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A Pragmatic Workflow for Gene Expression Analysis of Endothelial Cells Exposed to Shear Stress Using Multiple Parallel Plate Flow Chambers

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Dear Dr. Mukherjee,

Thank you for considering our manuscript titled: "**A Pragmatic Workflow for Gene Expression Analysis of Endothelial Cells Exposed to Shear Stress Using Multiple Parallel Plate Flow Chambers."** This manuscript includes our detailed protocols for experiments that involve the application of fluid shear stress used in our recent publication "Angiogenic Patterning by STEEL, an Endothelial-Enriched long noncoding RNA". These protocols were developed to minimize variability between experiments and we believe that strategies used in these protocols will be applicable in many settings.

We present unique elements of our workflow to increase accessibility of these experiments to labs less experienced in this field. The flow circuit is assembly is based on the excellent protocol by Lane W. et al in a previous issue of Jove. In this manuscript, we present several key adaptations that reflect differences in our experimental systems. Our experiments involved multiple conditions in endothelial cells exposed to fluid shear stress. To accommodate multiple concurrent experiments, we use a large heated unit that provides a standard environment for the operation of four or more simultaneous flow experiments. To standardize fluid shear stress between experimental conditions, we continuously monitor flow rates to each flow chamber. We include details of tubing size and luers that we have optimized to reduce leakage from the flow circuit. Similarly, we present details of our cell seeding procedures that we have found important for fluid shear stress experiments involving early passage endothelial cells. In our own experience, we have found that the implementation of these, and other steps in our protocols, crucial for the success of our experiments involving fluid shear stress.

For experiments that involve gene expression analysis with reverse-transcription quantitative PCR, we use an exogenous reference RNA to account for inter-sample differences in efficiency of RNA extraction and cDNA synthesis. In particular, cDNA synthesis is a process with quantitative variability. In this manuscript, we present our entire workflow for the implementation of an exogenous reference RNA from synthesis to analysis. This workflow is easy to implement and cost-effective. These protocols were used in our published fluid shear stress experiments and were particularly important because of the complex nature of these experiments. Nevertheless, this element of our manuscript will be widely applicable to gene expression analyses in all settings.

Overall, these protocols have facilitated experiments that have yielded significant insight into the regulatory mechanisms that control endothelial cell angiogenic potential in response to shear stress conditions. We hope that this pragmatic workflow, in whole or in part, can facilitate other such significant findings.

Thank you for your time and consideration.

Warmly,

Philip A. Marsden

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

Author response: Thank you for the formatting. We have retained the formatting in the document.

2. Please address all the specific comments marked in the manuscript.

Author response: We have made changes to the manuscript to address all the specific comments marked in the manuscript.

Lines 34-37: We have rephrased the summary to be less than 50 words.

Lines 105-106: We have removed commercial language – "ibidi". The use of this language was intended to address a specific reviewer comment that asked about these systems and we have tried to include general comments without commercial language that address the reviewer comment.

Lines 121, 125, 623, 629, 1320, 1321, 1378, 1396, 1398, 1401, 1413, 1437: We have converted dynes/cm2 to S.I. units. Line 623: We have included the conversion of S.I. units Pa to dynes/cm2 to facilitate unit conversion that is used in flow rate formulas or other manuscripts.

Line 166: We agree that a note is appropriate.

Line 168: We have re-organized this text to improve clarity of the steps.

Line 258: We have removed highlighting to keep the script within the length limits.

Line 268: We have removed highlighting to keep the script within the length limits.

Line 409: We have re-organized this section to improve the clarity of the steps.

Line 428: We have clarified the language to indicate a glass slide.

Line 440: We have re-organized this section to reflect the editor's suggestions and avoid redundant sentences.

Line 569: We have removed the commercial language "Oligofectamine".

Line 638, 1311: We have removed quotation marks as this is not a citation. It is a short form referring to our specific environment and set-up built for experiments involving laminar flow.

Line 687: We have moved the list of autoclaved materials to Table 2.

Line 1224, 1296: We have removed highlighting for steps that involve calculations.

3. Presently the protocol is more than 10 pages. Please reduce the protocol length to fit in 10 pages max (hard cut limit) including heading and spacings. Please do not use redundant sentences. Also please remove the list of equipment autoclaved and make a separate table for this.

Author response: We have reduced the length of the protocol to fit in 10 pages. We have removed the list of equipment autoclaved and made a separate table (Table 2) to refer to these items.

4. The highlighted text does not form a cohesive story. Also please remember that if you highlight a step, please highlight subsequent steps stating how the protocol is performed. Please ensure that the highlight is no more than 2.75 pages including heading and spacings.

Author response: We have amended our highlighted text to form a more cohesive story. We have focused the highlighted text on steps that are more specific to our use of endothelial cells in this setting and that include elements that are unique to our protocol. As such, we have included the cell seeding, and method of cell harvesting for RNA from endothelial cells, as well as our particular environment used for running multiple concurrent laminar flow experiments and the set-up of our flow monitors.

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Author response: All the illustrations in this manuscript are original artwork made for this manuscript. We have adapted the data for Figure 4 for this manuscript and cited in the figure legend Line 415. The figure itself is original for this JoVE manuscript. This link (http://www.pnas.org/page/authors/licenses) to the editorial policy outlines the journal's policy on use of materials.

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TITLE:

Gene Expression Analysis of Endothelial Cells Exposed to Shear Stress Using Multiple Parallel-plate Flow Chambers

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KEYWORDS:

Flow chamber, shear stress, laminar flow, parallel-plate, monitoring, endothelium, gene expression, gene regulation, quantitative PCR, normalization, luciferase, reference RNA

SUMMARY:

Here, a workflow for the culture and gene expression analysis of endothelial cells under fluid shear stress is presented. Included is a physical arrangement for simultaneously housing and monitoring multiple flow chambers in a controlled environment and the use of an exogenous reference RNA for quantitative PCR.

ABSTRACT:

We describe a workflow for the analysis of gene expression from endothelial cells subject to a steady laminar flow using multiple monitored parallel-plate flow chambers. Endothelial cells form the inner cellular lining of blood vessels and are chronically exposed to the frictional force of blood flow called shear stress. Under physiological conditions, endothelial cells function in the presence of various shear stress conditions. Thus, the application of shear stress conditions in *in vitro* models can provide greater insight into endothelial responses *in vivo*. The parallel-plate flow

chamber previously published by Lane *et al.*[9] is adapted to study endothelial gene regulation in the presence and absence of steady (non-pulsatile) laminar flow. Key adaptations in the set-up for laminar flow as presented here include a large, dedicated environment to house concurrent flow circuits, the monitoring of flow rates in real-time, and the inclusion of an exogenous reference RNA for the normalization of quantitative real-time PCR data. To assess multiple treatments/conditions with the application of shear stress, multiple flow circuits and pumps are used simultaneously within the same heated and humidified incubator. The flow rate of each flow circuit is measured continuously in real-time to standardize shear stress conditions throughout the experiments. Because these experiments have multiple conditions, we also use an exogenous reference RNA that is spiked-in at the time of RNA extraction for the normalization of RNA extraction and first-strand cDNA synthesis efficiencies. These steps minimize the variability between samples. This strategy is employed in our pipeline for the gene expression analysis with shear stress experiments using the parallel-plate flow chamber, but parts of this strategy, such as the exogenous reference RNA spike-in, can easily and cost-effectively be used for other applications.

INTRODUCTION:

Vascular endothelial cells form the inner cellular lining of blood vessels in the closed cardiovascular system of higher species. They form the interface between the blood and tissues and are characterized by luminal and abluminal surfaces. The endothelium is a diverse, active, and adaptive system that regulates blood flow, nutrient trafficking, immunity, and the growth of new blood vessels[1]. In the body, endothelial cells normally exist in an environment where they are exposed to the frictional force of circulation, shear stress[2]. Shear stress is an important regulator of endothelial cell gene expression[3], and endothelial cells attempt to maintain shear stress within a given range[2,4]. Endothelial cells demonstrate angiogenic patterning in the absence of shear stress[5] that can improve tissue perfusion. Regional patterns of disturbed flow and altered shear stress are associated with the expression of inflammatory genes[6] and the development of atherosclerosis[7,8]. Thus, models that include shear stress are a major component of understanding endothelial gene regulation.

