Journal of Visualized Experiments

Electrophysiological Recordings of Single-Cell Ion Currents Under Well-Defined Shear Stress

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59776R1
Full Title:	Electrophysiological Recordings of Single-Cell Ion Currents Under Well-Defined Shear Stress
Keywords:	Ion Channels; Shear Stress; Vascular Endothelium; Flow Chamber; electrophysiology; Mechanosensitive
Corresponding Author:	Ibra S Fancher University of Illinois at Chicago Chicago, IL UNITED STATES
Corresponding Author's Institution:	University of Illinois at Chicago
Corresponding Author E-Mail:	ifancher@uic.edu
Order of Authors:	Ibra S Fancher
	Irena Levitan
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.	Chicago, IL, USA

TITLE:

Electrophysiological Recordings of Single-Cell Ion Currents Under Well-Defined Shear Stress

AUTHORS AND AFFILIATIONS:

Ibra S. Fancher[1], Irena Levitan[1]

[1]Department of Medicine, Division of Pulmonary, Critical Care, Sleep and Allergy, University of Illinois at Chicago, Chicago, IL, USA

Corresponding author:

Ibra S. Fancher (ifancher@uic.edu)

Email address of co-author:

Irena Levitan (levitan@uic.edu)

KEYWORDS:

ion channels, shear stress, vascular endothelium, flow chamber, electrophysiology, mechanosensitive

SUMMARY:

The goal of this protocol is to describe a modified parallel plate flow chamber for use in investigating real time activation of mechanosensitive ion channels by shear stress.

ABSTRACT:

Fluid shear stress is well known to play a major role in endothelial function. In most vascular beds, elevated shear stress from acute increases in blood flow triggers a signaling cascade resulting in vasodilation thereby alleviating mechanical stress on the vascular wall. The pattern of shear stress is also well known to be a critical factor in the development of atherosclerosis with laminar shear stress being atheroprotective and disturbed shear stress being pro-atherogenic. While we have a detailed understanding of the various intermediate cell signaling pathways, the receptors that first translate the mechanical stimulus into chemical mediators are not completely understood. Mechanosensitive ion channels are critical to the response to shear and regulate shear-induced cell signaling thereby controlling the production of vasoactive mediators. These channels are among the earliest activated signaling components to shear and have been linked to shear-induced vasodilation through promoting nitric oxide production (e.g., inwardly rectifying K^+ [Kir] and transient receptor potential [TRP] channels) and endothelium hyperpolarizing factor (e.g., Kir and calcium-activated K^+ [KCa] channels) and shear-induced vasoconstriction through an undetermined mechanism that involves piezo channels. Understanding the biophysical mechanism by which these channels are activated by shear forces (i.e., directly or through a primary mechano-receptor) could provide potential new targets to resolve the pathophysiology associated with endothelial dysfunction and atherogenesis. It is still a major challenge to record flow-induced activation of ion channels in real time using electrophysiology. The standard methods to expose cells to well-defined shear stress, such as the cone and plate rheometer and closed parallel plate flow chamber do not

allow real time study of ion channel activation. The goal of this protocol is to describe a modified parallel plate flow chamber that allows real time electrophysiological recording of mechanosensitive ion channels under well-defined shear stress.

INTRODUCTION:

Hemodynamic forces generated by the blood flow are well known to play major roles in endothelial and vascular function[1,2]. It is also well known that several types of ion channels acutely respond to changes in shear stress[3-5] leading to the hypothesis that ion channels can be primary shear stress sensors. More recently, we and others showed that mechanosensitive ion channels play critical roles in several shear-stress sensitive vascular functions, including the vasoactive response to shear stress[6-8], and developmental angiogenesis[9]. The mechanisms of the shear-stress sensitivity of ion channels, however, are almost totally unknown. This gap of knowledge is likely to be due to the technical difficulty of performing electrophysiological recordings under well-defined shear stress. In this article, therefore, we provide a step by step detailed protocol routinely performed in our lab to achieve this goal[6,7,10,11].

The overall goal of this method is to allow the real-time investigation of ion channel mechanoactivation under well-defined shear stress in the physiological range. This is achieved by modifying a standard parallel plate flow chamber to allow an electrophysiological pipette to be lowered into the chamber and access cells grown on the bottom plate during the real time exposure to flow, providing a unique approach to achieve this goal[6,7,11]. In contrast, standard parallel plate flow chambers, described in prior publications can be used for the real time imaging analysis of cells exposed to shear forces[12] or other non-invasive approaches[13,14] but not for electrophysiology. Similarly, the cone and plate apparatus, another powerful approach to expose cells to shear stress[15,16] is also not suitable for electrophysiological recordings. Thus, these flow devices do not allow the investigation of shear stress sensitivity of ion channels. The difficulty in performing electrophysiological recordings under flow is the main reason for the paucity of information about the mechanisms responsible for these crucial effects.

In terms of the alternative approaches to achieve the same goal, there are none that are as accurate or controlled. Some earlier studies attempted to record ion channel activity under flow by exposing cells to a stream of liquid coming from another pipette brought to the vicinity of a cell from above[17,18]. This is highly non-physiological, as the mechanical forces generated under these conditions have little in common with the physiological profiles of shear stress in the blood vessels. Similar concerns apply to the attempts to simulate physiological shear stress by perfusion of open chambers. As discussed in detail in our earlier study[10], an open liquid-air interface creates multiple disturbances and recirculation, which are non-physiological. To address all these concerns, we have designed a modified parallel plate (MPP) flow chamber, also referred to as the “minimally invasive flow device” in our earlier studies[6,7,10,11], made from acrylic and extensively used in our lab. However, in spite of the fact that the original description of the design has been published almost 20 years ago and is the only flow device that allows performing electrophysiological recordings under well-defined shear stress, this methodology has not been adopted by other labs and there are only very few studies that attempt to record currents under flow. We believe, therefore, that providing a detailed description for using the

MPP flow chamber will be of great help to researches who are interested in mechanosensitive ion channels and vascular biology.

PROTOCOL:

The use of animals in our studies is approved by the University of Illinois at Chicago Animal Care Committee (#16-183).

1. Assembly of the modified parallel plate flow chamber

NOTE: Please refer to **Table 1** and **Figure 1** for MPP flow chamber piece IDs. Please refer to **Figure 1** for a schematic detailing the orientation of chamber pieces for assembly.

1.1. To adhere the rectangular cover glass, piece D, to the bottom of piece C, first make the silicone elastomer solution by thoroughly mixing 500 µL silicone elastomer curing agent into 5 mL of silicone elastomer base.

1.2. Apply a thin layer of the silicone elastomer solution around the edges of the rectangular space of piece C and gently place the rectangular cover glass piece D directly on the elastomer solution such that piece D completely covers the open rectangular space of piece C. Carefully wipe away excess silicone elastomer solution.

1.3. Repeat step 1.2 for adhering the rectangular cover glass, piece F, to the bottom of piece E and allow the silicone elastomer solution to cure over night at room temperature.

NOTE: Once cured, the rectangular cover glass will remain adhered for up to six months before needing to be replaced.

1.4. Beginning with the bottom chamber piece, piece E, assemble the MPP flow chamber by sequentially placing each piece on top of the previous in the following order: piece E (bottom), piece C, piece B, piece A (top).

1.5. Align the screw holes of each piece at the corners and tightly screw the pieces together to prevent leaks from occurring while administering flow to the MPP flow chamber.

2. Cell preparation and seeding into the MPP flow chamber

NOTE: Follow steps 2.1–2.7 for cultured endothelial cells. Follow the method detailed in steps 2.8–2.14 for isolating endothelial cells from the mouse mesenteric arterial arcade and preparation of freshly isolated endothelial cells.

2.1. In a 6-well plate, place four to five 12 mm cover glass circles/well and seed cells between 30% and 50% confluency such that single cells can be accessed for electrophysiological recordings.

2.2. Incubate cells under standard culture conditions (5% CO_2, 37 °C) for no less than 2 h to allow cells to adhere and no more than 24 h as endothelial cells in authors' experience become very flat and difficult to patch when seeded at sub-confluency for more than 24 h.

2.3. Remove a cover glass containing adhered cells from a well of the 6-well plate, quickly rinse in phosphate-buffered saline (PBS), and transfer to a 35 mm Petri dish containing 2 mL electrophysiological bath solution (**Table 2**) prior to transfer to the MPP flow chamber.

2.4. Transfer the cover glass circle to the rectangular cover glass, piece D, which is adhered to piece C of the MPP flow chamber being sure that adequate solution stays on the cover glass so that cells to not become exposed to air. Add the desired bath solution (~250 µL) to the cells so that the cover glass circle and cells are completely submerged in solution.

2.5. Position the cover glass circle such that it rests in the half closest to the vacuum reservoir side so that cells will be in line with the slit openings of piece B. Ensure that the glass cover circle adheres to piece D through solution-glass adhesion so that application of flow to the chamber does not disrupt the position of the cover glass circle.

