

Video Article

# Microperfusion Technique to Investigate Regulation of Microvessel Permeability in Rat Mesentery

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## Abstract

Experiments to measure the permeability properties of individually perfused microvessels provide a bridge between investigation of molecular and cellular mechanisms regulating vascular permeability in cultured endothelial cell monolayers and the functional exchange properties of whole microvascular beds. A method to cannulate and perfuse venular microvessels of rat mesentery and measure the hydraulic conductivity of the microvessel wall is described. The main equipment needed includes an intravital microscope with a large modified stage that supports micromanipulators to position three different microtools: (1) a beveled glass micropipette to cannulate and perfuse the microvessel; (2) a glass micro-occluder to transiently block perfusion and enable measurement of transvascular water flow movement at a measured hydrostatic pressure, and (3) a blunt glass rod to stabilize the mesenteric tissue at the site of cannulation. The modified Landis micro-occlusion technique uses red cells suspended in the artificial perfusate as markers of transvascular fluid movement, and also enables repeated measurements of these flows as experimental conditions are changed and hydrostatic and colloid osmotic pressure difference across the microvessels are carefully controlled. Measurements of hydraulic conductivity first using a control perfusate, then after re-cannulation of the same microvessel with the test perfusates enable paired comparisons of the microvessel response under these well-controlled conditions. Attempts to extend the method to microvessels in the mesentery of mice with genetic modifications expected to modify vascular permeability were severely limited because of the absence of long straight and unbranched microvessels in the mouse mesentery, but the recent availability of the rats with similar genetic modifications using the CRISPR/Cas9 technology is expected to open new areas of investigation where the methods described herein can be applied.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/53210/>

## Introduction

Microperfusion in the vasculature entails establishing controlled flow of an artificial perfusate of known composition via a micropipette in a blood vessel usually less than 40  $\mu\text{m}$  in diameter. The perfused vessel remains within its normal tissue environment and is perfused with the animal's blood up to the time of cannulation. When used in conjunction with a range of video imaging or fluorometric techniques, *in situ* microperfusion enables measurement of water and solute flows across the walls of microvessels under conditions where the driving forces for these flows are known and the permeability properties of the vascular wall can be directly evaluated. Further, by controlling the composition of the fluid surrounding the microvessel in the tissue (perfusate and superfusate), the regulation of microvessel permeability and exchange can be investigated by enabling the endothelial cells forming the microvessel wall to be exposed to a variety of experimental conditions (agonists, modified perfusion conditions, fluorescent indicators to measure intracellular composition and signaling) for precisely measured periods of time (sec to hr). In addition, ultrastructural or cytochemical evaluations of key cellular molecular structures regulating the barrier can be investigated in the same microvessels in which permeability is directly measured. The approach thereby forms a bridge between investigation of the cellular and molecular mechanisms to modify endothelial barrier function in cultured endothelial cell monolayers and investigation in intact microvessels. See the following reviews for further evaluation<sup>1-6</sup>.

A limitation of microperfusion is that it can be used only in microvascular beds that are thin, transparent and have sufficient structural integrity to enable cannulation with a glass micropipette. While early investigations used frog microvessels in mesentery and thin cutaneous pectoris muscle<sup>7,8</sup>, by far the most commonly used preparation in mammalian models is the rat mesentery<sup>9-15</sup>. Most investigations have focused on acute changes in vascular permeability studied over periods of 1-4 hr, but more recent investigations have been extended to measurements on individual vessels 24-72 hr after an initial perfusion<sup>12,16</sup>. The recently developed CRISPR technology, which promises to make more genetically modified rat models available for studying vascular permeability regulation<sup>17</sup> should enable the methods described in this communication to be applied in venular microvessels of the mesentery in these important new rat models.

The method requires an inverted microscope equipped with a custom built microscope stage large enough to hold both the animal preparation and at least three micromanipulators used to position microtools close to the perfused vessel and to align a perfusion micropipette with the vessel lumen. For example a custom platform for an x-y microscope stage (about 90  $\times$  60 cm) can be fabricated from a 1 cm thick steel plate

with a rust-proof coating. The stage is attached to an engineering index table or two dove-tail slides mounted at right angles and supported on Teflon pillars or ball transfers for movement in the horizontal plane. A typical rig (see **Figure 2**) has much in common with the microscope and micropositioning equipment used for a range of intravital microcirculation experiments such as those to measure single vessel blood flow and hematocrit, local oxygen delivery by blood perfused microvessels, regulation of vascular smooth muscle tone, and the local microvascular accumulation of fluorescent tracers injected into the whole circulation.<sup>18-26</sup>

The fundamental aspect of the technique is the measurement of volume flow ( $J_v$ ) across a defined surface area ( $S$ ) of the microvessel wall. To accomplish this via the modified Landis technique described herein a simple inverted microscope is adequate. A small video camera is mounted on the image port and the video signal, with an added time base, is displayed on a video monitor and recorded either in digital form on a computer or as a digital or analog signal on a video recorder. Once the microvessel is cannulated the portion of the microvessel visible to the camera can be changed by moving the stage and manipulators as a unit without disrupting the cannulation.

Measurement of transvascular flows may also be combined with more detailed investigations using a sophisticated fluorescence microscope with appropriate filters such as rigs used for measurements of solute permeability, fluorescent ratio monitoring of cytoplasmic calcium or other cellular mechanisms, and confocal imaging<sup>6,12,13,27</sup>. A key advantage of all microperfusion approaches is the ability to make repeated measures, on the same vessel, under controlled change of driving force such as hydrostatic and oncotic pressures, or induced change in vessel responses to inflammatory conditions. The most common design is a paired comparison of measured hydraulic conductivity ( $L_p$ ) on the same vessel with the vessel first perfused via a micropipette filled with a control perfusate and the red cell suspension to establish a baseline permeability state, then with a second pipette with the test agent added to the perfusate. Multiple cannulations are possible with the cycle repeated after reperfusion with the control pipette.