We describe a method for studying the gene regulation in vascular endothelial cells under shear stress. This system uses non-pulsatile flow and mimics fluid shear stress levels and oxygen concentration that model conditions for arterial endothelial cells. This protocol includes details of methods for the gene knockdown using RNA interference (RNAi), the set-up for the application of shear stress using the parallel-plate flow apparatus, and methods for the spike-in of an exogenous reference RNA prior to analysis by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). This pipeline is used for studying gene regulation in endothelial cells in the presence and absence of laminar shear stress and include an adaptation of the parallel-plate flow apparatus described by Lane *et al.*[9]. This particular set-up was designed to facilitate the simultaneous assessment of multiple experimental conditions that allows direct comparison of shear stress conditions, as well as the normalization of RNA analysis. A large heated unit with controlled humidity is utilized to allow multiple separate flow chambers and pumps to be running simultaneously with flow rates monitored for each flow chamber assembly in real-time. The application of this set-up is used for gene knockdown using RNAi in the setting of laminar

flow/shear stress, but aspects of this protocol can be applied to any assessment of RNA expression.

Common approaches to the application of shear stress for endothelial cells include microfluidic systems[10], a cone-and-plate viscometer[11], and a parallel-plate flow chamber[12]. Microfluidic systems from various manufacturers have been useful in studying mechanobiology and mechanotransduction in multiple cell and tissue types and a variety of biophysical stimuli. For endothelial cells, they have been used to study endothelial cells in isolation, as well as the interaction of endothelial cells and the trafficking of immune or tumor cells[10]. However, these systems are less suitable for the recovery of large numbers of cells[9]. Both the cone-and-plate viscometer and parallel-plate flow chambers allow the recovery of large numbers of cells in confluent monolayers[12]. These systems can generate a range of shear forces and patterns[12]. The parallel-plate flow chamber assembly[9] has the advantage that real-time imaging can be performed through the glass window to evaluate cellular morphology at any time point. Furthermore, the perfusate can be collected under sterile conditions. For the system presented here, the flow can also be monitored in real-time and in a multi-chamber set-up, which facilitates the maintenance of shear conditions between chambers.

For representative experiments, human umbilical vein endothelial cells (HUVEC), which represent a macrovascular endothelial cell type, are used, and the shear stress conditions we use (1 Pa) reflect arterial conditions (1 - 7 Pa). However, this protocol can be used with other endothelial cell types, and the shear stress conditions can be adjusted according to the experimental question. For example, the evaluation of human endothelial cells in conditions that model venous circulation would require lower levels of stress (1 - 6 dyne/cm^2) and studies that model microvascular circulation have utilized shear stress levels of 0.4 - 1.2 Pa[13,14]. In addition, shear stress can vary even between endothelial cells within the same blood vessel[6]. In the current set-up, a single monitoring system is used that can simultaneously monitor four separate flow loops. For labs that need more flow loops, there is space in the dedicated environment for an additional monitoring system.

RT-qPCR is used for the absolute quantitation of gene expression in the setting of shear stress. The relative expression of target genes is often used to compare RNA expression across conditions. Some RNA species can exist at very low quantities or be absent, thus complicating relative measurements. For example, long noncoding RNAs in endothelial cells can exert potent effects at relatively low copy numbers per cell[5]. In addition, differences in primer efficiency can lead to an inaccurate interpretation from utilizing the delta-delta cycle threshold (Ct) method to analyze the data. To address this concern, we perform absolute quantitation by generating a standard curve using a known quantity of plasmid DNA. Furthermore, complementary DNA (cDNA) synthesis is an inefficient process, and differences in cDNA efficiency can account for differences in RNA expression between conditions and between samples[15]. The application of shear stress and/or transfection reagents can affect cell proliferation, apoptosis, and viability, or add components that may interfere with RNA isolation and/or cDNA synthesis. To account for the possibility of bias from RNA isolation and cDNA synthesis, we use a spike-in RNA control synthesized in the lab, added at the time of RNA extraction and measured with each cDNA

synthesis *via* RT-qPCR. This allows not only the adjustment for technical differences in RNA extraction and cDNA synthesis but also allows the calculation of absolute quantities per cell, when the cell count is known.

This system uses additional steps to maintain similarity or account for technical differences between conditions. We particularly emphasize these steps because of the complex nature of these experiments, which involve multiple physical set-ups and experimental conditions that can lead to experimental variability.

PROTOCOL:

1. Preparation of Exogenous Reference RNA

Note: Choose an exogenous reference RNA that does not exist in the species or model of interest. For mammalian systems, firefly luciferase RNA may be used.

1.1 Linearization of exogenous reference RNA plasmid

1.1.1 Prepare exogenous reference RNA at least 48 h prior to the anticipated RNA extraction. Obtain or manufacture a cDNA clone of the chosen exogenous reference RNA, such as a firefly luciferase cDNA clone in a plasmid vector appropriate for *in vitro* transcription (see **Table of Materials**).

1.1.2 Perform restriction enzyme (RE) digestion of 1 µg of full-length plasmid (the firefly luciferase plasmid is pSP-luc+ which has 4100 bp) using single-cutter RE (*XhoI*) in 1.5-mL microfuge tubes. Choose an RE that is a single cutter (cuts plasmid only 1x) at the 3' end of the exogenous reference RNA sequence that leaves a 5' overhang or blunt end. For a typical preparation, perform seven plasmid linearization reactions (steps 1.1.4 - 1.1.7) in parallel to generate sufficient RNA concentration and quantity to complete one set of experiments or one project.

1.1.2.1 Measure the plasmid concentration using spectrophotometry or spectrofluorometry.

1.1.2.2 Prepare an RE mixture in each tube: add 4 µL of *XhoI* (20,000 units/mL), 8 µL of RE buffer, x µL of plasmid (1 µg), and sufficient H_2O to reach a total solution of 80 µL.

1.1.2.3 Incubate the RE mixture for 2 h at 37 °C. Use the RE according to the manufacturer's protocol, as any modifications can result in increased star activity or non-specific cleavage of target DNA.

1.1.2.4 Terminate the RE digest with ethanol precipitation in each tube. To the RE mix, directly add 4 µL of 0.5 M EDTA pH 8.0, 8 µL of 3 M sodium acetate pH 5.2, and 184 µL of 100% ethanol. Mix well and freeze the mixture at -20 °C for 30 min.

1.1.2.5 Spin down the mixture at 4 °C for 20 min at a relative centrifugal force (RCF) of 16,100 x *g*.

1.1.2.6 Remove the supernatant with a fine tip, without touching the pellet. Air-dry the pellet for 5 min and resuspend it in 6 µL of H_2O (warmed to 37 °C) by pipetting up and down 5x - 10x.

1.1.2.7 Confirm the linearization of luciferase plasmid by running the digested product on 2% agarose gel containing ethidium bromide (EtBr) along with a 1 kb+ ladder, a supercoiled ladder, and cut and uncut plasmid. Inspect the gel and proceed to the *in vitro* transcription if the lane for the cut plasmid shows a single band at ~4 kb (the uncut plasmid will have three bands; **Figure 1**).

CAUTION: EtBr is carcinogenic. Work in a chemical fume hood.

1.2 *In vitro* transcription of exogenous reference RNA plasmid

1.2.1 For each tube of digested plasmid products, perform *in vitro* transcription using an *in vitro* transcription method (see **Table of Materials**). Follow the manufacturer's instructions and use the appropriate phage RNA polymerase. If the method of cDNA synthesis requires a poly(A) tail, or other applications require a poly(A) tail, choose a method of *in vitro* transcription that includes a poly(A) tail addition (see **Table of Materials**).

1.2.2 Combine all *in vitro* transcribed products into a single 1.5-mL polypropylene tube prior to purification.

1.3 Purification of RNA from the *in vitro* transcription reaction

1.3.1 Purify *in vitro* transcribed products with an *in vitro* transcription clean-up kit (see **Table of Materials**). Please follow the manufacturer's protocol.

1.3.2 Assess the RNA concentration by measuring absorbance at 260 nm using spectrophotometry.

Note: RNA concentration is calculated using the Beer-Lambert law. This states that the absorbance of nucleic acids (which absorb light strongly at 260 nm) is proportional to the concentration. An absorbance of 1.0 is equal to 40 µg/mL of single-stranded RNA.

1.4 Aliquoting the stock RNA into PCR tubes for experimental use

1.4.1 Ensure the RNA concentration is 1 µg/µL.

1.4.1.1 If the RNA concentration is > 1 µg/µL, dilute the stock to 1 µg/µL. Aliquot 1 µL into PCR tubes and store them at -80 °C.