2.6. Assemble the MPP flow chamber by screwing the pieces together in the appropriate order, as described in steps 1.4 and 1.5 and as shown in **Figure 1**. Transfer the chamber to the microscope stage and immediately perfuse the chamber with bath solution such that solution reaches the vacuum reservoir for aspiration (~10 mL).

2.7. Identify a healthy cell for the experiment by identifying a cell with a dark border and obvious nucleus. Avoid cells that appear to be blebbing or cells that are in contact with other cells.

NOTE: In the authors' laboratory, human aortic endothelial cells and primary mouse mesenteric endothelial cells in culture are used. However, any other type of adherent cell that is of interest to specific research needs can be used in the same way.

2.8. Wash the isolated arterial arcade in dissociation solution (**Table 2**). Transfer the arcade to a 2 mL centrifuge tube containing 2 mL of pre-warmed (37 °C) dissociation solution (recipe for dissociation solution shown in **Table 2**) containing neutral protease (0.5 mg/mL) and elastase (0.5 mg/mL). Incubate for 1 h at 37 °C with gentle shaking every 10 min.

2.9. Remove 1 mL of the neutral protease/elastase dissociation solution and add 1 mg/mL collagenase type 1. Return the collagenase solution to the solution containing the arteries for a final collagenase type 1 concentration of 0.5 mg/mL. Incubate for 2−3 min at 37 °C.

2.10. Using 5 grade forceps, quickly move the arcade onto a 35 mm diameter Petri dish containing a 750-µL drop of fresh, chilled dissociation solution. Further dissociate the tissue by

using two 20 G syringe needles to mechanically liberate endothelial cells from enzymatically digested arteries.

2.11. Using a 9" disposable Pasteur glass pipet, triturate the cell solution 10x before transferring the cells to a new 1.5 mL centrifuge tube using the glass pipet.

2.12. Wash the Petri dish with another 750 µL of dissociation solution and transfer to the same tube. Using the glass pipette, further mechanically disperse the cells by pipetting at least 10x to create a single cell suspension being careful not to introduce bubbles that may damage endothelial cell integrity.

2.13. Add 750 µL of the endothelial cell suspension (~500−1,000 cells) directly to piece D of the MPP flow chamber on the half closest to the reservoir vacuum side. Allow the endothelial cells to adhere between 45 min and 1 h at room temperature.

2.14. Assemble the MPP flow chamber by screwing the pieces together in the appropriate order as described in steps 1.4 and 1.5 and as shown in **Figure 1**. Transfer the chamber to the microscope stage and immediately perfuse the chamber with bath solution such that solution reaches the vacuum aspiration (~10 mL). Identify accessible endothelial cells by their rough and round phenotype[19,20].

NOTE: A variety of digestion methods and enzyme cocktails have been used to isolate endothelial cells from different arterial beds. See **Table 3** for detailed descriptions of protocols that have been used by a variety of investigators to isolate endothelial cells for patch clamp electrophysiology of mechanosensitive ion channels. These methods are likely suitable for use in combination with the MPP flow chamber.

3. Controlling shear stress to the MPP flow chamber for electrophysiological recordings of shear-activated mechanosensitive ion channels

3.1. Set-up a gravity perfusion system by connecting a 30 mL graduated syringe cylinder to a 3-way luer lock fitted with microbore tubing (internal diameter: 0.05 in, outer diameter: 0.09 in) suited for insertion into the 3 mm diameter inlet hole of piece A of the MPP chamber.

3.2. Attach the graduated cylinder to the outer face of the Faraday cage surrounding the electrophysiology rig (**Figure 2**) using double-sided tape. Prior to inserting the tubing in the MPP chamber, pre-fill the syringe and tubing with bath solution (see **Table 2** for bath solution used for investigating inwardly rectifying K^+ channels in endothelial cells). Insert the tubing into the MPP flow chamber inlet hole of piece A.

3.3. Pre-fill the MPP flow chamber with solution such that solution is being removed in the vacuum reservoir. Stop flow to the chamber and refill the graduated cylinder to the top mark.

Calculate flow rates manually by allowing the solution to flow through the chamber and using a stop-watch to calculate mL/s at a given syringe cylinder height.

3.4. Raise or lower the syringe to alter flow, and thus shear in the chamber, and continue this process until a desired level of shear stress is found.

3.5. Calculate shear stress in a parallel chamber using the following equation[21]:

$$\tau = 6\mu Q/h^2w$$

where μ = fluid viscosity (g/cm·s), Q = flow rate (mL/s), and parallel plate chamber width (w = 2.2 cm) and height (h = 0.1 cm).

NOTE: In the current gravity perfusion system, at a syringe cylinder height (as measured from the top of the cylinder) of 57 cm above the microscope stage, the flow rate is 0.3 mL/s. The shear calculated in the chamber at this syringe cylinder height and flow rate is 0.7 dyn/cm^2. It should also be noted that other perfusion systems, such as a peristaltic pump, can be used to control flow to the MPP flow chamber. However, these devices may add unwanted turbulence and influence stability of the electrophysiology measurements under flow, therefore, using the gravity perfusion system described here is recommended.

3.6. Transfer an assembled chamber containing adhered cells to the microscope stage of the electrophysiology rig and insert the tubing pre-filled with bath solution into the hole of piece A. Simultaneously fill the chamber and wash the cells with 10 mL of bath solution by turning the 3-way luer lock such that solution flows to the chamber.

3.7. Once the desired patch configuration is successfully obtained allow channel currents to stabilize in a static bath at room temperature. Once currents have stabilized, apply shear in a step-wise fashion allowing increases in current to stabilize prior to the next step increase in shear stress.

NOTE: The authors find the most success with the perforated patch configuration when studying mechanoactivated ion channels in endothelial cells. To perform perforated whole-cell patch configurations, add 5 µL of a 60 mg/mL stock amphotericin B in dimethyl sulfoxide (DMSO) to 1 mL of 0.2 µm sterile filtered pipette solution. After generating a giga-ohm seal in the cell-attached configuration, perforated whole-cell patches form within 2−5 min.

3.8. Remove shear exposure to the cells by stopping flow to the chamber allowing mechanosensitive channel currents to return to baseline currents observed in the static bath.

3.9. Isolate mechanosensitive ion channel currents of interest by altering solution valence (e.g., 60 mM K^+ in bath solution with 0 Ca^{2+} in pipette solution to study inwardly rectifying K+ channels. **Table 2** shows example solution recipes) and/or pharmacological inhibition of potentially contaminating current sources.

REPRESENTATIVE RESULTS:

Multiple photographs showing different views of the MPP flow chamber on the microscope stage (upper panel) and a schematic representation of the MPP flow chamber (bottom panel) are shown in **Figure 1**. The schematic details the dimensions of the entire device and flow chamber. **Figure 2** shows a photograph of the gravity perfusion system to the MPP flow chamber in our laboratory (upper panel). Also shown is a schematic representation of the flow system (bottom panel) intended to highlight the steps which isolate the cells in the flow chamber from the rush of solution from the perfusion system and from the force of the vacuum removal of solution.

A hallmark of mechanosensitive ion channels is an abrupt return to baseline levels upon cessation of mechanical stimulus[3,6,7]. **Figure 3** shows as an example that removing the shear stress stimulus during electrophysiological recordings of Kir current from a freshly isolated endothelial cell results in a return to baseline currents initially recorded in a static bath. The return to baseline current levels after stopping flow to the MPP chamber occurred within ten seconds of flow cessation.

Whole-cell (WC) electrophysiological recordings, especially those taken from freshly isolated cells, often have obvious leak background currents that can mask channel activity. Some ion channels, such as those of the inwardly rectifying K^+ channel family, have biophysical properties that allow for subtracting the leak background current for more accurate analysis. **Figure 4** shows as an example the process from raw data (**Figure 4A**) to calculated and plotted linear outward leak (**Figure 4B**) to final, leak subtracted representative trace (**Figure 4C**). See a detailed explanation for calculating and subtracting leak from the raw perforated WC patch recording in the accompanying legend to **Figure 4**.

FIGURE AND TABLE LEGENDS:

Figure 1: MPP flow chamber and detailed schematic. Photographs of the assembled MPP flow chamber (upper panel) show the chamber on the microscope stage from three different views: the side as viewed during experiments (left), from the perfusion inlet (middle), and from the vacuum outlet (right) which is out of view in the photograph. The direction of flow is labeled in each. Notice that the ground wire can easily fit into one of the 2 mm slits when bent at a 90° angle. A detailed schematic (bottom) shows exact dimensions for replication of the apparatus.

Figure 2: The gravity perfusion system. A labeled photograph of the gravity perfusion system in our laboratory is shown in the upper panel. The two-step MPP flow chamber and gravity perfusion system are detailed in the bottom panel. The separation of the flow chamber containing cells and the two upper and lower reservoirs is highlighted. Step 1 allows solution to flow from the upper reservoir of piece B to piece D of the chamber where cells are seeded. Step 2 allows solution to flow from piece D down to the lower reservoir, piece F.

