

Video Article

# Simultaneous Measurements of Intracellular Calcium and Membrane Potential in Freshly Isolated and Intact Mouse Cerebral Endothelium

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## Abstract

Cerebral arteries and their respective microcirculation deliver oxygen and nutrients to the brain *via* blood flow regulation. Endothelial cells line the lumen of blood vessels and command changes in vascular diameter as needed to meet the metabolic demand of neurons. Primary endothelial-dependent signaling pathways of hyperpolarization of membrane potential ( $V_m$ ) and nitric oxide typically operate in parallel to mediate vasodilation and thereby increase blood flow. Although integral to coordinating vasodilation over several millimeters of vascular length, components of endothelium-derived hyperpolarization (EDH) have been historically difficult to measure. These components of EDH entail intracellular  $\text{Ca}^{2+}$  [ $\text{Ca}^{2+}_i$ ] increases and subsequent activation of small- and intermediate conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ ) channels.

Here, we present a simplified illustration of the isolation of fresh endothelium from mouse cerebral arteries; simultaneous measurements of endothelial [ $\text{Ca}^{2+}_i$ ] and  $V_m$  using Fura-2 photometry and intracellular sharp electrodes, respectively; and a continuous superfusion of salt solutions and pharmacological agents under physiological conditions (pH 7.4, 37 °C). Posterior cerebral arteries from the Circle of Willis are removed free of the posterior communicating and the basilar arteries. Enzymatic digestion of cleaned posterior cerebral arterial segments and subsequent trituration facilitates removal of adventitia, perivascular nerves, and smooth muscle cells. Resulting posterior cerebral arterial endothelial "tubes" are then secured under a microscope and examined using a camera, photomultiplier tube, and one to two electrometers while under continuous superfusion. Collectively, this method can simultaneously measure changes in endothelial [ $\text{Ca}^{2+}_i$ ] and  $V_m$  in discrete cellular locations, in addition to the spreading of EDH through gap junctions up to millimeter distances along the intact endothelium. This method is expected to yield a high-throughput analysis of the cerebral endothelial functions underlying mechanisms of blood flow regulation in the normal and diseased brain.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/58832/>

## Introduction

Blood flow throughout the brain is regulated by the coordination of vasodilation among cerebral arteries and arterioles in vascular networks<sup>1</sup>. Endothelial cells lining cerebral resistance arteries command changes in vascular diameter as needed to meet the metabolic demand of neurons<sup>1,2,3</sup>. In particular, during endothelium-derived hyperpolarization (commonly known as EDH), intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}_i]$ ) and electrical signaling in endothelial cells coordinate vasodilation among endothelial cells and their surrounding smooth muscle cells through gap junctions for arterial relaxation<sup>4</sup>. Physiological initiation of EDH sequentially entails stimulation of  $\text{G}_q$ -coupled receptors (GPCRs), an increase in  $[\text{Ca}^{2+}_i]$ , and activation of endothelial small- and intermediate- $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ ) channels to hyperpolarize cerebral endothelial membrane potential ( $V_m$ )<sup>5,6,7</sup>. Thus, the intimate relationship of endothelial [ $\text{Ca}^{2+}_i$ ] and  $V_m$  is integral to blood flow regulation and indispensable to cardio- and cerebrovascular function<sup>6,8</sup>. Throughout the broader literature, numerous studies have reported the association of vascular endothelial dysfunction with the development of chronic diseases (e.g., hypertension, diabetes, heart failure, coronary artery disease, chronic renal failure, peripheral artery disease)<sup>9,10</sup>, indicating the significance of studying endothelial function in physiological as well as pathological conditions.

Vascular endothelium is integral to the production of hyperpolarization, vasodilation, and tissue perfusion and thus, examination of its native cellular properties is crucial. As a general study model, preparation of the mouse arterial endothelial tube model has been published before for skeletal muscle<sup>11,12</sup>, gut<sup>13</sup>, pulmonary<sup>14</sup>, and recently for the brain<sup>6</sup>. Studies of simultaneous [ $\text{Ca}^{2+}_i$ ] and  $V_m$  measurements in particular have been published for skeletal muscle arterial endothelium<sup>15,16</sup> as well as lymphatic vessel endothelium<sup>17</sup>. In addition to primary studies utilizing the endothelial tube approach, a comprehensive review of its advantages and disadvantages<sup>9</sup> can be consulted to determine if this experimental tool is appropriate for a specific study. In brief, an advantage is that the key physiological components of endothelial cell function are retained (e.g.,  $\text{Ca}^{2+}$  influx and intracellular release, hyperpolarization of  $V_m$  up to the Nernst potential for  $\text{K}^+$  *via*  $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$  activation, and endothelial intercellular coupling *via* gap junctions) without confounding factors such as perivascular nerve input, smooth muscle voltage-gated channel function and contractility, circulation of blood, and hormonal influences<sup>8</sup>. In contrast, commonly used cell culture approaches introduce significant alterations in morphology<sup>18</sup> and ion channel expression<sup>19</sup> in a manner that can greatly obfuscate comparisons to physiological observations determined ex

*vivo* or *in vivo*. Limitations include a lack of integration with other essential components for regulating blood flow, such as smooth muscle and restricted flexibility in an experimental schedule, as this model is optimally tested within 4 h of intact vascular segment isolation from the animal.

