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TITLE:

Isolation and Functional Analysis of Arteriolar Endothelium of Mouse Brain Parenchyma

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SUMMARY:

Intensive preparation of intact mouse cerebral endothelial "tubes" from cerebral parenchymal arterioles is illustrated for studying cerebral blood flow regulation. Further, we demonstrate the experimental strengths of this endothelial study model for fluorescence imaging and electrophysiology measurement of key cellular signaling pathways, including changes in intracellular [Ca^{2+}] and membrane potential.

ABSTRACT

Cerebral blood flow is conveyed by vascular resistance arteries and downstream parenchymal arterioles. Steady-state vascular resistance to blood flow increases with decreasing diameter from arteries to arterioles that ultimately feed into capillaries. Due to their smaller size and location in the parenchyma, arterioles have been relatively understudied and with less reproducibility in findings than surface pial arteries. Regardless, arteriolar endothelial cell structure and function—integral to the physiology and etiology of chronic degenerative diseases—requires extensive investigation. In particular, emerging evidence demonstrates that compromised endothelial function precedes and exacerbates cognitive impairment and dementia.

In the parenchymal microcirculation, endothelial K^+ channel function is the most robust stimulus to finely control the spread of vasodilation to promote increases in blood flow to areas of neuronal activity. This paper illustrates a refined method for freshly isolating intact and electrically coupled endothelial "tubes" (diameter, ~25 µm) from mouse brain parenchymal arterioles. Arteriolar endothelial tubes are secured during physiological conditions (37 °C, pH 7.4)

to resolve experimental variables that encompass K^+ channel function and their regulation, including intracellular Ca^{2+} dynamics, changes in membrane potential, and membrane lipid regulation. A distinct technical advantage versus arterial endothelium is the enhanced morphological resolution of cell and organelle (e.g., mitochondria) dimensions, which expands the usefulness of this technique. Healthy cerebral perfusion throughout life entails robust endothelial function in parenchymal arterioles, directly linking blood flow to the fueling of neuronal and glial activity throughout precise anatomical regions of the brain. Thus, it is expected that this method will significantly advance the general knowledge of vascular physiology and neuroscience concerning the healthy and diseased brain.

INTRODUCTION:

Parenchymal arterioles directly deliver essential oxygen and nutrients throughout the brain[1]. While interfacing with capillaries, highly vasoactive arterioles respond to retrograde signaling initiated by capillary ion channels that sense metabolic signals from specific neuronal regions[2]. With brain parenchyma having historically received the bulk of investigation, a role for endothelial dysfunction has now emerged for clarifying pathological mechanisms associated with various cerebrovascular disorders that underlie dementia (e.g., ischemic stroke, Alzheimer's disease)[3-6]. The endothelium is integral to perfusion of the brain in accord with the heterogeneity of genetics, structure, and function throughout vascular segments[7]. Pial arteries have been extensively studied due to their relatively large size, high segmental vascular resistance, and role in blood flow distribution to the underlying cerebrum[8,9]. Thus, a better understanding of arteriolar endothelial mechanisms will likely enhance the understanding of brain blood flow regulation in health and disease towards the development of novel therapeutic regimens.

Emerging evidence highlights the importance of studying parenchymal arterioles in relation to different signaling pathways and diseases[8,10]. However, this approach has been limited to using intact pressurized arteriole[11] and/or capillary-parenchymal arteriole (CaPA) preparations[12]. Freshly isolated, native cerebral arteriolar endothelial cells devoid of other cell types and confounding factors have not been examined, likely due to technical difficulties in their isolation. This paper advances a previous technique highlighting the isolation of pial arterial endothelium[13] to now reliably and reproducibly isolate the endothelium of brain parenchymal arterioles (width: ~25 µm, length: ~250 µm). This technique helps achieve optimal resolution of electrically and chemically coupled cells in their individual orientation and cellular networks.

Key pathways of interest have included the interaction of intracellular Ca^{2+} ($[Ca^{2+}]_i$) signaling and hyperpolarization of membrane potential (V_m)[14,15]—integral to vasodilation[16]—to allow blood to enter the capillaries and deliver oxygen and nutrients to active parenchyma[17]. These preparations allow for real-time electrophysiological recordings of ion channels, including Ca^{2+}-permeant, transient receptor potential (TRP) and K^+ channels and/or fluorescent imaging of intracellular organelles within endothelial cell tubes in near-physiological conditions. This is a suitable technique for researchers interested in physiological cellular mechanisms that govern endothelial cell control of cerebral blood flow delivery to the brain parenchyma. Altogether, this technique will help researchers better understand fundamental endothelial signaling pathways

and network communication of arterioles embedded in brain parenchyma and address questions related to cerebrovascular physiology and pathology.

PROTOCOL:

Experimenters should ensure that designated use of animals and associated protocols are approved by their Institutional Animal Care and Use Committee (IACUC) and performed in accordance 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 and the University of Arizona has approved all protocols used for this manuscript for C57BL/6N and *3xTg-AD* mice (males and females; age range: 2–30 months). See **Figure 1** as an overview of the isolation and examination of arteriolar endothelial tubes freshly isolated from mouse parenchymal arterioles of the brain.

1. Materials and equipment

NOTE: See the **Table of Materials** for all reagents and materials required for this protocol. In addition, manuals and websites associated with the respective vendors can also be consulted as needed. Illustrations of dissection stations and experimental apparatuses have been previously provided[13].

1.1. Flow chamber

1.1.1. Fasten a superfusion chamber with a glass coverslip onto a platform composed of anodized aluminum. Secure the platform with the chamber onto an aluminum microscope stage.

1.1.2. Set a micromanipulator holding a pinning pipette at each end of the platform on the aluminum microscope stage.

NOTE: If necessary, use a transferable stage apparatus with a flow chamber unit to move a secured, isolated endothelial tube from one microscope apparatus to another for experimentation.

1.2. Microscopes

1.2.1. Use stereomicroscopes (5x to 50x magnification range) and fiber optic light sources for dissection procedures.

1.2.2. For isolation of endothelial tubes, use an inverted microscope rig equipped with phase contrast- or differential interference contrast (DIC)-compatible objectives (10x, 20x, and 40x) and an aluminum stage.

1.2.3. Have a microsyringe pump controller ready next to the apparatus dedicated to isolating endothelial tubes from partially digested blood vessels.

1.2.4. Set up the experimental apparatus by arranging an inverted microscope (objectives: 4x, 10x, 20x, 40x, and 60x) and a manual aluminum stage on a vibration isolation table.

1.3. Intracellular V_m recording equipment

1.3.1. Connect the electrometer to a compatible headstage. Use accessories, such as a function generator and stimulator, for protocols requiring current injection.

1.3.2. Connect amplifier outputs to a data digitizer system oscilloscope and audible baseline monitors. Secure the reference bath electrode (Ag/AgCl pellet) near the flow chamber exit.

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

1.3.5. Assemble a multichannel platform connected to a valve controller with an inline flow control valve to control delivery of solutions to endothelial tubes secured in the chamber.

1.4. Micropipettes and sharp electrodes

NOTE: The experimenter will need an electronic glass puller and a microforge for preparing trituration and pinning pipettes.

1.4.1. To separate the endothelial tube from the partially digested arteriolar segment, prepare fire-polished trituration pipettes (internal tip diameter: 30–50 µm) from borosilicate glass capillaries.

