Journal of Visualized Experiments

Microelectrode impalement method to record membrane potential from cannulated middle cerebral artery.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59072R2
Full Title:	Microelectrode impalement method to record membrane potential from cannulated middle cerebral artery.
Keywords:	Membrane potential, microelectrode, impalement, electrometer, cannulated vessels, vascular smooth muscle cells.
Corresponding Author:	MALLIKARJUNA Reddy Pabbidi, DVM, PhD UMMC Jackson, Mississippi UNITED STATES
Corresponding Author's Institution:	UMMC
Corresponding Author E-Mail:	MPabbidi@umc.edu
Order of Authors:	Joey T Reed
	Sumit P Sontakke
	Mallikarjuna Reddy Pabbidi, DVM, PhD
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Jackson, Mississippi, USA

TITLE:

Microelectrode Impalement Method to Record Membrane Potential from a Cannulated Middle Cerebral Artery

AUTHORS & AFFILIATIONS:

Joey T. Reed[1], Sumit P. Sontakke[1], Mallikarjuna R. Pabbidi[1]

[1]Department of Pharmacology and Toxicology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216.

Email Addresses of Co-Authors:

Joey T. Reed (jtreed@umc.edu)
Sumit P. Sontakke (ssontakke@umc.edu)
Mallikarjuna R. Pabbidi (mpabbidi@umc.edu)

Corresponding Author:

Mallikarjuna R. Pabbidi (mpabbidi@umc.edu)

KEYWORDS:

Membrane potential, microelectrode, impalement, electrometer, cannulated vessels, vascular smooth muscle cells.

SUMMARY:

The primary goal of this article is to provide details of how to record membrane potential (V_m) from the middle cerebral artery using the microelectrode impalement method. The cannulated middle cerebral artery is equilibrated to gain myogenic tone, and the vessel wall is impaled using high resistance microelectrodes.

ABSTRACT:

Membrane potential (V_m) of vascular smooth muscle cells determines vessel tone and thus blood flow to an organ. Changes in the expression and function of ion channels and electrogenic pumps that regulate V_m in disease conditions could potentially alter V_m, vascular tone, and blood flow. Thus, a basic understanding of electrophysiology and the methods necessary to accurately record V_m in healthy and diseased states are essential. This method will allow modulating V_m using different pharmacological agents to restore V_m. Although there are several methods, each with its advantages and disadvantages, this article provides protocols to record V_m from cannulated resistance vessels such as the middle cerebral artery using the microelectrode impalement method. Middle cerebral arteries are allowed to gain myogenic tone in a myograph chamber, and the vessel wall is impaled using high resistance microelectrodes. The V_m signal is collected through an electrometer, digitized, and analyzed. This method provides an accurate reading of the V_m of a vessel wall without damaging the cells and without changing the membrane resistance.

INTRODUCTION:

The membrane potential (V_m) of a cell refers to the relative difference of ionic charge across the plasma membrane and the relative permeability of the membrane to these ions. The V_m is generated by the differential distribution of ions and is maintained by ion channels and pumps. Ion channels such as K^+, Na^+, and Cl^- contribute substantially to the resting V_m. Vascular smooth muscle cells (VSMCs) express more than four different types of K^+ channels[1], two types of voltage-gated Ca^{2+} channels (VGCC)[2], more than two types of Cl^- channels[3-5], store-operated Ca^{2+} channels[6], stretch-activated cation channels[7,8], and electrogenic sodium-potassium ATPase pumps[9] in their plasma membranes, all of which may be involved in the regulation of V_m.

The V_m of VSMCs depends on lumen pressure. In non-pressurized vessels, V_m varies from -50 to -65 mV, however, in pressurized arterial segments, V_m ranges from -37 to -47 mV[10]. Elevation of intravascular pressure causes VSMCs to depolarize[11], decreases the threshold for VGCC opening, and increases calcium influx contributing to the development of myogenic tone[12]. On the contrary, in passive or non-pressurized vessels, membrane hyperpolarization, due to high K^+ channel activity, will prevent VGCC from opening, resulting in limited calcium entry and a decrease in intracellular calcium, contributing to less vascular tone[13]. Thus, V_m due to changes in lumen pressure appears to play an essential role in vascular tone development, and both VGCC and K^+ channels play a crucial role in the regulation of V_m.

V_m varies between vessel type and species. V_m is -54 ± 1.3 mV in guinea pig superior mesenteric arterial strips[14], -45 ± 1 mV in the rat middle cerebral arteries at 60 mmHg lumen pressure[12], and -35 ± 1 mV in rat parenchymal arteries at 40 mmHg lumen pressure[15]. The resting V_m recorded in unstretched rat lymphatic muscle is -48 ± 2 mV[16]. V_m of cerebral VSMCs is more negative than in peripheral arteries. In comparison, feline middle cerebral arteries were reported to have a V_m of approximately -70 mV, while mesenteric and coronary arteries were reported to have -49 and -58 mV, respectively[17,18]. Differences in the V_m across vascular beds may reflect the differences in the expression and function of ion channels and electrogenic sodium-potassium pumps.

Increases and decreases in V_m are referred to as membrane depolarization and hyperpolarization, respectively. These alterations in V_m play a central role in many physiological processes, including ion-channel gating, cell signaling, muscle contraction, and action potential propagation. At a fixed pressure, many endogenous and synthetic vasodilator compounds that activate K^+ channels cause membrane hyperpolarization, resulting in vasodilation[1,13]. Conversely, sustained membrane depolarization is vital in agonist-induced or receptor-mediated vasoconstriction[19]. V_m is a critical variable that not only regulates Ca^{2+} influx through VGCC[13] but also influences the release of Ca^{2+} from internal stores[20,21] and Ca^{2+}-sensitivity of the contractile apparatus[22].

