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The Journal of Visualized Experiments (JoVE)
Editorial Board

Subject: Revision of Invited Methods Manuscript

Dear Editorial Board of JoVE,

Please find the revised manuscript entitled “Simultaneous measurements of intracellular calcium and membrane potential in freshly isolated and intact mouse cerebral endothelium” to be considered for publication in JoVE. The authors would like to thank the time of the Senior Review Editor who handled the original submission and the selected Reviewers. We are pleased to acknowledge that all comments have been constructive for the improvement of the manuscript.

As an overview of what to expect with this revised manuscript, we have made major changes to the presentation of the Protocol and Discussion sections in particular. We have clarified details regarding the use of animals compatible with the protocol, specimen viewing and preparation, optimization of digestion cocktail composition vs. time of incubation, details of the trituration process, and tips for securing and examining the cerebral endothelial tube. Commercial details in the primary manuscript have now been removed and are included in the Table of Materials and Reagents only. Altogether, our hope is that the experimenter will build or adapt from the information provided in the manuscript to help us advance vascular biology and neuroscience in general.

We emphasize once more, that as the most popular and informative journal for Methods articles, we believe that JoVE is a suitable venue for publication of this manuscript. We look forward to your response following additional peer review.

Sincerely,

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TITLE:

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

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KEYWORDS:

Brain microcirculation, endothelial hyperpolarization, G-protein coupled receptors, Ca^{2+}-activated K^+ channels, Fura-2 photometry, intracellular microelectrodes, intercellular coupling

SUMMARY:

Demonstrated here are protocols for (1) freshly isolating intact cerebral endothelial "tubes" and (2) simultaneous measurements of endothelial calcium and membrane potential during endothelium-derived hyperpolarization. Further, these methods allow for pharmacological tuning of endothelial cell calcium and electrical signaling as individual or interactive experimental variables.

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 Ca^{2+} $[Ca^{2+}]_i$ increases and subsequent activation of small- and intermediate conductance Ca^{2+}-activated K^+ (SK_{Ca}/IK_{Ca}) channels.

Here, we present a simplified illustration of the isolation of fresh endothelium from mouse cerebral arteries; simultaneous measurements of endothelial $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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.

INTRODUCTION:

Blood flow throughout the brain is regulated by the coordination of vasodilation among cerebral arteries and arterioles in vascular networks[1]. Endothelial cells lining cerebral resistance arteries command changes in vascular diameter as needed to meet the metabolic demand of neurons[1-3]. In particular, during endothelium-derived hyperpolarization (commonly known as EDH), intracellular Ca^{2+} ($[Ca^{2+}]_i$) and electrical signaling in endothelial cells coordinate vasodilation among endothelial cells and their surrounding smooth muscle cells through gap junctions for arterial relaxation[4]. Physiological initiation of EDH sequentially entails stimulation of G_q-coupled receptors (GPCRs), an increase in $[Ca^{2+}]_i$, and activation of endothelial small- and intermediate-Ca^{2+}-activated K^+ (SK_{Ca}/IK_{Ca}) channels to hyperpolarize cerebral endothelial membrane potential (V_m)[5-7]. Thus, the intimate relationship of endothelial $[Ca^{2+}]_i$ and V_m is integral to blood flow regulation and indispensable to cardio- and cerebrovascular function[6,8]. 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)[9,10], 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[11,12], gut[13], pulmonary[14], and recently for the brain[6]. Studies of simultaneous $[Ca^{2+}]_i$ and V_m measurements in particular have been published for skeletal muscle arterial endothelium[15,16] as well as lymphatic vessel endothelium[17]. In addition to primary studies utilizing the endothelial tube approach, a comprehensive review of its advantages and disadvantages[8] 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.,* Ca^{2+} influx and intracellular release, hyperpolarization of V_m up to the Nernst potential for K^+ *via* SK_{Ca}/IK_{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[8]. In contrast, commonly used cell culture approaches introduce significant alterations in morphology[18] and ion channel expression[19] 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[12] and recent experimental developments in the interim[6,15,16], 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 ≥ 50 µm, length ≥ 300 µm) 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[20-22]. 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*"* (8th 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.

1.1. Flow Chamber

1.1.1. Fasten a superfusion chamber with a glass coverslip bottom into an anodized aluminum platform. Secure the platform with chamber into a compact aluminum stage.

1.1.2. 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.

1.2. Microscopes

1.2.1. Use stereomicroscopes (5X to 50X magnification range) equipped with fiber optic light sources for macro- and micro-dissection procedures.

1.2.2. 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.

1.2.3. To isolate endothelial tubes from partially digested blood vessels, place a microsyringe pump controller adjacent to the endothelial tube preparation apparatus.

1.2.4. 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.

1.3. Intracellular Recording Equipment

1.3.1. 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.

1.3.2. 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.

1.3.3. 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.

1.3.4. Use a temperature controller equipped with an inline heater to raise and maintain a physiological temperature (37 °C) throughout the experiment.

1.3.5. 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.

1.4. 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.

1.4.1. 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 µm) as appropriate, using borosilicate glass capillary tubes, and fire polish the tip.

1.4.2. 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 µm; prepared from thin-wall borosilicate glass tubes).

1.4.3. To record V_m of an endothelial cell, prepare sharp electrodes with a tip resistance of ~150 ± 30 MΩ from glass capillary tubes using an electronic puller.

2. Preparation of Solutions and Drugs

2.1. Physiological Salt Solution (PSS)

2.1.1. For one experiment with drug preparations, prepare a minimum of 1 L of PSS using: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 10 mM glucose.

2.1.2. To prepare the required solutions for dissection, isolation of cerebral artery, and preparation of endothelial tube, prepare PSS lacking $CaCl_2$ (zero Ca^{2+} PSS).

2.1.3. Prepare all solutions in ultrapure deionized H_2O, adjust the pH to 7.4, sterilize them through a filter (0.22 µm 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.

2.2. 10% Bovine Serum Albumin (BSA)

2.2.1. To prepare 10% BSA, dissolve 1 g of the lyophilized powder into a beaker of 10 mL zero Ca^{2+} PSS.

2.2.2. Cover the beaker with paraffin film and allow a period of several hours to pass, with slow stirring for the BSA to dissolve.