Building from a previous video protocol authored by Socha and Segal<sup>12</sup> and recent experimental developments in the interim<sup>6,15,16</sup>, we hereby demonstrate the isolation of fresh endothelium from posterior cerebral arteries and simultaneous measurements of endothelial  $[Ca^{2+}]_i$  and  $V_m$  using Fura-2 photometry and intracellular sharp electrodes, respectively. Additionally, this experiment entails continuous superfusion of salt solutions and pharmacological agents during physiological conditions (pH 7.4, 37 °C). We chose the posterior cerebral artery, as it yields isolated endothelium with the structural integrity (cells coupled through gap junctions) and sufficient dimensions (width  $\geq 50 \mu m$ , length  $\geq 300 \mu m$ ) amenable for intra- and intercellular signaling along and among endothelial cells. In addition, studies of the rodent posterior cerebral artery are substantially represented in the literature and encompass examination of fundamental endothelial signaling mechanisms, vascular development/aging, and pathology<sup>20,21,22</sup>. This experimental application is expected to yield a high-throughput analysis of cerebral endothelial function (and dysfunction) and will thereby allow for significant advancements in the understanding of blood flow regulation throughout aging processes and the development of neurodegenerative disease.

## Protocol

Before conducting the following experiments, ensure that all animal care use and protocols are approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accord with the National Research Council's "Guide for the Care and Use of Laboratory Animals" (8<sup>th</sup> Edition, 2011) and the ARRIVE guidelines. The IACUC of Loma Linda University has approved all protocols used for this manuscript for male and female C57BL/6 mice (age range: 3 to 30 mo).

## 1. Equipment and Materials

NOTE: Details of materials required for the protocol can be found in the **Table of Materials, Reagents**, and manuals or websites associated with the respective vendors.

### 1. Flow Chamber

1. Fasten a superfusion chamber with a glass coverslip bottom into an anodized aluminum platform. Secure the platform with chamber into a compact aluminum stage.
2. Set a micromanipulator at each end of the platform on the aluminum stage for holding the pinning pipette.  
Note: As a practical option, use this transferable stage apparatus with a flow chamber unit secured to facilitate the transition from isolation of the endothelial tube to performance of the experiments.

### 2. Microscopes

1. Use stereomicroscopes (5X to 50X magnification range) equipped with fiber optic light sources for macro- and micro-dissection procedures.
2. For preparation of endothelial tubes, use an inverted microscope equipped with phase contrast or differential interference contrast (DIC) compatible objectives (10X, 20X, and 40X) and an aluminum stage.
3. To isolate endothelial tubes from partially digested blood vessels, place a microsyringe pump controller adjacent to the endothelial tube preparation apparatus.
4. For the experimental apparatus, use an inverted microscope equipped with standard objectives (4X and 10X) in addition to fluorescent objectives (20X and 40X, numerical aperture 0.75) and a manual aluminum stage on a vibration isolation table.

### 3. Intracellular Recording Equipment

1. To record the  $V_m$  of endothelial cells in isolated endothelial tubes, connect the electrometer to the compatible headstage. If necessary, connect a function generator or stimulator to the electrometer for experiments that require a current injection.
2. Connect the amplifier outputs to a data acquisition system, audible baseline monitors, and an oscilloscope. Secure the reference electrode (Ag/AgCl pellet) near the flow chamber exit during experiments.
3. Use a photometric system with integrated components of a fluorescence system interface, high intensity arc lamp and power supply, hyperswitch, photomultiplier tube (or PMT), and camera to measure  $[Ca^{2+}]_i$  in endothelial cells.
4. Use a temperature controller equipped with an inline heater to raise and maintain a physiological temperature (37 °C) throughout the experiment.
5. Use a six-reservoir platform connected to a valve controller with an inline flow control valve to control the delivery of respective solutions to the endothelial tube secured in the chamber.

### 4. Micropipettes and Sharp Electrodes

NOTE: To manufacture pipettes for trituration and the mechanical stabilization of isolated endothelial tubes, use an electronic puller for pulling of the pipettes and a micro-forge for breaking and fire polishing.

1. To separate adventitia, smooth muscle, and the endothelial tube from the partially digested arterial segment, prepare trituration pipettes (each with an internal tip diameter of 80-120  $\mu m$ ) as appropriate, using borosilicate glass capillary tubes, and fire polish the tip.
2. To secure the endothelial tube against the bottom of the superfusion chamber, use pinning pipettes with a blunted, spherical end (heat-polished, OD of 100-150  $\mu m$ ; prepared from thin-wall borosilicate glass tubes).
3. To record  $V_m$  of an endothelial cell, prepare sharp electrodes with a tip resistance of  $\sim 150 \pm 30 M\Omega$  from glass capillary tubes using an electronic puller.