Figure 3: Representative traces of shear-induced current activation of Kir channels and return to baseline current levels upon removal of flow. A good positive control for mechanoactivation of ion channels is the return to baseline current levels initially observed in a static bath upon cessation of the mechanical stimulus. Inwardly rectifying K^+ (Kir) channel currents are activated within seconds by shear stress when gravity solution is allowed to flow to the MPP flow chamber. Upon cessation of flow to the chamber, Kir currents quickly return to baseline static levels observed prior to flow. A ramp of -140 mV to +40 mV was applied to the patch over 400 ms. The bath solution contained 60 mM K^+ and the reversal potential was ~-20 mV. The representative traces were generated from an endothelial cell freshly isolated from mouse mesenteric arteries.

Figure 4: Example of leak subtraction for accurate analysis of mechanoactivated Kir current. (**A**) Representative raw recording of Kir current from a primary mouse mesenteric endothelial cell with notable linear outward leak current (I_{leak}). A ramp of -140 mV to +40 mV was applied to the patch over 400 ms. The bath solution contained 60 mM K^+ and the reversal potential was ~-20 mV. (**B**) I_{leak} prevents analysis of real Kir channel activity. To subtract I_{leak}, first calculate the linear slope conductance of I_{leak} ($G_{slope} = (I_a-I_b)/(V_a-V_b)$). Multiply G_{slope} by corresponding voltages of the entire raw trace to plot I_{leak} on the raw data. The line should overlay the outward linear leak exactly. (**C**) Subtract the plotted I_{leak} from the entire trace so that the linear outward current is ~0 pA/pF and the real Kir current can be analyzed. Notice in panel C that the inward Kir current is approximately half that of the original raw data trace in panel A.

Table 1: Dimensions of the MPP (assembled and disassembled) and additional information specific to each part of the device.

Table 2: Examples of solutions with recipes used in the experiments.

Table 3: Methodology to study mechanosensitive ion channels using electrophysiological techniques.

DISCUSSION:

The vascular system is constantly exposed to active hemodynamic forces, which activate mechanosensitive ion channels[3,22] but the physiological roles of these channels in shear stress-induced mechanotransduction is only starting to emerge[4,6,8]. The mechanisms responsible for the mechanosensitivity of shear stress-activated channels remain unknown. The protocol detailed here describes the method for direct investigation of mechanosensitive ion channels exposed to laminar shear stress in real time.

The critical and essential step of this protocol is the use of a modified parallel plate flow chamber that has narrow openings to allow an electrophysiological pipette to be lowered into the chamber and access cells under flow. The general principle of this device is that if the openings are narrow enough, physiological shear stress (up to 15 dyn/cm^2) can be achieved without solution overflow due to surface tension forces[10]. In order to perform these experiments successfully, it is critical: (i) to seed cells in the area of the bottom plate that is

directly underneath the openings; (ii) establish a giga-ohm seal prior to the initiation of the flow or during very slow (<0.01 dyn/cm^2) background flow; (iii) maintain a stable seal while stepping up the flow. Dimensions of the four-piece acrylic apparatus used in our laboratory are provided along with detailed schematics (**Figure 1**) such that the MPP flow chamber and flow system (**Figure 2**) can be replicated for use in any laboratory investigating mechanosensitive ion channels. These dimensions can also be modified to increase the area seeded by cells and to change the height of the flow channel, which would change the relationship between the flow rate and the shear stress. A decrease in the height of the flow chamber would result in higher shear stress for the same flow rate, which could be beneficial for achieving higher shear stresses. The chamber can also be modified to include a flow separation barrier that induces a local region of recirculating disturbed flow[23].

The major advantages of using the MPP flow chamber include (1) real time investigation of shear-activated ion channels from endothelial cells in culture and cells freshly isolated from vascular tissue, (2) exposure of cells and the response of mechanosensitive ion channels to physiologically relevant levels of shear stress, and (3) easy assembly and disassembly with perfusion options for experimentation. With respect to other existing methods, the only other method that allows performing electrophysiological recordings under well-defined shear stress is seeding the cells inside a capillary end and inserting the recording pipette into the capillary opening, as was done in the early studies[3,22]. There are, however, multiple disadvantages compared to the method described here, such as the difficulty of seeding cells into capillaries, access to a very small number of cells that are close enough to the capillary end for the pipette to be able to reach them, and flow disturbances at the end of the flow channel (capillary in this case). Each of these disadvantages makes it difficult to perform electrophysiology under flow in cells cultured in the capillary opening and virtually impossible to patch or even seed cells freshly isolated from the vasculature. It is also impossible to introduce a step to generate an area of disturbed flow. All other existing methods that either employ open chambers[24,25] or puffing solutions directly on a cell[17,18] do not mimic the hemodynamic environment in the blood vessel.

The main limitation of this method, however, is a constraint to achieve shear stress at higher levels of the physiological range. Specifically, physiological shear stress levels have been estimated to reach up to 70 dyn/cm^2 in healthy arteries[26]. In contrast, the highest shear stress level that we could achieve in the current configuration of chamber was 15 dyn/cm^2, after which the surface tension forces become not sufficient to prevent the solution to spill out of the openings[10]. It is possible that decreasing the height of the flow channel might allow achieving higher shear stress levels. Maintaining a stable giga-ohm seal under high shear stress is another challenge but the success rate is reasonable with practice. We found that using perforated patch technique (including the antibiotic amphotericin B in the pipette solution as described above) yields more stable recordings than the standard whole cell configuration. Additionally, the MPP flow chamber and solutions used do not exactly replicate the shear of blood flow in arteries. Blood is a viscous, non-Newtonian fluid that we have not replicated in our in vitro experiments. Additionally, the chamber used here is a parallel chamber while shear stress in arteries is best calculated using the formula for shear in a cylinder.

There are important considerations and limitations for performing single-channel recordings under flow. This method is appropriate for a cell-attached configuration (pipette attached to the membrane of an intact cell), which yields stable seals. It is critical though to be aware that single channels whose activity is being recorded are not directly exposed to shear stress because they are shielded by the recording pipette, especially in the inside-out configuration[27]. We believe that the excised patch configurations are not the most reliable when used to test the sensitivity of ion channels to flow because the excised membrane is typically pulled into the recording pipette and thus is not exposed to a well-defined flow.

In terms of the type of cells that can be used in these experiments, vascular endothelial cells represent the most applicable cell type to studying shear stress and the mechanosensitive channels. Earlier studies focused primarily on cultured endothelial cells[3,28] that are easily available. We have tested and extended the use of this method to endothelial cells freshly-isolated from mouse resistance arteries[6,7]. Other cell types should definitely be considered. Vascular smooth muscle cells, for instance, can become exposed to shear stress during disease states where the endothelium has been damaged and removed[29,30]. This represents an intriguing area of study whereby mechanosensitive channels that reside in smooth muscle, which would otherwise be uninfluenced by shear stress, would now contribute to potentially pathophysiological disease mechanisms. Furthermore, the transfection of vehicle cell lines like HEK or CHO with the gene encoding the channel of interest is an excellent platform for the biophysical analyses of channels in combination with use of the MPP flow chamber.

It is also important to note that while the original intention for the MPP flow chamber was for the real time investigation of ion channel mechanoactivation, the application of the device may extend beyond this goal. Specifically, the same approach can be used for studies that use electrode sensors, such as a nitric oxide (NO) sensor to determine the release of NO in response to shear stress. Therefore, we provide a generalized methodology for those with interest in investigating mechanically regulated biological processes in real time and promote further modification of the MPP chamber to meet specific research needs for those studying mechanosensitive processes in a variety of fields.

ACKNOWLEDGEMENTS:

This work was funded by the National Heart, Lung, and Blood Institute (R01 HL073965, IL) and (T32 HL007829-24, ISF). The authors would also like to acknowledge the Scientific Machine Shop at the University of Illinois at Chicago for generating our latest MPP flow chambers.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Green, D. J., Hopman, M. T., Padilla, J., Laughlin, M. H., Thijssen, D. H. Vascular Adaptation to Exercise in Humans: Role of Hemodynamic Stimuli. *Physiological Reviews.* **97** (2), 495-528 (2017).