1.4.2. To secure the endothelial tube in the superfusion chamber, prepare heat-polished pinning pipettes with a blunted, spherical end (outer diameter: 50–70 µm) prepared from thin-wall borosilicate glass capillaries.

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

2. Solutions and drugs

2.1. Physiological salt solution (PSS)

2.1.1. 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. Prepare necessary solutions lacking $CaCl_2$ (zero Ca^{2+} PSS) for dissection of cerebral arterioles and isolation of endothelial tube.

NOTE: Prepare all solutions in ultrapure deionized H_2O, followed by filtration (0.22 µm). Ensure that the final product contains a pH 7.4 with osmolality between 290 and 300 mOsm.

2.2. Bovine serum albumin (BSA, 10%)

2.2.1. Dissolve 1 g of the lyophilized BSA powder in a beaker of 10 mL of zero Ca^{2+} PSS. Cover the beaker and allow at least 2 h with slow stirring for the BSA to dissolve.

2.2.2. Filter solution using a 10 mL syringe plus a 0.22 µm filter and prepare 1 mL aliquots to be stored at -20 °C.

2.3. Dissection solution

2.3.1.Add 500 µL of BSA (10%) to 49.5 mL of zero Ca^{2+} PSS for a total volume of 50 mL.

2.3.2. Transfer solution to a Petri dish for arteriolar dissections.

2.4. Dissociation solution

2.4.1. Add 5 µL of $CaCl_2$ (1 M) and 500 µL of BSA (10%) to 49.5 mL of zero Ca^{2+} PSS for a total volume of 50 mL. Incubate solution at room temperature for at least 1 h.

2.5. Enzymes

2.5.1. Prepare 1 mL of digestion solution with 100 µg/mL papain, 170 µg/mL dithioerythritol, 250 µg/mL collagenase (Type H blend), and 40 µg/mL elastase.

NOTE: Enzyme activities can vary, although successful protocols will likely require the following enzymatic activities: ≥ 10 units/mg for papain, ≥ 1 unit/mg for collagenase, and ≥ 5 units/mg for elastase.

2.6. Fura-2 and pharmacological agents

2.6.1. Prepare Fura-2 AM stock in dimethyl sulfoxide (DMSO; 1 mM). Prepare 500 µL of working concentration (10 µM) by adding 5 µL of the stock to 495 µL of PSS for loading.

2.6.2. Prepare at least 50 mL of working concentrations of pharmacological agents (e.g., MTA) in PSS or DMSO as appropriate.

2.7. Conducting solution

2.7.1. Prepare 2 M KCl by dissolving KCl in deionized H_2O (7.455 g of KCl in 50 mL of H_2O). Pass the solution through a syringe with a 0.22 µm filter prior to backfilling the sharp electrodes.

2.8. Fluorescent organelle trackers and antibodies

2.8.1. Prepare plasma membrane or organellar (e.g., nucleus, endoplasmic reticulum) trackers in the appropriate physiological saline solution according to the manufacturer's instructions.

2.8.2. Prepare primary and secondary antibodies according to the manufacturer's instructions in the appropriate physiological salt solution.

3. Dissection and isolation of mouse cerebral arterioles

NOTE: Stereomicroscopes and sharpened microdissection tools (e.g., fine-tipped forceps, Vannas-style dissection scissors) must be used for specimen magnification (up to 50x) in all these dissection procedures.

3.1. Isolation of mouse brain

3.1.1. Anesthetize a standard laboratory mouse (e.g., C57BL/6, *3xTg-AD*; 2–30 months old, male or female) using isoflurane inhalation (3% for 2–3 min), followed by immediate decapitation using sharp scissors or guillotine per IACUC approval. Place the mouse head in a Petri dish (diameter 10 cm, depth 1.5 cm) containing cold (4 °C) dissection solution.

3.1.2. While viewing under a stereomicroscope, remove the skin and hair over the skull and wash away excessive blood with cold dissection solution. Using the tips of standard dissection scissors (e.g., 24 mm blades), make an incision starting with the occipital bone and extending up through the nasal bone of the skull. Use coarse-tipped forceps to carefully the open skull along the incision, and separate connective tissue (meningeal membrane) to isolate the brain containing an intact Circle of Willis.

3.1.3. Wash the isolated brain with cold dissection solution in a beaker or Petri dish to remove remaining blood from the surface of the brain. Place the brain ventral side facing up in a chamber containing cold dissection solution for isolation of parenchymal arterioles (**Figure 2A**).

3.2. Isolation of parenchymal arterioles

NOTE: Arterioles arising from the middle cerebral arteries (MCAs) and embedded in brain parenchyma are chosen for this protocol. The arterioles are isolated as previously described[11], with modification.

3.2.1. Use steel pins (diameter: 0.2 mm, length: 11 to 12 mm) to secure the isolated brain in cold dissection solution in a Petri dish containing a charcoal-infused silicon polymer coating (depth ≥ 50 cm).

3.2.2. Using sharp and aligned dissection scissors, cut a rectangle of brain tissue (length: 5 mm, width: 3 mm) (**Figure 2B**) around the MCA while ensuring that the upper part of the tissue segment is past the branching point from the Circle of Willis. Cut another rectangle of brain tissue from the other hemisphere of the brain to access more arterioles if necessary.

3.2.3. Secure the separated brain tissue segment into the dish with the MCA facing upwards (distal from the Circle of Willis) using steel pins (diameter: 0.1 mm, length: 13 to 14 mm). Carefully make a shallow incision near the pins to remove the pia with small forceps, gently peeling from one end towards the other (**Figure 2C**). Remove the pia from the other tissue segment to access more arterioles if needed. Carefully secure the isolated pia with parenchymal arterioles branched from MCA in the dish with the pins, and dissect the parenchymal arterioles (**Figure 2D**), ensuring that the arterioles are not damaged.

NOTE: If the arterioles are not easily pulled out with the pia from the parenchyma, use fine forceps and dig into the brain, starting at the MCA branch point from the Circle of Willis. Locate the arteriole, carefully loosen the tissue around arterioles (**Figure 2E**), and gently pull the arteriole out of the parenchyma, holding the upper end of the arteriole (**Figure 2F**). Multiple arterioles (length: ~1.5–2 mm) can be isolated from one rectangle of brain tissue.

3.2.4. Ensure the arteriole is clean with no tissue attached to it, and cut off any remaining distal branches (**Figure 3A**). Use this clean, intact arteriole for enzymatic digestion. Alternatively, cut each arteriole into two pieces (length: 0.75–1 mm) for enzymatic digestion for the preparation of endothelial tubes if desired.

4. Digestion of parenchymal arterioles and preparation of endothelial tubes

4.1. Arrangement of equipment and pipettes

NOTE: Isolation of arterial endothelial tubes from the brain has been described previously[13,18]. The current protocol incorporates modifications for the isolation of endothelium from parenchymal (intracerebral) arterioles. Multiple arterioles or/and pieces of arterioles can be used together for enzymatic digestion.

4.1.1. Assemble the trituration apparatus[13] for preparing endothelial tubes using an aluminum stage supporting a chamber and micromanipulators. Assemble a microscope equipped with objectives (5x-60x) and a camera connected to a computer monitor. Secure a microsyringe with a pump controller next to the stage and specimen.