## 2. Preparation of Solutions and Drugs

### 1. Physiological Salt Solution (PSS)

1. For one experiment with drug preparations, prepare a minimum of 1 L of PSS using: 140 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 10 mM glucose.
2. To prepare the required solutions for dissection, isolation of cerebral artery, and preparation of endothelial tube, prepare PSS lacking  $\text{CaCl}_2$  (zero  $\text{Ca}^{2+}$  PSS).
3. Prepare all solutions in ultrapure deionized  $\text{H}_2\text{O}$ , adjust the pH to 7.4, sterilize them through a filter (0.22  $\mu\text{m}$  pore size), and ensure that the osmolality of the solutions is between 290 and 300 mOsm.  
NOTE: Solutions can be stored at 4 °C for approximately two weeks.
2. **10% Bovine Serum Albumin (BSA)**
  1. To prepare 10% BSA, dissolve 1 g of the lyophilized powder into a beaker of 10 mL zero  $\text{Ca}^{2+}$  PSS.
  2. Cover the beaker with paraffin film and allow a period of several hours to pass, with slow stirring for the BSA to dissolve.
  3. Filter the final solution using a 10 mL syringe plus a 0.22  $\mu\text{m}$  filter and make 1 mL aliquots to be stored at -20 °C.
3. **Dissection Solution**
  1. To prepare 50 mL of dissection solution, add 500  $\mu\text{L}$  of BSA (10%) to 49.5 mL of zero  $\text{Ca}^{2+}$  PSS.
  2. Immediately after preparation, transfer this dissection solution into a Petri dish for arterial dissection.
4. **Dissociation Solution**
  1. To prepare 50 mL of dissociation solution, add 5  $\mu\text{L}$  of  $\text{CaCl}_2$  (1 M) and 500  $\mu\text{L}$  of BSA (10%) to 49.5 mL of zero  $\text{Ca}^{2+}$  PSS. Allow the solution to sit at room temperature (RT).
5. **Enzymes**
  1. For partial digestion of the arterial segments, prepare 1 mL of digestion solution with 0.31 mg/mL papain, 0.5 mg/mL dithioerythritol, 0.75 mg/mL collagenase (Type H blend), and 0.13 mg/mL elastase.  
NOTE: Enzyme activities can vary; however, for our successful protocols, commercially supplied enzyme activities have been used as follows: papain  $\geq 10$  units/mg; collagenase  $\geq 1$  units/mg, for N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala or FALGPA substrate turnover; and elastase  $\geq 5$  units/mg.
6. **Preparation of Fura-2 and Pharmacological Agents**
  1. Prepare stock concentration (1 mM) of Fura-2 AM dye in DMSO. Prepare a working concentration (10  $\mu\text{M}$ ) by adding 5  $\mu\text{L}$  of stock to 495  $\mu\text{L}$  of PSS for loading.
  2. Prepare  $\geq 50$  mL of working concentrations of pharmacological agents (e.g., ATP) in PSS as appropriate.
7. **Conducting Solution**
  1. Prepare a conducting solution, 2 M KCl, by dissolving KCl in deionized  $\text{H}_2\text{O}$  (7.455 g KCl in 50 mL  $\text{H}_2\text{O}$ ).
  2. Filter the solution through a 0.22  $\mu\text{m}$  syringe filter prior to backfilling the sharp electrodes.
8. **Propidium Iodide (0.1%)**
  1. Dissolve 10 mg of lyophilized propidium iodide in 10 mL of 2 M KCl.
  2. Mix well and store at RT.

### 3. Dissection and Isolation of Cerebral Artery

NOTE: All dissection procedures require specimen magnification (up to 50X) *via* stereomicroscopes and illumination provided by fiber optic light sources. To perform dissection procedures for isolation of the brain and arteries, use sharpened dissection instruments. Microdissection tools to isolate and clean arteries include sharpened fine-tipped forceps and Vannas style dissection scissors (3 to 9.5 mm blades).

1. **Isolation of Mouse Brain**
  1. Anesthetize a C57BL/6 mouse (3-30 months old; male or female) *via* inhalation of isoflurane (3% for 2-3 min), then decapitate the animal immediately following complete induction of anesthesia. Place the head in a Petri dish (diameter 10 cm, depth 1.5 cm) containing cold (4 °C) zero  $\text{Ca}^{2+}$  PSS under a stereomicroscope.
  2. Viewing through the microscope, remove the skin and hair over the skull and remove excessive blood with cold zero  $\text{Ca}^{2+}$  PSS. Make an incision using only the tips of standard dissection scissors (e.g., 24 mm blade), starting with the occipital bone and extending up through the nasal bone of the skull.
  3. Open the skull carefully along the incision using coarse-tipped forceps, and separate connective tissue to isolate the brain with an intact Circle of Willis.  
NOTE: Alternatively, Littauer bone-cutting forceps can be used to open the skull and expose the brain.
  4. Gently wash the isolated brain with cold zero  $\text{Ca}^{2+}$  PSS in a beaker to remove blood. Place the brain ventral side facing up in a chamber containing cold dissection solution for isolation of cerebral arteries (**Figure 1A**).
2. **Isolation of Cerebral Arteries**  
NOTE: The posterior cerebral artery has been selected as a representative vascular endothelial study model for this protocol.
  1. Secure the isolated brain in cold dissection solution using stainless steel pins (diameter 0.2 mm, length ~11-12 mm) to be inserted into a charcoal-infused silicon polymer coating the bottom (depth  $\geq 5$  cm) of a glass Petri dish.
  2. To maintain a chilled temperature of PSS during dissection, use a dissection chamber cooled by a refrigerant system continuously cycling a 1:1 water-ethylene glycol mixture. Alternatively, dissection can be performed on fresh ice.
  3. Surgically isolate the posterior cerebral arteries (~0.3 to 0.5 cm segments, no axial tension) from the posterior communicating and basilar arteries using microdissection instruments Vannas style scissors and sharpened fine-tipped forceps (**Figure 1B**). Use stainless steel pins (diameter 0.1 mm, length ~13-14 mm) to secure both isolated posterior cerebral arteries in the dissection solution in the Petri dish (**Figure 1C**).

- Clean the isolated posterior cerebral arteries carefully by removing connective tissue using sharpened fine-tipped forceps (**Figure 1D**). Cut intact arteries into segments (length 1-2 mm) for enzymatic digestion (**Figure 1D**; inset).