2. Gimbrone, M. A., Jr., Topper, J. N., Nagel, T., Anderson, K. R., Garcia-Cardena, G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Annals of the New York Academy of Sciences.* **902** 230-239; discussion 239-240 (2000).
3. Olesen, S. P., Clapham, D. E., Davies, P. F. Haemodynamic shear stress activates a K+ current in vascular endothelial cells. *Nature.* **331** (6152), 168-170 (1988).
4. Barakat, A. I., Lieu, D. K., Gojova, A. Secrets of the code: do vascular endothelial cells use ion channels to decipher complex flow signals? *Biomaterials.* **27** (5), 671-678 (2006).
5. Beech, D. J. Endothelial Piezo1 channels as sensors of exercise. *Journal of Physiology.* **596** (6), 979-984 (2018).
6. Ahn, S. J. et al. Inwardly rectifying K(+) channels are major contributors to flow-induced vasodilatation in resistance arteries. *Journal of Physiology.* **595** (7), 2339-2364 (2017).
7. Fancher, I. S. et al. Hypercholesterolemia-Induced Loss of Flow-Induced Vasodilation and Lesion Formation in Apolipoprotein E-Deficient Mice Critically Depend on Inwardly Rectifying K(+) Channels. *Journal of the American Heart Association.* **7** (5), (2018).
8. Rode, B. et al. Piezo1 channels sense whole body physical activity to reset cardiovascular homeostasis and enhance performance. *Nature Communications.* **8** (1), 350 (2017).
9. Li, J. et al. Piezo1 integration of vascular architecture with physiological force. *Nature.* **515** (7526), 279-282 (2014).
10. Levitan, I., Helmke, B. P., Davies, P. F. A chamber to permit invasive manipulation of adherent cells in laminar flow with minimal disturbance of the flow field. *Annals of Biomed Engineering.* **28** (10), 1184-1193 (2000).
11. Fang, Y. et al. Hypercholesterolemia suppresses inwardly rectifying K+ channels in aortic endothelium in vitro and in vivo. *Circulation Research.* **98** (8), 1064-1071 (2006).
12. Shetty, S., Weston, C. J., Adams, D. H., Lalor, P. F. A flow adhesion assay to study leucocyte recruitment to human hepatic sinusoidal endothelium under conditions of shear stress. *Journal of Visualized Experiments.* 10.3791/51330 (85), e51330 (2014).
13. Man, H. S. J. et al. Gene Expression Analysis of Endothelial Cells Exposed to Shear Stress Using Multiple Parallel-plate Flow Chambers. *Journal of Visualized Experiments.* 10.3791/58478 (140), e58478 (2018).
14. White, L. A. et al. The Assembly and Application of 'Shear Rings': A Novel Endothelial Model for Orbital, Unidirectional and Periodic Fluid Flow and Shear Stress. *Journal of Visualized Experiments.* 10.3791/54632 (116), e54632 (2016).
15. Franzoni, M. et al. Design of a cone-and-plate device for controlled realistic shear stress stimulation on endothelial cell monolayers. *Cytotechnology.* **68** (5), 1885-1896 (2016).
16. Dewey, C. F., Jr., Bussolari, S. R., Gimbrone, M. A., Jr., Davies, P. F. The dynamic response of vascular endothelial cells to fluid shear stress. *Journal of Biomechanical Engineering.* **103** (3), 177-185 (1981).
17. Hoger, J. H., Ilyin, V. I., Forsyth, S., Hoger, A. Shear stress regulates the endothelial Kir2.1 ion channel. *Proceedings of the National Academy of Sciences of the United States of America.* **99** (11), 7780-7785 (2002).
18. Moccia, F., Villa, A., Tanzi, F. Flow-activated Na(+)and K(+)Current in cardiac microvascular endothelial cells. *Journal of Molecular and Cellular Cardiology.* **32** (8), 1589-1593 (2000).

19. Crane, G. J., Walker, S. D., Dora, K. A., Garland, C. J. Evidence for a differential cellular distribution of inward rectifier K channels in the rat isolated mesenteric artery. *Journal of Vascular Research.* **40** (2), 159-168 (2003).
20. Hannah, R. M., Dunn, K. M., Bonev, A. D., Nelson, M. T. Endothelial SK(Ca) and IK(Ca) channels regulate brain parenchymal arteriolar diameter and cortical cerebral blood flow. *Journal of Cereberal Blood Flow and Metabolism.* **31** (5), 1175-1186 (2011).
21. Lane, W. O. et al. Parallel-plate flow chamber and continuous flow circuit to evaluate endothelial progenitor cells under laminar flow shear stress. *Journal of Visualized Experiments.* 10.3791/3349 (59), e3349 (2012).
22. Lieu, D. K., Pappone, P. A., Barakat, A. I. Differential membrane potential and ion current responses to different types of shear stress in vascular endothelial cells. *American Journal of Physiology-Cell Physiology.* **286** (6), C1367-1375 (2004).
23. Le Master, E. et al. Proatherogenic Flow Increases Endothelial Stiffness via Enhanced CD36-Mediated Uptake of Oxidized Low-Density Lipoproteins. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **38** (1), 64-75 (2018).
24. Kim, J. G. et al. Measurement of Ion Concentration in the Unstirred Boundary Layer with Open Patch-Clamp Pipette: Implications in Control of Ion Channels by Fluid Flow. *Journal of Visualized Experiments.* 10.3791/58228 (143), e58228 (2019).
25. Kim, J. G. et al. Fluid flow facilitates inward rectifier K(+) current by convectively restoring [K(+)] at the cell membrane surface. *Scientific Reports.* **6**, 39585 (2016).
26. Malek, A. M., Alper, S. L., Izumo, S. Hemodynamic shear stress and its role in atherosclerosis. *Journal of the American Medical Association.* **282** (21), 2035-2042 (1999).
27. Jacobs, E. R. et al. Shear activated channels in cell-attached patches of cultured bovine aortic endothelial cells. *Pflugers Archiv: European Journal of Physiology.* **431** (1), 129-131 (1995).
28. Barakat, A. I., Leaver, E. V., Pappone, P. A., Davies, P. F. A flow-activated chloride-selective membrane current in vascular endothelial cells. *Circulation Research.* **85** (9), 820-828 (1999).
29. Fitzgerald, T. N. et al. Laminar shear stress stimulates vascular smooth muscle cell apoptosis via the Akt pathway. *Journal of Cellular Physiology.* **216** (2), 389-395 (2008).
30. Ueba, H., Kawakami, M., Yaginuma, T. Shear stress as an inhibitor of vascular smooth muscle cell proliferation. Role of transforming growth factor-beta 1 and tissue-type plasminogen activator. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **17** (8), 1512-1516 (1997).

Figure 1

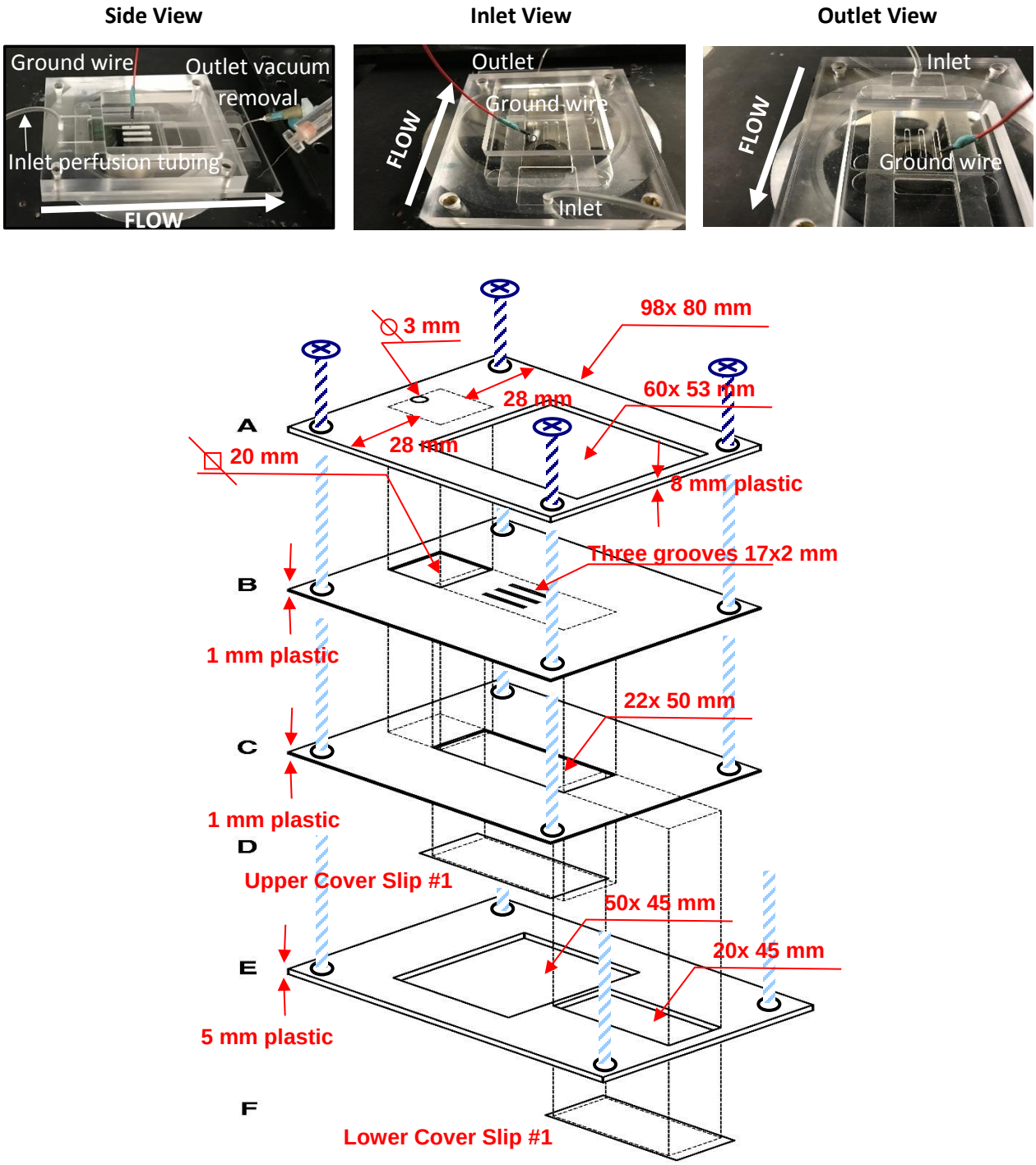
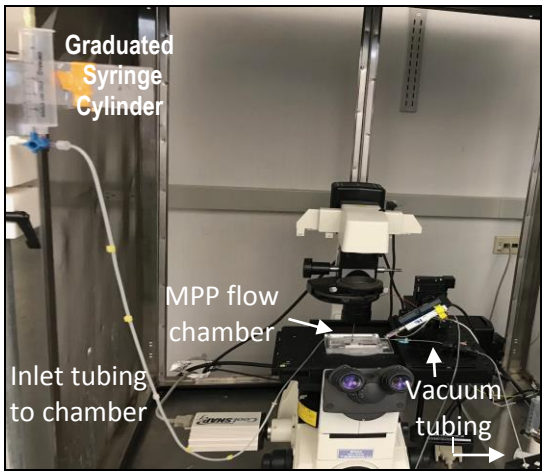