4.1.2. Secure a trituration pipette backfilled with mineral oil over the microsyringe piston. Use the microsyringe with the pump controller to withdraw the dissociation solution into the

trituration pipette (~130 nL) on top of the mineral oil while taking care to avoid air bubbles in the pipette.

4.2. Partial digestion of arteriolar segments

4.2.1. Place intact arteriolar segments into 1 mL of dissociation solution in a 10 mL glass tube containing 100 µg/mL papain, 170 µg/mL dithioerythritol, 250 µg/mL collagenase, and 40 µg/mL elastase. Incubate arteriolar segments at 34 °C for 10–12 min.

4.2.2. After digestion, replace the enzyme solution with 3–4 mL of fresh dissociation solution at room temperature. Use a 1 mL pipette to transfer one segment into a chamber containing the dissociation solution brought to room temperature.

4.3. Isolation of arteriolar endothelial tube

NOTE: Following digestion and replacement of the enzyme solution, transfer one or multiple partially digested segments into the dissociation solution in a superfusion chamber attached to a mobile platform. While viewing at 100x to 200x magnification, select one partially digested but unbroken vessel segment and focus on it under the microscope.

4.3.1. Place the trituration pipette attached with the microsyringe injector in the dissociation solution in the chamber and position it close to one end of the digested vessel segment. Set a rate within the range of 1–3 nL/s on the pump controller for gentle trituration.

4.3.2. While maintaining 100x to 200x magnification, withdraw the arteriolar segment into the pipette and then eject to dissociate the adventitia and smooth muscle cells (**Figure 3B**). Triturate the vessel segment until all smooth muscle cells are dissociated, and only endothelial cells remain as an intact "tube."

NOTE: If necessary during trituration, carefully use fine-tipped forceps to remove the dissociated adventitia and internal elastic lamina from the endothelial tube. Typically, only a few cycles of trituration yield an intact endothelial tube.

4.3.3. Use micromanipulators to secure each end of the endothelial tube on the glass coverslip in the chamber with borosilicate glass pinning pipettes (**Figure 3C,D**).

4.4. Securing the arteriolar endothelial tube

4.4.1. Replace the dissociation solution with superfusion solution (PSS containing 2 mM $CaCl_2$) while ensuring that nonendothelial material is washed out of the chamber.

4.4.2. Transfer secured endothelial tube in the mobile platform to the microscope rig designed for continuous superfusion and intracellular or fluorescent recordings/imaging.

4.4.3. Apply continuous delivery of PSS during the experiment. Use the inline flow control valve Manually set the flow rate (5–7 mL/min) throughout the experiment using the inline flow control valve, consistent with the laminar flow, while matching the feed of solution flow to the extent of vacuum suction. Allow ≥5 min of PSS flow to the chamber for superfusion of the endothelial tube before acquiring background data and/or dye loading.

5. Utilization of arteriolar endothelial tubes for the examination of cellular physiology

NOTE: Isolated and secured arteriolar endothelial tubes can be used for intracellular recordings of $[Ca^{2+}]_i$ dynamics and V_m using photometry and sharp electrode electrophysiology, respectively, as previously illustrated[13] (**Figure 4**). $[Ca^{2+}]_i$ and V_m can be measured as separate or combined experimental variables as needed[13] (**Figure 4**). However, arteriolar endothelial tubes are more delicate than arterial endothelium, and experimentation time should not exceed 1 h.

5.1. Measurement of $[Ca^{2+}]_i$

5.1.1. Turn on the equipment and software for $[Ca^{2+}]_i$ recordings while maintaining continuous superfusion at a flow rate of 5–7 mL/min.

5.1.2. Load the endothelial tube with the Ca^{2+} dye Fura-2 AM for 30 min at room temperature. Wash the cells with superfusion solution for another 20–30 min while gradually raising the bath temperature to 37 °C. Maintain the temperature at 37 °C throughout the experiment.

5.1.3. Manually adjust the imaging window using photometry software to focus on ~20 endothelial cells (**Figure 4A**). In the absence of light, turn the PMT on the fluorescence interface and begin acquisition of $[Ca^{2+}]_i$ by exciting Fura-2 alternately (≥10 Hz) at 340 nm and 380 nm while collecting fluorescence emission at 510 nm. Once a stable baseline recording of $[Ca^{2+}]_i$ is established, apply pharmacological agents (e.g., purinergic receptor agonists) per experimental objective (**Figure 4B**).

5.2. Measurement of V_m

5.2.1. Turn on the equipment and software for V_m recordings and set the data acquisition rate (≥10 Hz) while maintaining continuous superfusion at a flow rate of 5–7 mL/min. Gradually raise the bath temperature to 37 °C and maintain it until the end of the experiment.

5.2.2. Pull a sharp electrode using a borosilicate glass capillary, backfill with 2 M KCl, and secure it over a silver wire coated with chloride in the pipette holder attached to an electrometer, that in turn, is held by a micromanipulator.

5.2.3. While viewing through the 4x objective, use a micromanipulator to carefully position the sharp electrode tip just over a cell of the arteriolar endothelial tube into the flowing PSS in the chamber.

5.2.4. Gradually increase magnification to 400x and reposition the electrode tip as needed.

5.2.5. Using the micromanipulator, gently insert the tip of a sharp electrode into one of the cells of the endothelial tube and start recording V_m using an electrometer (**Figure 4A**).

5.2.6. Once the endothelial resting V_m is stable (−30 to −40 mV), apply the desired pharmacological agents per experimental objective (**Figure 4C**).

6. Cellular imaging

NOTE: Endothelial tubes secured in the chamber of the mobile platform can also be used for microscopic imaging in both live and fixed conditions[19] using standard fluorescence or confocal microscopy. Immunohistochemistry with different antibodies for receptors and ion channels can also be applied as previously described[20].

6.1. Load the endothelial tube with the fluorescence tracker for the plasma membrane or desired organelle (e.g., nucleus, endoplasmic reticulum) at 37 °C for 15–30 min.

6.2. Wash the cells with fresh superfusion PSS and image live cells under the microscope at the excitation wavelength of the respective dyes (**Figure 4D,E**).

NOTE: Immunohistochemistry can be performed on this tube model using antibodies of interest.

REPRESENTATIVE RESULTS:

A demonstration of the protocol is shown in **Figure 1** with arteriolar dissection and endothelial tube isolation steps as **Figure 2** and **Figure 3**, respectively. Here, endothelial function was assessed by measuring $[Ca^{2+}]_i$ and V_m using Fura-2 photometry and sharp electrode electrophysiology (**Figure 4A**) in response to a pharmacological agent [2-methylthioadenosine diphosphate (MTA), a potent purinergic receptor (P2YR) agonist] at 37 °C. Upon application of MTA (1 µM), $[Ca^{2+}]_i$ rapidly increases (**Figure 4B**) with a concomitant hyperpolarization (**Figure 4C**). These measurements are a reflection of the activation of G_q–protein-coupled receptors (P2YRs) followed by the activation of small- and intermediate-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}/IK_{Ca}), which initiate an endothelium-dependent hyperpolarization (EDH) vasodilatory pathway. Thus, endothelium isolated from a parenchymal arteriole is functional in isolation and can be used to study key components of pathways that involve mobilization of $[Ca^{2+}]_i$ and/or regulation of V_m via K^+ channels.