1.4.1.2 If the RNA concentration is < 1 µg/µL, additional precipitation using 5 M ammonium acetate (provided in kit) can be performed as per the manufacturer's protocol. If the RNA concentration is still < 1 µg/µL, proceed with aliquoting 1 µL into PCR tubes.

2. Slide Coating

Note: Steps 2.1 - 2.10 should be performed 24 - 48 h prior to the anticipated cell seeding.

2.1 Preheat the oven to 250 °C.

2.2 Using sterile gloves, wrap each glass slide 2x with aluminum foil. Avoid touching the surface of the slide directly.

2.3 Bake the slides in the oven for 1 h at 250 °C and allow the slides to cool to room temperature.

Note: This step is important for destroying any contaminating endotoxin.

2.4 While the slides are cooling, make a fibronectin stock solution of 1 mg/mL with distilled water and incubate it for 30 min at 37 °C to dissolve. Make 100-µL aliquots. Set aside the aliquots for immediate use and freeze the remaining aliquots for future use.

2.5 Unwrap the outer covering of aluminum foil before putting the glass slide into a biosafety cabinet. Perform steps 2.6 - 2.7 and 2.9 in the biosafety cabinet.

2.6 Place the glass slide into a sterile rectangular 4-well cell culture dish.

2.7 Dilute fibronectin stock solution 1:100 with distilled water. Coat each slide with 1 mL of diluted fibronectin, drop by drop, using a pipette. Make sure the whole slide is covered.

2.8 Incubate the slides in a tissue culture incubator at 37 °C for 24 - 48 h.

2.9 After the incubation, aspirate the fibronectin by tilting the 4-well cell culture dish. Avoid touching the slide directly with the aspirator.

3. Cell Seeding onto Glass Slides

3.1 Count the human endothelial cells at early passage (passage 2 - 5) and seed ~1.0 x 10^6 cells onto each fibronectin-coated glass slide with 1 mL of media (1.0 x 10^6 cells/mL media). Seed the cells 24 h prior to the anticipated application of laminar flow if no other treatment is to be performed.

Note: These numbers are used as a guide for experiments with 24 h of flow.

3.1.1. Adjust the seeding density to achieve a confluent monolayer of cells at the time of cell harvesting and RNA extraction.

3.2 Let the cells adhere to the slide for 15 min at 37 °C.

3.3 Add 3 mL of media in each well of the cell culture dish to cover the slide, and incubate the cells at 37 °C for 24 h with 5% CO_2.

4. Small Interfering RNA (siRNA) Transfection

4.1 Preparation of cells on glass slides for siRNA transfection and flow experiments

4.1.1 Follow the protocol in steps 2 (slide coating) and 3 (cell seeding onto glass slides) for glass slide preparation and coating for flow experiments.

4.1.2 Seed cells 24 h prior to siRNA treatment (48 h prior to the application of laminar flow).

4.1.3 Seed human endothelial cells in antibiotic-free media at 750,000 to 1 x 10^6 cells per slide to achieve 85% - 95% confluence the next day.

Note: HUVEC are used in this protocol.

4.2 Preparation of siRNA-lipid-based transfection reagent complexes (per slide)

4.2.1 Design custom siRNAs or order premade siRNAs for the desired gene of interest.

Note: Perform the following steps in a biosafety cabinet.

4.2.2 Add 6 µL of siRNA (20 µM stock) in 414 µL of reduced serum medium (see **Table of Materials**) and mix gently by pipetting.

4.2.3 Dilute 49.5 µL of gently mixed lipid-based transfection reagent (see **Table of Materials**) in 130.5 µL of reduced serum medium.

4.2.4 Mix gently and incubate at room temperature for 5 min.

4.2.5 Combine diluted siRNAs and lipid-based transfection reagent, mix gently, and incubate for 15 min at room temperature. Mix gently to avoid disruption of the lipid-based transfection reagent complexes.

Note: The solution may appear cloudy as complexes form.

4.2.6 While complexes are forming, remove the growth medium and wash the cells 1x with reduced serum medium.

4.2.7 Add 2.4 mL of reduced serum medium to each slide.

4.2.8 Add 600 µL of gently mixed siRNA-lipid-based transfection reagent complexes to each plate, and rock the plate back and forth to mix. Ensure the final concentration of siRNA is 40 nM. Ensure that the slide is completely covered.

4.2.9 Incubate the cells at 37 °C for 4 h.

4.2.10 Add 1 mL of antibiotics-free endothelial cell growth media containing 3x the normal concentration of fetal bovine serum (FBS) without removing the transfection mixture.

4.2.11 Incubate the cells at 37 °C until ready to use.

5. Calculation of the Flow Rate Based on the Desired Shear Stress[9]

5.1 Calculate the flow rate based on the desired shear stresses according to the following equation:

$$Q = \frac{\tau w h^2}{6\mu}$$

Here,
Q is the flow rate in mL/min;
τ is the desired shear stress in dynes/cm^2 (1 Pa = 10 dynes/cm^2);
w is the width of the parallel-plate flow chamber in cm;
h is the height of the parallel-plate flow chamber in cm;
μ is the viscosity of the media in cP (g/cm·s).

Note: Typical laminar shear stress experiments (non-pulsatile) in this workflow are conducted at τ = 1 Pa (10 dynes/ cm^2). μ can be measured using a viscometer such as a cone-and-plate viscometer and can vary depending on the contents of the media including the serum and additional dextran[9].

5.2 To achieve a specific τ (shear stress), adjust the flow rate and/or viscosity. At higher flow rates, adherent cells may dissociate from the slide. Sample flow rates with typical flow chambers are shown in **Table 1**.

6. Set-up of a Dedicated Environment for Monitoring System and Multiple Parallel-plate Flow Chambers (Figure 2)

6.1 Use a large heated unit/incubator with multiple shelves, internal electricity access, and glass doors—referred to as the BEACH (Built-in Environment with Adjustable CO2 and Heat)—to house multiple flow chambers simultaneously for experiments that require both shear stress and direct

comparison between two or more treatments or outputs (*i.e.*, DNA, RNA, and protein).

Note: The BEACH allows for frequent monitoring of the flow circuit, including the flow rate, without frequent disruption of the environment.

6.2 Ensure adequate CO_2 is available for the experiment and that the CO_2 monitor is functional. Ensure the water tray is appropriately filled, such that there will be humidified air.

7. Set-up of the Parallel-plate Flow Apparatus

Note: For the manufacturing of parallel plates, please see Lane *et al.*[9].

7.1 Autoclave the flow chamber plates, reservoir, dampener, tubing, and Luers for each parallel-plate flow chamber set-up as indicated in **Table 2**.

7.2 Set-up of the flow loop assembly (Figure 3).

7.2.1 Place sterile towels into the biological safety cabinet. Assemble the flow loop system, first without the parallel-plate flow chamber, in the biological safety cabinet.

7.2.2 Connect the tubing assembly for the reservoir:

7.2.2.1 Insert a #14 hard tube into one hole and two #14 soft tubes into the other two holes in the cap of the flow reservoir. Ensure that one of the soft tubes touches the bottom of the reservoir as **outflow** tubing.

7.2.2.2 Place a 1/16" male Luer at the end of the #14 hard tube and attach a sterile filter as an air vent.

7.2.2.3 Place a 1/16" female Luer at the end of the #14 soft **inflow** tube, coming from the reservoir, and attach a 4-way stopcock.

Note: For gene expression analysis or other studies where perfusates need not be collected, 2-way stopcocks can be used instead of 4-way stopcocks in this protocol.

7.2.2.4 Place a 1/16" male Luer at the end of the #14 soft outflow tube, coming from the reservoir.

7.2.3 Connect the reservoir outflow tubing to pump tubing: place a 1/16" female Luer at each end of a #13 hard tube (pump tubing). Connect the #14 soft outflow tube from the reservoir to the #13 hard tube by connecting 1/16" male and female Luers together.

7.2.4 Connect the pump tubing with 'dampener bridge' tubing: place a 1/8" male Luer and a 1/8" female Luer at each end of a #16 soft tube. Connect the 1/16" female Luer of the #13 hard tube (at the outflow end of the pump tubing) with the 1/8" male Luer of the #16 soft tube.

7.2.5 Assemble tubing for the flow dampener: place a 3/16" male Luer at one end of the #25 soft tubing and repeat this for the other side of the flow dampener. Attach the free ends of the #25 soft tubes to each side of the flow dampener.