Figure 2



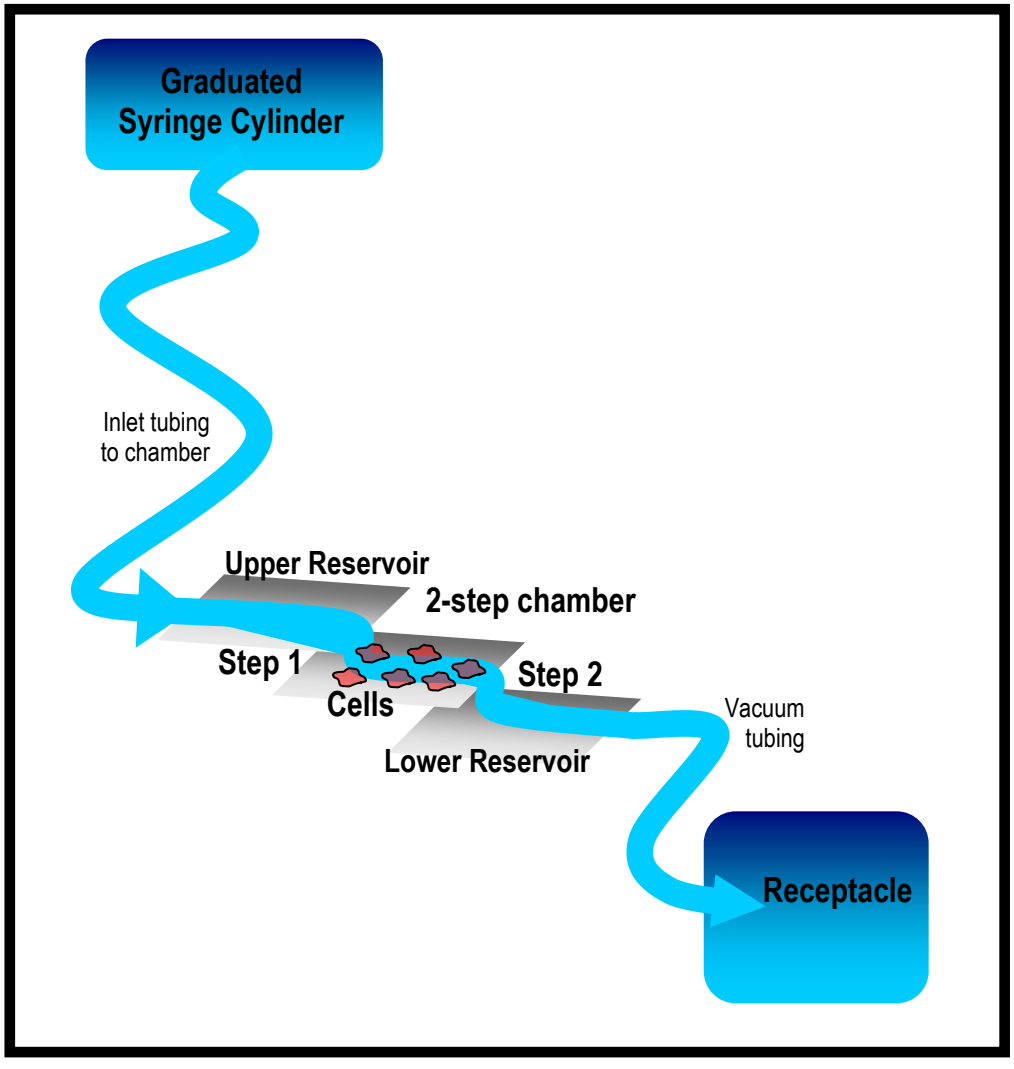


Figure 3

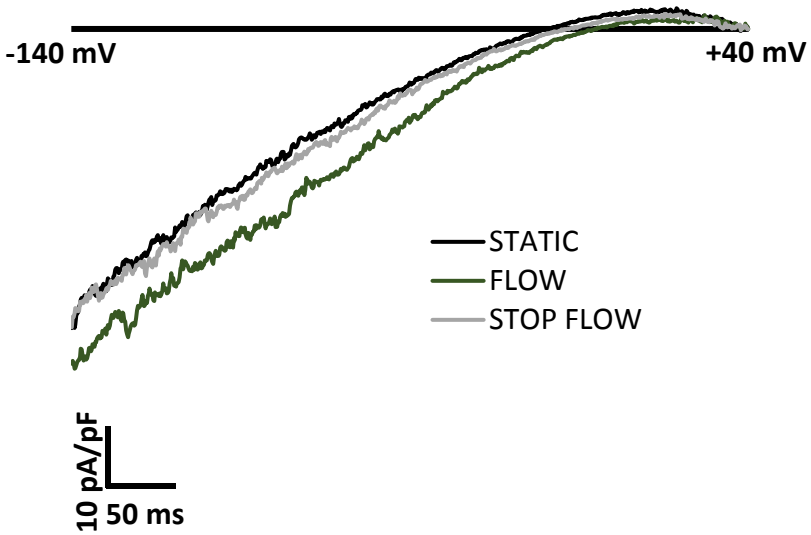


Figure 4

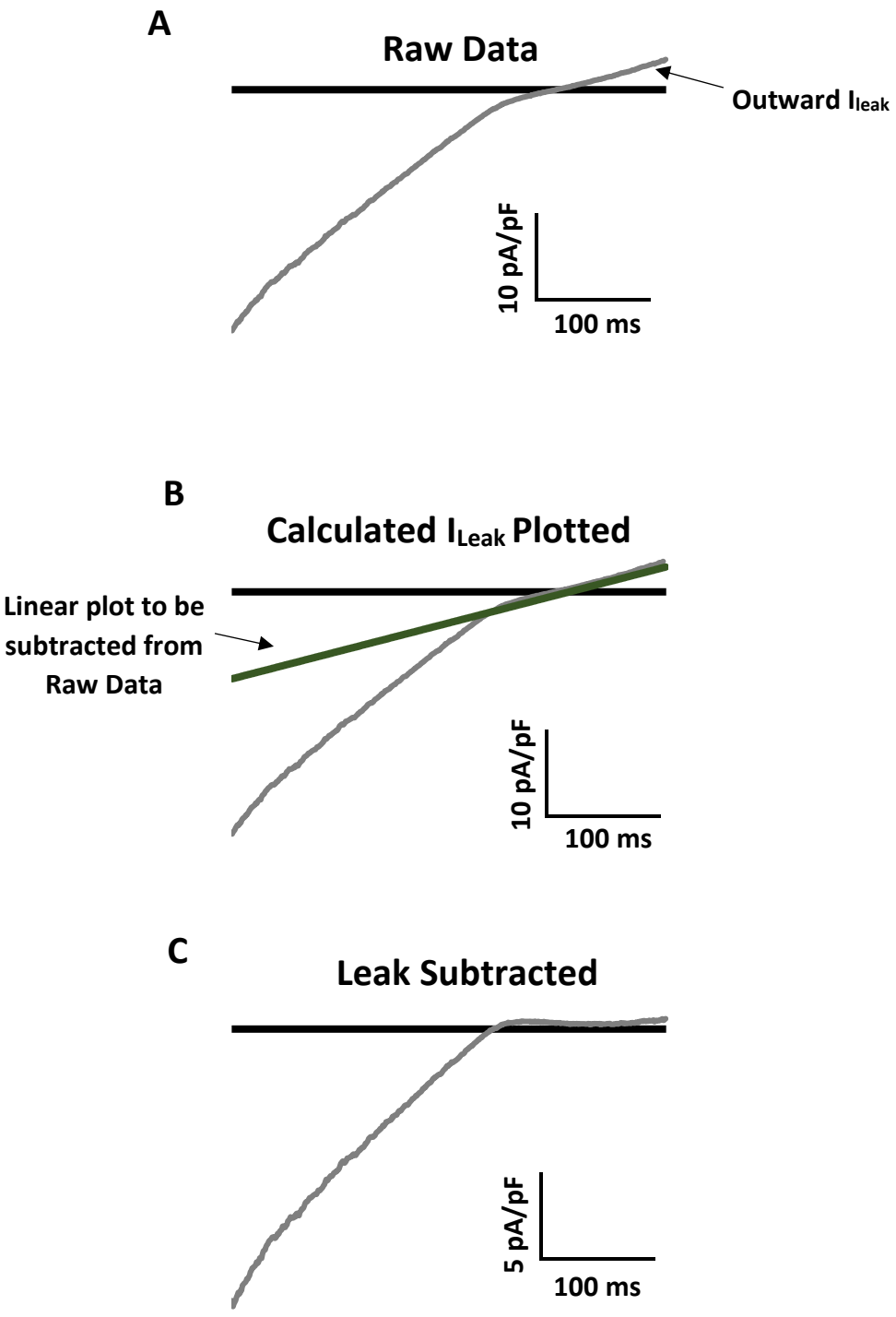


Table 1

Click here to access/download;Table;Table 1. MPP Dimensions.xlsx

	Height (mm)	Width (mm)	Length (mm)
Piece A	8	80	98
Piece B	1	80	98
Piece C	1	80	98
Piece D	0.16	24	40
Piece E	5	80	120
Piece F	0.16	24	50
Assembled MPP	15	80	120
Flow Chamber of Assembled MPP	1	22	42

Additional information

Contains a 3 mm diameter hole for inlet perfusion tubing (see **Table of Materials**) and 53 mm x 60 mm rectangula
Contains a space (20 mm diagonal) underneath perfusion hole of Piece A and three 2 mm x 17 mm slits for access
Contains a 22 mm x 50 mm space where Piece D is adhered using the silicone elastomer solution (see **Table of Ma**
Rectangular glass slide bottom of the flow chamber
Contains a 45 mm x 50 mm space to allow visualization of cells on Piece D. A 20 mm x 45 mm space is present for
Rectangular glass slide bottom of vacuum outlet reservoir
Flow chamber is separated from inlet perfusion and outlet vacuum by two steps to avoid disruption of well-define
Piece D is the glass slide bottom of the flow chamber

ır space for access to middle pieces and cells
to cells
aterials)

the reservoir vacuum outlet, Piece F, to be adhered

ed laminar shear

Table 2

Solution
Dissociation
(Cell isolation)
Bath (Electrophysiology)
Pipette (Electrophysiology)

Recipe (in mM)	**pH**
55 NaCl, 80 Na-glutamate, 6 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 10 glucose, 10 HEPES	7.3
80 NaCl, 60 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES	7.4
5 NaCl, 135 KCl, 5 EGTA, 1 $MgCl_2$, 5 glucose, 10 HEPES	7.2

Table 3

Click here to access/download;Table;Table 3. Methodology in Literature.xlsx

Channel	Patch configuration
Kir2.1	Perforated whole-cell
Kir channels	Whole-cell
Kir2.1	Two-electrode voltage clamp whole-cell
Kir channels	Whole-cell
Piezo1	Outside-out single channel

Shear/flow device

MPP flow chamber

MPP flow chamber

Solution from a pipette tip with exit diameter 1.5x that of the cell

Cells cultured and patched at the end of a capillary tube which receives flow

Patches were positioned near a capillary tube which delivered flow to the patch

Cultured cells vs. isolation

Cultured mesentery microvascular endothelial cells; freshly isolated mesentery endothelial cells

HAECS; freshly isolated porcine aorta endothelial cells

Oocytes

BAECs

Freshly isolated 2nd order mesentery endothelial cells

Endothelial cell isolation protocol

Cocktail of neutral protease and elastase (0.5 mg/mL each; 1 h at 37 °C) followed by collagenase type I (0.5 mg/m

Gentle mechanical scraping of a 5-cm^2 region located at the inner wall of the descending thoracic aorta

NA

NA

Collagenase type IA (1 mg/mL) for 14 min at 37 °C

References

6, 7

11

17

3

8

Table of Materials

Click here to access/download;Table of Materials;Copy of JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 µm sterile syringe filters	VWR	28145-501	Used for filtering electrophysiolgoical pipette solution
5 grade forceps	Fine Scientific Tools	1252-30	Used for transferring digested arteries to fresh solution
9" Pasteur Pipet	Fisher Scientifc	13-678-20D	Used for mechanically disrupting digested arteries and trans
12 mm diameter Cover glass circles	Fisher Scientifc	12-545-80	For use with studies involving cultured cells and multiple tre
24 x 40 mm Rectangluar Cover glass	Sigma-Aldrich	CLS2975224	Cover glass to be added to MPP flow chamber pieces C (Figu
24 x 50 mm Rectangluar Cover glass	Sigma-Aldrich	CLS2975245	Cover glass to be added to MPP flow chamber E (Figure 1)
20 gauge syringe needles	Becton Dickinson and Co	305175	For use in mechanical disruption of digested mesenteric arte
35 mm Petri dish	Genesee Scientific	32-103	For use in mechanical disruption of digested mesenteric arte
Amphotericin B solubilized	Sigma-Aldrich	A9528-50MG	Used for generating the perforated whole-cell patch configu