Furthermore, the intact endothelium was incubated at 37 °C with fluorescent trackers for visualization to examine cellular morphology. Live endothelial cell imaging shows co-staining for plasma membrane (green) and nuclei (red) (**Figure 4D**). In **Figure 4E**, the plasma membrane (green) was stained together with an endoplasmic reticulum (ER; red) fluorescent stain, whereby areas of apparent overlap of ER within proximity of the plasma membrane appear orange (**Figure 4E**). These fluorescent images further reveal the cellular integrity of arteriolar endothelium and their possible use to study signaling microdomains located at junctional membrane complexes.

FIGURE AND TABLE LEGENDS:

Figure 1: **Examination of arteriolar endothelial tube freshly isolated from mouse parenchymal arteriole.** As depicted, the brain is isolated from a mouse, and a piece of rectangular brain tissue containing the MCA was removed. Parenchymal arterioles branching from the MCA and entering the brain parenchyma are isolated. Arterioles are enzymatically digested and triturated, producing an intact endothelial tube for study. The prepared endothelial tube enables measurement of intracellular Ca^{2+} dynamics and V_m while also enabling cellular imaging for fluorescence imaging and immunohistochemistry. Abbreviations: MCA = middle cerebral artery; V_m = membrane potential.

Figure 2: Dissection and isolation of parenchymal arterioles. (**A**) Isolated mouse brain with intact Circle of Willis. (**B**) A dissected rectangular piece of brain tissue containing the MCA (white arrow). (**C**) Isolation of pia from the upper part of the tissue (arteriole, blue arrow). (**D**) Arterioles (outlined with blue arrowheads) connected to middle cerebral artery (yellow arrows). (**E**) Identified arteriole (blue arrow) in the brain tissue. (**F**) Pulling out the arteriole (blue arrow) from the loosened tissue. Note that panels **E** and **F** indicate an alternative method for isolation of the arteriole (see protocol step 3.2.3). Scale bars = 0.2 mm (**D**) and 2 mm for all other images.

Figure 3: Preparation of endothelial tubes from parenchymal arterioles. (**A**) Isolated and clean arteriole (blue arrow). (**B**) Trituration of partially enzyme-digested arteriole. (**C**) Intact endothelial tube (magnification: 100x) prepared and secured on the glass coverslip using pinning pipettes. (**D**) Endothelial tube at 200x magnification. Scale bars = 0.1 mm (**B, C**) and 0.2 mm (**A, D**).

Figure 4: Application of endothelial tube for physiological examination of pathways related to blood flow regulation. (**A**) A sharp electrode (purple arrow) is positioned in a cell of an endothelial tube focused in the data acquisition window for photometry. (**B**) Representative recording of intracellular Ca^{2+} using Fura-2 photometry in response to MTA (1 µM). (**C**) Representative recording of V_m simultaneous with intracellular Ca^{2+} trace in panel **B**. (**D**) Representative image of plasma membrane (green) and nuclei (red) of live endothelial cells stained with fluorescent dyes. (**E**) Representative image of plasma membrane (green) and endoplasmic reticulum (ER, red) of live endothelial cells stained with fluorescent dyes. Areas of proximity for ER and plasma membrane are indicated in orange. Images were acquired using a monochrome camera and were pseudo-colored. Scale bars = 20 µm (**D**), 10 µm (**E**). Abbreviations: MTA = 2-methylthioadenosine diphosphate; F_{340}/F_{380} = Fura-2 dye ratio signal; V_m = membrane potential; PM = plasma membrane; ER = endoplasmic reticulum.

DISCUSSION:

Growing evidence suggests that cerebrovascular disease (CVD), aging, and Alzheimer's disease are strongly correlated and are a current topic of dementia research[4,8,14,21]. Thus, it is obvious that studies of the cerebrovascular network would have a broad impact on health while requiring continued extensive investigation under conditions of health and disease. As a significant point of vascular resistance for cerebral perfusion, the general importance of parenchymal arterioles in the etiology and development of CVD has been highlighted for over 50 years[22].

However, scientific examination tools have not been adequately developed to study intact parenchymal arterioles and their composite cell types until the recent onset of *ex vivo* approaches such as the isolated, pressurized parenchymal arteriole[11] and CaPA[12] preparations. The cerebrovascular physiology field would also benefit from complementary techniques in homocellular study models (smooth muscle, pericytes, and endothelium) to focus on specific cell types and their genetic and pharmacological characteristics underlying cerebral blood flow regulation and blood-brain barrier permeability.

The endothelium is a central regulatory organ that "feeds" all other organs throughout the body, including the brain[23]. Although all vascular segments (arteries, arterioles, capillaries, venules, veins) are essential for cerebral perfusion, the endothelium of arterioles requires dedicated investigation as a central component to cerebrovascular autoregulation and neurovascular coupling[24]. Further, Ca^{2+} signaling[25] and K^+ channel activity[17,26] appear to be enhanced during the regulation of myogenic tone in parenchymal arterioles relative to pial arteries. Thus, we have now extended the previously established method for the pial cerebral arteries (posterior)[13] to parenchymal arterioles.

Relative to these previously illustrated approaches[11,13], this protocol was refined by empirically adjusting various steps (e.g., enzymatic treatment, trituration) to reproducibly yield intact endothelial tubes from parenchymal arterioles. In addition, this protocol demonstrates the use of this study model to optimize the application of cellular photometry, electrophysiology, and fluorescent imaging (**Figure 4**). With general strengths and limitations of Fura-2 photometry and sharp electrode electrophysiology previously described in detail[13], the experimenter is encouraged to try an array of other Ca^{2+} signaling and ion channel assays to test specific hypotheses of interest. Further, the advancement and generation of novel genetic models with endothelial cell-specific genetically encoded indicators (e.g. $[Ca^{2+}]_i$[27] and voltage[28] indicators) can expand the usefulness of this preparation.

For investigative context, this isolated endothelial model is useful for research questions that require studying the role of the arteriolar endothelium in regulation of vascular dynamics, whereby *en face* measurements are not experimentally feasible due to their small size. Endothelial regulation of cerebral blood flow begins with the activation of various G-protein coupled receptors (e.g., purinergic, muscarinic)[18] and ion channels (TRPV3[29], TRPA1[5], NMDA receptors[8]). Activation of G_q-type receptors entails phosphatidylinositol 4,5-bisphosphate (PIP_2) lipolysis to generate inositol trisphosphate (IP_3) and diacylglycerol for $[Ca^{2+}]_i$ mobilization that can then mediate K^+ channel activation for changes in V_m[15]. K^+ channel activity, and thereby V_m alone, can also be studied directly under physiological conditions[2] or via select pharmacological targeting[30]. Moreover, the arteriolar endothelial tube enables enhanced fluorescent visualization of select plasma membrane remodeling (e.g., Filipin-III), intracellular organelles (e.g., ER tracker), and cell-to-cell coupling (e.g., propidium iodide)[18,19]. Thus, another feature of the study model entails effective pairing of cellular morphology and histology with parameters of function, such as Ca^{2+} and electrical signaling, in aging and the development of chronic diseases.