2.2.3. Filter the final solution using a 10 mL syringe plus a 0.22 µm filter and make 1 mL aliquots to be stored at -20 °C.

2.3. Dissection Solution

2.3.1. To prepare 50 mL of dissection solution, add 500 µL of BSA (10%) to 49.5 mL of zero Ca^{2+} PSS.

2.3.2. Immediately after preparation, transfer this dissection solution into a Petri dish for arterial dissection.

2.4. Dissociation Solution

2.4.1. To prepare 50 mL of dissociation solution, add 5 µL of $CaCl_2$ (1 M) and 500 µL of BSA (10%) to 49.5 mL of zero Ca^{2+} PSS. Allow the solution to sit at room temperature (RT).

2.5 Enzymes

2.5.1. 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 ≥ 10 units/mg; collagenase ≥ 1 units/mg, for N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala or FALGPA substrate turnover; and elastase ≥ 5 units/mg.

2.6. Preparation of Fura-2 and Pharmacological Agents

2.6.1. Prepare stock concentration (1 mM) of Fura-2 AM dye in DMSO. Prepare a working concentration (10 µM) by adding 5 µL of stock to 495 µL of PSS for loading.

2.6.2. Prepare ≥ 50 mL of working concentrations of pharmacological agents (*e.g.*, ATP) in PSS as appropriate.

2.7. Conducting Solution

2.7.1. Prepare a conducting solution, 2 M KCl, by dissolving KCl in deionized H_2O (7.455 g KCl in 50 mL H_2O).

2.7.2. Filter the solution through a 0.22 µm syringe filter prior to backfilling the sharp electrodes.

2.8. Propidium Iodide (0.1%)

2.8.1. Dissolve 10 mg of lyophilized propidium iodide in 10 mL of 2 M KCl.

2.8.2. 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).

3.1 Isolation of Mouse Brain

3.1.1. 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 Ca^{2+} PSS under a stereomicroscope.

3.1.2. Viewing through the microscope, remove the skin and hair over the skull and remove excessive blood with cold zero 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.

3.1.3. 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.

3.1.4. Gently wash the isolated brain with cold zero 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**).

3.2. Isolation of Cerebral Arteries

NOTE: The posterior cerebral artery has been selected as a representative vascular endothelial study model for this protocol.

3.2.1. 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 ≥ 50 cm) of a glass Petri dish.

3.2.2. 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.

3.2.3. 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**).

3.2.4. 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[12], with modifications for the cerebral artery[6].

4.1.1 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**).

4.1.2. 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.

4.1.3. 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.

4.1.4. 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.

4.1.5. 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).

4.1.6. 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**).

4.1.7. 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**).

4.1.8. Wash-out dissociated adventitia and smooth muscle cells from the chamber and replace the dissociation solution with 2 mM $CaCl_2$ PSS. Transfer the mobile platform with secured endothelial tube onto the microscope of superfusion and experimental rig.

4.1.6. 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

5.1. Turn on all components of the photometry system for measuring $[Ca^{2+}]_i$. 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.

5.2. 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).

5.3. To measure $[Ca^{2+}]_i$ 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.

5.4. 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 $[Ca^{2+}]_i$ and V_m

6.1. Turn on all other equipment and the electrometer software suite for measuring V_m (see **Figure 3, Figure 4**). Adjust data acquisition rate accordingly (≥ 10 Hz).

6.2. 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.

6.3. 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.

6.4. Set the resting V_m 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.

6.5. 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.

6.6 Gently place the electrode into one of the cells of the endothelial tube using the micromanipulator and wait ≥ 2 min for the resting V_m to stabilize. Similarly, insert a second electrode in another cell (distance ≥ 100 µm) from the first cell impaled with first electrode.

6.7. Once resting V_m is stable at its expected values (-30 to -40 mV)[6], turn on the PMT on the fluorescence interface in the absence of light and begin acquisition of intracellular $[Ca^{2+}]_i$ 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_m can be recorded with another electrometer as "Site 2" (distance ≥ 100 µm).

6.8. Once simultaneous measurements of V_m and $[Ca^{2+}]_i$ are established, allow ~5 min for superfusion of endothelial tube with PSS at a constant laminar flow rate before application of drugs.

6.9. 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.

6.10. Once the experiment is done, withdraw electrode from the cell using the micromanipulator and notice V_m ~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

7.1. 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 µm in width and ≥ 300 µm 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 µM). A significant hyperpolarization of V_m (≥ 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 (± 0.5 to 3 nA, ~20 s pulses) can be found in our recently published work (Figure 1 in the publication[6]). 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 LEGENDS:

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 µm).

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.

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.

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.

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 µM).

DISCUSSION:

In light of recent developments[6,15-17], 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[6]). 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[11,12]. 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[12], 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[12]. 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 µm; length ~100 µm; thickness ~0.5 µm)[8]. 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 typical last ≥ 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[12,23].

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 ~150 ± 30 MΩ. 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.

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DISCLOSURES:

The authors declare no conflicts of interest.