While there are several methods to record V_m from different cell types, data collected from the microelectrode impalement method of cannulated vessels appears to be more physiological than data obtained from isolated VSMCs. When recorded from isolated VSMC using current clamp methods, V_m is seen as spontaneous transient hyperpolarizations in VSMCs[24]. Isolated VSMCs are not in the syncytium, and the changes in the series resistance may contribute to the oscillatory behavior of V_m. On the other hand, oscillatory behavior is not observed when V_m is recorded from intact vessels, probably due to cell-cell contact between VSMCs that are in syncytium in the artery

and are summated throughout the vessel leading to a stable V_m[24]. Thus, measurement of V_m from pressurized vessels using standard microelectrode impalement technique is relatively close to the physiological conditions.

Recording V_m from cannulated vessels could provide vital information, since V_m of VSMCs that are in syncytium is one of the major determinants of vascular tone and blood flow, and modulation of the V_m could provide a way to dilate or constrict blood vessels. Thus, it is essential to understand the methodology involved in recording V_m. This article describes intracellular recording of V_m from cannulated middle cerebral arteries (MCAs) using a microelectrode impalement method. This protocol will describe how to prepare MCAs, microelectrodes, set up the electrometer and perform the impalement method to record V_m. Also, representative data, common issues that were encountered when using this method and potential issues are discussed.

PROTOCOL:

The male rats were housed in the Animal Care Facility at UMMC, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals had free access to food and water throughout the study. Animals were maintained in a controlled environment with temperature at 24 ± 2 °C, humidity levels of 60–80% and 12 h light/dark cycles. All protocols were approved by the Animal Care and Use Committee of UMMC.

1. Preparation of equipment

1.1. Place a dual channel differential electrometer amplifier (see the **Table of Materials**) close to the vessel chamber and at the desired location.

1.2. Connect the output of the amplifier channel A or B to the channel input of the digitizer with a BNC-BNC cable.

1.3. Mount the probe in the micromanipulator and place it near the microscope and the myograph. The recording setup must be installed on a vibration-free table.

1.4. Place the knobs and switches on the front of the amplifier in positions that configure it for this experiment as described in the manual.

1.5. Connect the bath ground to the circuit ground of the amplifier via with an appropriate electrode. Similarly, ensure that the cage is grounded to the chassis of the amplifier.

2. Preparation of microelectrodes and assembly

2.1. Use borosilicate glass microelectrodes (see the **Table of Materials**) and pull the glass tip to have a 8–10 mm taper, diameter of <1 µm and resistance of 80–120 MΩ when filled with 3 M KCl.

2.1.1. Use a standard puller to achieve a short gradual taper using the following settings: heat = 650; velocity = 20; pull = 25; time = 250 and loop twice for higher resistances and smaller tips. See the **Table of Materials** as the settings are instrument-specific.

NOTE: Tip diameters <1 µm will cause minimal damage to the cell when impaled.

2.2. Fill the microelectrode with 3 M KCl using a microfiber syringe (see the **Table of Materials**).

2.2.1. Slowly pull the plunger of the microfiber syringe up while injecting the 3 M KCl into the microelectrode to allow space for the fluid to fill and to prevent the formation of air bubbles inside the microelectrode.

2.2.2. Fill the microelectrode until full and ensure that there are no air bubbles before placing it in the microelectrode holder. If bubbles are present, gently tap the microelectrode with a finger to remove the bubbles.

2.3. Exercising care, firmly push the electrode shank into the holder through the bored hole. If excess fluid is present, remove it with a tissue.

2.4. Connect the electrode holder assembly to the amplifier probe. Conduct an electrode test, adjust the input offset, verify zero setting and check the probe input leakage as per the amplifier manual.

2.5. Measure electrode resistance using an electrode test as shown in **Table 1**.

2.6. Note that a working electrode displays a positive DC voltage shift of 1 mV/MΩ at the channel output. On the other hand, if a large voltage appears at the channel output and on the meter, this indicates a blocked or broken electrode.

2.7. Open the recording software, assign a name to the file and save it for future analysis in a storing software.

3. **Isolation and cannulation of the middle cerebral artery**

3.1. Preparation the reagents.

3.1.1. Prepare normal and low calcium physiological salt solution (PSS) as described in **Table 2**.

3.2. Prepare the myograph.

3.2.1. Rinse the myograph chamber (see the **Table of Materials**) with distilled water multiple times to keep it free of debris. Load the chamber with 5 mL of normal PSS.

3.2.2. Fill both glass cannulas with filtered normal PSS using a 5–10 mL syringe. Carefully fill the entire cannula and the attached tubing without introducing any air bubbles.

3.2.3. Prepare two monofilament nylon sutures (10-0, 0.02 mm) with a half-knot each using blunt forceps.

3.2.4. Place the partially closed suture knots on both cannulas slightly away from the tip using dissection forceps under a dissection microscope. Later these knots will be slid off and tied carefully onto the cannulated arterial ends to secure the vessel.

3.3. Isolate and cannulate the middle cerebral artery.

3.3.1. Induce deep anesthesia in a Sprague Dawley rat by using 2–4% inhaled isoflurane.

3.3.2. Decapitate the rat using guillotine under deep anesthesia.

3.3.3. Carefully remove the skull using a bone cutter and a scissor.

3.3.4. Remove the brain from the skull and place it in 5 mL of low calcium PSS on ice.

3.3.5. Identify and dissect out an unbranched segment of rat middle cerebral artery (MCA) with an inner diameter of 100–200 µm from the brain using spring scissors and forceps.

3.3.6. Mount the MCA onto the glass cannulas using fine forceps and secure by tightening the sutures in the myograph containing normal PSS.

3.3.7. Close off the distal cannula so that there will be no flow within the MCAs.

3.3.8. Connect the inflow pipette to a reservoir holding PSS to allow for control of intraluminal pressure which will be monitored with an in-line pressure transducer.

3.3.9. Visualize the cannulated MCAs using a charge-coupled device camera (see the **Table of Materials**) mounted on an inverted microscope and an imaging software.

3.3.10. Set the axial length of the MCA to an approximate length where it should appear neither rigid nor flaccid.

3.3.11. Equilibrate the bath solution with O_2 (95%) and CO_2 (5%) at 37 °C to provide adequate oxygenation, temperature and to maintain pH at 7.4.