collagenase, type I	Worthington Biochemical	100 mg - LS004194	Enzyme used in our laboratory as a brief digestion following
Dimethyl Sulfoxide (DMSO)	Fisher Scientifc	67-68-5	Solvent for Amphotericin B used in perforated whole-cell pa
elastase, lyophilized	Worthington Biochemical	25 mg - LS002290	Enzyme used in our laboratory in a cocktail with neutral pro
Falcon Tissue culture Plate, 6-well, Flat Bottom with Low Evaporation Lid	Corning	353046	For use with studies involving cultured cells and multiple tre
neutral protease/dispase	Worthington Biochemical	10 mg- LS02100 50 mg - LS02104	Enzyme used in our laboratory in a cocktail with elastase to
SylGard	World Precision Instruments	SYLG184	Silicone elastomer for adhering the rectangular cover slip to
Tygon ND 10-80 tubing	Microbore Tubing	AAQ04133	ID: 0.05 in, OD: 0.09 in, inlet perfusion tubing for adminsiter

sferring freshly isolated endohtelial cells

eatments. Cells adhered to the cover glass are used for patch clamp analyses

ure 1)

eries
eries
uration.

the initial cocktail of neutral protease and elastase
atch clamp

tease/dispase to begin digestion of arteries for endothelial cell isolation.

eatments

begin digestion of arteries for endothelial cell isolation

the MPP flow chamber pieces C and E (Figure 1)
ring flow to the chamber

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:	Electrophysiological recordings of single-cell ion currents under well-defined shear stress
Author(s):	Ibra S Fancher and Irena Levitan

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ 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. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **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.

10. **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.

11. **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

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

ARTICLE AND VIDEO LICENSE AGREEMENT

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 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.

12. **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.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of 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.

14. **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 is required per submission.

CORRESPONDING AUTHOR

Name:	Ibra S Fancher
Department:	Department of Medicine, Division of Pulmonary, Critical Care, Sleep and Allergy
Institution:	University of Illinois at Chicago
Title:	Postdoctoral Research Fellow
Signature:	Ibra S. Fancher IV
Date:	1/31/2019

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

1. Upload an electronic version 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 02140

Editorial comments:

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Done.

2. Please provide an email address for each author. Done.

3. The current Long Abstract is over the 150-300-word limit. Please rephrase the Long Abstract to more clearly state the goal of the protocol. Done. The goal of this protocol is to describe a modified parallel plate flow chamber that allows real time electrophysiological recording of mechanosensitive ion channels under well-defined shear stress.

4. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points - Done.

5. Please revise the Introduction to include all of the following: - Done, the Introduction is significantly revised to address all of the Editors suggestions and concerns. We believe that the revised Introduction describes much more clearly the goal, the rationale and advantages and limitations of the approach, as well as the general physiological and methodological context.

a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

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 or dashes. Done.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly.

Done. The descriptive text detailing the MPP flow chamber in the original submission can now be found in the new Table 1. We refer to this Table and Figure 1 prior to the protocol section to help readers easily identify the individual pieces of the chamber.