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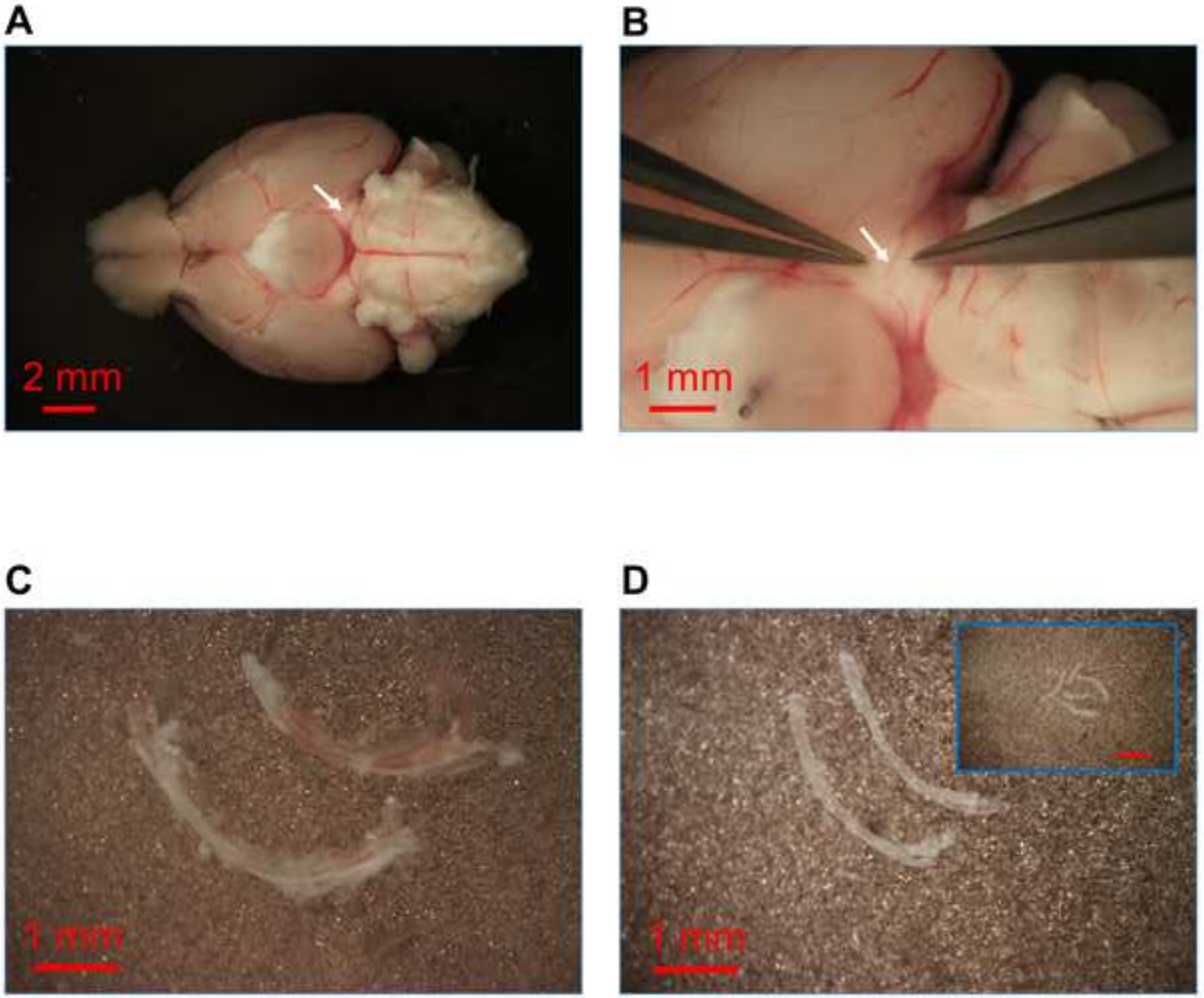
Figure



Figure

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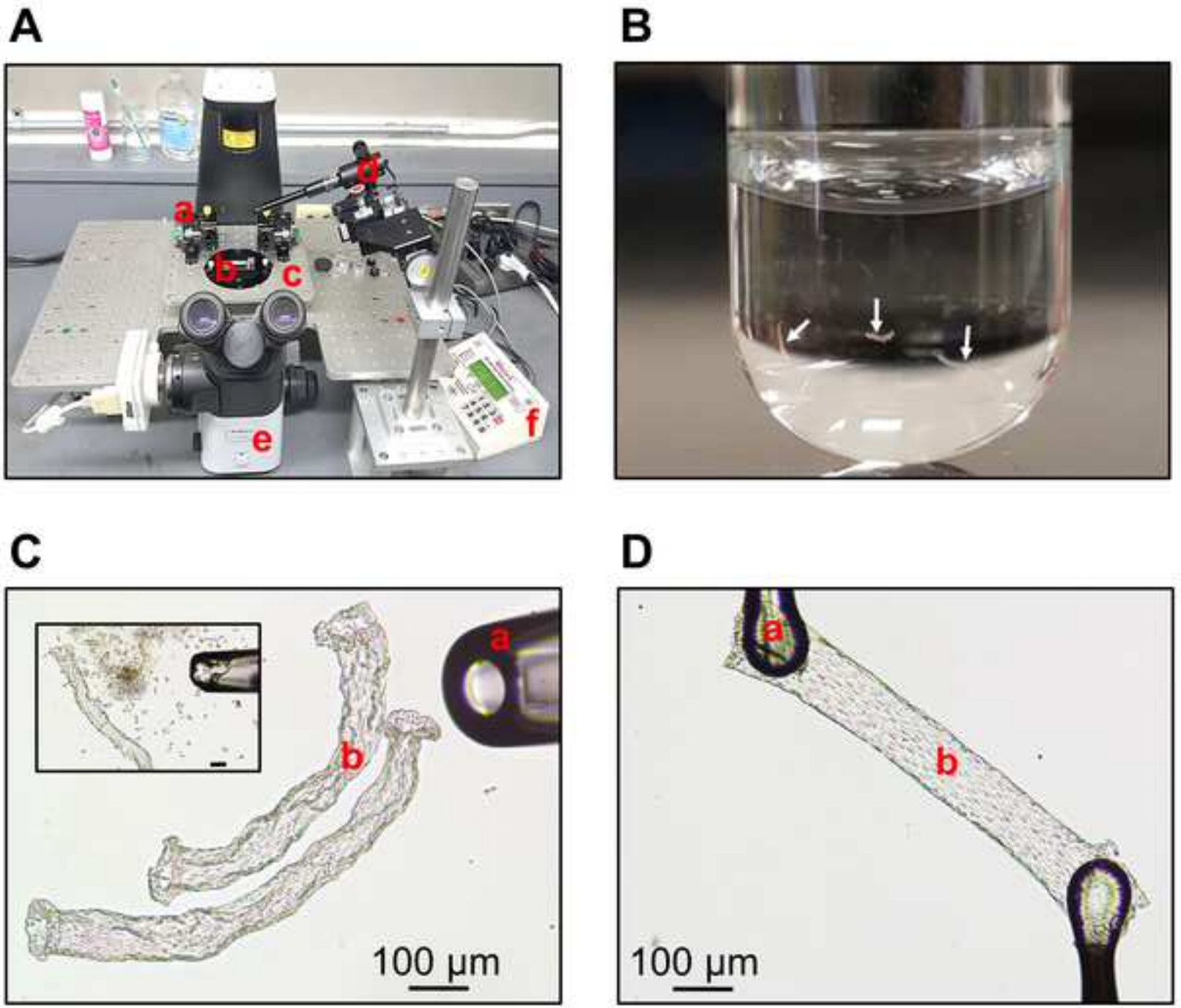