The present protocol demonstrates the cannulation and microperfusion of a venular vessel in rat mesentery to record water fluxes across the microvessel wall and measure the  $L_p$  of the vessel wall, a useful index of the permeability of the common pathway for water and solutes across the intact endothelial barrier. The procedure is called the modified Landis technique because the original Landis principle of using the relative movement of red cells as a measure of transvascular fluid exchange after perfusion is blocked is preserved<sup>28</sup>, but the range of experimental conditions (e.g., the hydrostatic and albumin oncotic pressure differences across the microvessel wall) available after microperfusion is far greater than in uncannulated blood perfused microvessels<sup>8,29</sup>.

## Protocol

Ethics Statement: All procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

### 1. Preliminary Fabrication of Micropipettes, Restrainers, and Blockers

1. Pull several clean borosilicate glass capillary tubes in half using an electronic puller adjusted so that, when pulled, the stretched portion of the tube is about 1 cm in length and the two halves are somewhat symmetric. Ensure that the taper is consistent with the dimensions in **Figure 1**. Use both halves for micropipettes, restrainers and blockers.
  1. Bevel the micropipettes using an air driven grinding wheel<sup>30</sup> with 0.5  $\mu\text{m}$  abrasive paper bathed with water. Set the angle of the micropipette holder so that it is about 30 degrees from horizontal.
  2. Thoroughly wash and dry the micropipettes by using suction to pull clean acetone through the tip and up the shaft. Inspect the micropipettes (**Figure 1A**) using a small upright microscope with 4 $\times$  and 40 $\times$  objectives fitted with an alligator clip micropipette holder and an eyepiece reticle.
  3. Select micropipettes with tip opening about 50  $\mu\text{m}$  long with a bevel whose length/width ratio is between 3.1 and 3.5 and with a sharply tapered point which helps penetrate the tough collagen fibers in the mesentery.
  4. Store 15-20 micropipettes of slightly varying sizes in a dust-free box.
2. Make restrainers using pulled capillary tubes prepared in step 1.1). Hold a pulled glass capillary tube briefly near a microflame to form a blunt end (**Figure 1B**). Store several in a dust-free box.
3. Make microoccluders using pulled capillary tubes prepared in step 1.1). Hold a pulled capillary tube under a microflame and gently bend (using a tubing adapter, e.g., 23G) the fine tip (about 4 mm from tip) to make an angle of close to 120 degrees to the shaft (**Figure 1C**). Make and store several.

### 2. Animal Preparation and Surgery

1. Anesthetize male (350-450 g) or female Sprague-Dawley rats (200-300 g) with pentobarbital sodium (80-100 mg/kg, Sigma-Aldrich, P3761) via subcutaneous injection; protect the mesentery by not using intraperitoneal injection.
2. Throughout surgical procedures and experimental protocols, maintain anesthesia by supplemental injections (30 mg/kg) as needed. Determine depth of anesthesia by toe pinch or corneal reflex. After completion of the experimental procedure, euthanize the rat via pentobarbital overdose or intra-cardiac injection of saturated potassium chloride under anesthesia.

### 3. Prepare Solutions and Erythrocytes for use as Flow Markers

1. Prepare mammalian Ringer's solution for superfusate and control perfusate solution (typically mammalian Ringer's solution containing 10 mg/ml fatty acid free bovine serum albumin, BSA).
  1. Filter perfusate through 0.2  $\mu\text{m}$  syringe filters to remove tiny particles that might block the micropipette tip; filter into small clean container.

2. Prepare erythrocytes by drawing about 0.2 ml blood from the tail vein of the anesthetized rat into a 20G needle on a small syringe containing 0.05 ml heparin (1,000 U/ml) and transfer to a 15 ml centrifuge tube containing about 14 mL mammalian Ringer's solution solution.
  1. Centrifuge to gently pack red cells (about max 200 × g for 7 min). Draw off the supernatant and re-suspend the red cells in Ringer's solution.
  2. After centrifuging and removing supernatant three times leave the packed red cells in the bottom of the tube for later use. Note that this procedure reduces platelets in the final perfusates to less than 0.1% of normal levels<sup>31</sup>.

#### 4. Arrange the Tissue on the Animal Tray

1. Shave the abdomen of the anesthetized rat from below navel to xiphoid process. Ensure that the shaved area extends around the right side (for rat placed on right side) so that hair does not form a wick to draw the superfusate out of the tissue well. Remove cut hairs with a wetted gauze pad or tissue.
  1. Hold the skin with a blunt serrated forceps and make a center-line incision (2-3 cm long) through the skin using a sturdy scissors. Using a fine (iris) scissors make a similar incision through the linea alba, lifting the abdominal wall with serrated forceps to avoid damaging the gut.
  2. With the animal on its side on the animal tray, gently pull out the gut from the abdominal cavity using cotton-tipped applicators (wood stick) soaked in Ringer's solution and blunt serrated forceps. Arrange the gut in the tissue well so that the mesentery is draped over the pillar for viewing through the microscope taking care to avoid touching the mesentery (potentially damaging microvessels) with either forceps or cotton.

