Decrease fluorescein-streptavidin permeation time. • Use a lower concentration (higher dilution) of fluorescein-streptavidin for permeability testing.
Poor imaging focus	• Determine working (focal) distance of objective lenses used during microscopy and take into account when designing microscopy setup (i.e., imaging through glass slide/plate bottom vs. cover glass) – high magnification/oil immersion lenses may require thinner cover glasses (e.g., No. 0) for optimal focus. • Multiple imaging wavelengths (for fluorescein, Cy3, DAPI) may have different planes of focus depending upon extent of chromatic aberration correction in imaging lenses – consider imaging at multiple focal planes for optimal focus at each wavelength.
Absence of fluorescein-streptavidin signal in area lacking cells	• This is likely due to loss of cells during processing. Decrease washes number or duration. Omit permeabilization if intracellular staining not performed.
Poor staining quality (signal intensity, background)	• Increase concentration of blocking serum or protein in fluorescent staining buffer to decrease background. • Increase staining time or primary antibody/ secondary antibody/ DAPI final staining concentrations to increase signal intensity. (Note: Higher staining intensity may be required for microscopic techniques involving high intensity illumination/rapid photobleaching, e.g., confocal or other laser illumination.) • DAPI is modestly cell permeant –presence and/or increased concentration of permeabilization agent (e.g., Triton X-100) in fluorescent staining buffer will improve signal. • Protect fluorescent samples from light at all times and/or use anti-fade reagent (oxygen radical scavenger) in mounting media to minimize photobleaching. • Optimize imaging conditions (magnification, numerical aperture, exposure time, gain, illumination intensity) for enhanced signal.

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