Methods for Forming Human Microvascular Tubes In Vitro and Measuring Their Macromolecular Permeability

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Abstract

This chapter describes a protocol for forming open endothelial tubes in vitro and quantifying their permeability to macromolecules. These tubes consist of confluent monolayers of human microvascular endothelial cells in perfused microfluidic collagen gels. The cylindrical geometry of the tubes mimics the shape of microvessels in vivo; it allows simultaneous and/or repeated measurements of permeability coefficients and detection of focal leaks. We have used these in vitro models to test the effects of agonists on microvascular permeability and are developing arrays of microvascular tubes to enable large-scale testing.

Key words: Microvascular tissue engineering, Endothelial cells, Collagen, Permeability, Focal leaks

1. Introduction

This chapter describes methods recently developed by our group to quantify the barrier function of engineered human microvascular tubes in vitro (1, 2). It provides step-by-step instructions for forming endothelial tubes within channel-containing collagen gels, measuring their permeabilities to macromolecules, and determining their number of focal leaks. We assume that the reader is familiar with standard cell culture techniques and with our complementary review on methods to form cylindrical channels within collagen (3).

Our methods are adapted from those originally designed for use in intact or explanted microvessels from mice, rats, and frogs (4–8). In vivo, macromolecular permeability can be quantified with two metrics: (1) an effective permeability coefficient P_d, which describes the ability of a solute to escape uniformly from the vascular lumen and (2) the density of focal leaks, which are localized regions of high permeability (9). The reflection coefficient σ,

which describes the oncotic contribution of a solute, provides a third measure of barrier function (10, 11); we do not describe how to measure this quantity in our system.

Traditionally, the permeability of *human* endothelium has been examined in planar cell cultures in vitro (12). These cultures lack physiologically relevant shear stress, and the permeability coefficients are averaged over large areas and long times. In contrast, the open cylindrical architecture of our vessels allows constant perfusion, which enables real-time measurement of permeabilities and focal leaks, just as in cannulated microvessels. The small footprint of these tubes may eventually enable the development of microvascular arrays for high-throughput assays of human microvascular barrier function.

2. Materials

2.1. Endothelial Cell Culture

1. Human dermal blood microvascular endothelial cells (BECs; Lonza) or human dermal lymphatic microvascular endothelial cells (LECs; Lonza).

2. Sterile phosphate-buffered saline (PBS; Invitrogen).

3. 60-mm-diameter tissue culture polystyrene dishes (Corning).

4. 0.1% gelatin from pig skin (Sigma) in PBS, filter-sterilized, autoclaved, filter-sterilized again, and stored at 4°C.

5. MCDB 131 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin–streptomycin-glutamine (Invitrogen), 1 µg/mL hydrocortisone (Sigma), 80 µM dibutyryl cyclic AMP (db-cAMP; Sigma), 25 µg/mL endothelial cell growth supplement (ECGS; Biomedical Technologies), 2 U/mL heparin (Sigma), and 0.2 mM L-ascorbic acid 2-phosphate (Sigma).

6. Complete medium (see Subheading 2.1, item 5) supplemented with 3% 70 kD dextran (dex; Sigma) and filter-sterilized ("3% dex media").

7. Trypsin/EDTA (Invitrogen).

8. Dispase (Invitrogen), *optional*.

2.2. Formation of Microvascular Tubes

1. Culture media (see Subheading 2.1, item 5).

2. Trypsin/EDTA.

3. 1.7-mL microcentrifuge tubes (Costar).

4. 3% dex media (see Subheading 2.1, item 6).

5. Collagen gels with channels, assembled between silicone housings and glass coverslips (as described in 11.3.1–11.3.4 of (3)).

6. Silicone lids with attached tubing (as described in 11.3.5.2 of (3)).

2.3. Measurement of Permeability

1. Alexa Fluor 594-conjugated bovine serum albumin (BSA-594; Invitrogen).

2. Alexa Fluor 488-conjugated 10 kD dextran (dex-488; Invitrogen).

3. 3% dex media (see Subheading 2.1, item 6) supplemented with 50 μg/mL BSA-594 and 20 μg/mL dex-488.