## 4. Preparation of Endothelial Tube and Superfusion

NOTE: The endothelial tube is prepared as described previously<sup>12</sup>, with modifications for the cerebral artery<sup>6</sup>.

- Prepare the trituration apparatus using a microscope equipped with objectives (10X, 20X, and 40X), a camera, and an aluminum stage holding a chamber and micromanipulators. Secure a microsyringe with a pump controller adjacent to the stage and specimen (**Figure 2A**).
- Completely backfill a trituration pipette with mineral oil and secure it over the micro-syringe piston. Then, using the micro-syringe with pump controller, withdraw PSS into the pipette (~130 nl) on top of the mineral oil while ensuring the absence of air bubbles in the pipette.
- Place intact arterial segments into 1 mL of dissociation solution in a 10 mL glass tube (**Figure 2B**), containing 0.31 mg/mL papain, 0.5 mg/mL dithioerythritol, 0.75 mg/mL collagenase, and 0.13 mg/mL elastase. Incubate at 34 °C for 10-12 min for partial digestion.
- Following the digestion, replace the enzyme solution with ~5 mL of fresh dissociation solution. Using a 1 mL pipette, transfer one segment into a chamber containing the dissociation solution at RT.
- Place the pipette into the dissociation solution in the chamber and position it close to one end of the digested vessel. Set a rate within the range of 2 to 5 nl/s on the pump controller for gentle trituration (**Figure 2C**; inset).
- While viewing through 200X-400X magnification, withdraw and eject the arterial segment to dissociate smooth muscle cells while producing an endothelial tube. If necessary, carefully use fine-tipped forceps to separate dissociated adventitia and internal elastic lamina from the endothelial tube. Confirm that all smooth muscle cells are dissociated and that only endothelial cells remain as an intact "tube" (**Figure 2C**).
- Using micromanipulators, secure each end of the endothelial tube on the glass cover slip of the superfusion chamber using borosilicate glass pinning pipettes (**Figure 2D**).
- Wash-out dissociated adventitia and smooth muscle cells from the chamber and replace the dissociation solution with 2 mM CaCl<sub>2</sub> PSS. Transfer the mobile platform with secured endothelial tube onto the microscope of superfusion and experimental rig.
- Use 6 clean 50 mL reservoirs (**Figure 3A**) for continuous delivery of PSS and respective drug solutions during the experiment, as appropriate. Use the inline flow control valve to manually set the flow rate throughout as consistent with laminar flow while matching flow feed to vacuum suction. Deliver PSS to the chamber (**Figure 3B**) for the superfusion of the endothelial tube for ≥ 5 min before recording the background data and dye loading.

## 5. Dye Load, Wash-Out, and Temperature Settings

- Turn on all components of the photometry system for measuring [Ca<sup>2+</sup>]<sub>i</sub>. Use the microscope on the experimental rig (**Figure 3C**) to view the endothelial tube at 400X magnification, and focus on cells in the photometric window using the photometry system software suite.
- Measure the diameter of the endothelial tube and record background autofluorescence values in accord with 510 nm emission during alternate excitation at 340 and 380 nm (≥ 10 Hz).
- To measure [Ca<sup>2+</sup>]<sub>i</sub> responses, load the endothelial tube with Fura-2 AM dye (10 μM final concentration) at RT and allow ~30-40 min in the absence of light.
- Restart superfusion of the endothelial tube with fresh PSS for ~30-40 min to wash-out excess dye and allow intracellular Fura-2 AM to de-esterify. During the wash-out period, raise the temperature gradually from RT to 37 °C using the temperature controller (**Figure 3A**) with an inline heater, then maintain at 37 °C throughout the experiment.

NOTE: The recommended incremental transition between RT to 37 °C entails three steps (e.g., 5 °C) with a ≥ 5 min equilibration time at each step.

## 6. Simultaneous Measurement of [Ca<sup>2+</sup>]<sub>i</sub> and V<sub>m</sub>

- Turn on all other equipment and the electrometer software suite for measuring V<sub>m</sub> (see **Figure 3**, **Figure 4**). Adjust data acquisition rate accordingly (≥ 10 Hz).
- Pull a sharp electrode and backfill with 2 M KCl. For studies of intercellular coupling *via* dye transfer, backfill the microelectrodes with 0.1% propidium iodide dissolved in 2 M KCl.
- Secure the electrode over a silver wire coated with chloride in the pipette holder attached to an electrometer head stage secured with a micromanipulator. Use the micromanipulator to briefly position the tip of the electrode into the flowing PSS in the chamber while viewing through the 4X objective.
- Set the resting V<sub>m</sub> to 0 as consistent with grounded bath potential. Position the tip of electrode just over a cell of the endothelial tube. If desired, use audible baseline monitors linked to electrometers to associate sound pitch with potential recordings.
- Increase magnification to 400X using the 40X objective and re-position the tip of the electrode as needed. Adjust the photometric window using the photometry software to focus on ~50 to 80 endothelial cells.
- Gently place the electrode into one of the cells of the endothelial tube using the micromanipulator and wait ≥ 2 min for the resting V<sub>m</sub> to stabilize. Similarly, insert a second electrode in another cell (distance ≥ 100 μm) from the first cell impaled with first electrode.
- Once resting V<sub>m</sub> is stable at its expected values (-30 to -40 mV)<sup>6</sup>, turn on the PMT on the fluorescence interface in the absence of light and begin acquisition of intracellular [Ca<sup>2+</sup>]<sub>i</sub> by exciting Fura-2 alternately (10 Hz) at 340 and 380 nm while collecting fluorescence emission at 510 nm.