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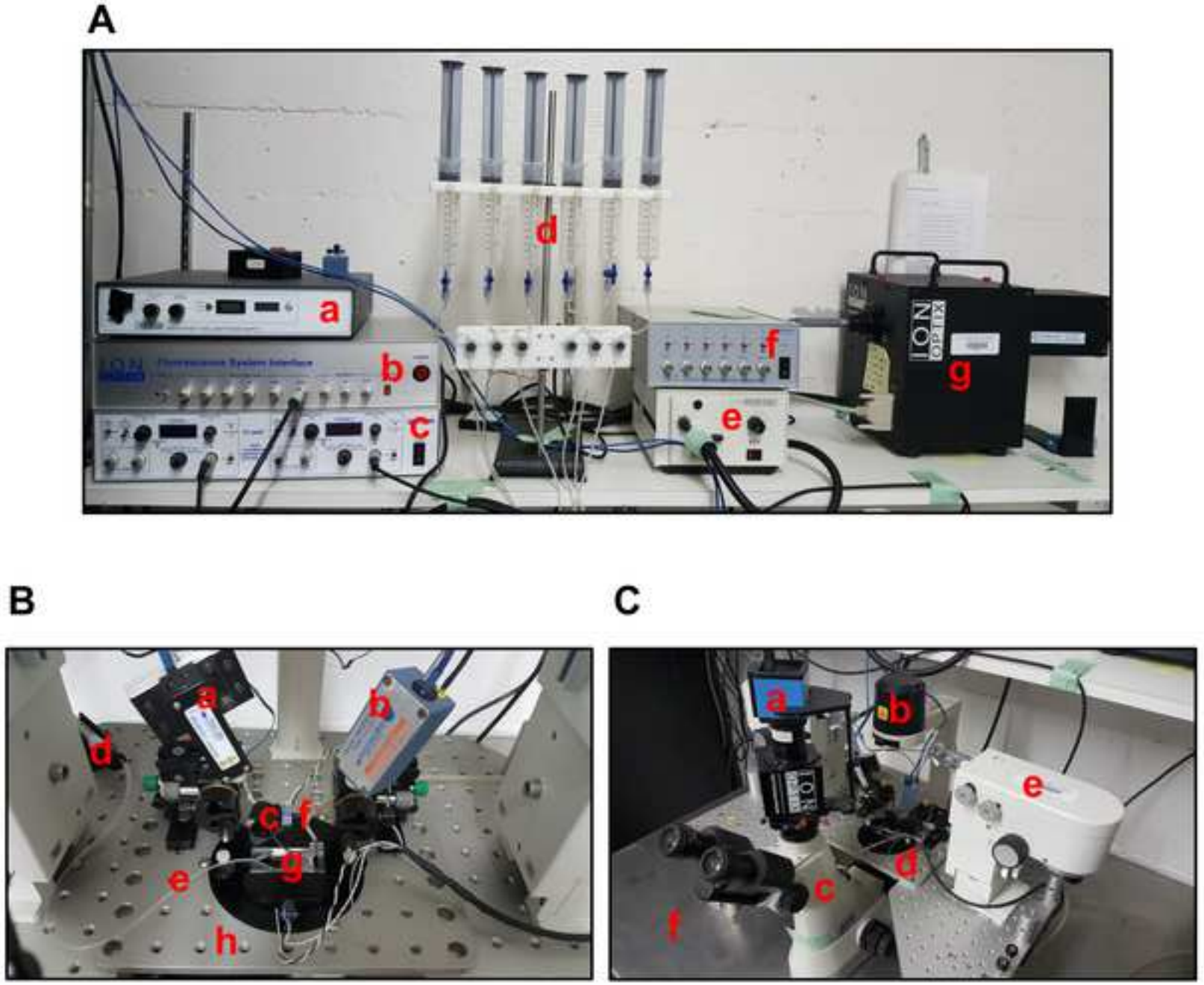