8. The Protocol should contain only action items that direct the reader to do something. In this regard please convert step 1 to action step. Done.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
More details have been added to each section of the protocol relevant to the use and assembly of the MPP flow chamber with endothelial cells from different sources (culture vs. fresh isolations).

10. 2.1: Please name the type of cell used in this case. How many cells are seeded per coverslip, do you leave it to adhere in an incubator? Please write exactly how you perform your experiment.
It is now specified that we use endothelial cells and we provide two alternative protocols for cultured and for freshly isolate cells. It is also noted, however, that any other type of adherent cells can be used in the same way (see Note at 2.7). We have also included relevant %confluency (Page 3) and number of cells seeded (Page 5) for appropriate protocols.

11. 2.2: After how long? Which solution is added, how much?
The protocol section has been updated to include these details. It is our intention to provide a general methodology to be used by experienced electrophysiologists using any desired version of bath and pipette solution they may require. We believe to better reflect this intention in the protocol section. We also provide examples of the solutions used in our laboratory in the new Table 2.

12. 3: What is the desired configuration in your case?
It is now specified that our studies are performed in the whole cell configuration in Note 3.7.

13. 4: How are these steps performed? 14. Please provide all the button clicks, knob turns etc.
We apologize but a full manual of how to perform an electrophysiological recording is far beyond the scope of the current Method paper.

15. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
Done.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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.
We have highlighted the section where cells from culture will be used for experimentation in the MPP flow chamber (~2.75 highlighted pages).

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to

your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
All figures and photographs presented in the current manuscript are new and do not appear as published material elsewhere.

18. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.
Done.

19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We have significantly reworked the Discussion section to cover the appropriate details.

20. Please do not make points in the discussion and use paragraph style instead.
We corrected this formatting style. The points are now included in the relevant paragraph.

21. Please expand the journal title in the reference section. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.
Done.

22. Please alphabetically sort the materials table. Done.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Fancher & Levitan wish to publish a manuscript (+ video) that illustrates a method for the design and use of a custom flow chamber for real time electrophysiological recordings of cultured or

freshly isolated endothelial cells undergoing shear stress. Investigation of the underlying mechanisms of vascular sheer stress is an important topic per our understanding of the physiological regulation of blood flow and treatment/prevention of pathology development (e.g., atherosclerosis). Readers of the broader scientific community may benefit from the use of the modified parallel plate flow chamber as detailed in Figure 1. In brief, the manuscript does not sufficiently present how the current apparatus is an improvement from earlier approaches and prototypes (developed in the authors' lab as well as others) accompanied by appropriate citations. Also, in this regard (lack of appreciable acknowledgement and discussion of internal/external efforts), there are several other JoVE publications illustrating methods for examining endothelial sheer stress and mechanosensitivity (e.g., PMID #30394398, #30663667, #27842359, #24686418). Thus, it is not clear how the current apparatus/method is building from past approaches to introduce modern advantages.

Respectfully, we would like to point out that none of the previous JOVE manuscripts listed by the Reviewer describe a method to perform electrophysiological recordings under well-defined shear stress conditions. Three out of four papers describe closed and more standard flow chambers that cannot be used for electrophysiological recordings and one paper describes an open chamber design that does not generate a well-defined laminar flow profile. These points are clarified in the Introduction.

In addition, the manuscript is lacking in descriptions of several key details (e.g., cellular sources, compositions of solutions, microscopes/magnification, electrometers) and a discussion of protocol advantages vs. limitations coupled with critical steps for troubleshooting.

Many of the requested details have been included in the revised manuscript. We did not include information regarding microscopes and general electrophysiology protocols as these lie outside the specific scope of the present JOVE manuscript detailing the use of the MPP flow chamber.

Please see specific comments below:

(1) Abstract; Line 31, "…essential…"
Do the authors really intend "essential" here or "potential" instead?
We agree that “potential” is more appropriate. The edit has been included.

(2) Abstract; Line 38 & Line 53, "…freshly isolated cells…"
It would be ideal to specify endothelial cells as "…from culture or freshly isolated endothelial cells…" here.
For clarity and focus in the abstract, we removed this sentence. The introduction has been significantly reworked, as well. We save this particular distinction of “..from culture or freshly isolated..” for the protocol and discussion sections.

(3) Introduction; General comment
A description of the development of the current apparatus from earlier approaches and prototypes (+ citations) over the past 20+ years is lacking. Thus, it is not clear how the current

apparatus/method is significantly building from past approaches to introduce modern advantages.
The Introduction is significantly revised to clarify the uniqueness and the advantages of this approach as compared to other approaches. We are not claiming, however, that this approach is a novel development. It was developed in our lab 20 years ago and the description of the chamber was published in 2000 (Levitan I, *Ann Biomed Eng*, 2000). In spite of this, however, and most likely due to significant technical difficulty of performing these experiments, there are only very few studies that attempted to perform electrophysiological recordings under flow and in most cases, these studies use approaches that do not simulate the laminar shear stress correctly. The goal of this JOVE paper is to help researches in the field to overcome the barrier of the technical difficulty and perform high quality recording under well-defined flow. These considerations are clarified in the Introduction and in the Discussion sections of the manuscript.

Also, the authors should describe the interaction between specific examples of relevant mechanosensitive channels and the distinct profiles of sheer stress to blood flow whether physiological or pathological.
This is far beyond the scope of the current Method paper. The readers are referred to a review by Barakat et al (*Biomaterials*, 2006) that provides up-to-date information on this topic.

Finally, the reader will need a better idea of how this open flow/sheer apparatus may relate to conditions of blood flow regulation in vivo.
This is apparatus is not open flow but a closed one with only minimal narrow openings to allow introducing the pipette. We refer the reader to our previous paper that described the flow profiles in this chamber (Levitan I, *Ann Biomed Eng*, 2000). This is also clarified in the Discussion.

(4-6) Introduction
We thank the Reviewer for the suggestions and we revised the Introduction to clarify all the previous concerns

(7) Protocol; General Comment
Important details regarding types of microscopes and magnification/resolution for establishing electrophysiological recordings are missing. Other required components (e.g., microscope stage dimensions, vibration isolation table, valve controller, types of electrometers, manufacturing glass pipettes) are also missing.
Respectfully, it is not our intention to provide a full manual on how to perform an electrophysiological recording. We believe this to be beyond the scope of the current Methods paper. We intend for this protocol to detail use of the MPP flow chamber so that experienced electrophysiologists may investigate mechanosensitive channels using this device.

(8) Protocol; Line 104, "…appropriate tubing…"
Specify examples of "appropriate" tubing (e.g., general material, size).
We have included the specific tubing along with catalog numbers (See the Material Table).

(9) Protocol; Line 108, "…flow rates a different heights."
It would be ideal to provide a range of heights as they pertain to range of flow rates in ml/s. Also, replace the typo "a" with "at".
We have included the height and corresponding flow rate of the graduated syringe cylinder at which we perform our experiments (See Note 3.5). As we currently use only one shear stress level for our experiments investigating Kir channels, we have altered our text to reflect the process of determining a desired value of shear stress by changing cylinder heights/flow rates, and the calculation for the corresponding shear stress levels. While we agree it would be ideal to report on and show a step-increase, "dose-response" to flow, we do not have this data currently available. As such, we have curbed our language to reveal this and discuss, as a limitation, the shear levels that can be reached in the MPP flow chamber.
The typo is corrected.

(10) Protocol; Line 114-115, "…frequency noise…cumbersome…".
Pulsatory mechanical movement of the pump may also challenge stability of electrophysiology measurements. A better description in replace of "cumbersome" (e.g., Too big? Inaccessible for control?) should be used as well.
We have significantly changed the Protocol section and now focus primarily on gravity perfusion system to the MPP flow chamber, as performed in our lab. As suggested, we do mention the potential drawbacks of the peristaltic pump in Note 3.5.

(11) Protocol; Lines 119-125, Sheer stress equation.
Please provide the appropriate reference(s) supporting this equation as the standard calculation of sheer stress.
A reference showing the calculation for shear stress in a parallel chamber has been included.

(12) Protocol; Line 125, "…rinsed…"
Typo: replace "rinsed" with "rinse". Corrected.

(13) Protocol; Line 135, "…electrophysiological bath solution…"
What is the solution's composition and desired experimental temperature and pH?
Example solutions are now presented in the new Table 2 and include desired solution pH values. We have also included a statement that these experiments are to be performed at room temperature.

(14) Protocol; Line 139, "…covered…"
Do the authors mean "submerged?"
Yes, thank you. "Covered" is now "submerged" in the text.

(15) Protocol; Lines 143-145, 157, Identification of cell health: "…endothelial cells…rough…round".
This procedure for identifying endothelial cells is vague. Can the authors use a more precise description coupled with an actual image? Also, it would be ideal for the authors to illustrate healthy vs. "blebbing" cells (per Lines 143-145) using an actual image as well.