The components of the protocol that deserve special attention are arteriolar dissection, enzymatic digestion, trituration, and securing of the arteriolar endothelial tube for measurements. During dissection from the brain, damage to the parenchymal arteriole should be avoided at all costs to ensure successful digestion and trituration for isolating an intact and functional endothelial layer. Further, excessive stretching while securing the endothelial tube in the perfusion chamber will also destroy the preparation, effectively negating all prior protocol steps.

Viability tests should be applied to ensure that arteriolar endothelial tubes are functional with respect to key physiological components such as Ca^{2+} signaling (influx and/or intracellular release) following stimulation of G-protein coupled receptors, the extent of V_m hyperpolarization, and robust intercellular coupling through gap junctions[18,23]. Examples of such controls include reversible purinergic stimulation using MTA (1 µM; $\Delta[Ca^{2+}]_i$ ~ 300 nM; ≥10 mV hyperpolarization; **Figure 4B,C**), reversible SK_{Ca}/IK_{Ca} activation using NS309 (1 µM; ≥20 mV hyperpolarization), and/or correspondence between current injection (e.g., ~-1 nA) into one cell and V_m responses (~-10 mV) of another at a distance of ≥250 µm[18,31]. With practice, care, and experimental rigor, the entire protocol is reproducible if the learner has patience and perseverance.

This method has key limitations to address, such as the insufficient abundance of samples (e.g., nucleic acids, proteins, and lipids) and relative fragility for biochemical experiments and live-cell recordings, respectively. However, the molecular biology caveat can be avoided by pooling from several animals and/or devising quantitation methods of higher sensitivity and throughput. If the physiological temperature is to be maintained at 37 °C, short experimental timeframes (≤1 h) will be required with focused pharmacological interventions only. In addition, it would be extremely challenging to isolate arteriolar endothelial tubes from near-term or newborn (<1-month-old) animals, potentially precluding studies of cerebral vascular development.

Other considerations encompass the precise composition of cerebral vascular endothelial tubes, potentially requiring select approaches of immunofluorescence and electrophysiology. For example, the basement membrane (composed primarily of collagen IV, laminin, and fibronectin) runs along the cerebral vascular tree. It is known for its contribution to the integrity of the blood-brain barrier (BBB) formed by brain capillary endothelial cells[32]. It should be noted that this study model may not be appropriate for BBB permeability experiments as basement membrane components are substrates of the enzyme cocktail used for partial digestion of arteriolar endothelial tubes, in particular, the collagenase H blend. Further, application of a selective enzyme cocktail, removal of the internal elastic lamina, and clear identification of endothelial cells per structure (parallel arrangement vs. circumferential for smooth muscle cells), morphology (“teardrop” shape of endothelial cells vs. spindle-shaped smooth muscle cells), and electrophysiology (e.g., hyperpolarization to GPCR stimulation), eliminates physiological contributions of smooth muscle cells and pericytes. Finally, the experimenter must take note of endothelial cell heterogeneity (gene expression profile, structure, function) per organ type (e.g., brain, heart, lung[33]) in addition to the brain region (e.g., cortex[9,34], hippocampus[10]) and respective vascular segmentation (e.g., arteries, arterioles, and capillaries[9,34,35]). Thus, a comprehensive study of cerebral blood flow regulation will also require complementary *ex vivo* (e.g.,

intravascular pressure myography, capillary–parenchymal arteriole preparation) and *in vivo* (e.g., intracerebral Laser Doppler) approaches of intact vascular segments and networks.

In summary, this paper presents an advanced technique demonstrating how to prepare an intact endothelial tube freshly isolated from mouse brain parenchymal arterioles. We anticipate that this approach will complement and/or build from *in vivo* and intact vascular study models. The overall goal is to generate new information for understanding mechanisms underpinning cerebral blood flow at the fundamental level for physiology and select transitions towards pathology for therapy.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