7.2.6 Connect the 'dampener bridge' tubing with tubing for the flow dampener: connect a #25 soft tube from the flow dampener with the #16 soft tube of the 'dampener bridge' using the 1/8" female Luer from the #16 'dampener bridge' side and the already placed 3/16" male Luer from the #25 soft dampener tube side.

7.2.7 Assemble 'chamber bridge' tubing:

7.2.7.1 Place a 1/8" female Luer and a 1/8" male Luer at each end of a #16 soft tube ('chamber bridge' tubing).

7.2.7.2 Connect the 1/8" female Luer of the 'chamber bridge' with the 3/16" male Luer at the #25 soft tube from the free end of the flow dampener.

7.2.7.3 At the 1/8" male Luer (free end) of the 'chamber bridge' tubing, place a 4-way stopcock.

7.2.8 Connect the 4-way stopcock from the 'chamber bridge' free end to the 4-way stopcock from the reservoir inflow soft tubing (from step 7.2.2.3). Close the stopcocks.

7.2.9 Add media to the reservoir and dampener. For the 48-h exposure to shear stress, add 35 mL of media to the reservoir and 25 mL to the dampener. Adjust the volume of the media based on the duration of the flow experiment and the number of cells seeded.

7.2.10 Bring the assembled flow loop system to the pump in the BEACH. Place the #13 hard tube (pump tubing) into the pump head and secure it. Open the stopcocks.

7.2.11 Turn on the pump and slowly increase the pump speed. Let the media circulate through the loop system. Check for any leakage or blockage (*e.g.*, pressure build-up). Ensure that the media is flowing back to the reservoir.

7.3 Set-up of the flow chamber assembly

7.3.1 Place a 1/8" female Luer at one end of a #16 soft tube and attach a 4-way stopcock. Attach the free end of the tube to the **right** side of the top plate (**inflow** side).

7.3.2 Place a 1/8" male Luer at one end of a #16 soft tube and attach a 4-way stopcock. Attach the free end of the tube to the **left** side of the top plate (**outflow** side).

7.3.3 Place a 1/8” male Luer and a 1/8” female Luer at each end of a #16 soft tube (bubble trap tubing). Attach the tubing to bubble trap *via* the 1/8” male Luer. Attach a 4-way stopcock to the other end of the tubing *via* the 1/8” female Luer.

7.3.4 Using sterile tweezers, transfer the cell-seeded glass slide from the 4-well cell culture dish to the recess on the bottom plate. Ensure the cell-seeded side of the glass slide is facing up.

7.3.5 Using a 10-mL syringe, add 10 mL of warm media to the bottom plate within the red gasket line around the plate. Allow the media to flow through the slide and cover the cells. Avoid adding media directly onto the slide.

7.3.6 Gently place the top plate onto the bottom plate, aligning from one side to the other. Avoid the introduction of air bubbles. Screw the plates together tightly.

7.3.7 Remove air bubbles from the bubble trap by opening the stopcock on the right side (inflow) of the plate and gently flushing 20 mL of warm media using a 30-mL syringe. Make sure the stopcock on the left side (outflow) of the plate is closed and that the media is flowing through the bubble trap stopcock (open). Discard the flushed media.

7.3.8 Close the stopcock on the bubble trap and open the stopcock on the left side (outflow) of the chamber. Elevate the left side (outflow) of the chamber to a 45° angle and gently flush 20 mL of warm media using a 30-mL syringe from the right side (inflow) of the chamber to remove air bubbles from the chamber. Bubbles can be visualized through the window. Discard the flushed media.

7.3.9 Close the stopcocks on both sides of the chamber and cap. Inspect the cells by microscopy.

7.3.10 Transport the chamber to the BEACH with the previously assembled loop system.

7.3.11 Slowly decrease the pump speed and pause the pump. Close the stopcocks on the flow loop system to prevent leakage.

7.3.12 Connect the chamber and loop system together *via* stopcocks. Open all the stopcocks.

7.3.13 Slowly increase the pump speed and examine the set-up for any leakage or blockage. Ensure the media circulates in one direction and returns to the reservoir.

7.3.14 Place the flow sensor on the ‘chamber bridge’ tubing located on the inflow side of the chamber. Make sure the sensor is oriented properly in the direction of flow.

7.3.15 Adjust the pump speed to obtain the flow rate that was calculated previously, based on the desired shear stress.

Note: The flow rate may vary depending on the height and width of each chamber, as well as the viscosity of the media.

7.3.16 Turn on the CO_2 tank to achieve 5% CO_2 and place a water tray inside the BEACH.

8. Harvesting of the Cells and Extraction of RNA from the Flow Chamber

8.1 Once the desired time-period of flow is complete, slowly turn down the peristaltic pump speed to 0 and turn off the power. Quickly close all the open stopcocks and remove the chamber and the attached tubing with a stopcock on each end.

8.2 Take the chamber to a clean benchtop and gently unscrew all screws to remove the top plate. Using needle-nose tweezers, remove the glass slide from the bottom plate and place it into a 10-mm^2 tissue culture dish.

8.3 Wash the slide with 10 mL of cold phosphate-buffered saline (PBS) -/- and check the cells under a microscope to confirm cell adherence and alignment in the direction of flow.

8.4 Aspirate the PBS from the plate and transfer the slide to a clean 10-mm^2 dish. Add 350 µL of lysis buffer from the RNA extraction kit (see **Table of Materials**), containing 1/100 of beta-mercaptoethanol, to the slide.

CAUTION: Add beta-mercaptoethanol in a chemical fume hood.

8.5 Scrape the cells off the slide using a polyethylene-bladed cell scraper. Tilt the tissue culture dish, enabling the liquid to pool at the bottom, and remove the glass slide with forceps. Pipette the cell lysate into a 1.5-mL tube and keep it on ice.

8.6 Dilute stock exogenous reference RNA (luciferase RNA) to 0.0025 ng/µL through serial dilution prior to adding it to the sample. For example, if the stock concentration is 1 µg/µL, a dilution of 1/400,000 is required to achieve 0.0025 ng/µL. Add 10 µL of diluted luciferase RNA to each sample of cell lysate for downstream RNA isolation and analysis.

8.7 Proceed with the RNA extraction protocol as per the manufacturer's instructions, or freeze the lysate at -80 °C until able to proceed with the extraction.

9. Calculation of the Efficiency of the RNA Extraction and cDNA Synthesis

Note: Calculate the luciferase efficiency after RT-qPCR by comparing the theoretical yield and the experimental yield.

9.1 Determine the theoretical yield for luciferase RNA.

9.1.1 Determine the total amount of luciferase copies added per sample. (Adding 0.025

ng/sample = 2.73 x 10^7 copies of luciferase RNA per sample prior to RNA extraction.)

9.1.2 Calculate the molecular mass of the luciferase RNA by using an average molecular mass per nucleotide of 330 g/mol and multiplying it by the length of the firefly luciferase RNA, 1652 nucleotides.

9.1.3 Divide the amount of luciferase RNA added (0.025 ng) for each sample prior to RNA extraction by the molecular mass to yield the molar quantity. Then, multiply that by Avogadro's number to yield the copies added per sample.

9.1.4 Calculate the theoretical yield for the luciferase copies for each RT-qPCR reaction by using the equation:

$$= \frac{(\frac{Copies}{\mu L\ RNA})\ x\ (Volume\ of\ RNA\ used\ for\ First\ Strand\ cDNA\ synthesis)}{(Final\ volume\ for\ First\ Strand\ cDNA\ synthesis)\ x\ (\frac{2\ \mu L}{well\ on\ RT\ plate})}$$

9.2 Calculate the experimental yield for the luciferase copies for each RT-qPCR reaction by using the luciferase plasmid to generate a standard curve for RT-qPCR.

9.3 Calculate the luciferase efficiency (%) using the equation:

$$Luciferase\ Efficiency\ (\%) = \frac{Experimental\ yield}{Theoretical\ yield} \times 100$$

REPRESENTATIVE RESULTS:

Successful linearization of luciferase plasmid using restriction enzymes was confirmed by running digested products on an agarose gel (**Figure 1**). The size of the linearized product was confirmed using DNA ladders and by comparison with uncut plasmid.

We have adapted the parallel-plate flow chamber set-up from Lane *et al.*[9] for experiments that require multiple conditions/treatments with shear stress or multiple shear stress conditions. We use a dedicated environment, the BEACH, that can house multiple, fully-assembled flow circuits that all have monitored flow rates (**Figure 2**). The flow rate is monitored just upstream of the parallel-plate assembly (**Figure 3**). The flow circuits and rates can be monitored directly through glass doors without causing fluctuations in temperature, humidity, or gas content within the BEACH.