Note: The animal tray (**Figure 2A**) can be constructed from Plexiglas and requires a tissue well (about 3 mm deep), an optically clear pillar (e.g., polished quartz about 2 cm dia. and 5 mm high), and a warming pad adjusted to maintain the animal's temperature. The thickness of the pillar must not exceed the working distance of the objective.
2. Position the animal tray on the microscope stage so that the mesentery is visible through the eyepieces.
3. Position a gravity-fed drip line to continuously superfuse the mesentery with mammalian Ringer's solution maintained at 35–37 °C. Use gauze pads to hold the gut in place, help retain moisture, and wick excess Ringer's solution off the surface. Adjust the flow and aspirate the effluent to maintain a consistent superfusate thickness.
4. Identify target venular microvessels, which are unbranched segments downstream of convergent flow, one or two branches distal to true capillaries. Find an unbranched vessel (ideally, 600 to 1,000 µm in length) having brisk blood flow and free of white cells sticking on the vessel wall.
5. Position the test microvessel in the center of the microscope field and move the restrainer into position near the chosen cannulation site.

#### 5. Fill Micropipette and Mount in Holder

1. Just before cannulating, suspend the red cells in the perfusate. Add 7 µl of packed red cells to 0.5 ml perfusate in a clean borosilicate test tube. Note: Hematocrits of 1-3% can be used, but consistent hematocrit will lead to consistent perfusate concentrations of any substances released from the red cells.
2. Fit a 1 ml syringe with a 23G tubing adapter and PE 50 tubing (5-15 cm length). Draw the perfusate/red cell mixture into the syringe. Invert syringe to mix and expel all bubbles from tubing and adapter. For increased efficiency, fit tubing to several syringes before the experiment starts and keep them in a dust free box or plastic bag.
3. Fill the micropipette by advancing the tubing into the large end of the micropipette until it abuts the tapered region. Apply a gentle quick push on the syringe plunger to fill the micropipette tip. Note: A tiny stream or droplet should be visible at the tip to evidence complete filling.
4. Withdraw the tubing while gently pushing on the plunger to fill the wider part of the micropipette shaft. Remove small bubbles in the wide shaft by carefully flicking the micropipette shaft. Note: A similar procedure has been illustrated<sup>32</sup>.
5. Place the micropipette into a pipette holder with a side port which allows a continuous fluid connection to a water manometer. Ensure that the holder, which is attached to a hydraulic drive used to control very fine advancement of the micropipette, is at a slight angle (15-25°) to the horizontal so that the edge of the micropipette taper does not hit the gut or tray.
6. Mount the hydraulic drive on a moveable micromanipulator, which has x, y, and z drives to enable close alignment to the test vessel (**Figure 2**).
7. Adjust the hydrostatic pressure applied to the fluid in the micropipette using a syringe or water-filled rubber bulb to change the height of fluid in the water column of the manometer to about 40 cmH<sub>2</sub>O above the mesentery.
8. Cannulate the microvessel as soon as possible after filling the pipette; red cells settle to the bottom of the pipette, so that after about 40 min very few red cells will flow into the vessel.

#### 6. Microcannulation and Microvascular Pressure Measurement

1. Before positioning the filled micropipette under the microscope, gently press the restrainer onto the tissue near the microvessel applying sufficient force to grip the tissue. Draw the restrainer back, slightly stretching the tissue in line with the microvessel so that the stresses in the mesenteric collagen fibers are aligned with the vessel.
  1. Align the micropipette with the vessel and lower it into view through the eyepieces. Place the tip just upstream of the chosen cannulation site and lower it onto the tissue so that it partially obstructs flow within the vessel, but does not occlude it.
2. Cannulate the vessel by driving the pipette tip slowly into the vessel lumen using the hydraulic drive. Be careful not to push through the lower side of the vessel.
 

Note: As the micropipette enters the lumen, the perfusate rapidly washes the blood in the vessel out of the lumen and perfusate freely flows into the animal's circulation distal to the test microvessel, displacing some of the normal blood perfusion in the downstream vessels.

3. Lower the perfusion pressure by adjusting the manometer until the blood (as indicated by the animal's red cells) is drawn back into the perfused segment; this determines the balance pressure where the red cells gently oscillate in the vessel lumen and measures the hydrostatic microvessel pressure ( $P_c$ ) at the distal end of the microvessel segment. Maintain vessel perfusion at all pressures above this balance pressure.

## 7. Microocclusion and Collection of Data

1. Optional step: Before using the micro-occluder to block the vessel, press it into the tissue in a non-used area of mesentery far from any vessel so that the tip accumulates a fine pad of tissue that protects the experimental vessel during repeated micro-occlusions.
2. Place the blocker above the perfused vessel near the lower edge of the microscope field.
3. Record the following with video and audio.
  1. Verbally record the location of the block site and the lower screen edge as seen on the eyepiece reticle.
  2. With the tip of the blocker placed just above the microvessel, use the fine z control of the micromanipulator to gently lower the blocker until the flow in the vessel is occluded. Note the manometer pressure ( $P_c$ ) on audio. Apply occlusion typically for 3-10 sec, and then release, restoring free perfusion. Move the block site up the vessel toward the pipette tip in 5-10  $\mu\text{m}$  steps based on time (>5 min after initial block at a site), number of occlusions (3-5), to prevent vessel wall damage at the block site.