NOTE: To test intercellular electrical coupling, current (± 0.5-3 nA, ~20 second pulse duration) can be delivered *via* one electrometer as "Site 1", and changes of V<sub>m</sub> can be recorded with another electrometer as "Site 2" (distance ≥ 100 μm).

- Once simultaneous measurements of V<sub>m</sub> and [Ca<sup>2+</sup>]<sub>i</sub> are established, allow ~5 min for superfusion of endothelial tube with PSS at a constant laminar flow rate before application of drugs.

9. Apply the drug (e.g., ATP) prepared in PSS to the superfusion chamber with the constant flow rate. After ~3 min (or a period of time appropriate for the kinetics of the drug), wash with PSS until  $V_m$  and the  $F_{340}/F_{380}$  ratio return to their baseline conditions. Mark respective recordings as temporally synchronized across the photometer and electrometer software suites during each transition of solutions.
10. Once the experiment is done, withdraw electrode from the cell using the micromanipulator and notice  $V_m \sim 0$  mV as referenced by the bath electrode. Stop respective recordings of  $V_m$  and  $[Ca^{2+}]_i$  and save the records on file for data analysis.

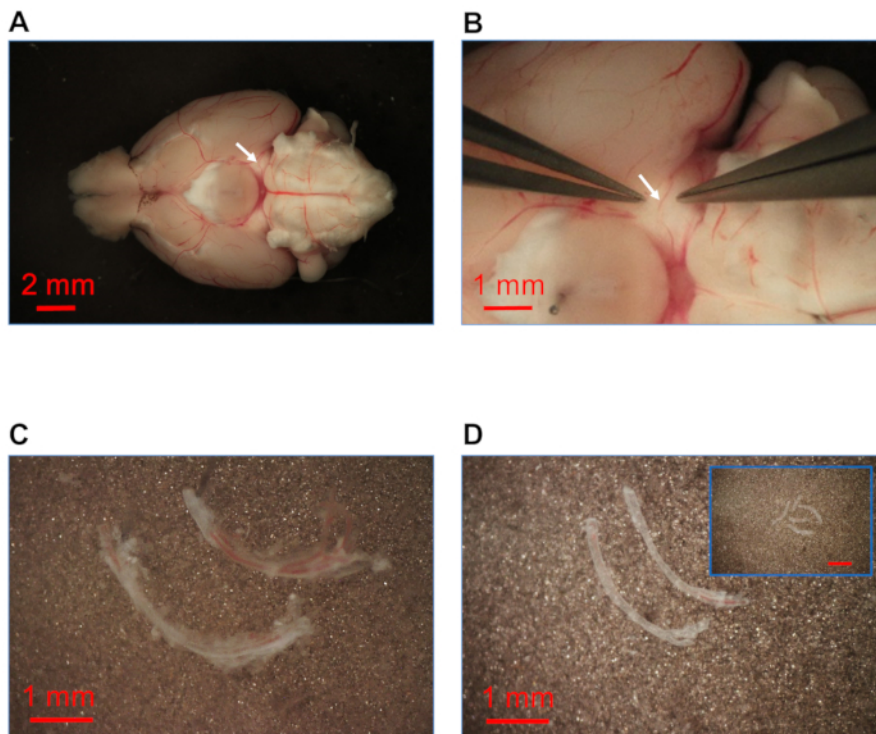
## 7. Visualization of Cell-to-Cell Coupling

1. To visualize cell-to-cell coupling via propidium iodide, use a 40X or 60X fluorescent objective with a relatively high numerical aperture (e.g., 0.95) and rhodamine filter set with solid-state illumination, a high-resolution camera (e.g., 16-megapixels), and an imaging software suite.