Manufacturing processes can lead to small variations in chamber height. Thus, flow rates must be calculated for each chamber to achieve the same shear stress (**Table 1**). In theory, chambers with identical heights can use identical flow rates to achieve the same shear stress and can be used in series. Typical experiments with endothelial cells use shear stress of 0 - 1.5 Pa. Laminar shear stress of 1 Pa was used in this workflow to model arterial endothelial shear stress. There

563 can also be variations between pump head settings and within pump heads over time with use.
564 Using the flow meter can account for these differences.
565
566 Experiments using the application of shear stress often involve multiple shear stress conditions,
567 treatment conditions, and time points. Where possible, we use an endogenous reference RNA to
568 account for any variabilities in the experimental set-up. For some experiments, finding an
569 endogenous reference RNA with quantitative stability is not feasible[16]. Furthermore, quantitative
570 stability or instability of endogenous reference genes between samples can be attributed to
571 either stimulus-dependent effects on cellular expression levels or variations in efficiencies of RNA
572 extractions or reverse transcription. To account for these inefficiencies, and in the setting where
573 an endogenous reference gene is not quantitatively stable, we use a spike-in exogenous
574 reference gene. For experiments incorporating laminar shear stress in mammalian endothelial
575 cells, we use a firefly luciferase RNA as an exogenous RNA spike-in (**Figure 4A**).
576
577 **Figure 4** shows analyzed RT-qPCR experimental data from shear stress experiments assessing
578 Krüppel-like factor 2 (KLF2) loss-of-function using siRNA. KLF2 is a transcription factor
579 upregulated by laminar flow in endothelial cells and a major transcriptional mediator of
580 endothelial gene expression in the setting of laminar flow[2].
581
582 **Figure 4A** shows luciferase efficiencies for three separate experiments, each using two flow
583 chambers. Luciferase efficiencies can be similar between samples of an experiment (Experiment
584 1) or show some variability (Experiments 2 and 3) (**Figure 4A**). These results are especially
585 valuable in experimental systems where only small absolute changes are seen. The use of an
586 exogenous reference gene may be particularly important in experiments where experimental
587 treatments can interfere with the efficiency of reverse transcription or PCR[17]. The results
588 depicted in **Figure 4A** are typical. A luciferase efficiency of 5% indicates that 5% of the luciferase
589 RNA (*i.e.*, the initial starting amount of RNA) added to the sample prior to RNA extraction is
590 detected by RT-qPCR. Between samples or conditions within a single experiment, luciferase
591 efficiencies are usually ± 50%. Results should be interpreted with caution if the variability of
592 luciferase efficiencies is > 50% and should include a review of the experimental procedures and
593 conditions.
594
595 **Figure 4B** shows typical experimental results of RT-qPCR from repeated shear stress experiments,
596 each using multiple parallel-plate flow chambers. Within each experiment, KLF2 mRNA
597 expression is normalized in three ways. The first normalization uses an endogenous reference
598 RNA, Cyclophilin A (CycA). The second normalization uses an exogenous reference RNA, firefly
599 luciferase (Luc). The third normalization uses both the endogenous and exogenous reference
600 RNA. Within each experiment shown in **Figure 4B**, all three normalization methods
601 (normalization to the endogenous reference gene, the exogenous reference gene, and both
602 endogenous and exogenous reference genes together) yields similar results. If the normalization
603 method significantly changes the results (*e.g.*, leads to > 50% difference), the results should be
604 interpreted with caution. When there is considerable variability between the methods of
605 normalization, the endogenous reference gene(s) should be reviewed, as it may be a dependent
606 variable in the experimental system. Similarly, the experimental procedures and conditions

should be reviewed. In **Figure 4B**, KLF2 knockdown using siRNA yields similar knockdown efficiency between three separate experiments (Experiment 1, 2, and 3). We used three distinct biological samples for these experiments, using two simultaneously running flow chambers with shear stress at 1 Pa for each experiment.

FIGURE AND TABLE LEGENDS:

Figure 1: Agarose gel image of the linearization of exogenous luciferase plasmid. Supercoiled and 1 kb+ DNA ladders are used as markers to determine both uncut and cut luciferase plasmid sizes in kilobases (kb).

Figure 2: Schematic overview of multiple flow circuit assemblies within a dedicated environment (the BEACH). The flow rates in both flow circuits are easily monitored in real-time, without disturbing the environment, and both circuits can run simultaneously.

Figure 3: Schematic overview of a single flow circuit assembly. The tubing sizes and Luers used are indicated in this figure. Ensure that the flow meter is oriented in the direction of the flow and placed upstream of the flow chamber.

Figure 4: Representative results from KLF2 loss-of-function experiments in human endothelial cells exposed to shear stress (1 Pa) for 24 h. (**A**) This panel shows the quantification of exogenous luciferase RNA in three separate flow experiments. Luciferase efficiencies can be similar between samples of an experiment (Experiment 1) or show some variability (Experiments 2 and 3). **Luciferase (absolute copies)** is the copy number of luciferase RNA detected by reverse-transcriptase quantitative PCR (RT-qPCR) by absolute quantitation using a standard curve. **Luciferase efficiency** is the experimental luciferase copies divided by the theoretical luciferase copies for each sample multiplied by 100 (see step 8 of the protocol). **Relative luciferase efficiency** is the luciferase efficiency of each sample divided by the reference condition (Flow + Ctlsi) within each experiment. (**B**) These panels show the normalization of gene expression in a set of sample shear stress experiments using both endogenous and exogenous reference genes. The results are from reverse-transcriptase quantitative PCR (RT-qPCR). Knockdown of KLF2 mRNA expression is shown in the presence of laminar flow with shear stress of 1 Pa for 24 h. FC = fold change; CycA = cyclophilin A, used as an endogenous reference RNA; Luc = luciferase, used as an exogenous reference RNA. The data adapted from Man *et al.*[5].

Table 1: Chamber heights and examples of flow rates for various flow chambers to achieve shear stress of 1 Pa.

Table 2: Parts to be autoclaved in step 6.2 of the protocol.

DISCUSSION:

Shear stress is a physiologic condition that modulates endothelial function, in part, by affecting steady-state gene expression[2,5]. Models of gene regulation in various shear stress conditions will contribute to a greater understanding of endothelial function. This pragmatic workflow includes

a flow circuit using a parallel-plate flow chamber adapted from Lane *et al.*[9] and represents laminar, non-pulsatile flow. The overall set-up was designed to facilitate experiments that require multiple flow chambers and minimize experimental variability in this setting.

The flow circuit assembly is a major component of this workflow and is adapted from Lane *et al.*[9]. Several adaptations of this assembly and protocol were made to reflect differences in the experimental systems. A large heated unit, the BEACH, is an adaptation that facilitates the simultaneous operation and monitoring of several flow circuits within the same environment. This system has been used successfully for the application of shear stress to endothelial cells for various time periods, from 1 h to 7 days, and at several levels of shear stress (*e.g.*, 1.0, 1.5, and 2.0 Pa). This system was also used for gene knockdown studies to assess the function of a flow-responsive endothelial gene in the setting of shear stress (**Figure 4**)[5]. There is considerable variability between pumps and pump heads, which may also change over time due to normal wear and tear. To account for these differences, we use a flow sensor situated proximal to the flow chamber to continuously monitor flow rates. Various sizes of tubing and Luers are used to ensure a tight fit for each component and to prevent any leakage during experiments. We counted cells and seeded approximately 1,000,000 cells per glass slide, dropwise, and then let the cells incubate at 37 °C for 15 min to increase adherence efficiency. Compared to endothelial progenitor cells, which can be seeded for a short time prior to the application of shear stress[9], we seed human endothelial cells for at least 24 h prior to the application of shear stress. Shorter durations can lead to the dislodging of cells during flow, or a discontinuous layer of endothelial cells, even with the appropriate seeding density. We emphasize the inspection of the slides for a confluent monolayer of endothelial cells both prior to and after the application of shear stress. We find that slides coated with fibronectin, a natural extracellular matrix component[18], maintain the endothelial monolayer more consistently compared to sides coated with gelatin (denatured fibrillar type I collagen). Finally, the flow dampeners in this protocol are optimized to use 30 mL of media, compared to 190 mL of media for other manufacturers.