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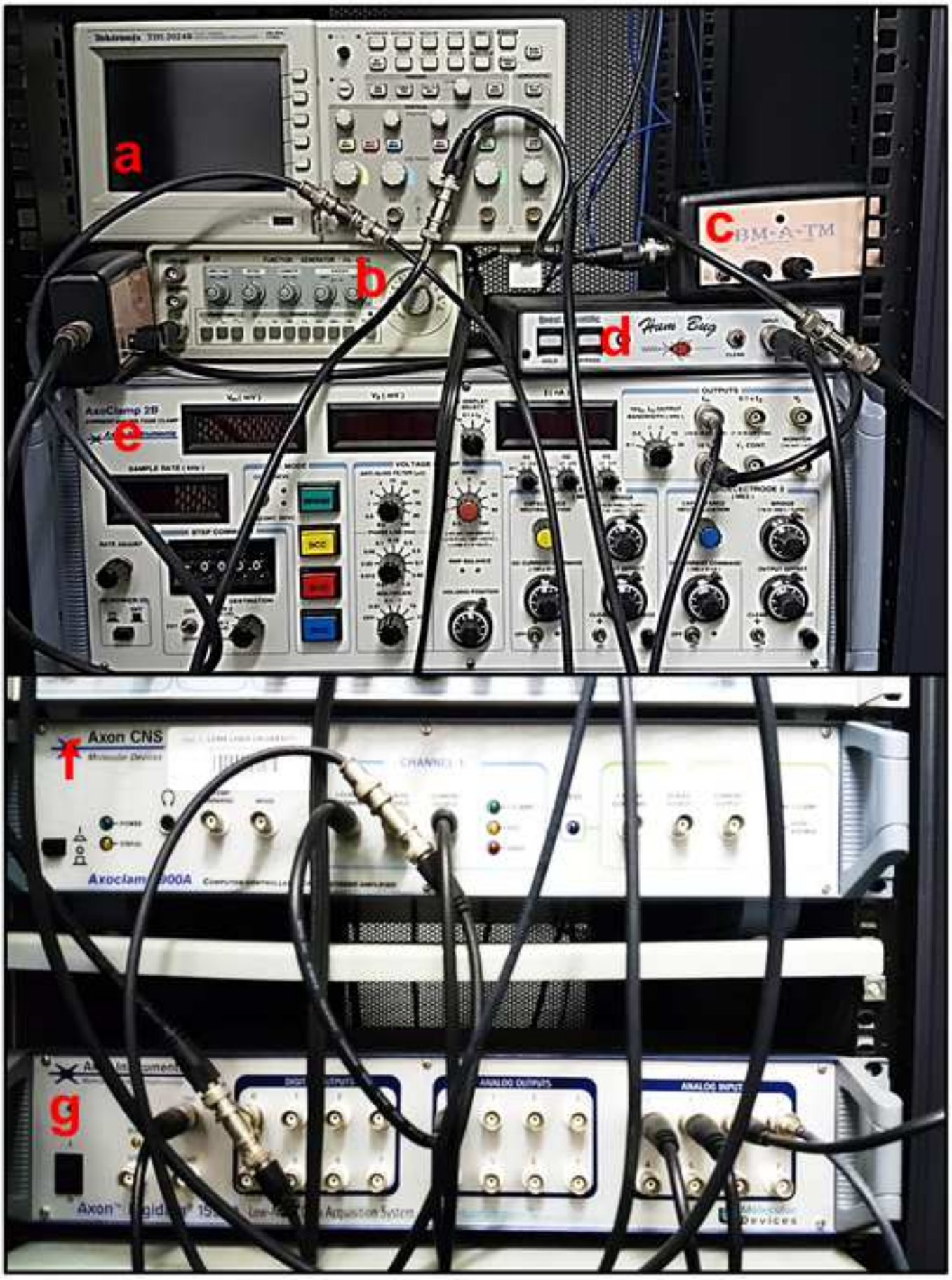