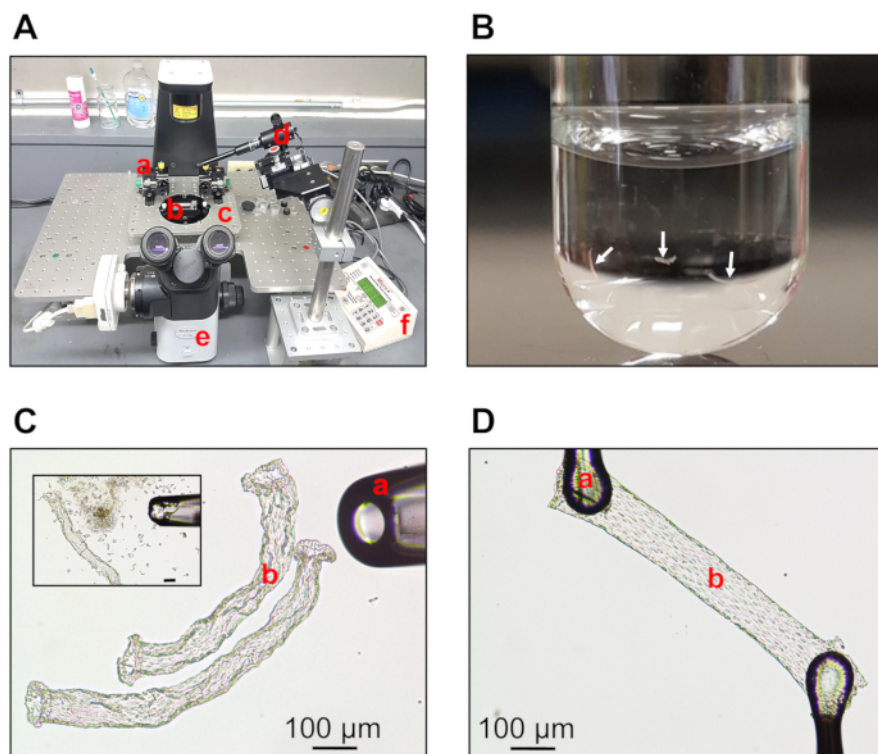
### Representative Results

The schematic demonstration of the protocol described above is shown in the attached figures. A brain isolated from a young adult male C57BL/6N mouse (5 mo) is shown in **Figure 1A**. Posterior cerebral arteries are carefully isolated from the Circle of Willis, removed without connective tissue, and cut into segments (**Figure 1B-D**). From partially digested arterial segments, the intact endothelial tube is produced and secured on the glass cover slip using pinning pipettes. Gentle mechanical stretch is applied using two pinning pipettes to approximate linear *in vivo* length without vessel tortuosity. This has been further clarified in the manuscript (**Figure 2A-D**). Endothelial tubes isolated from posterior cerebral arteries are ~90-100  $\mu$ m in width and  $\geq 300$   $\mu$ m in length. The tube is loaded with Fura-2 AM followed by wash-out with PSS. Simultaneous measurement of  $[Ca^{2+}]_i$  and  $V_m$  in the endothelial tube is performed by  $Ca^{2+}$  photometry as well as sharp electrode electrophysiology (experimental rig shown in **Figure 3** and **Figure 4**).

Functional assessment of the endothelial tube is performed by applying a classical endothelial GPCR agonist such as ATP into the flow chamber under physiological conditions. As shown in **Figure 5**,  $[Ca^{2+}]_i$  and  $V_m$  are recorded simultaneously in response to ATP (100  $\mu$ M). A significant hyperpolarization of  $V_m$  ( $\geq 5$  mV) occurs concomitantly with an intracellular  $[Ca^{2+}]_i$  increase, indicating that a rise in  $[Ca^{2+}]_i$  activates  $SK_{Ca}/IK_{Ca}$  channels and thereby allows  $K^+$  efflux across the plasma membrane to produce EDH. Evidence of intercellular coupling using current injection ( $\pm 0.5$  to 3 nA, ~20 s pulses) can be found in our recently published work (**Figure 1** in the publication<sup>6</sup>). Note that propidium iodide has a mass of ~668 Da in reference to second messengers known for passing through gap junctions such as inositol trisphosphate ( $IP_3$ , ~420 Da), cyclic adenosine monophosphate (cAMP, ~329 Da).

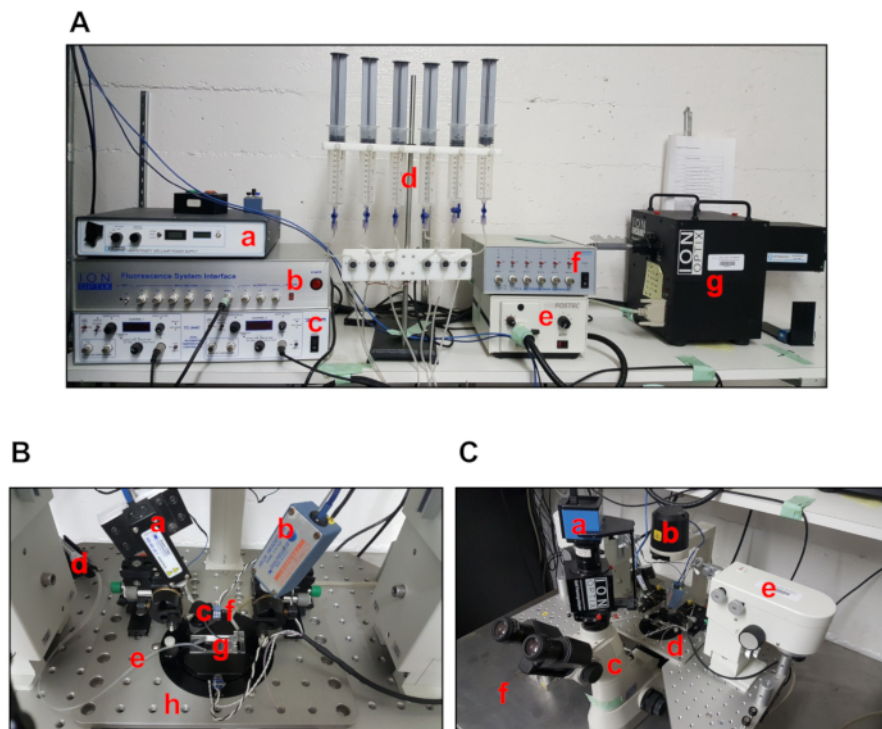


**Figure 1: Isolation of cerebral artery from brain.** (A) Brain isolated with intact arteries (white arrow indicates the posterior cerebral artery) in dissecting solution. (B) Magnified view of posterior cerebral artery (white arrow; ~3X vs. Panel A). (C) Isolated posterior cerebral arteries secured with stainless steel pins in specimen dish. (D) Posterior cerebral arteries following removal of connective tissues. Insert shows cut segments of arteries (scale bar represents 500  $\mu$ m). [Please click here to view a larger version of this figure.](#)



**Figure 2: Preparation of cerebral endothelial tube.** (A) Equipment used for triturating the partially digested arterial segments; a = micromanipulator, b = chamber for preparing tube, c = aluminum stage, d = microsyringe, e = microscope, f = microsyringe pump controller. (B) Arterial segments (white arrows) in solution for enzymatic digestion. (C) Endothelial tubes prepared by trituration; a = trituration pipette, b = intact endothelial tube. Insert shows the trituration process to remove adventitia and smooth muscle cells (scale bar represents 100 μm). (D) An intact endothelial tube secured on the glass cover slip with pinning pipette; a = pinning pipette, b = intact endothelial tube with diameter of ~100 μm.