3.4. Impale (penetrate the cell plasma membrane) the vascular smooth muscle cells.

3.4.1. Connect the ground electrode and keep it immersed in the PSS of the myograph.

3.4.2. Illuminate the vessel chamber and look through the microscope to visualize the tip of the microelectrode in the bath solution.

NOTE: Alternatively, one can visualize the MCA and microelectrode on a computer having an imaging software.

3.4.3. Use the controls of the micromanipulator to move the tip of the microelectrode close to the outer wall of the blood vessel. The micromanipulator and the tip of the microelectrode must be in a stable position in relation to the tissue.

NOTE: Before beginning experiments, confirm that the membrane voltage has stabilized. If the measured voltage is unstable, the connection between the electrode and the cell is not sealed, indicating a leak.

3.4.4. Begin the recording.

3.4.5. Slowly move the tip of the microelectrode towards the vessel, aiming for the center of the vessel using course or fine control of the micromanipulator.

NOTE: Occasionally, a small deflection in the recording may be observed when the microelectrode tip contacts a muscle fiber membrane.

3.4.6. When the tip comes in close proximity to the vessel, advance the electrode forward in one rapid motion using the micromanipulator to impale the membrane of the muscle.

3.4.7. At this point, one can begin observing the changes in V_m being recorded. Do not touch the micromanipulator when the microelectrode impales the membrane of the cell.

NOTE: The difference in voltage between the recording and reference electrode decreases from 0 mV to between -40 mV and -75 mV depending on the level of intravascular pressure or other excitatory or inhibitory stimuli. These readings characterize the transmembrane potential difference of the current cell.

3.4.8. Perform multiple impalements on a single vessel in different areas of the vessel without damaging VSMCs in order to get accurate measurements.

3.4.9. After recording, use the manipulator to remove the microelectrode in one rapid movement.

3.4.10. Stop the recording and save data files for further analysis.

REPRESENTATIVE RESULTS:

The presented method can be reliably used to record V_m in cannulated vessels. A brief procedure describing how to isolate MCA from the brain is presented in **Figure 1A**. After separating the brain

from the skull, the MCA was dissected out and placed in a Petri dish containing low calcium PSS. Part of the connective tissue that was attached was also dissected along with MCA using spring scissors and forceps to prevent damage to MCA during the isolation. Carefully, connective tissue was also removed, and the dissected MCA was ready to transfer to the myograph. MCA was mounted on the cannulas and tied using suture knots on both ends of the cannulas in the myograph. A schematic representation of a typical microelectrode impalement method setup is shown in **Figure 1B**. A microelectrode filled with 3 M KCl was connected to the electrometer via a holder in the probe. Channel output of the electrometer was connected to an analog input channel of a digitizer using a BNC-BNC cable. Digitizer output was further connected to an oscilloscope to visualize the signal in the recording software. The ground is established using an AgCl pellet wire that was extended from the chassis of electrometer to the bath solution in the myograph. Finally, digital traces were visualized in the recording software on the computer monitor.

The MCA was then incubated in freshly prepared warm PSS and was pressurized to 60 mmHg. Only vessels that gain tone were used for V_m recording. After the vessel gained significant tone, the microelectrode was advanced into the vessel wall. Arterial diameter and impalement of the artery were visualized using video microscopy. Impalement is considered successful when there is a rapid deflection to negative values, V_m is stable for ≥30 s, and the voltage returns abruptly to 0 mV upon removal of the electrode as shown in **Figure 2**[12,15]. Our results suggest that, in an MCA that is pressurized to 60 mmHg, the V_m is ~ -43.2 ± 2.9 mV.

After a successful impalement and V_m stabilization, the drugs that change the V_m were perfused in the bath and changes in the V_m were recorded. We used 20 mM KCl to depolarize and 20 µM NS1619, a synthetic large conductance potassium channel opener, to hyperpolarize the membrane. Our results suggest that perfusion of the chamber with KCl depolarized the membrane by ~ 5.8 ± 0.18 mV. On the other hand, perfusion with NS1619 hyperpolarized the membrane by ~ 3.8 ± 0.4 mV.

FIGURE AND TABLE LEGENDS:

Figure 1: An illustration of isolation of middle cerebral arteries and recording of membrane potential using microelectrode impalement method. (**A**) Using spring scissors, cut the brain or connective tissue along the dotted lines. Transfer and mount the MCA between the two glass cannulas and secure it using sutures in the myograph chamber filled with PSS. (**B**) A microelectrode filled with 3 M KCl is inserted into the holder and is connected to the probe. Changes in transmembrane potential travel from the probe to an electrometer and to the digitizer via a BNC cable (BNC cable is connected from channel output of electrometer and to an analog input of a digitizer). The digitized potential is seen in the recording software on the monitor. The ground is established using an AgCl pellet wire connected from the electrometer to the bath solution in the myograph. MCA = middle cerebral artery; PSS = physiological salt solution; AgCl = silver chloride.

Figure 2: Recording of membrane potential using microelectrode impalement method from

cannulated middle cerebral arteries. Representative trace of V_m. Impalement is considered successful if there is an abrupt deflection to negative values upon electrode entry, V_m is stable for ≥30 s, and the voltage returns abruptly to 0 mV upon removal of the electrode. The X-axis represent the time, and the Y-axis represents the membrane potential. The dotted line represents the adjusted baseline before the impalement of the vessel. Sec = seconds.

Figure 3: Recording of changes in the membrane potential using microelectrode impalement method when vessels are exposed to vasoactive agents. V_m was recorded using microelectrode impalement method from cannulated middle cerebral arteries before and after exposure to vasoactive agents. Sample trace represents (**A**) depolarization in response to 20 mM KCl and (**B**) hyperpolarization in response to 20 µM NS1619—a large conductance potassium channel agonist. (**C**) Summary bar graph of the changes in V_m before and after application of KCl and NS1619. The dotted line represents resting V_m. The number in the parenthesis represents the number of vessels used in the study. Note that V_m reached baseline as soon as the drug is washed out. Error bar represents the standard error of the mean. Sec = seconds.