4. A microscope environmental chamber (Zeiss) that maintains temperature at 37°C.

5. Image-processing software, such as ImageJ (NIH freeware).

6. Silicone blocks with channels (as described in 11.3.3.1 of (3)), with the same dimensions as the channel-containing gels in Subheading 2.2, item 5.

7. Silicone lids with tubing (see Subheading 2.2, item 6).

3. Methods

As shown in Fig. 1, we form human endothelial tubes by seeding BECs or LECs into collagen gels that contain open channels and allowing the seeded cells to form confluent linings on the channels
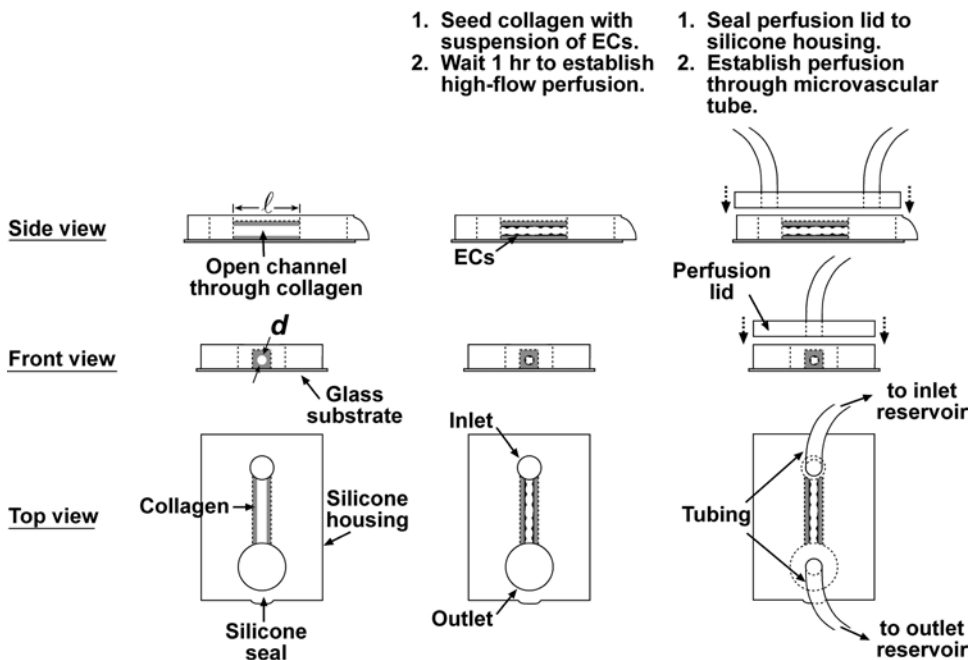

Fig. 1. Schematic diagram of the formation of a perfused microvascular tube from a channel-containing collagen gel. *Left panel*, a channel within a collagen gel. The collagen is surrounded by a silicone housing and a glass substrate (3). The parameters "*d*" and "*l*" refer to the diameter and length of the channel, respectively. *Middle panel*, a channel seeded with endothelial cells (ECs). *Right panel*, a seeded tube coupled to a lid that establishes high-flow perfusion. The tubing of the lid aligns with the centers of the inlet and outlet wells adjacent to the tube.

under constant perfusion. Once the cells reach confluence, the lumen widens with the inlet and outlet diameters increasing by ~40 and ~10%, respectively (1, 2). Figure 2 illustrates a typical time-course of this expansion. Tubular widening takes place over 2 days, and we usually perform permeability assays on the third day after seeding.