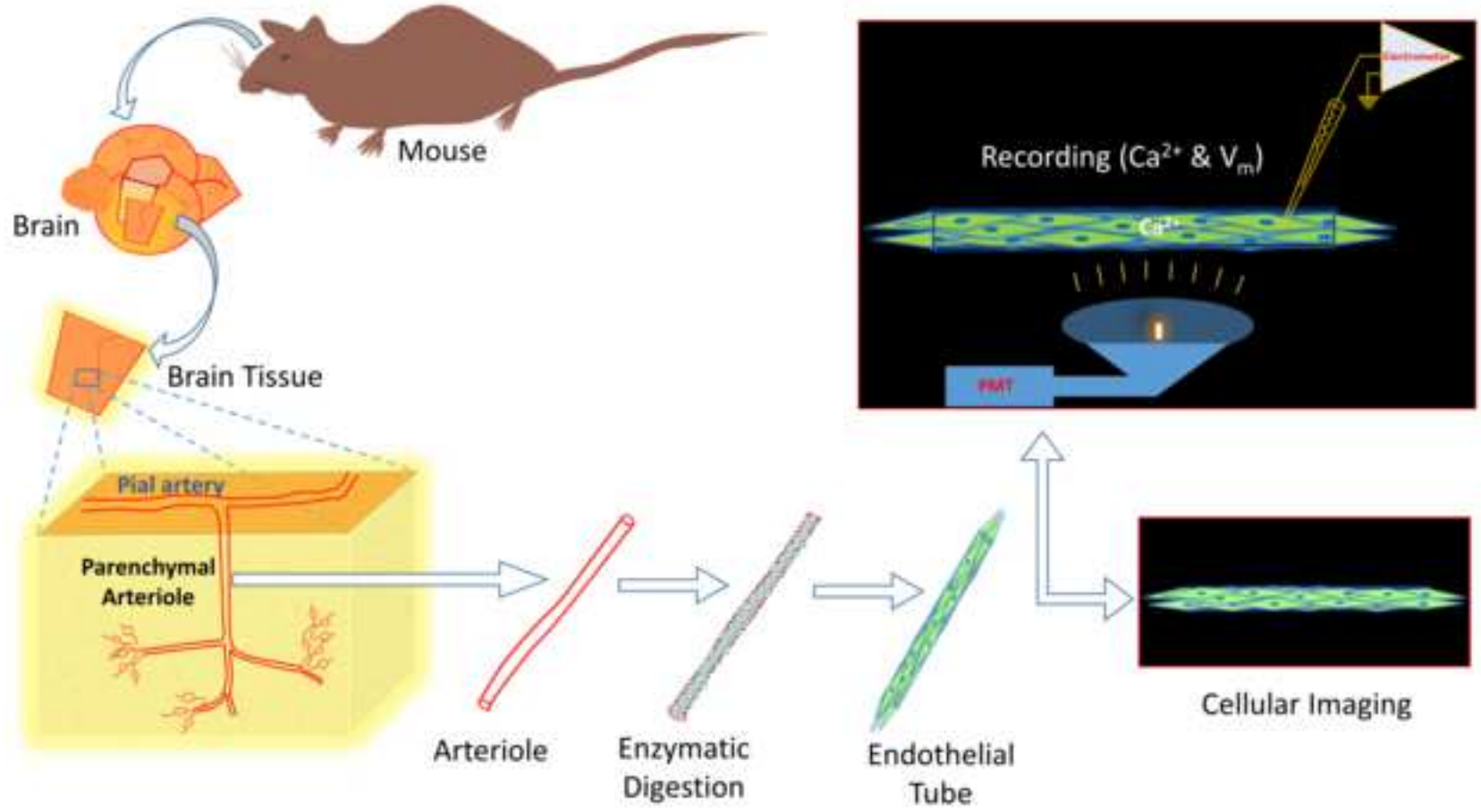

Figure 2

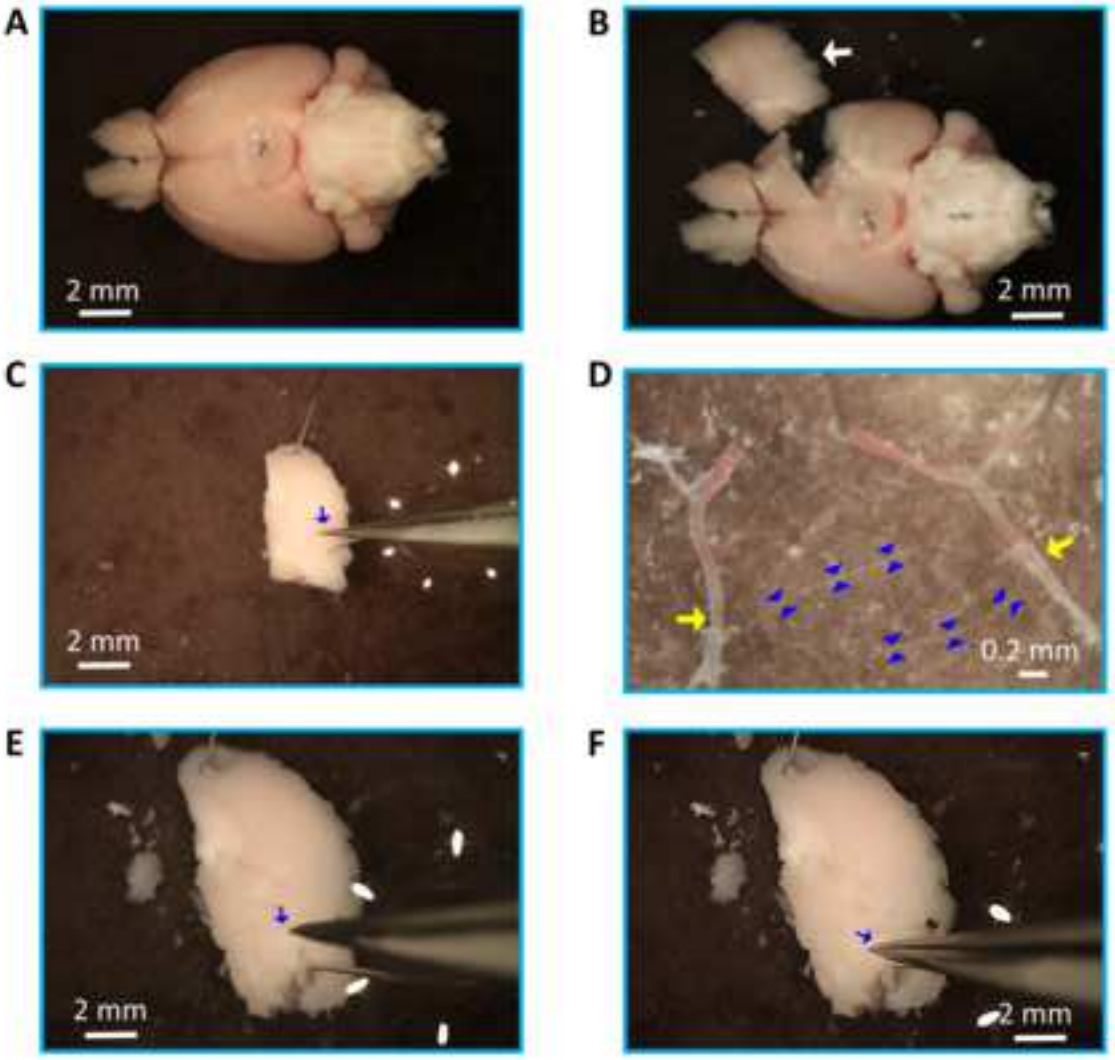

Figure 3

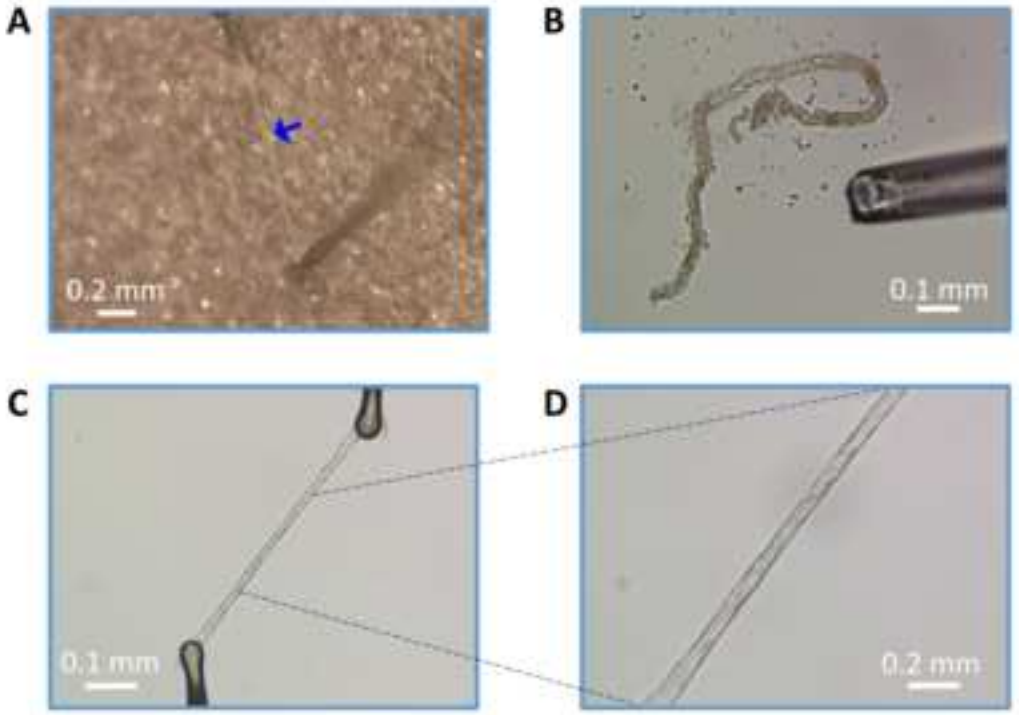

Figure 4

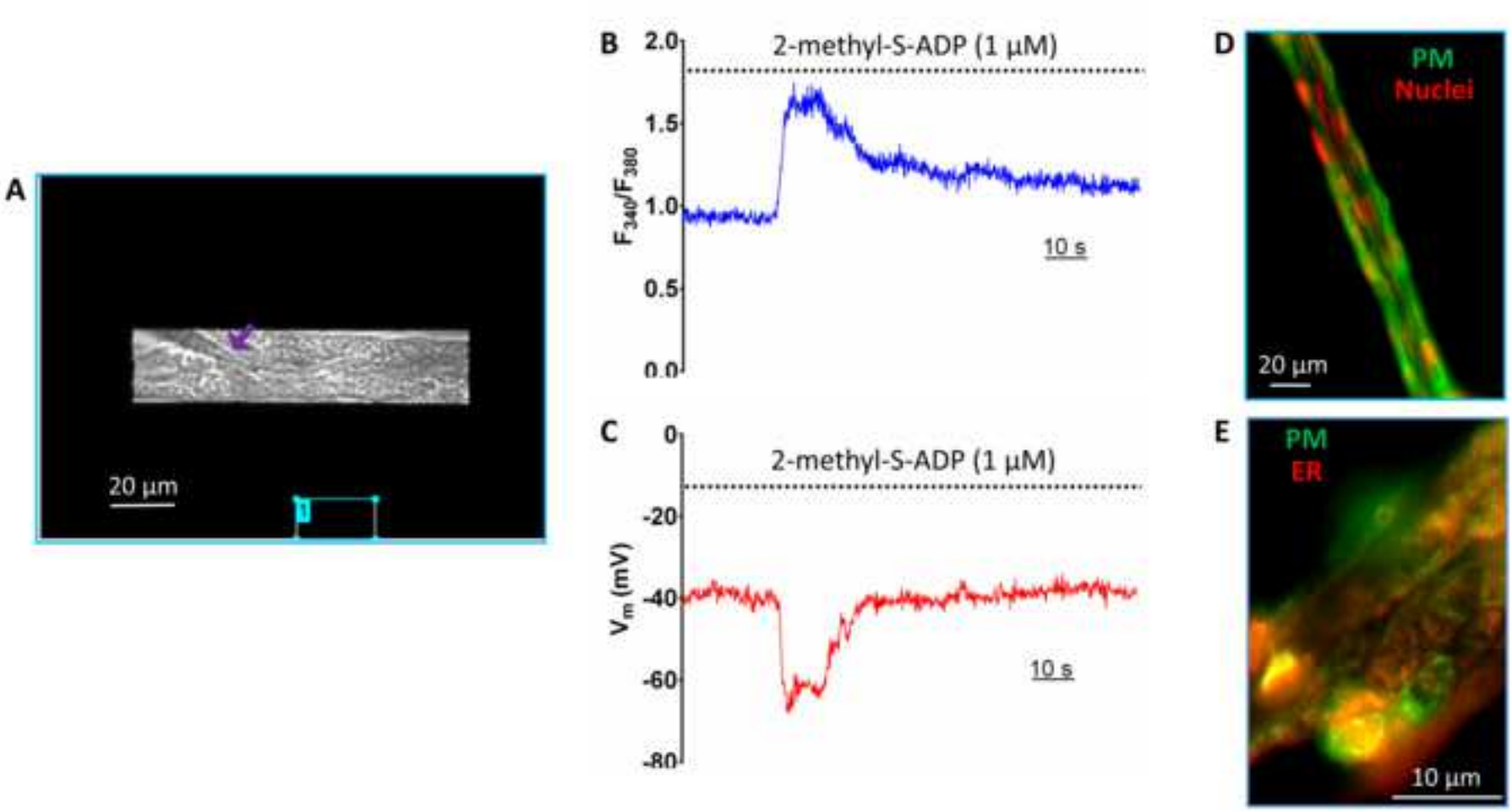

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Table of Materials

Table of Materials_Alphabetical Order_11Jan2022_JoVE2.xlsx

Subject: Manuscript ID JoVE63463_R1, Journal of Visualized Experiments

Manuscript title: Isolation and functional resolution of arteriolar endothelium of mouse brain parenchyma

Editorial Changes:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Authors: We have now thoroughly proofread the manuscript.

2. Please provide a summary between 10-50 words.
Authors: Yes, we originally provided a 50-word summary (indicated in between "Keywords" and "Abstract") per JoVE format.

3. Please also include in the Introduction the following with citations:
a) The rationale behind the development and/or use of this technique
Authors: The rationale for the development and use of the study model/technique is presented as paragraphs 1 and 2 of the Introduction respectively.
b) A description of the context of the technique in the wider body of literature
Authors: Using 17 citations (e.g., PMIDs 27286481, 31904015, 30735188, 21179072), we described the technique in the context of the broader literature.
c) Information to help readers to determine whether the method is appropriate for their application
Authors: We have now added a sentence in the Introduction (Par. 2) to clarify suitability of the technique.

4. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
Authors: We have completely revised the Protocol section while moving information to the Discussion as needed.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
Authors: The numbering has been revised accordingly.

6. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
Authors: We have now simplified as advised accordingly.

7. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
Authors: We have simplified, moved, and/or deleted "Notes" in the Protocol section as requested.

8. Please describe the dissociation fluid, cold solution, superfusion solution composition either in the table of materials or as a separate table.
Authors: We have now incorporated such details from PMID 30735188 as Step 2 (Solutions & Drugs) to avoid confusion while maintaining continuity throughout the current protocol.

9. Line 112: Please provide more details as to how the cranial bones and connective tissues were removed and the brain was isolated.
Authors: We have now described this step with sufficient detail as step 3.1.2.

10. Line 160: How was the apparatus prepared and assembled? Please provide more details.
Authors: We have now incorporated such details from PMID 30735188 as Step 1 (Equipment & Materials) to avoid confusion while maintaining continuity throughout the current protocol.

11. Line 256: How was cellular imaging? There are currently no protocol steps for this heading.
Authors: We have now clarified steps for cellular imaging as Step 6.

12. For all software steps, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.