Table 1: Settings of electrometer to measure electrode resistance.

Table 2: Reagents used in the preparation of low calcium and normal physiological salt solution.

DISCUSSION:

This article provides the necessary steps on how to use a sharp microelectrode impalement method to record V_m from a cannulated vessel preparation. This method is widely used, and offers high-quality, consistent recordings of V_m that answer a wide range of experimental questions.

Some critical considerations and troubleshooting steps are described here to ensure success of the method. The quality of the microelectrode (its sharpness and resistance) and the cellular process it penetrates influence the stability and accuracy of V_m. If the signal continuously drifts or is above or below the amplifier's recording range, it is most likely that the electrode is blocked, or the tip is broken. If the impalement is only partial, insufficient, or damages the cell, the recorded potential climbs back to 0 mV resulting in an unstable signal or no signal. In some cases, the contents of the cell can also block the very high resistance electrode. All these problems can be overcome by merely replacing the electrode with a new one.

For successful impalement, one must ensure that the total resistance of the cell membrane is unaltered, and the measured membrane potential is stable with no leaks between the electrode and the membrane. It is critical that the electrode is advanced towards the cell by a stable micromanipulator. When advanced to within a few micrometers of the cell, the tip potential will change resulting in a slight deflection in the detected voltage. To avoid damaging the tip of the electrode or stretching of the cell membrane, rapid advancement of the electrode is required. Successful impalement is characterized by a rapid drop in membrane potential followed by a stabilization around the resting potential of the membrane. If the potential reading fluctuates, it is possible that the tip of the electrode has disrupted the membrane causing sodium to leak into

the cell and potassium to leak out of the cell, resulting in progressive depolarization. Another problem in this method is that junction potentials and electrode tip potentials can add an unnecessary artifact to the V_m recording. Junction potentials occur when differing conductors come into contact. There are two different types of junction potentials, liquid-metal, and liquid-liquid. Liquid-metal junctions are formed when the tip of the probe contacts the electrolyte in the micropipette. Liquid-liquid junctions, also called liquid junction potential, occur when two solutions of varying concentrations come into contact. The diffusion of the ions between the solutions contributes to the development of the potential. Besides, the properties of the glass electrode tip interacting with liquid can generate tip potentials. To minimize the junction as well as tip potentials, zeroing the measured potential in the bath could reduce the unwanted bias. During impalement, one cannot rule out the possibility that endothelial, rather than smooth muscle, V_m could inadvertently be sampled in some experiments. However, studies indicate that the endothelium and VSMC layers are electrically coupled in small arterioles and exhibit similar V_m responses[23].

Ultimately, three steps in this process are critical to achieving successful recordings. First, vessels must be appropriately prepared, ensuring that they are not damaged in the process. Second, microelectrodes must be pulled to the right electrical properties. Finally, rapid impalement of the membrane without breaking the tip is crucial for accurate results. The investigator must understand electrophysiology, how to create viable microelectrodes, and how to set up and use an electrometer.

Despite its importance, this method has various limitations. First, the aggregate cost to procure all the equipment is high (~$30–40,000). Second, fresh vessels are needed for all these experiments; hence an animal is euthanized for each experiment, adding to the overall cost. Third, dissection of cerebral arteries, cannulation of the vessels is tedious, expensive and has a learning curve. Fourth, preparing microelectrodes, impalement, and recording V_m requires a thorough understanding of the electrophysiology. Finally, to establish this set up in the lab requires dedicated staff, time and effort.

V_m is an essential electrophysiological property that determines the vascular tone and thus blood flow to an organ. V_m could be altered by several vasoactive chemicals that are released from neurons, endothelium and blood components. While vasoconstrictors depolarize the membrane, dilators hyperpolarize the membrane. Several proteins including K^+ channels, VGCC, sodium potassium ATPase, Ca^{2+} ATPase, Cl^- channels, store-operated and stretch channels regulate the V_m. Alterations in any of these proteins in disease conditions could potentially alter V_m and thus vascular tone and blood flow. The microelectrode impalement method is useful to record resting as well as changes in V_m in response to vasoconstrictors and dilators. So, this method could be used reliably to understand normal and altered V_m associated with disease, and may be useful in the development of pharmacological agents designed to modulate V_m, vascular tone and blood flow.

ACKNOWLEDGMENTS:

This work was supported in part by grants from the Intramural support research program (IRSP)

from UMMC, AHA Scientist Development Grant (13SDG14000006) awarded to Mallikarjuna R. Pabbidi.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Nelson, M. T., Quayle, J. M. Physiological roles and properties of potassium channels in arterial smooth muscle. *American Journal of Physiology.* **268** (4 Pt 1), C799-822, (1995).

2 Hughes, A. D. Calcium channels in vascular smooth muscle cells. *Journal of Vascular Research.* **32** (6), 353-370, (1995).

3 Large, W. A., Wang, Q. Characteristics and physiological role of the Ca(2+)-activated Cl- conductance in smooth muscle. *American Journal of Physiology.* **271** (2 Pt 1), C435-454, (1996).

4 Nelson, M. T., Conway, M. A., Knot, H. J., Brayden, J. E. Chloride channel blockers inhibit myogenic tone in rat cerebral arteries. *Journal of Physiology.* **502** (Pt 2), 259-264, (1997).

5 Yamazaki, J. et al. Functional and molecular expression of volume-regulated chloride channels in canine vascular smooth muscle cells. *Journal of Physiology.* **507** (Pt 3), 729-736, (1998).

6 Gibson, A., McFadzean, I., Wallace, P., Wayman, C. P. Capacitative Ca2+ entry and the regulation of smooth muscle tone. *Trends in Pharmacol Sciences.* **19** (7), 266-269, (1998).

7 Davis, M. J., Donovitz, J. A., Hood, J. D. Stretch-activated single-channel and whole cell currents in vascular smooth muscle cells. *American Journal of Physiology.* **262** (4 Pt 1), C1083-1088, (1992).