The operating principle behind our permeability assay is to measure the rate at which a fluorescently labeled macromolecule (bovine serum albumin or dextran) escapes from the endothelial lumen. This idea has been implemented previously for intact or explanted microvessels (5). Application of this assay to engineered endothelial tubes requires two modifications: First, because our tubes consist solely of an endothelial monolayer, they are not
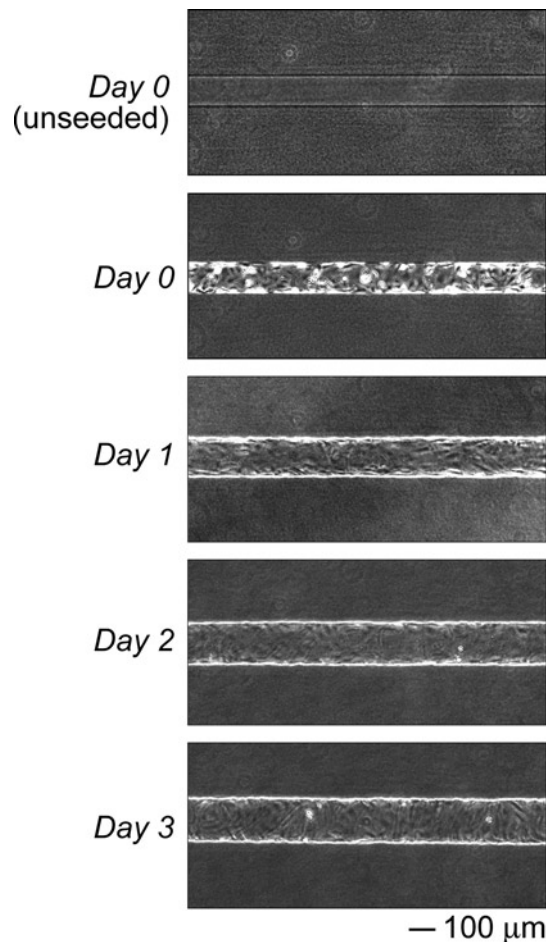

Fig. 2. Maturation of tubes over a span of 3 days. The *top row* shows phase-contrast images of a middle segment of an unseeded channel. The *second row* displays spreading endothelial cells in a channel a few hours after seeding (day 0). The *subsequent rows* show changes in tubular morphology as the cells grow to confluence: By day 1, endothelial cells have formed a confluent monolayer. By day 2, the tube has widened. By day 3, morphological changes have stabilized.

mechanically strong enough to withstand direct cannulation. The introduction of fluorescent molecules thus has a lag time on the order of several minutes, as a dead volume is flushed out of the inlet well and tubing. Second, the perfusing medium contains bleachable solutes (possibly, flavins) that can interfere with imaging, which limits the frequency of measurement. With these caveats in mind, we can obtain P_d for each macromolecule. Manual counts of focal leaks provide complementary data on the nonuniformity of leakage.

Calibration of the permeability assay is absolutely essential to obtain meaningful values of P_d. The methods described below confirm that the fluorescence signal is proportional to the concentration of solute, and that light collection efficiency does not depend on the location of fluorophore (e.g., above or below the mid-plane of the tube). We use a standard inverted epifluorescence microscope (Zeiss), with a Plan-Neo 10×/0.30 NA objective, $1,388 \times 1,040$ resolution AxioCam HRm camera, and flat-field correction software. The fluorescent solutes we use are conjugated to Alexa Fluor dyes, which do not photobleach appreciably with our exposure doses. Our calculation of P_d assumes that the tube is cylindrical in geometry; this assumption is valid within each ~1-mm-wide measuring window. The assay also takes the perfusion rate into account to ensure that the lumenal concentration of fluorescent solute is constant during imaging. Imaging systems that do not satisfy the above requirements (e.g., by using highly bleachable dyes such as fluorescein) require extensive mathematical compensation to obtain accurate values of P_d (6, 13).

The equation we use for P_d calculates the *effective* permeability coefficient, which includes diffusional and convective contributions (10, 14). Since the tubes are enclosed in an impermeable housing (silicone and glass), we assume that transendothelial water flux – and, therefore, convective transport – is negligible. The convective contribution to permeability cannot be ignored if a large water flux exists across the endothelium (e.g., due to a large transendothelial pressure), and a more complex analysis must be used (5, 14, 15).