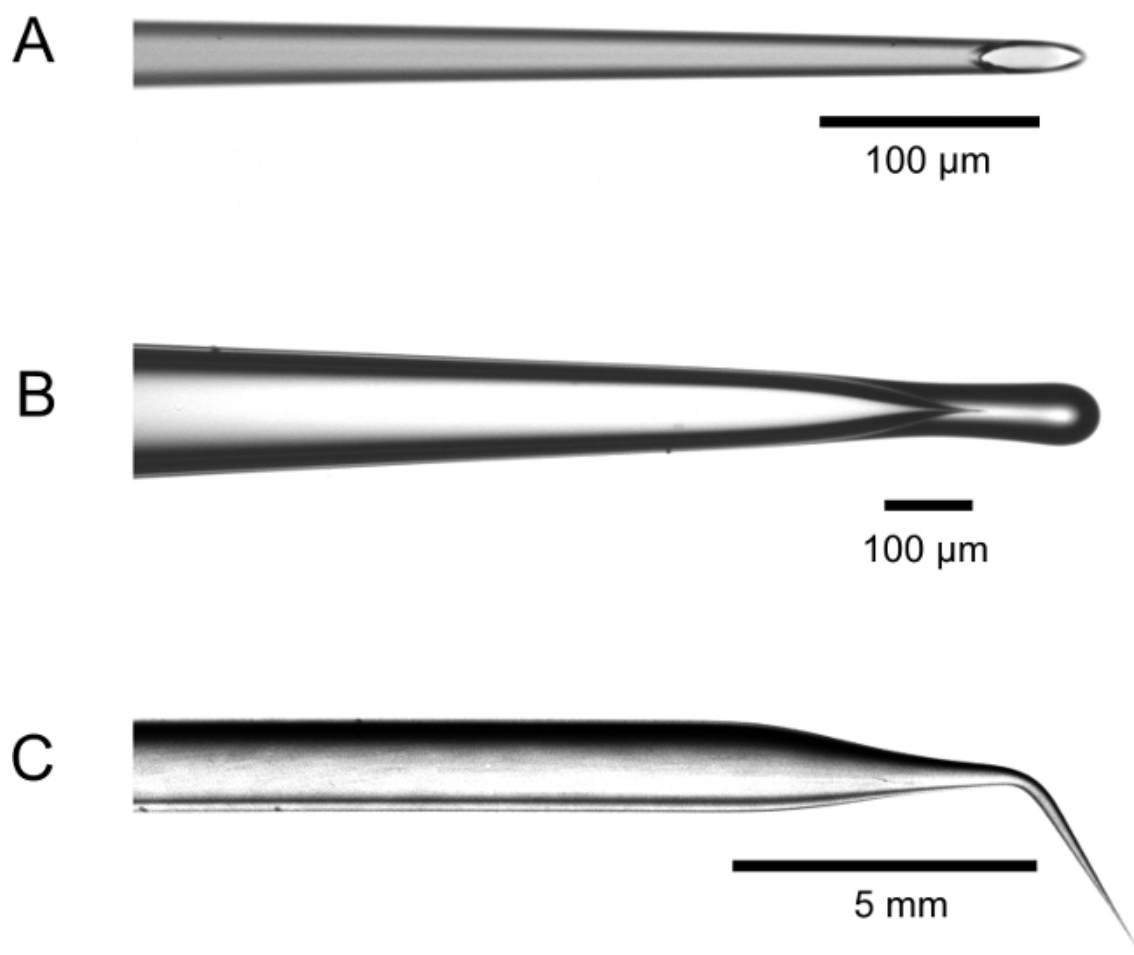
## 8. Analysis of Data and Measurement of $L_p$ (Water Permeability)

1. During video playback, determine the initial distance ( $l_0$ ) from a marker red cell to the occlusion site by use of an image of a stage micrometer and the known positions on the images. Determine the initial velocity ( $dl/dt$ ) of the marker cell from its position in several frames during the 3-10 sec of recorded images. Determine the vessel radius ( $r$ ) from the images taken during occlusion (**Figure 3**).
2. Calculate  $J_v/S$  for each occlusion as  $(dl/dt) \times (1/l_0) \times (r/2)$ . Calculate  $L_p$  at a constant pressure as  $(J_v/S)$  divided by the driving pressure (microvessel pressure – effective colloid osmotic pressure; see Discussion).