Authors: Various software suites may operate differently and we have described and illustrated details about the general software steps in our previous JoVE protocol (PMID 30735188). Here, we provide the key steps necessary for the general isolation and examination of arteriolar endothelial tubes.

13. Please include a single line space between each step, substep, and note in the protocol section. Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Authors: We revised the format of the Protocol while having highlighted only the necessary steps for the video not illustrated in our previous JoVE protocol (PMID 30735188).

14. Please remove the embedded figures from the manuscript.
Authors: The Figures have been removed from the manuscript, and submitted separately.

15. Please do not abbreviate journal names in the references.
Authors: The references are updated as suggested.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

This is an interesting manuscript with a fascinating technique advancing the authors' previous approach and isolating EC "tubes" that are intact and electrically coupled, from mouse PAs within the MCA trajectory. The importance of PAs in the contribution of cerebral blood flow regulation and brain perfusion in cerebral vascular disease and AD/ADRD have been intensively studied, but the heterogenetic effects of ECs in the brain have been limited to using pressurized PAs and CaPA preparation. Thus, the technique described in this manuscript will help researchers better understand fundamental endothelial signaling pathways and network communication of arterioles embedded in brain parenchyma towards addressing unfulfilled questions of cerebrovascular physiology and pathology. The manuscript is well-written and well-designed. However, some minor concerns remain.

Authors: We thank the reviewer for understanding our work and sharing their concerns with us, which have further improved the manuscript.

Major concerns: validation is not sufficient.

a. Although collagenase and elastase are used in the digestion step, the authors did not validate the "purity" of EC "tubes." Are they pure "EC tubes" or basement membrane tubes attached with ECs? This could be discussed as a limitation, or stain with basement membrane markers.

Authors: The basement membrane is composed primarily of collagen IV and laminin, which are substrates of the enzyme cocktail, in particular of the collagenase type H blend, which contains a mix of 7 different collagenases, thus it is likely that the basement membrane is mostly digested in this preparation. However, we have not performed a qualitative assessment of possible remnants of basement membrane in our EC tubes, and we have included this limitation in the Discussion. Further, as the basement membrane is integral to the blood brain barrier (BBB), we have clarified in the Discussion (Par. 6) that this model is not appropriate for BBB permeability measurements.

b. Markers of VSMCs and pericytes are needed on the EC "tubes" to prove that these cells are fully removed.