3.1. Formation of Endothelial Tubes

3.1.1. Endothelial Cell Culture

1. Coat polystyrene dishes with gelatin for 40 min at room temperature. Wash with sterile water and dry.

2. Plate human BECs or LECs in the gelatin-coated dishes. Routinely passage confluent cultures at a 1:4 dilution using trypsin/EDTA or dispase. A confluent 60-mm-diameter dish typically provides enough cells to seed 3–4 channels.

3.1.2. Seeding Channels in Collagen Gels

1. Form collagen gels that contain single open channels, following the directions described in our recent work (1, 3). Condition the gels with 3% dex media for at least 1 h at 37°C

by adding media to the inlet well and removing it from the outlet well. Do not allow any media to overflow on top of the silicone housing as this media will interfere with perfusion under high pressures.

2. Treat a culture of BECs or LECs with trypsin/EDTA, collect the cells in ~1 mL dextran-free media, and pellet them at $200 \times g$ for 2 min (see Note 1).

3. Aspirate the media from the vial of pelleted cells and gently resuspend the cells in 20 µL of 3% dex media.

4. Aspirate media from the inlet and outlet wells of a conditioned collagen gel.

5. Add 1–2 µL of cell suspension to each of the inlet and outlet wells. A steady, dense stream of cells should begin flowing through the collagen channel.

6. Modulate the flow velocity by tilting the dish that contains the collagen gel until the cells are nearly stationary within the channel. Hold the dish steady for 30–40 s to allow cells to settle and adhere to the collagen channel. We typically perform this step while constantly viewing the channel with a 10× objective on an inverted microscope.

7. If the seeding is sparse, level the dish to add more cell suspension to the channel as needed. Seeding should require ≤5 min for a single channel (see Note 2).

8. Gently wash the inlet and outlet wells twice with 3% dex media. Avoid disturbing the collagen, else the gel may detach from the surrounding silicone or glass.

9. Add a large drop of 3% dex media to the inlet well and add a small drop to the outlet. Place the seeded tube in a 5% CO_2 incubator at 37°C. The cells should visibly spread within the channel after 15 min (see Note 3). Wait at least 1 h before establishing perfusion under high pressure (see below).

3.1.3. Perfusing Seeded Tubes

1. Assemble perfusion lids and associated tubing and dishes by following step 13.3.5.2 described in our recent review (3).

2. Fill reservoir dishes with 30 mL of warm 3% dex media and aspirate media through the tubing.

3. Place a lid on a seeded channel to establish fluidic contact between the tubing and the inlet and outlet wells (see Note 4).

4. Set the height difference of the reservoirs to ~6 cm. A pressure difference of 6 cm H_2O should yield a flow rate of 0.5-1 mL/h for subconfluent tubes and ~1 mL/h for confluent, widened ones.

5. Regenerate the pressure difference by pipetting media from the outlet reservoir to the inlet reservoir every ~12 h (see Note 5). The same media may be reused to perfuse a microvascular tube

for 3 days (by which we usually perform the permeability assay), after which the media should be replaced (see Notes 6–11).

3.2. Control Experiments and Calibration of Permeability Assay

All control experiments and calibrations should be performed under conditions as close as possible to those used in the actual permeability assay (e.g., with environmental chamber warmed to 37°C, in the dark, etc.).

3.2.1. Signal Detection

1. Place a drop of BSA-594 or dex-488 between two #1½ glass coverslips. Capture fluorescence images using flat-field correction, and measure the average light intensity (e.g., with ImageJ). Focus at various planes to ensure that the intensity of the collected light does not change with focus; the range of focus should span ~1 mm.

2. Repeat the above step with different solute concentrations to create a standard curve of fluorescence intensity vs. concentration. The solute concentrations used in all subsequent sections should be within the linear range of this curve. We typically find a linear range of 0.2–50 µg/mL for BSA-594 and dex-488.

