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).