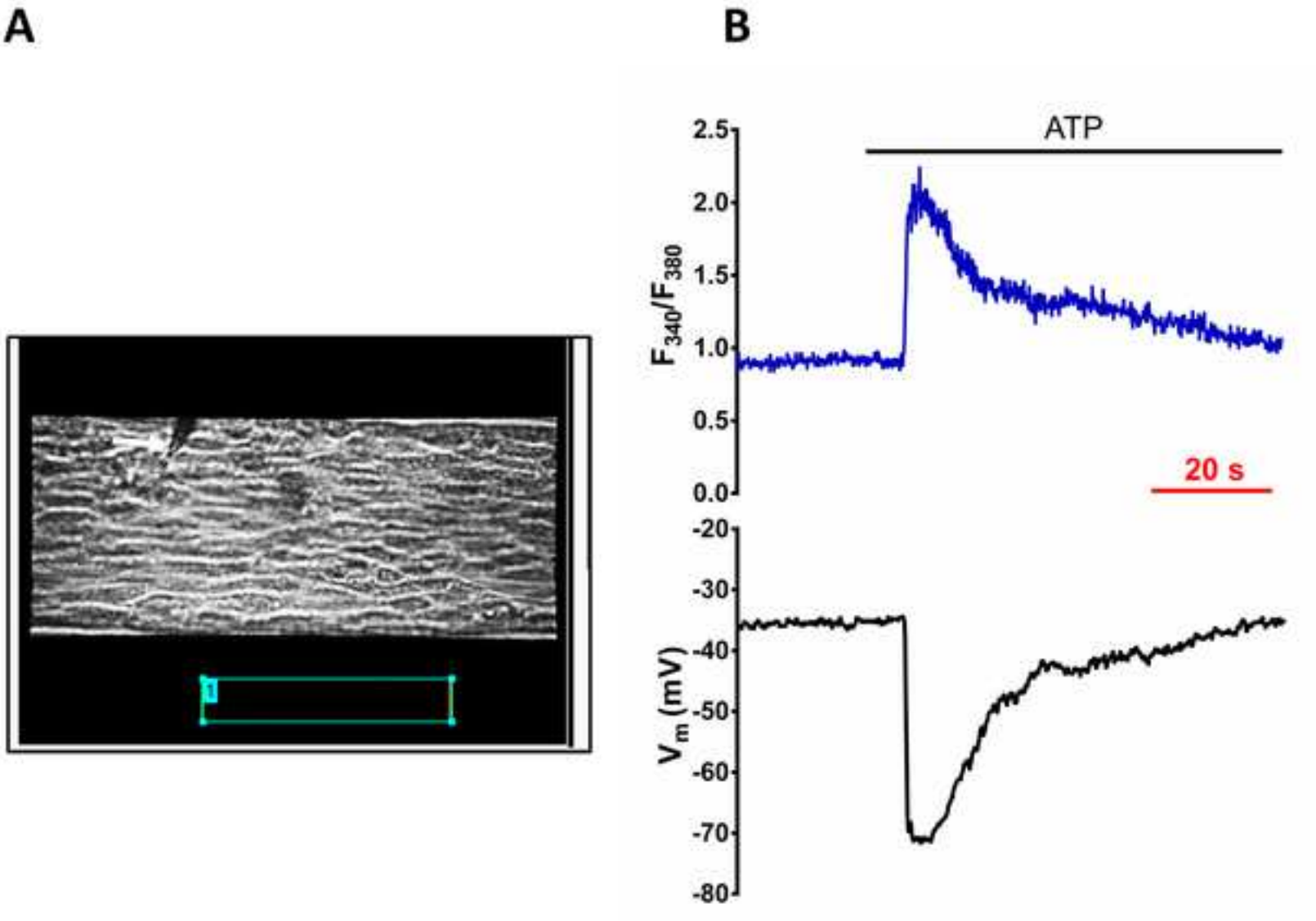

Name of Material/Equipment
Glucose
NaCl
MgCl2
CaCl2
HEPES
KCl
NaOH
ATP
HCl
Collagenase (Type H Blend)
Dithioerythritol
Papain
Elastase
BSA
Propidium iodide
DMSO
Fura-2 AM dye
Recirculating chiller (Isotemp 500LCU)
Plexiglas superfusion chamber
Glass coverslip bottom (2.4 × 5.0 cm)
Anodized aluminum platform (diameter: 7.8 cm)
Compact aluminum stage
Micromanipulator
Stereomicroscopes
Fiber optic light sources
Nikon inverted microscope
Phase contrast objectives
Fluorescent objectives
Nikon inverted microscope
Microsyringe pump controller (Micro4)
Vibration isolation table
Amplifiers
Headstages
Function generator
Data Acquision System
Audible Baseline Monitors
Digital Storage Oscilloscope
Fluorescence System Interface, ARC Lamp + Power Supply, Hyperswitch, PMT
Temperature Controller
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Borosilicate Glass Tubes (Trituration)
Borosilicate Glass Tubes (Pinning)
Borosilicate Glass Tubes (Sharp Electrodes)
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Glass Petri Dish + Charcoal Sylgard
Vannas Style Scissors (3 mm & 9.5 mm)
Scissors 3 & 7 mm blades
Sharpened fine-tipped forceps

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Date:	7/25/2018

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Response to Reviewers: Manuscript JoVE58832

We appreciate the time invested by the editor in handling our manuscript while having three reviewers evaluate the manuscript. All comments have been constructive towards the improvement of the manuscript. Individual comments are addressed below:

Editorial comments:

Changes to be made by the Author(s) regarding the manuscript:

(1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript and, to our knowledge, there are no more spelling or grammar issues.

(2) Please provide an email address for each author.

The following email addresses are provided here: ebehringer@llu.edu (Erik J. Behringer) and mhakim@llu.edu (Md A. Hakim). As the corresponding author, Dr. Behringer's e-mail address is in the manuscript but it is not clear whether Dr. Hakim's e-mail address needs to be included in the manuscript as well.

(3) JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Plexiglas, Warner, Siskiyou, Micro4, WPI, Nikon Eclipse, Grass S48, Axoclamp 2B, Digidata 1550A, Molecular Devices, IonOptix, Narishige, Sylgard, etc.

We have now removed all commercial names and adjusted text throughout the manuscript. Also, we have added a statement in the Protocol directing the reader to the Table of Materials and Reagents for commercial details (Pg. 2, Lines 115-116).

(4) Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have now included the animal ethics statement at the beginning of the protocol (Pg. 2, Line 108-112). Also, with respect to Reviewer #1 (comment #7), we have added specific information regarding all types of animals, ages, and genders that have been successful for fulfilling the protocol in our experience.

(5) Listing approximate volumes for all solutions to be set up would be helpful.

Where reasonable, we have now clarified all volumes of solutions throughout the protocol.

(6) 4.1.1: What is the concentration of isoflurane? How large is the petri dish?

We have now clarified the concentration of isoflurane and the size of petri dish (Pg. 5, step 3.1.1, Line 222-225).

(7) After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have now highlighted the primary steps of the protocol for video presentation (Pgs. 5-8).

(8) Discussion: Please discuss any limitation of the technique.

We have further clarified limitations of the protocol, particularly with respect to the isolation and fixture of the endothelium for experimentation (Discussion, Pg. 10, Lines 434-455).

(9) References: Please do not abbreviate journal titles.

We have now updated the references accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The submitted manuscript describes the isolation of EC tubes from cerebral artery with subsequent Ca and V_m measurement by Fura 2 and sharp electrode, respectively. The premise for the paper is well developed and the manuscript in generally very clear and well-written.

The authors thank Reviewer #1 for their time and apologize for some oversight in reporting salient protocol details in the original submission. Revisions in accord with comments have significantly increased the quality of the manuscript.

(1) Introduction: Page 1, paragraph 1: provide references for statement regarding cerebral EC Ca and IK/SK activation in EDH.

The appropriate references have been added (Pg. 1, Lines 60-61).

(2) Introduction: Page 1, paragraph 2: change "intercellular coupling via gap junctions..." to "intercellular coupling (EC:EC) via gap junctions..." to distinguish between intercellular coupling between EC and SMC.

We have now clarified this statement as "…endothelial intercellular coupling…" (Pg. 1, Lines 79-80). The authors would like to avoid use of the "EC" abbreviation in the manuscript if possible.

(3) Introduction and Discussion: Authors refer to aging and age-dependent neurodegenerative disease for the significance of the method. However, it is not stated whether this method has been attempted/validated in aged mice (e.g. 20 month). Differences with digestion and cell viability, etc. may not be trivial with aged vessels. Have the authors done these studies? If so, how does the protocol need to be modified in aged cerebral arteries?

In our experience, age (from 3 mo to a maximum of 30 mo) has not been a significant barrier to the preparation of isolated endothelium from the cerebral arteries at least. We do not alter the composition of the enzyme cocktail or time of enzyme digestion with respect to age. We specify the complete age and gender range of animals in our Animal Ethics statement (Pg. 2, Line 112) and the lack of alterations needed for the enzyme digestion (Pg. 10, Lines 441-443) in the Discussion.

(4) Protocol: Page 4, 2.2: If stirring is specifically contra-indicated, indicate "without stirring" - otherwise specify "with stirring" "with slow stirring" etc.

We have now specified preparation of BSA solution "with slow stirring" (Pg. 4, step 2.2, Line 189).

(5) Protocol: Page 4, 2.4: Add 5 ul $CaCl_2$ of what stock concentration?

We have now mentioned the stock concentration of $CaCl_2$ as 1 mol/L (Pg. 4, step 2.4, Line 196).