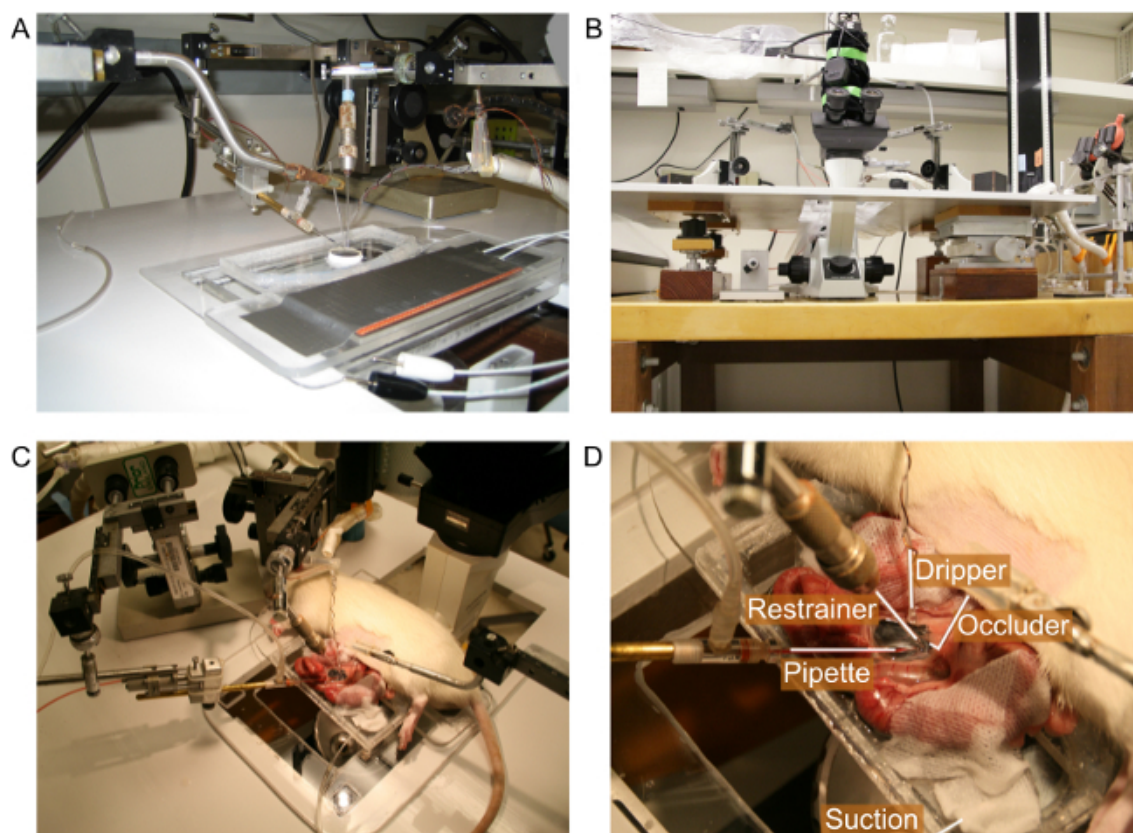
## Representative Results

**Figure 4** shows the results from measuring the time course of changes in  $L_p$  in a rat venular microvessel cannulated successively with four perfusates.<sup>33</sup> The magnitude of  $L_p$  calculated at a constant pressure was used as a measure of changes in microvessel wall permeability, first in the control state with a perfusate containing 1% bovine serum albumin then when the vessel was exposed to the inflammatory agent bradykinin (Bk) using a second micropipette containing 10 nM Bk. Bradykinin caused a transient increase in  $L_p$  that returned towards control value over the subsequent 10-15 min. The vessel was then re-perfused with control perfusate for 30 min to re-establish a stable baseline. When the microvessel was perfused using a fourth micropipette with the same concentration of Bk as well as 1  $\mu\text{M}$  sphingosine-1-phosphate (S1P) in the perfusate, the response to Bk was significantly attenuated. The degree of attenuation when Bk and S1P were perfused at the same time was found to be similar to that measured in other experiments where S1P was added to the perfusate up to 20 min before exposure to Bk. These results demonstrate the power of being able to make multiple measurements of  $L_p$  on a single vessel, and show that the action of an agent such as S1P to stabilize the vessel wall in the presence of an inflammatory agent can be resolved to time periods on the order of a few seconds. In separate vessels control studies were carried out with a similar protocol in the absence of S1P to show that the attenuation of the Bk response was not due to anaphylaxis.

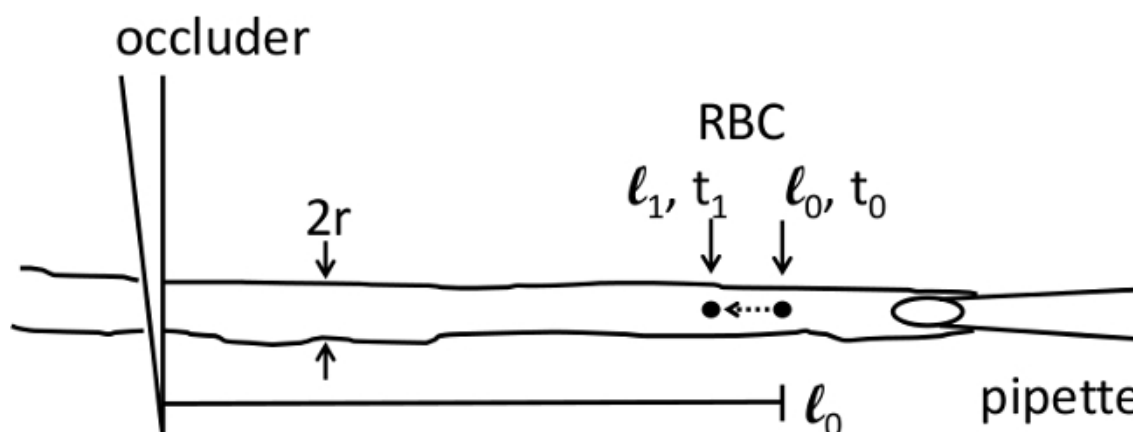
For some experimental designs it is important to estimate both the solute reflection coefficient for a macromolecule ( $\sigma$ ) and the  $L_p$ . This is accomplished best by measuring  $J_v$  at multiple pressures on each individual microvessel. **Figure 5** shows an experiment in which both  $L_p$  and the effective oncotic pressure of 5% albumin in the perfusate ( $\pi_{\text{albumin}} = 27 \text{ cmH}_2\text{O}$  at  $37^\circ\text{C}$ ) were estimated under conditions when there was no albumin in the tissue surrounding the rat microvessel. In these experiments  $J_v/S$  was measured at three different pressures. The  $L_p$  is the slope of the relation between  $J_v/S$  and pressure, and the intercept on the pressure axis is the effective oncotic pressure difference across the vessel wall ( $\sigma\Delta\pi$ ).