3.2.2. Determination of Assay Sensitivity

1. Form channels in silicone by following step 11.3.3.1 of our review on microfluidic gels (2).

2. Establish perfusion exactly as described for endothelialized tubes (see Subheading 3.1.3) with fluorescent 3% dex media.

3. Capture several fluorescence images in succession. Since silicone is impermeable to proteins, the intensity of the images should theoretically not vary over time. The range of values indicates the inherent noise of the imaging system (e.g., due to lamp flicker) and sets a bound on the precision of our permeability measurements (see Note 12).

3.2.3. Determination of Lag Time

1. Establish perfusion in a silicone channel as in Subheading 3.2.2, but with nonfluorescent 3% dex media.

2. Supplement the media in the inlet reservoir with 50 µg/mL BSA-594 and 20 µg/mL dex-488.

3. Capture fluorescence images every minute as the fluorescent solute flows through the tubing into the inlet well and through the silicone channel. The lag time is the time at which the intensity of fluorescent solute reaches a maximum (within the sensitivity determined in Subheading 3.2.2). Increasing the length of tubing, increasing the size of the inlet well, or decreasing the perfusion rate will increase the lag time. With our standard setup and perfusion conditions, we observe a lag time of <20 min.

3.2.4. Determination of Imaging Frequency That Minimizes Photobleaching

1. Perfuse a seeded tube as described in Subheading 3.1.3 (do not add any fluorescent solutes to the media).

2. Capture two consecutive fluorescence images of the tube (see Note 13). If the second image is photobleached (i.e., has a lower average intensity) compared to the first one, repeat the process in another tube with a longer delay between capturing the two images. The recovery time is the time interval that routinely yields the same intensities (discounting noise) in consecutive images. We have found 6 min to be sufficient for recovery from photobleaching with our perfusion conditions.

3.3. Measurement of Diffusional Permeability Coefficient P_d

1. Warm the microscope environmental chamber to 37°C.

2. Capture background fluorescence images of a perfused tube (do not add any fluorescent solutes to the media). The image should appear black.

3. Draw a measuring window that extends the width of the collagen gel, with the lumen of the tube in the middle, as shown in Fig. 3a. The average signal in the measuring window is the background fluorescence intensity I_b.

4. Replace the media in the inlet reservoir with fluorescent media and gently pipette to mix the fluorescent media with any residual media. Be careful not to introduce bubbles into the inlet tubing.

5. Place the tube back in the incubator for 10 min (half of the lag time found in Subheading 3.2.3).

6. Transfer the tube back to the environmental chamber for an additional 10 min (the remainder of the lag time). This time allows the tube to recover from any agitation during the transfer.

7. Capture the initial fluorescence images of the tube (see Note 14). We obtain images for BSA-594 and dex-488 in parallel, which allow us to use the same focal leak data for both solutes and decreases the time needed for the permeability assay (see Note 15). We typically image a region ~4.5 mm downstream from the inlet.

8. Wait 6 min (the recovery time found in Subheading 3.2.4) and capture another set of images of the tube. Repeat as desired.