Several steps in the flow circuit assembly require additional care. An even monolayer of endothelial cells should be established prior to the application of shear stress. It is important to seed cells onto the glass slide dropwise to increase the number of cells that adhere to the slide and, thus, the overall slide coverage. The wait time between cell seeding and adding additional media to the slide generally improves seeding efficiency as it provides sufficient time for cells to adhere to the slide rather than be washed away into the multi-slide tray. Inspect cells visually before, during, and after the application of shear stress. A microscope can be placed in the BEACH for this purpose. The flow sensor must be attached in the correct orientation and the target flow rate should be checked for each individual flow chamber. The flow loop system should be perfused without the chamber to ensure no media leakage or other problems, such as pressure build-up, to avoid perturbing the cells during the actual experiments. Inspect for and eliminate bubbles in the system. While testing the flow loop system, ensure the stopcocks are all open before turning on the peristaltic pump to allow uninterrupted, unidirectional flow. Ensure that all stopcocks are closed prior to attaching the flow chamber to the loop and fully opened prior to restarting the pump.

We find that the addition of an exogenous reference gene is helpful in a variety of scenarios. Endothelial cell media often contains heparin, which is an inhibitor of PCR[17]. While some RNA extraction protocols incorporate steps to remove heparin, trace amounts can cause differences in PCR efficiencies between samples. Potent treatments may also preclude the identification of an endogenous reference gene that is quantitatively stable. Our lab has created an efficient protocol to synthesize 5'-capped and poly-A-tailed luciferase RNA for use as an exogenous reference RNA. This strategy has proved to be a cost-effective approach compared to purchasing a commercially available RNA. During the preparation of exogenous reference RNA, it is important to aliquot RNA into single-use aliquots to avoid multiple freeze-thaw cycles. Thorough mixing and accurate pipetting are critical to maintaining inter-aliquot consistency. Typical experiments show a luciferase efficiency in the range of 5% ± 2.5% but can range from 1% - 10%. It is prudent to correct RT-qPCR results for both the exogenous reference RNA efficiency and an endogenous reference (housekeeping) gene.

For the experiments we conduct, firefly luciferase sequences are used as a non-mammalian spike-in reference RNA in mammalian models. In experiments where firefly luciferase is expressed in cells, this would not be an appropriate reference gene. Other species-specific reference genes can be used, including the *Caenorhabditis elegans* miRNA cel-miR-39[19] and ribulose bisphosphate carboxylase plant RNA[20].

This flow circuit models a 2-D monolayer of endothelial cells grown on tissue culture plastic or glass, which is quite stiff. Matrix stiffness can influence the endothelial response to fluid shear stress[21]. This model system uses a relatively high oxygen concentration more similar to arterial than venous oxygen concentrations. This model closely resembles straight segments of larger vessels in a closed cardiovascular system and provides a relatively homogenous environment for endothelial cells on the slide. Other specific conditions in 3-D structures, such as bifurcations or curvatures of vessels, are not represented with this model. Other systems can model other flow patterns, including those in curved regions of the vasculature, but yield fewer cells than the system described in this protocol. Similarly, other assemblies may be more appropriate if > 1 x 10^6 cells are required, or if a single-cell analysis is required. Our current application models non-pulsatile laminar flow. Yet, this model can be used to generate other waveforms, including pulsatile or oscillatory waveforms, with consistency as the flow rates are monitored continuously.

Overall, this pragmatic workflow provides a system for the simultaneous application of shear stress to multiple flow chambers with monitored flow rates. Materials and procedures throughout this workflow are designed to minimize experimental variability between samples and conditions. This workflow has been successfully used for RNAi experiments in the setting of laminar flow and can be also used for any experiments requiring multiple conditions with laminar shear stress, or multiple laminar shear stress magnitudes and/or time points, including alternative waveforms.

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DISCLOSURES:

The authors have nothing to disclose.

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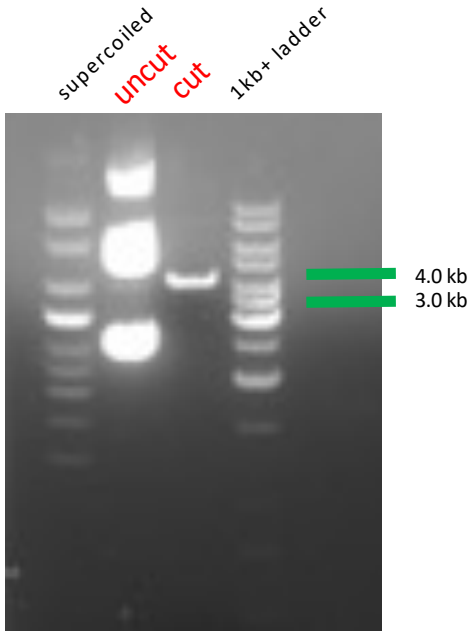
Figure 1