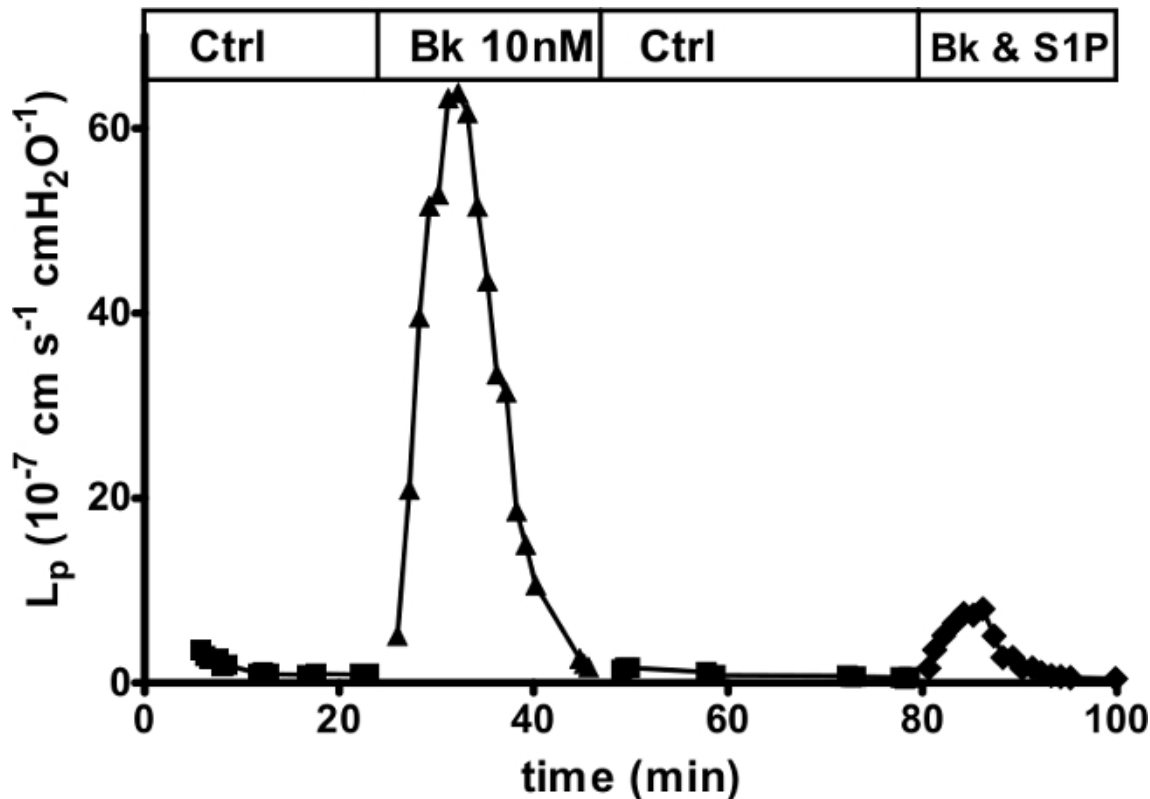
**Figure 1. Microtools for perfusion of individual microvessels.** (A) A micropipette for cannulation of rat microvessels with a beveled tip. (B) A restrainer for holding mesentery tissue stable near the site of cannulation. (C) An occluding rod, or blocker, used to press down on the mesentery and cannulated microvessel to occlude the flow in the vessel. [Please click here to view a larger version of this figure.](#)



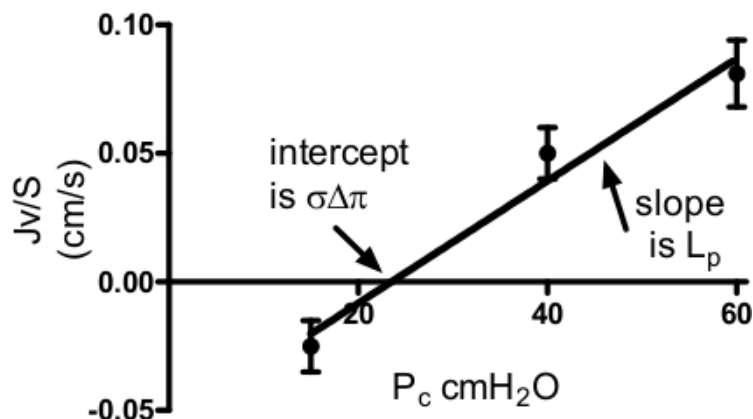
**Figure 2. Intravital rig for microperfusion.** (A) Overview of animal tray with micromanipulators and microtools oriented over glass pillar. (B) Large metal stage mounted on engineering table and supporting bearings. (C) Anaesthetized rat with microtools in position for microperfusion of a microvessel. (D) Close view of mesentery during an experiment. [Please click here to view a larger version of this figure.](#)



**Figure 3. Measurement of filtration rate per unit area using modified Landis technique.** This schematic shows a single microvessel cannulated with a micropipette and an occluding rod positioned over the vessel. Immediately after occluding the microvessel, the distance ( $l_0$ ) between the marker RBC travelling on the centerline of the occluded microvessel and the occlusion site is recorded so that the cell trajectory can be followed for 5-10 sec after occlusion. The initial position ( $l_0$ ) and initial velocity ( $dL/dt$ ) of the cell are used to calculate the average filtration rate per unit area of microvessel wall between the marker cell and the site of occlusion. The microoccluder is then raised, and free perfusion established before additional measurements are made. [Please click here to view a larger version of this figure.](#)



**Figure 4. Representative data show that sphingosine-1-phosphate (S1P) inhibition acts very fast.** Bradykinin (Bk; 10 nM) caused a transient increase in microvessel  $L_p$ . S1P applied as concurrent with second bradykinin (Bk) test inhibited the acute Bk response relative to first Bk. These data demonstrate that the time of exposure to a test agent can be modified to evaluate the kinetics of activation of an inhibitory response. Specifically, S1P applied concurrent only with Bk strongly inhibited the Bk response. S1P was 1  $\mu$ M and Bk was 10 nM. [Please click here to view a larger version of this figure.](#)