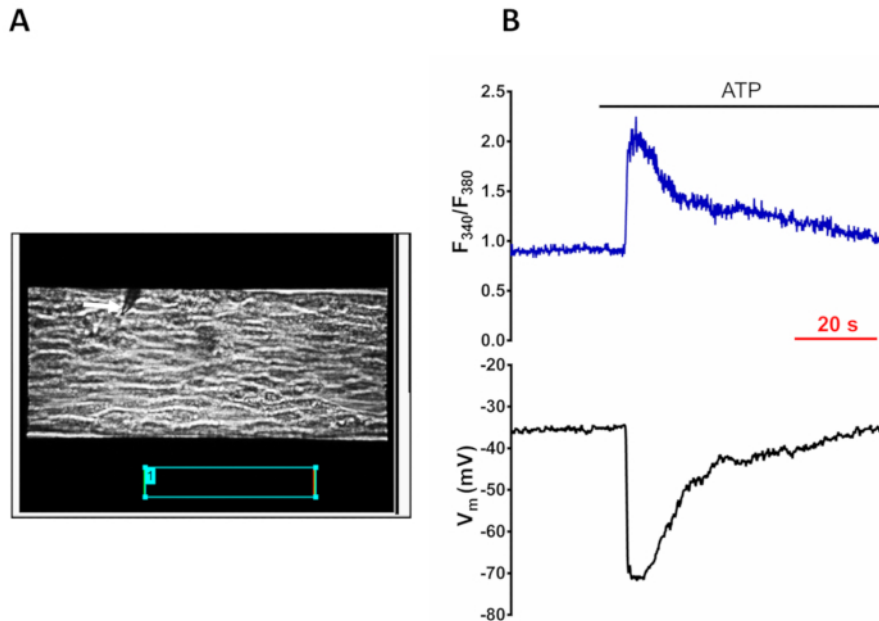
[Please click here to view a larger version of this figure.](#)



**Figure 3: Equipment for superfusion and simultaneous  $[Ca^{2+}]_i$  and  $V_m$  measurements.** (A) Equipment for superfusion of the endothelial tube and temperature control, a = high intensity ARC lamp power supply, b = fluorescence system interface, c = temperature controller, d = six-reservoirs of superfusion system, e = fiber optic light sources, f = valve controller, g = hyperswitch of  $[Ca^{2+}]_i$  photometry system. (B) Superfusion chamber apparatus; a and b = headstages, c = ground electrode, d = inline heater, e = superfusion tubing, f = vacuum suction, g = superfusion chamber, h = aluminum stage holding the chamber. (C) Experimental platform on a vibration isolation table; a = cell framing adaptor with camera, b = microscope light, c = microscope, d = aluminum stage, e = micromanipulator for headstage control, f = vibration isolation table. [Please click here to view a larger version of this figure.](#)



**Figure 4: Equipment for running electrophysiology and data recording.** a = oscilloscope, b = function generator, c = audible baseline monitor, d = 50/60 Hz noise filter, e and f = electrometers, g = data acquisition system. [Please click here to view a larger version of this figure.](#)



**Figure 5: Simultaneous  $[Ca^{2+}]_i$  and  $V_m$  recordings.** (A) Brightfield image (400X) of a mouse cerebral arterial endothelial tube adjusted for experiment in photometric window using a 40X objective. A sharp electrode is placed into a cell as shown at the top of the image (white arrow). (B) Raw traces for simultaneous measurement of  $F_{340}/F_{380}$  ratio (top) and  $V_m$  (bottom) in response to ATP (100  $\mu M$ ). [Please click here to view a larger version of this figure.](#)

## Discussion

In light of recent developments<sup>6,15,16,17</sup>, we now demonstrate the method to isolate mouse cerebral arterial endothelium in preparation for simultaneous measurement of  $[Ca^{2+}]_i$  and  $V_m$  underlying EDH consistently for ~2 h at 37 °C. Although technically difficult, we can measure cell-to-cell coupling as well (see **Figure 1** in previous publication<sup>6</sup>). In this manner, we can measure discrete and conducted signaling events simultaneously. For an account of the general challenges faced while isolating mouse arterial endothelium consult references<sup>11,12</sup>. We emphasize critical steps for isolating mouse cerebral arteries, our particular measurements of endothelial  $[Ca^{2+}]_i$  and  $V_m$ , suggestions for troubleshooting, and considerations for alternative methods in light of experimental strengths/weaknesses below.

The posterior cerebral artery requires careful isolation from the Circle of Willis and connective tissue without damage to smooth muscle and endothelial cells. Surgical tools (forceps and scissors) should be suitable for mouse microcirculation dissection procedures while maintained with clean, sharp edges. Relative to skeletal muscle<sup>12</sup>, we reduced the time period for enzymatic incubation by two-thirds and the concentrations of papain, collagenase, and dithioerythritol by half while adding use of an empirically optimized concentration of elastase. For consistent success in isolating cerebral arterial endothelial tubes, it is important to order enzymes in batches (2 to 4 vials) from a highly reputable vendor with consistent manufacturing practices in accord with frequency of use and shelf-life. Even with the potential for variation in enzyme activity from one lot of enzyme to the next, it is suggested that enzyme concentrations be maintained while optimizing the time of digestion within 10 to 12 min. It is worth noting that neither the gender nor age of the animal (3 to 30 months) has been a significant barrier to the preparation of isolated endothelium from the cerebral arteries in our experience. Thus, it is recommended that the composition of the enzyme cocktail and time of enzyme digestion remain the same in studies on age and gender.