9. Draw a measuring window as shown in Fig. 3b. The positions of the measuring windows with respect to the tube should be exactly the same at both time points. The average initial intensity in the measuring window for a given fluorophore is I_1 and the average intensity after 6 min is I_2. If the tube has nonuniform leakage (i.e., focal leaks), choose a location for the measuring window as far from the nonuniform regions as possible to minimize their effects on the calculation of P_d (see Fig. 3c).
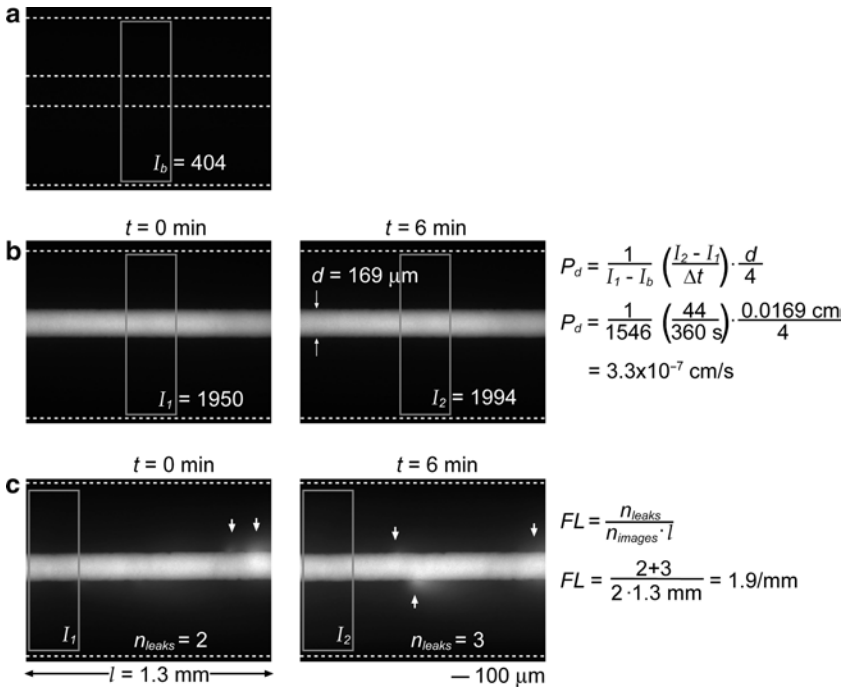
Fig. 3. Quantifying the barrier function. (**a**) Fluorescence image of the background intensity. The tube is indicated by the *dotted lines* in the *middle* of the image, while the collagen borders are represented by the *dotted lines* at the *top* and *bottom*. The average intensity within the measuring window (*gray box*) is the background intensity I_b (scale from 0 to 16,383 using a 14-bit CCD). (**b**) Fluorescence images of a tube at 0 and 6 min (solute is BSA-594). The average intensities in the measuring window for each time point are I_1 and I_2, respectively. The diameter of the tube within the measuring window is d. (**c**) Fluorescence images of a tube with poor barrier function at 0 and 6 min (solute is BSA-594). The *arrows* indicate focal leaks along the vessel wall. The measuring window is placed away from leaks.

10. Compute P_d as follows (5):

$$P_d = \frac{1}{I_1 - I_b}\left(\frac{I_2 - I_1}{\Delta t}\right) \cdot \frac{d}{4}$$

where $\Delta t = 6$ min is the recovery time (determined in Subheading 3.2.4) and d is the diameter of the tube in the middle of the measuring window. Figure 3b shows a sample calculation of P_d (see Notes 12 and 16–19).

3.4. Quantifying Focal Leaks

1. Maximize the contrast in the images taken for the permeability assay. Examine the images for any nonuniformities (typically, regions of excess intensities). Figure 3c shows a microvascular tube that has focal leaks in both the initial and final images. Since counts of focal leaks can be somewhat subjective, the same experimenter should count the number of leaks for all images.

2. Sum the number of focal leaks in the images at both time points, irrespective of the position of the focal leaks. If the same focal leak is present in the initial and final images, then that leak is counted twice.

3. Compute the number of focal leaks per mm of microvascular tube (FL) as follows (2):

$$FL = \frac{n_{\text{leaks}}}{n_{\text{images}} \cdot l}$$

where n_{leaks} is the total number of focal leaks summed over the different time points, $n_{\text{images}} = 2$ is the number of time points imaged, and l is the length of the tube in the viewing window. Figure 3c shows a sample calculation of FL.

3.5. Repeating Permeability Measurements

1. Remove fluorescent media from the inlet reservoir and exhaustively wash the reservoir with 3% dex media without fluorescent solutes.

2. Place the tube back in the incubator for 2–3 h.

3. Remove the media from the outlet reservoir and exhaustively wash the reservoir with 3% dex media without fluorescent solutes.

4. Replace the media in the inlet reservoir, and perfuse the tube for ≥12 h before repeating the permeability assay. A new background measurement must be taken, since residual fluorescent molecules are always present in the collagen gel or in the endothelium.