**Figure 5. Measurement of  $J_v/S$  at multiple pressures enables both  $L_p$  and the effective oncotic pressure to be measured.** The filtration flux,  $J_v/S$ , was measured in this vessel at various microvessel hydrostatic pressures during perfusion with BSA (50 mg  $\text{ml}^{-1}$ ;  $\pi = 27 \text{ cmH}_2\text{O}$ ) while the mesentery was bathed with protein-free Ringer's solution. The slope of the relation between  $J_v/S$  and pressure is the  $L_p$  and the intercept on the pressure axis indicates the effective oncotic pressure difference across the vessel wall. In this experiment the  $L_p$  was  $1.2 \times 10^{-7} (\text{cm sec}^{-1} \text{ cmH}_2\text{O}^{-1})$  and the  $\sigma\Delta\pi$  was 24  $\text{cmH}_2\text{O}$ . [Please click here to view a larger version of this figure.](#)

## Discussion

**Details of  $L_p$  calculations.** Although transvascular fluid movement occurs while the vessel is freely perfused, such exchange is far too small to be measured during free perfusion because it is typically less than 0.01% of the vessel perfusion rate. However, when perfusion is transiently stopped by occluding the microvessel, transvascular flow (*i.e.*, filtration) is measured from the movement of marker red cells in the lumen as the column of fluid between a marker red cell and the site of occlusion shortens as shown in **Figure 3**. Depending on the complexity and length of the experimental protocol an unbranched vessel 600 to 1,000  $\mu$ m in length is needed.

When the vessel is not occluded and the manometer pressure is driving flow through the microvessel, the pressure drop across the pipette tip is large so that microvessel pressure during free perfusion is typically only a few  $\text{cmH}_2\text{O}$  above the downstream pressure. In contrast, during

an occlusion the pressure drop across the pipette is very small due to fluid slowly entering the vessel lumen to replace fluid filtering across the vessel wall. Thus during occlusion the hydrostatic pressure in the vessel lumen ( $P_c$ ) equals that set by the manometer. The oncotic pressure in the mesenteric tissue is negligible, so immediately after occlusion, transvascular fluid movements from marker red cells are measured where the hydrostatic pressure is known and the oncotic pressure in the vessel lumen is equal to that set in the perfusate. With successive measurements of  $L_p$ , vessel length is lost each time the block site is advanced. In addition, successive recannulation reduces available vessel length.

The transvascular fluid flow per unit area of vessel wall ( $J_v/S$ ) is estimated from the rate that a measured length of the column of water in the microvessel lumen between a marker red cell and the occlusion site either shortens (net filtration) or lengthens (net reabsorption). The assumption is that neutrally buoyant red cells moving along the centerline of the vessel lumen can be used to track the mean velocity of the water column surrounding the cell (the ratio of red cell velocity to mean water velocity is close to 1.8 for a 6  $\mu\text{m}$  red cell in a 30  $\mu\text{m}$  diameter vessel)<sup>8,14</sup>. Thus if the velocity of a marker cell (in  $\mu\text{m}/\text{sec}$ ) towards the blocker is  $V$   $\mu\text{m}/\text{sec}$  over the first 2-5 sec after occlusion, and the vessel radius is  $r$ , the volume of fluid filtered across the microvessel wall between that red cell and the site of occlusion ( $J_v$ ) is  $\pi r^2 V$   $\mu\text{m}^3/\text{sec}$  assuming the microvessel is cylindrical. Further the area of a cylindrical microvessel ( $S$ ) between that marker cell and the occlusion site is  $2\pi r\ell$ , where  $\ell$  is the initial distance between the marker cell and the occlusion site. Thus the average initial transvascular flow per unit area ( $J_v/S$ ) at the pressure set by the water manometer  $Vr/(2\ell)$ .

The simplest estimate of vessel  $L_p$  is made if the oncotic pressure of the perfusate has been set low relative to the microvessel pressure (typically 3.6  $\text{cmH}_2\text{O}$ , the oncotic pressure of bovine serum albumin at a concentration of 10  $\text{mg}/\text{ml}$  and a capillary pressure greater than 30  $\text{cmH}_2\text{O}$ ).  $L_p$  is calculated as  $(J_v/S)/(P_c-3)$   $\text{cm}/\text{sec}/\text{cm H}_2\text{O}$  because the albumin reflection coefficient ( $\sigma_{\text{albumin}}$ ) in the control state is close to 0.9. With this approach rapid changes in  $L_p$  with a time resolution of the order of 1 min can be measured by establishing free flow between successive measures of  $J_v/S$  at constant pressure as shown in the representative results in **Figure 4**. The above approach ignores small changes in the oncotic pressure of albumin which fall to less than 1  $\text{cmH}_2\text{O}$  when the reflection coefficient falls below 0.5, but the error in the estimate of  $L_p$  remains less than 5% when  $P_c$  is greater than 30  $\text{cmH}_2\text{O}$ .

When accurate measurement of both  $L_p$  and reflection coefficient is required,  $J_v/S$  is measured at a series of microvessel pressures below the expected effective oncotic pressure of the test macromolecule. The slope of the relation between  $J_v/S$  and pressure measures  $L_p$  and the intercept on the pressure axis when net flow is zero measures  $\sigma\pi$  (**Figure 5**). The demonstration that  $J_v$  is linearly related to pressure is also an important validation of the use of single pressure measurements using the simpler protocol described above (**Figure 4**) when the oncotic pressure difference is low relative to hydrostatic pressure.

**Possible sources of error.** There are several problems that compromise accurate measurement. Failure to properly occlude the vessel or damage to the vessel wall at the occlusion site results in fluid leak past the occlusion site, and movement of marker cells towards the occlusion site faster than that due to transvascular exchange. Whereas marker cells normally move slowly toward the occlusion site, but do not reach it, a leak is indicated if marker cells track all the way to the occluder. On the other hand, failure to seal the pipette into the microvessel lumen at the cannulation site results in underestimation of  $J_v/S$ . Fluid leak from the pipette to the superfusate or to the vessel lumen upstream of the cannulation site causes a significant pressure drop across the pipette tip. Such a leak is detected when cells in the micropipette tip flow at a higher velocity than those in the downstream vessel lumen of the occluded vessel. Careful repositioning of the micropipette usually seals such a leak. A different issue arises if the  $L_p$  of the vessel wall is not uniform, usually having with one or more sites of localized damage or inflammation. Marker cells track to the site if most transvascular flow is concentrated there. Red cell movement still measures transvascular flow, but the measure  $J_v/S$  is not representative of the most of the wall area within the test segment.