The description of isolated endothelial cells as having a “rough and round phenotype” is common and makes them easily distinguished from smooth muscle cells which are thin and elongated (see references 19 and 20). In addition, smooth muscle cells lack expression of inwardly rectifying channels in our vascular bed of choice (mouse mesenteric arteries) and so the presence of this current type in our recordings is further evidence that we are indeed identifying endothelial cells.
As we intend to include the cell culture protocol in the video portion of the JoVE article, the healthy vs. “blebbing” cells can be further addressed in this way, without including an additional figure.

(16) Protocol; Line 151, "…isolated cells (≥750 µl)"
The volume that the chamber can accommodate is clear but is there an actual number of cells (or a range of cell numbers or % confluence) per unit of volume that would be optimal for the successful use of the apparatus? It would ideal to list commercial and experimental animal sources that are best for successful completion of this protocol (see comment #22).
We have updated the protocol to include %confluency (cells from culture) and number of cells seeded (fresh isolation). We believe the MPP flow chamber could be used with any number of animal models and sources of cells and have added these thoughts to the Discussion.

(17) Protocol; Line 165, "…apply sheer in a step-wise…"
What is the range of flow rates used/recommended?
We have updated the protocol to include our flow rate and shear used (see Note 3.5) and recommend not going above 15 dynes/cm^2 for any specific research applications. We discuss the latter as a limitation in the Discussion as physiological shear stress values can reach levels well beyond 15 dynes/cm^2 (Malek AM, *JAMA,* 1999).

(18) Protocol; Lines 167, 168, & 173, "…physiologically relevant levels…0.7 dynes/cm2 shear…" What would be a physiological to pathological range of shear forces for this procedure? It would be ideal to implement and include raw data for more than one shear force application per Figures 3 & 4 (see comment #21).
We of course agree with the Reviewer that it would be great to obtain data for multiple shear stress levels from the same cell but due to the difficulty of obtaining stable recordings under flow, we use only one shear stress level in each experiment. Please see additional explanation in Comment 9 above.

(19) Protocol; Lines 178-179, "…(e.g. supraphysiological K+ bath solution with 0 Ca2+ in pipette solution to study Kir channels)". It is not clear as to what an example of "supraphysiological" K+ bath solution is. A recommendation is to include compositions of bath and pipette solutions in Table 1 per respective channel investigation and patch configuration.
While we do not think it reasonable to include the recipes for every bath and pipette solution for the references listed in Table 1 (now Table 3), we have included the recipes that we use for our bath and pipette solutions in the new Table 1. We also replace “supraphysiological” in the text with the concentration of 60 mM K^+ used in our bath solution for investigating inwardly rectifying K^+ channels.

(20) Representative Results, Figure 2. Instead of a cartoon, show an actual image of the entire apparatus with labeled components.
We agree that showing an actual image of the gravity perfusion system is beneficial and have included such a labeled photograph of the entire set-up in the new Figure 2. We also elected to keep the cartoon because the photograph alone does not convey the two-step system in the MPP flow chamber to isolate flow from inlet and outlet disturbances. This is a critical component of the chamber design.

(21) Representative Results, Figure 3 & 4. It would be ideal to illustrate Kir activity in response to a range of physiological to pathological flow rates/patterns (see comment #18).
Please see our response above.

(22) Discussion; General Comments
The Discussion does not clearly indicate the critical steps of the protocol for performing a successful experiment and tips for troubleshooting accordingly. The limitations of the method are not considered either. In this manner, a sufficient discussion of the variety of cellular sources (e.g., commercial cell lines, species/gender/age of experimental animals) as suitable (or not) for critical cellular adherence to the coverslip and stable patch clamp measurements during flow is also missing.
We have addressed and explained the critical steps of the protocol in the revised Discussion. Respectfully, we also must place some responsibility on the other investigators to know whether their cell type of interest is adherent (or not) or suitable for such an application. We have not tested every scenario to be confident in making such claims.

(23) Discussion; Lines 216-221, Protocol Advantages
The manuscript does not indicate how the current protocol fulfills unprecedented experimental abilities in a significant manner using the literature. Thus, it is difficult to ascertain whether the declared advantages (real-time investigation, physiological relevance, ease of assembly/disassembly) are distinct from other published efforts.
We believe to have adequately addressed these concerns in the Discussion.

(24) Discussion; Line 227, "…physiological range…"
The authors do not provide any information in the manuscript about the range of physiological shear stress and do not explain the difference between shear-stress imposed via solution in their apparatus vs. shear-stress due to blood flow in arteries/arterioles.
We believe to have addressed the Reviewer's concerns regarding ranges of shear stress in the revised manuscript. Clearly, the MPP flow chamber and solutions used do not exactly replicate the shear of blood flow in arteries. As the Reviewer correctly points out, blood is a viscous, non-Newtonian fluid that we have not replicated in our in vitro experiments. Additionally, the chamber used here is a parallel chamber while shear stress in arteries would be calculated using the formula for shear in a cylinder. We have added these thoughts to the Discussion.

Reviewer #2:

Manuscript Summary:
Flow-shear stress is a crucial regulator of endothelial function under healthy and disease conditions. Several receptors/ion channels that mediate the flow/shear stress effect on endothelial cells have been identified. For therapeutically targeting the interaction between shear stress and ion channels, it is important to understand the biophysical features of the interaction. The study describes a modified parallel plate flow chamber that can be used for real time studies of flow/shear stress-induced activation of ion channel currents. This chamber can be used for studies in cultured or freshly isolated endothelial cells. The methodological details are very well-explained, and representative results are convincing. This new methodology will find a lot of interest in the field, and the paper will be well-cited.

Major Concerns:
None

Minor Concerns:

1. A comment on the range of shear stress that can be used on this chamber without altering the gigaohm seal will be helpful.
We added such comments that highlight the upper limit of shear stress to be 15 dynes/cm^2 (please see Pages 8 and 9 of the Discussion) before loss of the gigaohm seal and solution overflow from the chamber.

2. Please specify the cells that have been used in figure 3.
We added the specific cell type to the Figure Legend of Figure 3.

3. As is commonly the case with endothelial cell patch clamp, it appears that the studies have been performed at room temperature. If so, please specifiy.
Yes, this is the case and we added this information to the protocol section 3.7.

4. It will be beneficial to know whether perfusion under gravity is better than using peristaltic pump (which is likely to introduce turbulence) for this purpose?
We have significantly revised our manuscript to focus on our use of the gravity perfusion system and make mention in Note 3.5 that this is indeed our preference for use with the MPP flow chamber. We included the underlying reasons for our preference as added turbulence and the potential for reduced patch stability when using a peristaltic pump.

Reviewer #3:

Manuscript Summary:
This is a concise manuscript that describes the development of a new modified parallel plate flow chamber for studies defining the effects of shear stress on the activity of mechanosensitive ion channels. The development of this tool may enhance the study of mechanosensitive ion

channels to specific shear stress conditions in native cells. Thus, the dissemination of this technique is warranted. I only have some minor comments.

Minor Concerns:

1) In the abstract section, there is a reference to shear-induced vasoconstriction through an undetermined mechanism and then there is a reference to piezo channels. This is confusing. Please clarify whether piezo channels are the part of the mechanism or not.
We believe to have clarified this sentence in the abstract to convey that piezo channels *are* involved in the mechanism of shear-induced vasoconstriction.

2) In the Protocol section, it will be clearer to identify the different parts of the new chamber first, and then use that new ID to provide a description of the chamber and the procedure. Referring to pieces with no other context is confusing.
Thank you, we agree. As requested by the Editor, we have significantly revised the Protocol section to be written in the imperative tense and have converted the original Section 1 of the protocol, which contained a detailed description of the MPP flow chamber pieces, into the new Table 1. We reference the reader to Table 1 and Figure 1 for Piece IDs with the hope that it clearly identifies each part of the chamber and supplements the new text in the Protocol section.

3) It is not clear from the discussion section why previous approaches to study ion channel regulation by shear stress could only use cultured cells. Perhaps a more extensive discussion on this issue will enhance the importance of this new study.
We believe to have addressed this concept in the Discussion. Briefly, the other main technique for studying mechanosensitive channels under flow is by culturing them in the opening of a capillary tube and subsequently perfusing the tube with solution to implement a shear force over the cell and patch. The difficulty of seeding cells into capillaries, access to a very small number of cells that are close enough to the capillary end for the pipette to be able to reach them, and flow disturbances at the end of the flow channel makes it difficult to perform electrophysiology under flow in cells cultured in the capillary opening and virtually impossible to patch or even seed cells freshly isolated from the vasculature.

4) In Figure 1, additional pictures of the chamber from different angles will be helpful.
We agree and have added two new photographs of the chamber at different angles; an inlet view and an outlet view now accompany the original side view of the MPP flow chamber in Figure 1.

5) It is unclear what page 16 is all about.
Page 16 is the required Table of Materials. It is now updated to include the materials added to the revised Protocol.