(6) Protocol: Page 4, 2.5: The vendor, product number for enzymes shown in table, however, the table needs to be fixed for odd formatting. For collagenase, indicate which collagenase type. Suggest indicating the enzyme activity (or range) of your successful enzyme experiments. Something like, "Enzyme activity varies, however, for these studies, the enzyme activity for papain was …., for collagenase was…., and for elastase was…." This gives readers a head-start on the optimization procedure.

We have corrected the Table of Materials and Reagents, indicated the collagenase type, and have added more descriptions about enzyme activities. We have now clarified details of enzyme activities in the Protocol (Pg. 4, Step 2.5, Lines 199-204). Thank you for raising this concern as it is indeed a crucial step in the protocol. The key is to order enzymes in batches (2 to 4 vials) from a highly reputable vendor with consistent manufacturing practices (e.g., Sigma) to the extent reasonable in accord with frequency of use and shelf life in a -20 °C to -30 °C freezer. Even if there is the potential for variation in enzyme activity

from one lot of production to the next, we prefer to keep enzyme concentrations the same while optimizing the time digestion within the 10-12 min digestion time frame. Further details of how to handle this crucial step for a successful endothelial tube isolation has been added to the Discussion (Pg. 10, Lines 434-443).

(7) Protocol: Page 5, 4.1.1: What is the age range of the mice used for this procedure? I.e. for which you know this procedure will work?

See response to Comment #3. We have now mentioned the age range of mice that is successful for use of this protocol (Pg. 2, Line 112) & (Discussion; Page 10, Lines 439-441).

(8) Protocol: Page 6, 5.1.4: Does this result in sucking the artery segment into the mineral oil within the pipette? If so, indicate this and perhaps a reason for why mineral oil versus another solution (e.g. PSS/BSA) is used.

We apologize for this confusion. The viscous mineral oil serves as a hydraulic medium between the piston of the microsyringe and the aqueous PSS in order to withdraw or eject vessel segments bathed in PSS. Note that if during the unfortunate occasion that a vessel segment comes into contact with the mineral oil, this step must be repeated with a clean trituration pipette and sequential backfilling of mineral oil and PSS. We have further clarified this step accordingly in the Protocol (Page 6, step 4.1.4, Line 261-270) and the Discussion (Pg. 10, Lines 444-450).

(9) Protocol: Page 6, 5.1.4: Trituration is a critical and nuanced step. Please provide some additional description of this step.

We agree and apologize for the oversight. Additional clarification has been added regarding the sequential backfilling of mineral oil and PSS, the approximate volume of PSS, and rates of gentle withdrawal and ejection of vessel segments (Page 6, step 4.1.4, Line 261-270).

(10) Protocol: Page 7, 6.3: What is the approximate rate of temperature increase? Do you step it up gradually or do you simply raise the set temp to 37?

The temperature is gradually increased from room temperature (≈22 °C) to 37 °C with three incremental steps of ≈5°C increases with at least 5 min equilibration at each step. This has now been clarified in the manuscript (Page 7, step 5.3, Line 295-297).

(11) Results: Page 8, paragraph 1: Indicate sex and age here. Also, indicate substrain (eg. BL/6J or BL/6N…)

We have included the information here for the representative Results (Pg. 8, Lines 354-355).

(12) Results: Page 8, paragraph 1: Is stretch applied to the EC tube? Can you describe your criteria for straightening or stretching (or lack thereof)?

Gentle mechanical stretch is applied using two pinning pipettes to approximate linear *in vivo* length with the absence of vessel tortuosity. This has been further clarified in the manuscript (Results, Pg. 8, Line 358-360 & Discussion, Pg. 10, Lines 449-455).

Reviewer #2:

The authors thank Reviewer #2 for their time and apologize for an unclear Protocol in the original submission. We have addressed all comments and believe that revisions have significantly increased the quality of the manuscript.

Manuscript Summary:

(1) Needs a bit more detail. I also think that more emphasis should be placed on the Vm measurements and the calcium fluorimetry as those are the "new" pieces beyond what has already been published in JoVE.

We agree as clarified in the Abstract (Pg. 1, Lines 33-37), Introduction (Pg. 2, Lines 89-93) and Discussion (Pg. 11, Lines 420-428).

(2) Lines 29-33 - Long and confusing sentence - suggest that it be divided into at least two sentences and clarified.

It is indeed a long sentence and have chosen to divide it with a semicolon (Abstract, Pg. 1, Lines 29-33).

(3) Line 38 - What enzymes are used?

We have chosen to clarify the composition of the enzyme cocktail in the protocol itself (Pg. 4, Step 2.5, Line 199-204) & (Pg. 6, Step 4.1.2, Line 255-257) and not the Abstract.

(4) Line 41 - How are tube secured? More detail required for statement under a microscope.

We have clarified the process for securing the endothelial tube in the Protocol (Pg. 6, Step 4.1.5, Line 271-272).

(5) Lines 45-47 - Not sure what you are getting at with this sentence - please revise for clarity.

We apologize for this vague sentence. We have now corrected it to simply read “Illustration of this method is expected to yield a high-throughput analysis of cerebral endothelial function underlying mechanisms of blood flow regulation in the normal and diseased brain” (Abstract, Pg. 2, Lines 45-47).

(6) line 122 - What magnifications needed for stereoscope? Suggested scopes?

The magnification range recommended is from 5X to 50X magnification. We have now clarified this in the protocol (Pg. 2, Step 1.2.1, Line 127) and have specified the suggested microscopes in the Table of Materials and Reagents.

(7) lines 124-125 - Example scope? Would DIC also be acceptable? Suggest that you remove the "To set up" verbage here and elsewhere in the Equipment and the Materials and just list the scopes required.

Any inverted microscope equipped with standard objectives (10X, 20X, & 40X) with phase contrast or DIC compatible objectives will work depending on the experimenter's preference. We have clarified this in the Protocol (Pg. 2, Step 1.2.2, Lines 130-132). The commercial examples of microscopes/objectives are indicated in the Table of Materials and Reagents.

The text for Step 1.2.2 has been corrected according the reviewer's suggestion (Pg. 2, Step 1.2.2, Line 130). "To set up…" has been eliminated elsewhere in the manuscript as well.