**Significance of the method with respect to existing methods.** The two most commonly used methods to investigate the regulation of endothelial barrier permeability and transvascular exchange are (1) *in vitro* measurement of exchange across cultured endothelial cell monolayers and (2) measurement of tracer accumulation in a tissue sample after the tracer is injected intravenously into the whole animal. Experiments in cultured endothelial cell monolayers are favored because the cellular functions can be more directly modified and there is sufficient cellular material available for detailed evaluation of molecular events in endothelial cells. However under most culture conditions the endothelial cells do not usually form the barrier representative of vascular permeability *in vivo*. The modified Landis technique described above has been demonstrated to be a robust and reliable technique to investigate the regulation of endothelial barrier function in intact venular microvessels. In the hands of an experienced investigator paired measurements of permeability under control and test conditions where some of the approaches used in cell culture (e.g., imaging of cellular components, modification of signaling pathways) can be applied. The importance of experiments in intact microvessels has been demonstrated by showing significant differences between the mechanisms that regulate barrier permeability in individually perfused microvessels and those often described in cultured endothelial monolayers. Examples include responses to shear, thrombin exposure, and the contribution of contractile mechanisms to inflammatory gap formation<sup>2-6,9,16</sup>. Measurements of tracer accumulation in an intact vascular bed preserves more normal physiological function but direct investigation of real changes in vascular permeability are compromised because tracer accumulation may increase as the result of increased perfusion (increased surface area for exchange) with or without an increase in the permeability of the wall. Thus the extent of a permeability increase may be overestimated when vasodilation increases the number of perfused vessels and thereby total solute accumulation is greater than that due to increase in vascular permeability alone<sup>34</sup>.

**Future applications.** The range of options to modify cellular structure and function under the same conditions as permeability of individual vessels is directly measured during microperfusion is expected to increase. Furthermore it is expected that the application of the methods in genetically modified rat mesentery may solve a long-term problem that has existed for genetically modified mice. Specifically, when genetically modified mice became available it was expected that microperfusion could be extended to investigate microvessels in their mesenteric vessels. While some microperfusion experiments have been completed in microvessels of mouse mesentery<sup>35</sup>, mice generally have fewer microvessels in their mesenteric tissue than rats, and these vessels are often short and highly branched, as opposed to more suitable longer (> 500  $\mu\text{m}$ ) straight vessels that can be found in rats. Thus experiments to investigate the modulation of transvascular exchange in genetically mice have relied on assays such as the Miles<sup>34</sup> or the technically demanding, but more reliable tracer experiments which measure changes in both vascular and extravascular accumulation of fluorescent or radiolabelled test probes<sup>36</sup>.

**Modifications of the method.** In the above protocols, changes in perfusate composition required change of micropipettes. An alternate approach is to refill the micropipette *in situ* using a refilling apparatus. This procedure has been accomplished as described in detail<sup>37</sup>. The limitation is

that the time to change the composition of the perfusate in the pipette is not as fast as achieved by pipette replacement, but the procedure is especially useful if it is necessary to perfuse a microvessel for extended periods of time.

The same microperfusion approach can be extended to measure permeability coefficients to fluorescently labeled solute<sup>31,38-40</sup>. In this case the single barreled micropipette is replaced by a double barreled (theta) pipette<sup>41</sup> and the vessel is perfused alternately with the test fluorescent solute to measure tissue accumulation relative to lumen content, and the same perfusate without the fluorescent solute to clear the tissue and allow repeated measurement of solute accumulation. When measurements of transvascular water flows are to be combined with measurements of solute permeability coefficient using fluorescently labeled test solutes, fluorescent indicators of intracellular composition, or confocal imaging of cellular components, a more sophisticated computer controlled fluorescence microscope is required. Since these investigations usually require lenses with higher magnification and much shorter working distance, the quartz pillar used to hold the mesentery is replaced by a glass cover slip glued to a Plexiglas support within the tissue well on the microscope tray. The increased availability of 3-D printers, will allow high precision manufacture of custom trays, which meet the narrow tolerance required to position larger diameter lenses with shorter working distance relative to selected microvessels.

The availability of usable microvessels in the mesentery varies with age, gender, and strain. Therefore, the choices of strain of rat and the use of male or female will depend on the specific questions being addressed. For many of our recent studies we have used male Sprague-Dawley rats which are acclimated in our animal facility at least one week before use at about age 3-4 months at which time we find they generally have available long, straight mesenteric microvessels. By contrast, our colleagues have successfully used female Sprague-Dawley rats of 2-3 months age<sup>13</sup>.

**The role of the red cells.** The primary role of red cells as described above was to track transvascular water movement assuming the cell acted as inert, neutrally buoyant indicators of water flows after the microvessels were occluded. In addition to this fundamental role, it is now recognized that the red cells contribute to the stability of the barrier by supplying S1P to the perfusate when albumin is present<sup>31,42,43</sup>. There are several important implications of these observations. In the absence of red cells or with the use of alternate inert flow markers the baseline permeability is likely to be less stable. It is recommended that measurements of S1P should be made in all perfusates used in microperfusion experiments.

## Disclosures

The authors have nothing to disclose.

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