As emphasized, caution is required during gentle trituration and isolation of the endothelium from adventitia and spindle-shaped smooth muscle cells in order to avoid damage to endothelial cells<sup>12</sup>. For the trituration step, the viscous mineral oil serves as a hydraulic medium between the piston of the micro-syringe and the aqueous PSS in order to withdraw or eject vessel segments bathed in PSS. It should be noted that if the vessel segment contacts the mineral oil, this step must be repeated using a clean trituration pipette with sequential backfilling of mineral oil and PSS. Before an experiment, it is recommended that an approximately linear *in vivo* length be restored to the endothelial tube to the extent possible, whereby individual cells assume a "flat", longitudinal morphology (e.g., diameter ~5  $\mu m$ ; length ~100  $\mu m$ ; thickness ~0.5  $\mu m$ )<sup>8</sup>. However, axial tension should not be applied to the extent where cells at the edges of the specimen are pulling away from underneath respective pinning pipettes and thereby compromising mechanical stability for reliable cellular measurements during the experiment.

The photometric  $[Ca^{2+}]_i$  measuring system used in this technique is suitable for continuous measurements of endothelial  $[Ca^{2+}]_i$  using the ratiometric Fura-2 dye. Individual protocols can typically last  $\geq 10$  min without significant photobleaching. Further, we commonly use this approach for measuring  $[Ca^{2+}]_i$  because it is amenable to timely pauses in data acquisition and frequent movement of microscope objectives as needed to secure sharp electrodes for  $V_m$  measurements. Note that this is a photometric approach that only captures global, averaged  $[Ca^{2+}]_i$  among multiple cells. In general, for  $[Ca^{2+}]_i$  measurements, we recommend photometry for the initial characterization of endothelial cell responses to pharmacological agents (e.g., time course of peak and plateau phases) while securing plans to proceed to quantitate microdomain signaling events using standard confocal or multiphoton microscopy<sup>12,23</sup>.

The sharp electrode electrophysiology approach is amenable for integrated  $V_m$  recordings in physiological multicellular preparations. The measurement of intracellular  $V_m$  is by far the most technically challenging with numerous critical steps that can either facilitate or

obliterate success of measurements. First, the experimenter needs to determine the optimal program conditions on a glass puller for consistently manufacturing sharp electrodes with a tip resistance of  $\sim 150 \pm 30$  M $\Omega$ . Using a filament heat ramp test reading as a reference, it is recommended that the experimenter determine parameters empirically, particularly with respect to variation in the "heat" setting while maximizing the time of pull. Next, mechanical stability of the micromanipulators, headstages, pipette holders, and specimen is absolutely critical. Aside from the obvious necessity for a vibration isolation table, the experimenter will need to ensure that all fittings are secure and that the stage area around and underneath the manipulators is clean. Ideally, extraneous noise should be reduced to the extent that the resolution of sharp electrode recordings is within 1 mV. In our experience, the most common source of noise is silver wire secured in the pipette holder that is in need of a sufficient chloride coating or replacement altogether. If common 50/60 Hz noise is present, "hum silencing" technology can be used and comes standard with a modern electrometer. If noise appears completely unmanageable, check to see whether there is a leak in the bath or overflow where PSS will come into contact with resistors for temperature control. If complications remain with acquiring a stable  $V_m$  signal, check the integrity of electrical cords and t-connectors.

Altogether, if establishing a sharp electrode recording is unsuccessful, it is commonly due to an inappropriate electrode, mechanical instability, or poor cellular health. Alternatively, the experimenter may also want to consider a configuration of the patch-clamp technique on individual, electrically uncoupled cells where information on whole cell currents and single ion channel recordings can be obtained. Although faced with the general challenges of using a fluorescent dye (e.g., heterogeneous intracellular loading, bleaching, calibration using ionophores, modest detection resolution), voltage-sensitive dyes such as Di-8-ANEPPS are also commercially available.

Our understanding of the endothelium in blood flow regulation to and throughout the brain is crucial with a rapidly aging human population and the rising incidence of chronic cardiovascular and neurodegenerative diseases. Thus, we provide a method for isolating intact endothelium from a vital cerebral artery, which may be adapted to other vascular structures in the brain. In addition, we show a useful approach for the simultaneous measurements of underlying  $[Ca^{2+}]_i$  and  $V_m$ . Finally, using dual intracellular electrodes, cell-to-cell coupling through gap junctions can also be assessed. With carefully planned pharmacological and/or genetic interventions, the current protocol reasonably and quantitatively encompasses study of cerebral endothelial function in its native form. Our expectation is that experimenters will build from and adapt this information to advance vascular biology and neuroscience in general. In particular, it would be satisfactory to see common experimental struggles involved with electrical measurements minimized altogether. Although this protocol is not a stand-alone set of techniques by any means, it can be used as a complementary approach to determine precisely how endothelial biophysical determinants translate into changes in vascular resistance and how they fine-tune regulation of blood flow relative to conditions representative of health vs. disease.

## Disclosures

The authors declare no conflicts of interest.

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