8 Setoguchi, M., Ohya, Y., Abe, I., Fujishima, M. Stretch-activated whole-cell currents in smooth muscle cells from mesenteric resistance artery of guinea-pig. *Journal of Physiology.* **501** (Pt 2), 343-353, (1997).

9 Shelly, D. A. et al. Na(+) pump alpha 2-isoform specifically couples to contractility in vascular smooth muscle: evidence from gene-targeted neonatal mice. *American Journal of Physiology-Cell Physiology.* **286** (4), C813-820, (2004).

10 Coca, A., Garay, R. *Ionic Transport in Hypertension New Perspectives*. (Taylor & Francis, 1993).

11 Harder, D. R. Pressure-dependent membrane depolarization in cat middle cerebral artery. *Circulation Research.* **55** (2), 197-202, (1984).

12 Knot, H. J., Nelson, M. T. Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure. *Journal of Physiology.* **508** (Pt 1), 199-209, (1998).

13 Nelson, M. T., Patlak, J. B., Worley, J. F., Standen, N. B. Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *American Journal of Physiology.* **259** (1 Pt 1), C3-18, (1990).

14 Harder, D. R., Sperelakis, N. Action potentials induced in guinea pig arterial smooth muscle by tetraethylammonium. *American Journal of Physiology.* **237** (1), C75-80, (1979).

15 Nystoriak, M. A. et al. Fundamental increase in pressure-dependent constriction of brain parenchymal arterioles from subarachnoid hemorrhage model rats due to membrane depolarization. *American Journal of Physiology-Heart and Circulatory Physiology.* **300** (3), H803-

812, (2011).
16 von der Weid, P. Y., Lee, S., Imtiaz, M. S., Zawieja, D. C., Davis, M. J. Electrophysiological properties of rat mesenteric lymphatic vessels and their regulation by stretch. *Lymphatic Research and Biology.* **12** (2), 66-75, (2014).
17 Harder, D. R. Comparison of electrical properties of middle cerebral and mesenteric artery in cat. *American Journal of Physiology.* **239** (1), C23-26, (1980).
18 Harder, D. R. Heterogeneity of membrane properties in vascular muscle cells from various vascular beds. *Federation Proceedings.* **42** (2), 253-256, (1983).
19 Cogolludo, A. et al. Serotonin inhibits voltage-gated K+ currents in pulmonary artery smooth muscle cells: role of 5-HT2A receptors, caveolin-1, and KV1.5 channel internalization. *Circulation Research.* **98** (7), 931-938, (2006).
20 Ganitkevich, V., Isenberg, G. Membrane potential modulates inositol 1,4,5-trisphosphate-mediated Ca2+ transients in guinea-pig coronary myocytes. *Journal of Physiology.* **470**, 35-44, (1993).
21 Yamagishi, T., Yanagisawa, T., Taira, N. K+ channel openers, cromakalim and Ki4032, inhibit agonist-induced Ca2+ release in canine coronary artery. *Naunyn-Schmiedeberg's Archives of Pharmacology.* **346** (6), 691-700, (1992).
22 Okada, Y., Yanagisawa, T., Taira, N. BRL 38227 (levcromakalim)-induced hyperpolarization reduces the sensitivity to Ca2+ of contractile elements in caninse coronary artery. *Naunyn-Schmiedeberg's Archives of Pharmacology.* **347** (4), 438-444, (1993).
23 Xia, J., Little, T. L., Duling, B. R. Cellular pathways of the conducted electrical response in arterioles of hamster cheek pouch in vitro. *American Journal of Physiology.* **269** (6 Pt 2), H2031-2038, (1995).
24 Jaggar, J. H., Porter, V. A., Lederer, W. J., Nelson, M. T. Calcium sparks in smooth muscle. *American Journal of Physiology-Cell Physiology.* **278** (2), C235-256, (2000).