pSP-luc+ = 4100 bp

Figure 2

Click here to download Figure Figure3(1).ai.ps

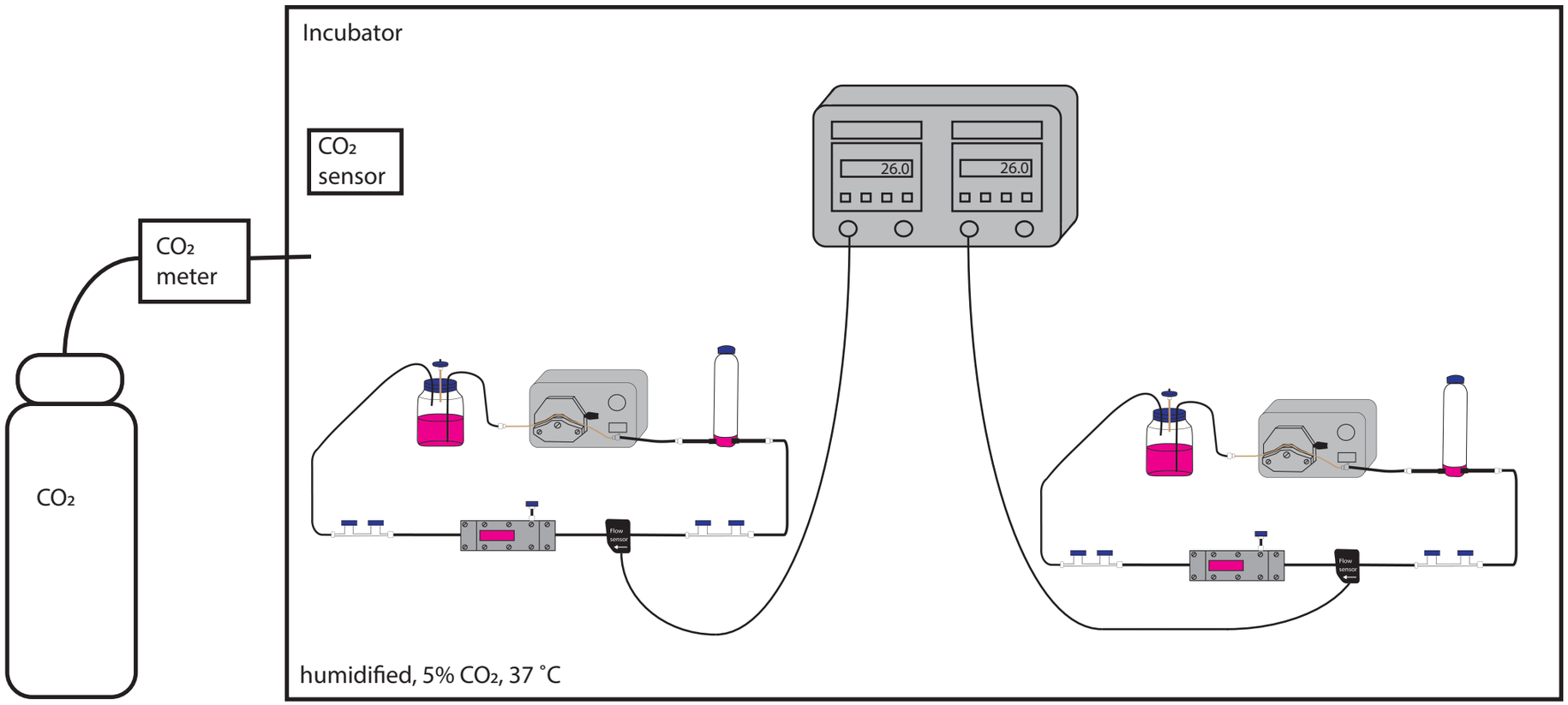


Figure 3

Click here to download Figure Figure2(1).ai.ps

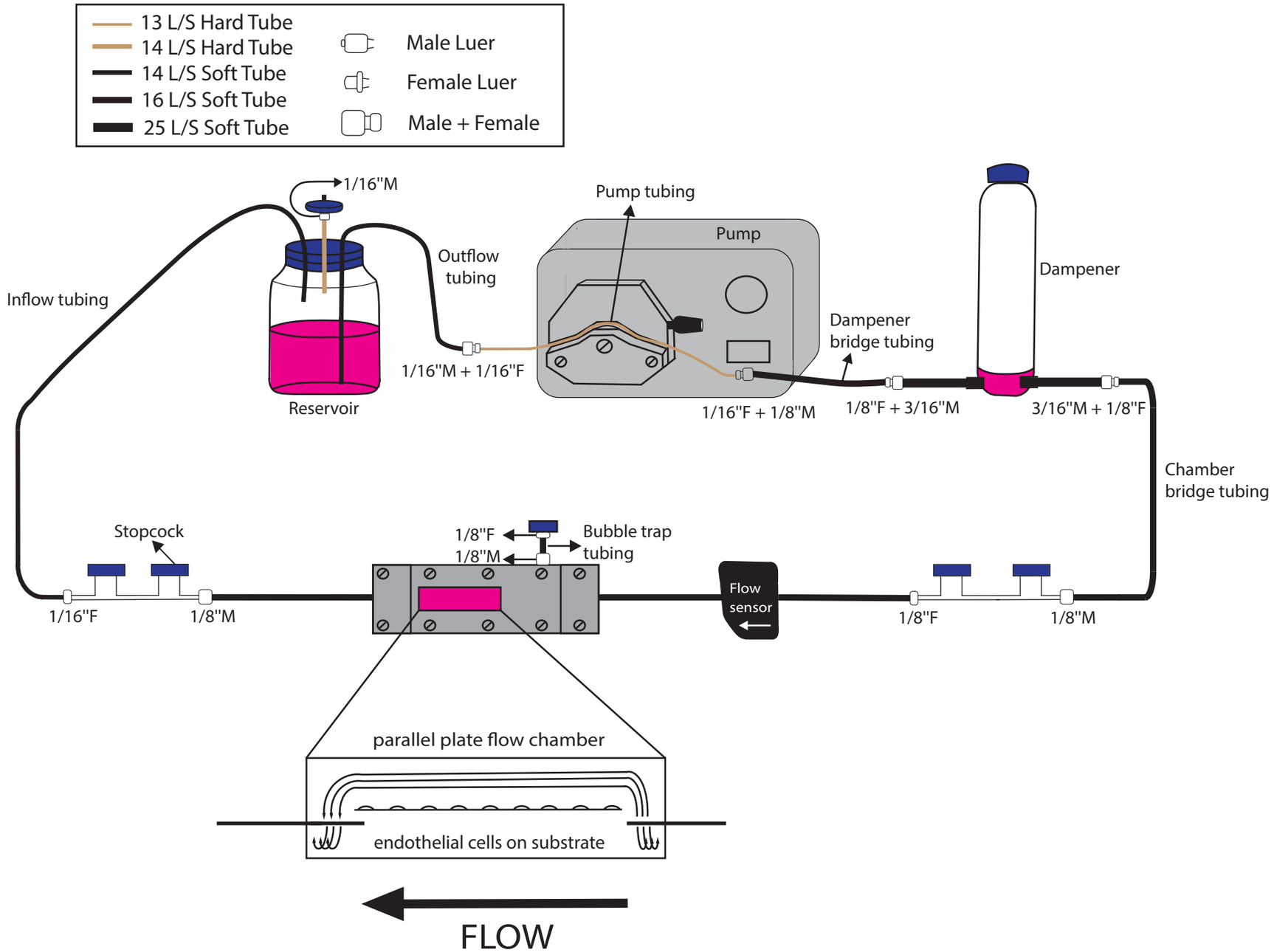


Figure 4

A

Experiment 1	Luciferase (absolute copies)	Luciferase efficiency (%)	Relative Luciferase efficiency (vs. Ctlsi for each Experiment)
Flow+Ctlsi	2569.3	3.3	1.0
Flow+KLF2si	1957.9	3.0	0.9
Experiment 2			
Flow+Ctlsi	9906.0	6.9	1.0
Flow+KLF2si	3277.3	2.4	0.3
Experiment 3			
Flow+Ctlsi	6797.7	5.3	1.0
Flow+KLF2si	7247.4	3.8	0.7

B

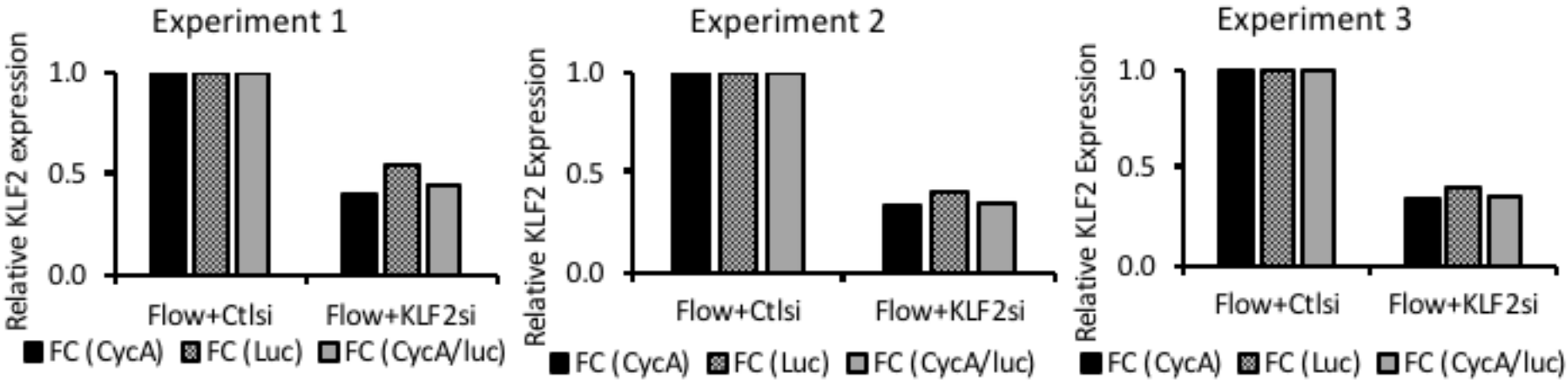


Table 1

Chamber Height (microns; μm)	Chamber Width (centimeters; cm)	Flow Rate (mL/min) for 1 Pa shear stress	Viscosity (centipoise; cP)
303.80	1.90	19.48	0.90
326.10	1.90	22.45	0.90
344.84	1.90	25.10	0.90
319.06	1.90	21.49	0.90

J cloths
Tweezers
Reservoir Bottle and Cap
Dampener and Cap

Flow Loop
1/16" Male Luer x 3
1/16" Female Luer x 3
1/8" Male Luer x 2
1/8" Female Luer x 4
3/16" Male Adaptor x 2
14 L/S Hard Tube (2 inches) x 1
14 L/S Soft Tube (5 inches) x 2
16 L/S Soft Tube (3 inches) x 2
25 L/S Soft Tube (3 inches) x 2
13 L/S Hard Tube (10 inches) x 1

Flow Chamber
1/8" Male Luer x 2
1/8" Female Luer x 2
16 L/S Soft Tube (3 inches) x 3
Top and Bottom Plates
Screws

Table of Materials

Click here to download Table of Materials Materials Table .xls

Name of Material/Equipment	Company
0.05% Trypsin-EDTA	gibco
10 mL Syringe	BD
10 mm^2 Culture Dish	Sarstedt
30 mL Syringe	BD
4-Way Stopcocks	Discofix
Aluminum foil	
BEACH	Darwin Chambers Company
Cell Scrapers	
CO_2 Meter	BioSphenix, Ltd.
CO_2 Sensor	BioSphenix, Ltd.
Distilled water	gibco
Dulbecco's phosphate-buffered saline (DPBS) -/-	gibco
Endothelial Cell Growth Medium 2	Promo Cell
Endothelial Cell Growth Medium 2 Supplement Mix	Promo Cell
Fibronectin (pure)	Sigma-Aldrich
Filter (0.20 um)	Sarstedt
Flow Dampener and Cap	U of T glass blowing shop
Flow Meter: 400 Series Console	Transonic Scisense Inc.
Flow Meter: 400 Series Tubing	Transonic Scisense Inc.
Flow Reservoir and Cap	U of T glass blowing shop
Flow Sensor	Transonic Scisense Inc.
Isotemp 737F Oven	Fisher Scientific
J cloth	J cloth
Microscope Slide (25 x 75 x 1 mm)	Fisherfinest
Paper sterilization pouch	Cardinal Health
Pump (Masterflex L/S Economy Drive)	Cole-Parmer
Pump Head (Masterflex L/S Easy Load)	Cole-Parmer
Rectangular 4 Well Dish	Thermo Scientific
Tweezers	