4. Notes

1. Cells that are overtrypsinized or harvested from a subconfluent dish usually seed poorly.

2. If cells clog the tube during seeding, it is sometimes possible to dislodge the clog by *gently* tapping the dish. Vigorous tapping can cause the collagen gel to detach from the surrounding silicone and glass. We strongly recommend seeding a fresh channel if a clog forms. It is always possible to reseed a sparsely seeded tube but very difficult to repair an overseeded one.

3. If seeded cells do not adhere to the collagen gel or do not begin to spread within 15 min of seeding, the channel may need to be conditioned with media for a longer period of time. If cells do not adhere to well-conditioned channels, then the particular lot of endothelial cells may be unusable for making tubes. We always use primary cells and have found that the immortalized endothelial cell line HMEC-1 (16) does not form well-behaved tubes.

4. The tubing should never become crimped or have air bubbles or dust in it, since the flow rate will decrease and the barrier function can weaken.

5. The media in the inlet reservoir should be renewed regularly so that the driving pressure head does not decrease too much as media flows through the tube.

6. On day 0 (the day of seeding), the endothelial cells should adhere and completely spread out within 1–2 h of seeding as shown in Fig. 2.

7. By day 1, the cells should have grown to confluence within the tube. Gaps should not be visible along the endothelial wall in profile.

8. By day 2, the tube should have an expanded diameter although tubes with weak barrier function may not widen much. Expansion of the tube is not uniform: the region near the inlet expands more than that near the outlet, causing the tube to have a gradually sloping conical geometry. The cells should have a cobblestone appearance without any preferential alignment.

9. By day 3, the tube should have stabilized and usually will not expand further.

10. We have successfully maintained tubes for ≥1 week. Perfusion rates slowly decrease in tubes maintained for longer than 4 days, for reasons we do not yet understand.

11. Perfusing the tube under conditions that greatly weaken barrier function may prevent it from widening or growing to confluence and may cause the profile of the tube to appear wavy. We normally culture tubes under standard conditions for 1–2 days and then switch to the experimental condition (e.g., low flow rates) only after the tubes reach confluence.

12. In our hands, the dynamic range of this permeability assay is 5×10^{-8}–5×10^{-6} cm/s. Near the lower limit, lamp flicker limits the sensitivity of the assay. Near the upper limit, the tubes invariably contain many focal leaks, and measurements of background intensity I_b become inaccurate.

13. An acute change in the perfusion rate can alter the barrier function of the tube, so it is important that the height difference between the inlet and outlet reservoirs be kept constant. While transporting the tube (e.g., to and from the incubator, to the microscope, etc.), we always hold the inlet and outlet reservoirs in the same positions with respect to one another and with respect to the tube.

14. Cells may start to grow between the collagen gel and the silicone or glass, causing a decrease in perfusion or a leak of fluorescent material around the gel when performing the permeability assay. This ingrowth can be caused by detachment of the collagen gel and may gradually develop in tubes maintained for ≥1 week.

15. The selectivity of the endothelial barrier can be calculated by taking the ratios of P_d for dex-488 over P_d for BSA-594. The higher the selectivity, the stronger the barrier function.

16. As a general principle, tubes that do not widen will display large permeability coefficients.

17. For LEC tubes perfused under the standard conditions described in Subheading 3.1.3, P_d averages $3.0^{+1.0}_{-0.7} \times 10^{-7}$ cm/s for BSA-594 and $6.2^{+0.9}_{-0.8} \times 10^{-7}$ cm/s for dex-488 (geometric means $\pm$ 95% CI) (2). Surprisingly, we have noticed that BEC tubes tend to have slightly higher P_d compared with LEC tubes under identical perfusion conditions.

18. Comparisons of P_d should always use a nonparametric test (e.g., Mann–Whitney U-test), since the data do not follow a normal distribution.

19. Typically, permeability values from ~10 pairs of tubes are needed to detect a twofold difference in P_d with statistical significance of 0.05.

Acknowledgments

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