Figure 1

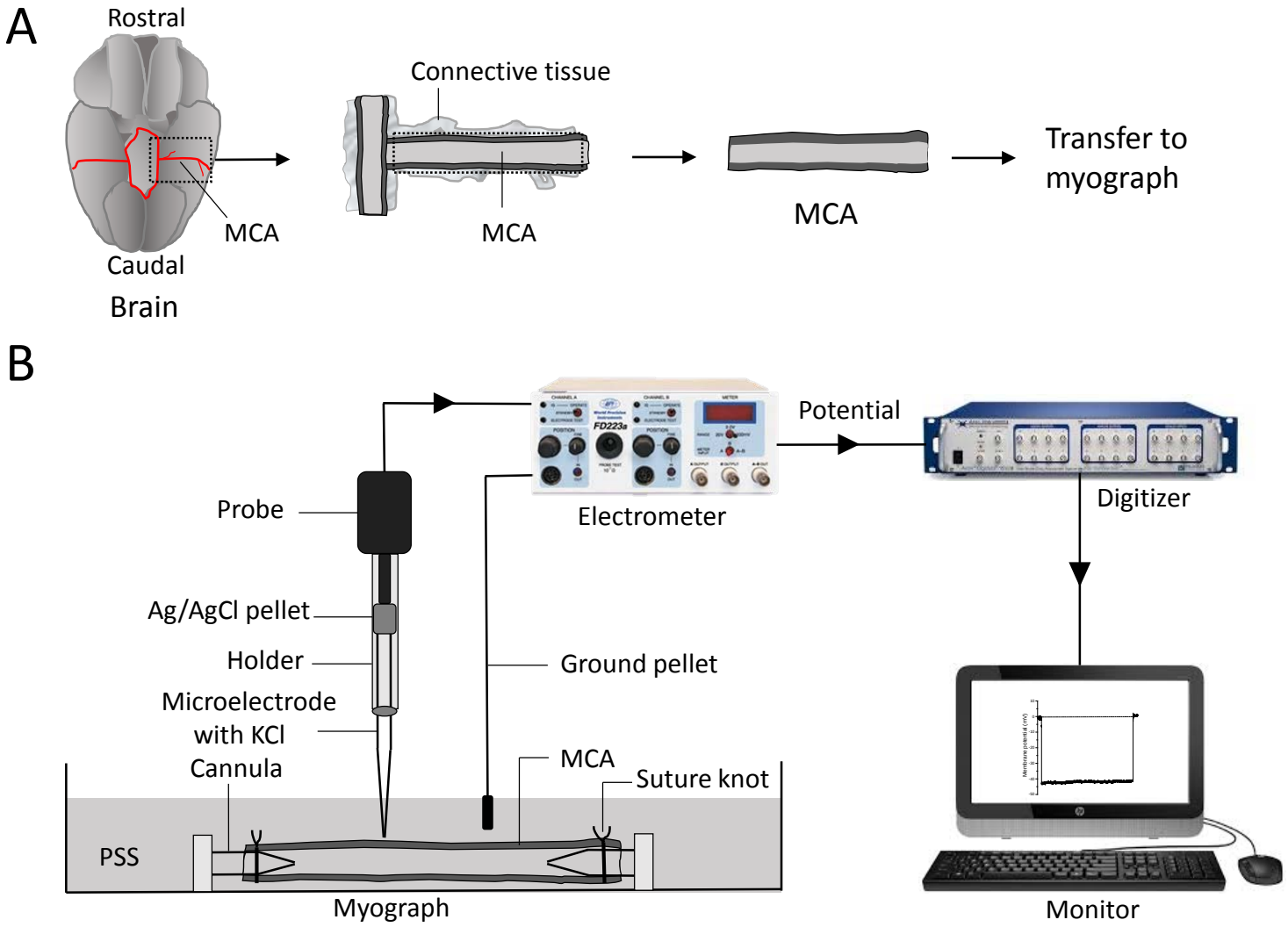


Figure 2

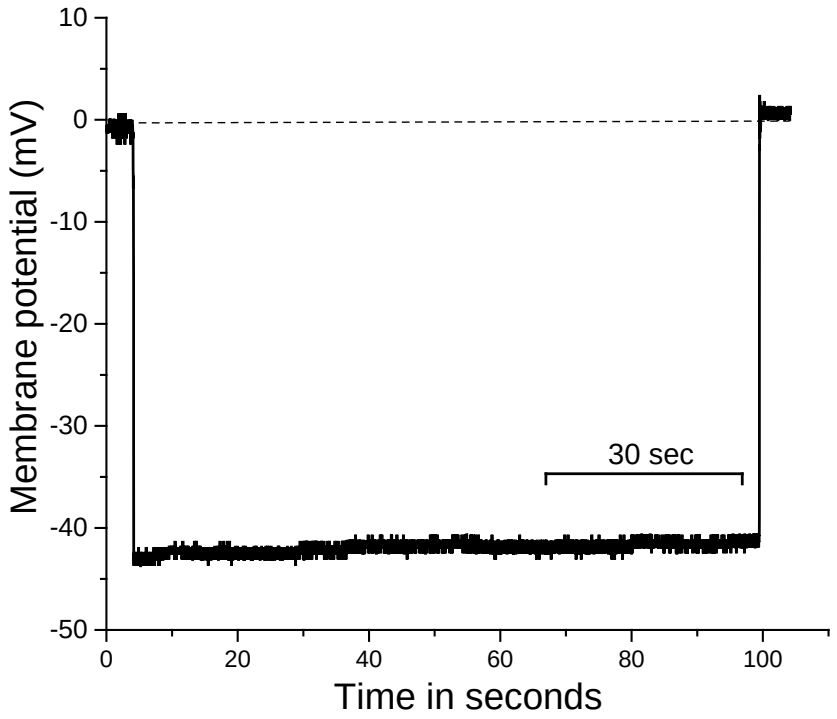
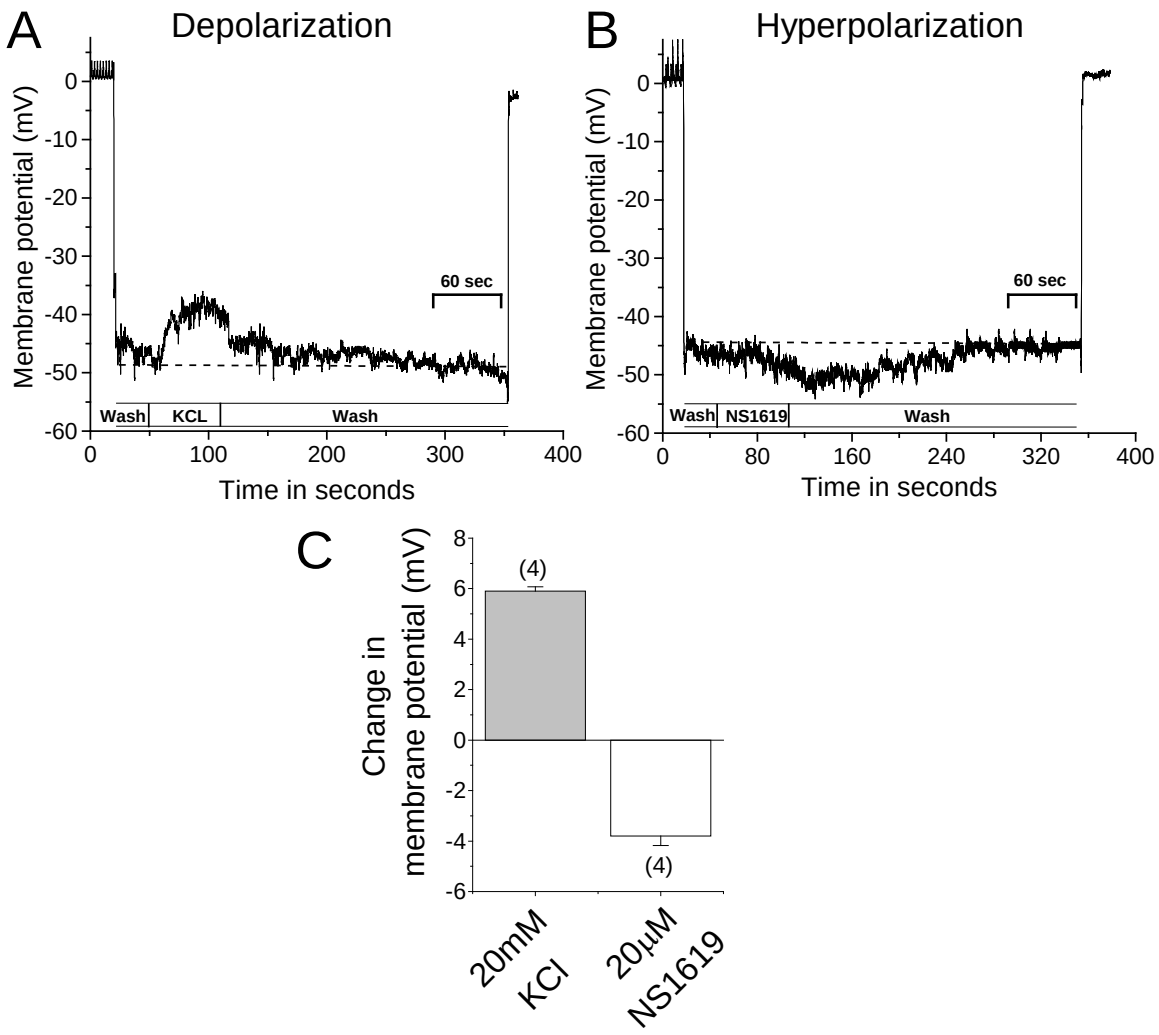