(8) lines 134-137 - Not clear why an additional scope is "needed" here if the inverted scope above had the correct objective and illumination system. It is suggested that the authors think more broadly about how to do this prep in general and not how they have their lab set up.

Yes, we agree. An additional microscope is indeed not necessary. Step 1.2.5 has now been removed and general information regarding objectives, illumination system, camera and imaging software suite have been provided as Section 7 of the Protocol (Pg. 8, Step 7, Line 349-351).

(9) line 180 - Should give sources for all chemicals, drugs and enzymes.

Per Editor's instructions, sources of all chemicals, materials, and equipment have been provided as the Table of Materials and Reagents only. This has been clarified in the beginning of the Protocol (Pg. 2, Lines 115-116).

(10) lines 224-225 - More detail of what instruments are needed.

Some specifications have now been clarified (Pg. 5, Step 3, Lines 219-220). We can only specify the instruments that have been successful us in the Table of Materials and Reagents.

(11) line 232 - What kind of scissors (specifically) and source.

Please see response to Comment #10.

(12) lines 233-235 - What instruments are used to open the skull and remove the brain - more detail needed here.

Use of the general tools to open the skull and remove the brain have now been clarified (Pg. 5, Step 3.1.2, Lines 226-231). Although, as stated throughout responses to the Reviewer, commercial information is only allowed in the Table of Materials and Reagents.

(13) line 242 - charcoal Sylgard is lab jargon - please provide source and recipe.

We have removed the Sylgard jargon and have clarified as "…charcoal-infused silicon polymer…" instead (Pg. 5, Step 3.2.1, Lines 238). Our laboratory has purchased charcoal Sylgard coated Petri dishes from Living Systems Instruments and this product is specified in the Table of Materials and Reagents. If the experimenter wishes to make their own, there are recipes available online to do so.

(14) lines 243-245 - This section is confusing and non-informative - please provide sources.

We apologize for this vague statement. We have now clarified a general procedure for maintaining a chilled temperature for the specimen during dissection (Pg. 5, Step 3.2.1, Line 239-241). Pertinent sources are provided Table of Materials and Reagents.

(15) lines 248-249 - How are the isolated arteries secured?

We have now described how the brain and isolated arteries are secured using stainless steel pins (Pg. 5, Step 3.2.1, Lines 237-239; Step 3.2.2, Lines 244-246).

(16) lines 270-272 - How much tension should be applied (how much should they be stretched?)

Gentle mechanical stretch is applied using two pinning pipettes to approximate linear *in vivo* length with the absence of vessel tortuosity. This has been further clarified in the manuscript (Results, Pg. 8, Line 359-361 & Discussion, Pg. 10, Lines 450-456).

(17) line 328 - How distant? Revise to clarify.

This depends on the precise application of the experimenter but different cells separated by a distance of ≥ 100 µm will suffice. This has been clarified in the Protocol (Pg. 7, Step 6.5, Line 321; Page 8, Step 6.7, Line 331).

(18) lines 345-346 - Do you mean to temporally synchronized? Please revise to clarify.

Yes, recordings are to be temporally synchronized across software suites (Pg. 8, Step 6.9, Lines 339-341).

Minor Concerns:

(19) line 251 = "attached with" should be "attached to"

We have corrected this statement accordingly (Page 5, Step 3.2.3, Line 247-249).

(20) line 311 - Not sure why you have the worded updated in this sentence? Please clarify.

We have removed the word “updated” and corrected the statement (Page 7, Step 6.1, Lines 301-302).

(21) line 327 - change "get" to "insert"

We have changed it to “insert” (Page 7, Step 6.5, Line 320).

(22) line 330 - Should state that these are expected Vm values and give a reference

We have clarified the expected typical V_m range for cerebral endothelial cells with a reference (Page 7, Step 6.6, Line 323).

(23) line 344 - time kinetics is redundant

Yes, we have removed “…time…” (Pg. 8, Step 6.9, Line 337).

(24) lines 345-346 - Do you mean to temporally synchronized? Please revise to clarify.

See response to Comment #18. Yes, the software suites are temporally synchronized.

(25) line 349 - Change "remove electrode out of the cell" to "withdraw electrode from the cell using the micromanipulator.

We have adjusted this statement accordingly (Page 8, Step 6.10, Line 342-343).

(26) line 445 - Use...useful is redundant - please revise.

We have revised this statement accordingly. Major revisions have been made to the Discussion.

(27) line 461 - recommend should be recommended

This has been corrected to “…recommended…”. Major revisions have been made to the Discussion.

(28) line 462 - tests should be test

This statement has been revised (Discussion, Pg. 11, Line 473-475).

(29) lines 468-469 - Not an English sentence, please revise.

The sentence has been revised (Discussion, Pg. 11, Lines 480-482).

(30) line 485 - Not an English sentence, please revise.

The sentence has been revised (Discussion, Pg. 11, Lines 497-499).

Reviewer #3:

Manuscript Summary:

The manuscript by Erik Behringer group present a novel method and illustration of isolation of fresh endothelium from cerebral arteries, simultaneous measurements of endothelial intracellular calcium concentration ([Ca2+]i) and endothelial membrane potential (Vm). The authors showed that enzymatic digestion of posterior cerebral arteries followed by gentle trituration yielded endothelial tubes. Utilizing these tubes they demonstrated changes in [Ca2+]I and Vm, spread of endothelium-dependent hyperpolarization through gap junctions. Thus, the authors demonstrated the potential utility of the methods for high throughput analysis of endothelial function in normal and disease brain. The manuscript was well written providing details of each step involved and the equipment necessary to perform the experiments. The method described provides unique technique to study endothelial function in freshly isolated cells.

The authors thank Reviewer #3 for their time. We have addressed all comments and believe that revisions have significantly increased the quality of the manuscript.

(1) Abstract. Mention that the arteries isolated were from mice.

We have now specifically mentioned the use of mice in this protocol in the Abstract (Line 34).

(2) Space permitting, authors are encouraged to include potential issues that might come up and how to troubleshoot them.

We have further clarified limitations of the protocol, particularly with respect to the isolation and fixture of the endothelium for experimentation (Discussion, Pg. 10, Lines 434-455).