Authors: Application of a selective enzyme cocktail, removal of internal elastic lamina, and clear identification of endothelial cells per structure (parallel arrangement vs. circumferential for smooth muscle cells), morphology ("teardrop" shape of endothelial cells vs. spindle-shaped smooth muscle cells), and electrophysiology (e.g., hyperpolarization to GPCR stimulation), eliminates physiological contributions of smooth muscle cells and pericytes. Thus, we have now clarified that comprehensive study of cerebral blood flow regulation will also require complementary *ex vivo* (e.g., intravascular pressure myography) and *in vivo* (e.g., intracerebral Laser Doppler) approaches of intact vascular segments and networks (Discussion, Par. 6).

Minor concern: Expand the discussion on the heterogeneity of ECs is suggested to enhance the importance of this manuscript.

a. Difference in arterial, arteriolar, and capillary ECs in previous studies can be included.

Authors: Thank you for this suggestion. We have now discussed consideration of the heterogeneity of endothelial cells along cerebral vascular segments in the Discussion (Par. 6).

b. Difference in PAs within the MCA trajectory and hippocampal arterioles could be discussed.

Authors: Likewise, using select references, we have also mentioned the general possibility of different cerebral vascular function among brain regions as well.

Reviewer #2:

Manuscript Summary:
This is an interesting paper that expands on currently, limited techniques to study mice parenchymal arterioles. The authors describe a method to isolate endothelial tubes from cerebral parenchymal arterioles. The study demonstrates the successful isolation of endothelial tubes and their use to record Ca^{2+} imaging and electrophysiological variables. The methodology is of interest to the vascular researcher and can provide an advanced tool to investigate the intrinsic properties of endothelial cells in health and disease. The manuscript can be improved with the addition of methodological and tool details.

Major Concerns:
1. A suggestion is to expand on the limitations of the study and specify what types of controls are needed to ensure that the functional state of the endothelial tubes is representative of the state of the animal model as opposed to an artifact resulting for the delicate manipulation of these vessels.
Authors: We have expanded the manuscript to include discussion of controls for cellular health (Discussion, Par. 4) and various experimental limitations/considerations (Par. 5 & 6).

2. Also, while the authors state that other details of the techniques can be found in prior publications, given the nature of the approach it would be important to include those in this article. Specifically, the list of each solution with corresponding concentrations. The same would apply for the solutions used for electrophysiological recordings and the concentration of dyes used. I would also suggest converting the lists (e.g., Catalog number item) to tables where the item, catalog number and any potential reference can be found. Tables would facilitate the reader access to resources.
Authors: To avoid confusion, we have incorporated salient details (e.g., compositions of solutions) from the prior JoVE publication (PMID 30735188) into the current manuscript. Details of materials required for the protocol can be found in Table 1 in addition to manuals and websites associated with the respective vendors.

Reviewer #3:

Manuscript Summary:
This manuscript describes an experimental method for isolating endothelial tubes from parenchymal arterioles in the mouse brain. Vascular endothelial cells of parenchymal arterioles play an essential role in the local regulation of cerebral blood flow to meet neural demand (i.e., neurovascular coupling), and furthermore a key role in the pathology of cerebrovascular diseases and neurodegeneration. The reproducible and standardized methods for fluorescent imaging and electrical measurements of endothelial cell signaling, established by the authors, will contribute significantly to basic research in the areas of neuroscience and neurovascular physiology.

Major Concerns:
This study is considered an extension of the previously established methods to study vascular endothelial cell signaling in the pial arteries of the mouse brain. Because the authors have much experience and knowledge about the functions of endothelial cells in both pial and parenchymal arteries/arterioles, please briefly describe the differences between the endothelial cells in these

arteries, such as in relation to genetics, morphology, ion channels, and other functions (not just proximal to neurons and glia). This contrast of phenotypic differences between these arterial endothelial cells provides a basis for studies needed to understand endothelial cell signaling in parenchymal arterioles.
Authors: We thank the reviewer for their understanding of our expertise while providing valuable comments. We have now expanded the Discussion (Par. 6) for general considerations regarding brain region and vascular segmentation. We have added pertinent references accordingly but a satisfactory answer to the requested information remains a work in progress. Perhaps the current study model will help advance information for endothelial heterogeneity (gene expression, structure, function).

Minor Concerns:
In the protocol, the authors specified the use of the animals from 3 to 30 months of age. Please explain why the experiments are not appropriate for the use of younger animals or infants, such as for the study of vascular development.
Authors: Thank you for making this point. In our experience, we have done these experiments using animals between ages from ~2 months to 30 months. Thus, we have now updated the age of mice in the manuscript (2 to 30 mo) in the manuscript. We have not used animals younger than 1 month but speculate that it would be extremely challenging to adapt this method for studying endothelial cells from artery/arteriole during vascular development. To do so, the protocol will likely require modifications since the developing artery/arteriole would be presumably even more delicate for preparing endothelial tubes. We have now clarified this limitation in the Discussion (Par. 5, Final sentence). In addition, it would be extremely challenging to isolate arteriolar endothelial tubes from near-term or newborn (<1 month) animals, potentially precluding studies of cerebral vascular development.

In the Discussion, the authors highlighted the permeability of the blood-brain barrier (BBB) as a pivotal function of brain vascular endothelial cells. Could you comment on how this model can assist in the study of BBB integrity or signaling mechanisms?
Authors: See the revised Discussion (Par. 6). Provided that the enzyme cocktail may degrade proteins of the basement membrane (e.g., collagen, laminin), we clarify that this model may not be suitable for BBB integrity or permeability measurements.

Lines 144-152; 1.5-2 mm "in length", 0.75-1 mm "in length", "long arterioles" what is the rough length?
Authors: Sorry for this confusion. In the revised protocol, we have clarified approximate length of arterioles in steps 3.2.3 & 3.2.4.

Line 237; The authors recommended the use of calcium dye Fura-2 AM. What is the reason this dye was chosen, or what type of indicator is appropriate or inappropriate for the study of calcium signaling in vascular cells.
Authors: At least for initial characterization, we recommend Fura-2 AM because it provides experimental strengths as a ratiometric excitation, ultraviolet wavelength excitation, and potential for calculation of cytoplasmic Ca^{2+} concentrations. However, other calcium dyes (e.g., Fluo-4) or even a V_m dye (e.g., Di-8-ANEPPs) can be potentially used to study arteriolar endothelial tubes. We have now clarified in the Discussion (Par. 2, Final sentence) that general strengths and

limitations of Fura-2 photometry and sharp electrode physiology have been previously described in detail (PMID 30735188).

Line 410; "all vascular segments" why not include venules and veins?
Authors: Yes, we have now included venules and veins in that statement as well (Discussion, Par. 2, First sentence).

Figures 2, 3; Please describe what the arrows on the picture point to.
Authors: We have now indicated in Figures 2 & 3 that arrows point to parenchymal arterioles.

Figure 4; Please provide a description of how the cell membrane and nuclei were stained.
Authors: We have now added step 6 (Cellular Imaging) in the protocol. Commercial information for membrane and organelle trackers are specified in Table 1.