Figure 3



Settings of electrometer to measure electrode resistance

Meter Input	Channel A or B
Position toggle	In
Meter range	toggle to 200 mV
Electrode	In the bath
Mode	Operate to Electrode test
Meter indication	1 mV/MΩ

Table 2

	Chemicals	low calcium PSS mM	Normal PSS mM
1	NaCl	119.00	119.00
2	KCl	4.70	4.70
3	$MgSO_4$	1.17	1.17
4	$CaCl_2$	0.05	1.60
5	HEPES	5.00	5.00
6	Glucose	10.00	10.00
7	NaH_2PO_4	1.18	1.18
8	$NaHCO_3$	18.00	18.00
		pH: 7.4	pH: 7.4

Company	Catalog
Sigma	S7653
Sigma	P4504
Sigma	M7506
Sigma	C3881
Sigma	H7006
Sigma	G7021
Sigma	S0751
Sigma	S5761

Name	Company	Catalog
Dissection instruments		
Aneshetic Vaporiser	Parkland scientific	V3000PK
Dissection microscope	Nikon Instruments Inc., NY	Eclipse Ti-S
Kleine Guillotine Type 7575	Harvard Apparatus, MA	73-198
Littauer Bone Cutter	Fine science tools	16152-15
Moria MC40 Ultra Fine Forceps	Fine science tools	11370-40
Surgical scissors Sharp-Blunt	Fine science tools	14008-14
Suture	Harvard Apparatus	72-3287
Vannas Spring Scissors	Fine science tools	15018-10
Electrophysiology Instruments		
Charge-coupled device camera	Qimaging, , BC	Retiga 2000R
Differential electrometer amplifier	WPI	FD223A
In-line pressure transducer	Harvard Apparatus, MA	MA1 72-4496
Micromanipulator	Thor labs	PCS-5400
Microelectrodes	Warner Instruments LLC, CT	G200-6,
Micro Fil (Microfiber syringe)	WPI	MF28G67-5
Microelectrode holder	WPI	MEH1SF
Myograph	Living Systems Instrumentation, VT	CH-1-SH
Puller	Sutter Instrument, San Rafael, CA	P-97
Vibration-free table	TMC	3435-14
Softwares		
Clampex 10	Molecular devices	
p Clamp 10	Molecular devices	
Imaging software	Nikon, NY	NIS-elements
Chemicals		
NaCl	Sigma	S7653
KCl	Sigma	P4504
$MgSO_4$	Sigma	M7506
$CaCl_2$	Sigma	C3881
HEPES	Sigma	H7006
Glucose	Sigma	G7021
NaH_2PO_4	Sigma	S0751
$NaHCO_3$	Sigma	S5761

Commemts

jove JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Microelectrode impalement method to record membrane potential from cannulated middle cerebral artery.

Author(s): Joey T. Reed, Sumit P. Sontakke, Mallikarjuna R. Pabbidi

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Mallikarjuna R. Pabbidi, DVM, Ph.D.

Department: Pharmacology and Toxicology

Institution: University of Mississippi Medical Center

Article Title: Microelectrode impalement method to record membrane potential from cannulated middle cerebral artery

Signature: [signature] Date: 09/06/2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

March 8th , 2019
Alisha Dsouza
Senior Review Editor
JoVE

Dear Dr. Dsouza,

Enclosed is a revised version of our manuscript (JoVE59072R1) "Microelectrode impalement method to record membrane potential from cannulated middle cerebral artery." We thank the reviewers for the valuable critiques. We have addressed all the major and minor concerns raised by the reviewers. Please find the responses for the respective queries 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. **Response:** Reviewed and ensured there are no spelling or grammar issues.
2. Please provide an email address for each author. **Response: Provided on title page, line 11.**
3. Please expand your Introduction to include the following: advantages of the method over alternative techniques with applicable references to previous studies and Information that can help readers to determine if the method is appropriate for their application. **Response: Expanded introduction on page 2 from lines 129 to 138.**
4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. **Response: Included on page 3 from lines 149 to 153.**
5. 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: Warner Instruments LLC, Sutter Instrument, MicroFil (WPI), Clampex 10, molecular devices, Living Systems Instrumentation, Milli-Q, Nikon Instruments Inc., Harvard Apparatus, etc. **Response: All commercial language is deleted.**
6. 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. Please refrain from using bullets, dashes, or indentations. **Response: Adjusted the numbering.**
7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion. **Response: Revised the protocol accordingly.**
8. A schematic of the equipment and the setup as Figure 1 would greatly aid in the protocol.

Response: We revised the Figure 1 accordingly. We have included a schematic representation of the equipment and also explained in the figure legend and in results section.

9. Lines 191-193: Please specify what animals are used here. Are animals anesthetized before the procedure? Also, please describe how the surgical procedure is done and specify all surgical tools used. **Response: Specified the animal, anesthesia and surgical procedure on page 4 from lines 211 to 216. All surgical tools are provided in the table of materials.**

10. Please include single-line spaces between all paragraphs, headings, steps, etc. **Response: Included.**

11. 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. **Response: Highlighted.**

12. 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. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia. **Response: Highlighted only imperative sentences.**

13. 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. **Response: Highlighted the details also.**

14. Figure 1: Please change "Agcl" to "AgCl". **Response: Changed.**

15. Figure 2: Please include an x-axis and explain what the dotted line represents. **Response: included and explained on page 7 from line 311 to 313.**

16. Figure 3: Please include a space between numbers and their corresponding units (100 nM). Please change the time unit "sec" to "s". Please define the error bars and the asterisk symbol in the figure legend. **Response: This figure is deleted in the revised version of the article.**

17. Table 1: Please include a space between numbers and their corresponding units (200 mV, 1 mV/MΩ). Please remove the period after "mV/MΩ" in the last row. **Response: Included.**

18. Table 2: Please apply subscript formatting to the number "2" in CaCl2. **Response: Applied.**

19. Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the name of material/equipment. **Response: Revised the Table of Materials accordingly.**

20. References: Please do not abbreviate journal titles. **Response: Complete Journal title is provided on page 9-10.**

Reviewer #1:

Manuscript Summary:

This is a well-thought out protocol paper investigating membrane potentials in whole middle cerebral arteries. This is very beneficial for the vascular community taking this one step further from cell culture to essentially an organ culture patch. Utilizing electrophysiological derived techniques, the authors are able to record membrane potentials in whole arteries. Only minor concerns exist.

Major Concerns: NA

Minor Concerns:
Line 108/109, Can this technique be done in other isolated artery preparations? **Response: Yes, previous studies have shown and used this method in isolated mesenteric arterial strips, lymphatic vessels (Harder and Sperelakis 1979 and Von der Weid et al., 2014).**
Line 154, catalog number of MicroFil **Response: Catalog number is provided in the table of materials in table 2.**
Line 251 and others in the representative results, please correlate to the fig. **Response: Revised results to correlate with the figures on page 6-7 from line 297 to 306.**
Line 254 did you show how to isolate MCA. That would be helpful utilizing an illustration. **Response: An illustration showing the dissection of MCA is incorporated into the figure 1 and shown as figure 1A. Description is provided in the legend on page 6 from line 298 to 300 as well as in the results on page 6 from line 263 to 269.**

Reviewer #2:
Manuscript Summary:
This manuscript should provide a useful adjunct for the JOVE video. However, the authors should address the following requests for clarification.

Major Concerns:

1. Line 137 - The electrometer manufacturer should be mentioned here. **Response: Mentioned in table of materials table 2.**

2. Line 150 - Insert "(when filled with 3M KCl)" after "resistances". **Response: Inserted on page 3 from line 170 to 171.**
3. Line 195 - A comment regarding setting of the axial length of the isolated artery would be useful as this will likely contribute to impalement success rate. **Response: Commented on page 5 from line 224 to 225.**
4. Line 206 - change "soaked" to "immersed". **Response: changed on page 5 from line 230.**
5. Line 212 - The brand/type of manipulator should be mentioned here. **Response: Mentioned in table of materials table 2**
6. Line 227 - Insert "depending on the level of intravascular pressure or other excitatory or inhibitory stimuli" after "-75mV". **Response: Inserted on page 5 from line 251 to 253.**
7. Line 227 - Delete "resting potential" and insert "transmembrane potential difference" . **Response: Inserted on page 5 from line 251 to 253**
8. Line 250 - Insert "the" after "to". **Response: Inserted on page 6 line 275.**
9. Lines 283-289, lines 339-350, and Figure 3 - The topic of this methods article is measurement of vascular smooth muscle membrane potential in intact arteries. The data in figure 3 are patch clamp data using isolated cells. These results are out of context, and the relevance and importance of the discussion (lines 339-350) are vague and speculative. These data and this discussion should be omitted. **Response: Deleted this figure. Both results and discussion are**

revised accordingly.
10. Lines 362 - 364 - This sentence is somewhat vague. Suggest changing to "Thus, this method can be used reliably to understand normal and altered Vm associated with disease, and may be useful in the development and application of pharmacological agents designed to modulate Vm, vascular tone and blood flow. **Response: Changed on page 8 from line 393 to 395.**

Reviewer #3:
Manuscript Summary:
Joey T et al. used microelectrode impalement method to record membrane potential from cannulated middle cerebral artery and it is really important in electrophysiology study. The method is useful for researchers to start the experiment. However, some problems should be addressed and additional data should be shown to clarify this completely method.

Major Concerns:
1: For equipment, I strongly suggest author provide a picture to show the equipment and point out which one is what? **Response: Modified Figure1 accordingly. Equipment is shown in the figure 1.**
2: It is better to provide parameter of P-97 puller for pull 80-120 MΩ resistances microelectrode. **Response: Parameters are provided on page 3 line 174 to 175.**
3: In representative results, authors should show the resting membrane potential when the microelectrode come into the vascular smooth muscle cell, but should show some positive response like high K+ solution-induced depolarization and K+ channel activation-induced hyperpolarization. **Response: 20mM KCl is used to induce depolarization and 20µM NS1619 a BK channel opener is used to elicit hyperpolarization. Both representative traces and data is shown in figure 3. Figure legends, and result section are also modified accordingly.**

4: In representative results, it is strange to show a result of perforated whole-cell patch clamp. The result is completely not related to the microelectrode method. **Response: This data is deleted in the revised version of the article.**

Minor Concerns:
Some redundant blanks are in the text: line 284, 256…. **Response: Deleted.**

Between unit and number should have a blank: line 260, 269, 270, 286…… **Response: Included space between unit and number across the manuscript.**