Tubing

Masterflex C-Flex L/S 25 Soft Tubing	Cole-Parmer
Masterflex C-Flex L/S 14 Soft Tubing	Cole-Parmer
Masterflex C-Flex L/S 16 Soft Tubing	Cole-Parmer
Masterflex PharMed BPT L/S 13 Hard Tubing	Cole-Parmer
Masterflex PharMed BPT L/S 14 Hard Tubing	Cole-Parmer

Luer

3/16" Male Luer	Cole-Parmer
1/8" Male Luer	Cole-Parmer
1/8" Female Luer	Cole-Parmer
1/16" Male Luer	Cole-Parmer
1/16" Female Luer	Cole-Parmer

Knockdown reagents

Oligofectamine Reagent	Invitrogen
Opti-MEM I Reduced Serum Medium	gibco

***In vitro* transcription**

Generuler 1kb+ DNA ladder	Thermo Scientific
MEGAclear Kit	Ambion
mMESSSEGE mMACHINE SP6 Transcription Kit	Ambion
pSP-luc+	Promega
Supercoiled DNA Ladder	New England BioLabs Inc.
UltraPure Agarose	Invitrogen
UltraPure Ethidium Bromide	Invitrogen
XhoI Restriction Enzyme	New England BioLabs Inc.

RNA extraction

Beta-mercaptoethanol	Sigma
RNeasy Mini Kit	Qiagen

Catalog Number	Comments/Description
25300-062	
302995	
83.3902	
302832	
D500	
MN: HO85, SN: 4947549	
MN: P120, SN: 0342	
MN: C700, SN: 52852	
15230-170	
14190-144	
C-22011	
C-39216	
11051407001	
83.1826.001	
T402	
TS410	
ME4PXL	
FI-737F	
12-544-4	
92713	
7554-90	
7518-00	
267061	

06424-25
06424-14
06424-16
06508-13
06508-14

45518-08	For #25 tubing
30800-24	For #16 tubing
30800-08	For #16 tubing
45518-00	For #14 tubing
45508-00	For #14 tubing

12252-011
31985-070

SM1331
AM1908
AM1340
E4471
N0472S
16500-500
15585011
R0146S

M3148-100mL
74104

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Institution: University of Toronto

Article Title: A Pragmatic Workflow for Gene Expression Analysis of Endothelial Cells

Signature: [signature] Date: May 15, 2018

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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.

Author response: We have reviewed the manuscript for spelling and grammar issues.

2. Please check either Standard Access or Open Access in the Author License Agreement (ALA) form. Please then scan and upload the form to your Editorial Manager account.

Author response: We have re-uploaded the Author License Agreement and checked "Standard Access".

3. Please revise lines 217-222 to avoid previously published text.

Author response: We have revised lines 217-222 to avoid any redundancy from previously published text.

4. Figure 2: Please include a space between the number and its unit (37 °C).

Author response: We have included a space between the number and its unit.

5. Please shorten the title if possible.

Author response: Thank you for the suggestion. We were unable to convey the essence of our protocol with a shorter title.

6. Please define all abbreviations before use (RCF, etc.).

Author response: We have reviewed the manuscript for abbreviations and defined all abbreviations before use.

7. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Author response: We have revised the manuscript to use SI abbreviations for all units.

8. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Author response: We have reviewed the manuscript and included a space between all numbers and their corresponding units.

9. 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.

Author response: We have adjusted the number of the Protocol to follow the JoVE Instructions for Authors.

10. 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. For example: CutSmart, MEGAclear, Promega, Ambion, AM1340, Sigma-Aldrich, Dharmacon, Darwin Chambers Company, Qiagen, QIAshredder, etc.

Author Response: We have revised our manuscript to remove commercial language. In some situations, our protocol is based on specific commercial products. Wherever possible, generic terms have been used instead and commercial products are referenced in the Table of Materials.

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Author Response: We have removed personal pronouns.

12. Please revise the protocol so 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. Please include all safety procedures and use of hoods, etc.

Author Response: We have revised the protocol such that actions are described in the imperative tense wherever possible.

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

Author Response: We have substantially reduced the number of Notes from 23 → 10

14. For buffer and elution/binding solution etc., please provide composition. If they are purchased, please cite the materials table.

Author Response: We have revised the manuscript to include solution composition or included purchased materials in the materials table.

15. Lines 139-141, 271-272: Please add more details to this step. This step does not have

enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.
Author Response: We have added detail to lines 139-141 by and included details about the cDNA clone for the exogenous reference RNA. We have added details to line 271-272 by referencing previous sections for cell seeding and by including more detail in the title.

16. Lines 163-164: The Protocol should contain only action items that direct the reader to do something. Please move the expected results to the Representative Results section.

Author Response: Thank you for the comment. Our previous wording was not clear. These lines were intended as a checkpoint step in the protocol, prior to proceeding. We have rephrased lines 163-164 to better reflect a step in the protocol and included an example in the representative results.

17. Lines 168-171, 194-199, 255-256, 324-329: Please write the text in the imperative tense.

Author Response: We have written text in the imperative tense for these lines.

18. Line 177: Please specify centrifugation conditions (force and time).

*Author Response: Centrifugation conditions (*10,000-15,000 RCF for 5 s
) have been added.

19. Line 223: Please mention how RNA yield is calculated.

Author Response: Our original protocol used the term "yield " in error. We have revised the manuscript and used the term "concentration" which is the intended term.

20. Line 320: Please add a numbered superscript for citing the reference.

Author Response: We have revised the line to add the reference using the Jove endnote style.

21. Lines 474-497: 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 and a maximum of 4 sentences per step.

Author Response: We have revised this section of the protocol and broken down this paragraph into a number of small steps.

22. Please include single-line spaces between all paragraphs, headings, steps, etc.

Author Response: We have revised the protocol to include single-line spaces between major steps and major sub-steps.

23. There is a 10 page limit for the Protocol. Please revise the protocol section to meet this page limit.

Author Response: Within the manuscript as a whole, the protocol is ~ 9 pages.

24. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Author Response: We have highlighted 2.75 pages of the protocol to tell the most cohesive story possible.

25. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing euthanasia.

Author Response: We have highlighted complete sequences and highlighted actions steps in imperative tense wherever possible.

26. 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.

Author Response: Wherever possible, we have highlighted all relevant details required to perform the step.

27. References: Please do not abbreviate journal titles.

Author Response: We have revised our references in EndNote using the JoVE endnote style.

28. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available.

Author Response: We have removed trademarks and registered symbols in the Table of Equipment and Materials. No antibodies are listed in the Table If Equipment and Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this study, authors described a workflow for analysis of gene expression form endothelial cells subjected to steady laminar flow using multiple monitored parallel-plate flow chambers (PPFCs). Overall, this work is well organized and can be considered for publication. However, the following issues should be addressed:

Major Concerns:
1. The sections for PPFCs setup and cell seeding were introduced by Lane, W et al. in an earlier issue of JoVE. They may need to be rephrased;

Author Response: Thank you for the suggestion. While the sequence of steps for the PPFCs setup is similar in general, the text from these sections was newly written and we have also revised these sections such that we provide more details than previously published work with minimal overlap in the text.

2. The functions of 4-way stopcocks in the two sides of flow chamber are convenient for extracting perfusion fluids for detecting active factors secreted by cells, these stopcocks are unnecessary in the study of gene expression in this work;

Author Response: Thank you for your comment. While we use 4-way stopcocks in our protocols, we agree with the reviewer that they are unnecessary for the gene expression work described here and have amended the protocol accordingly. Line #411-412 "Note: For gene expression analysis or other studies where perfusates need not be collected, 2-way stopcocks can be used instead of 4-way stopcocks in this protocol."

3. At least three times of cell experiments at one condition should be conducted to verify the reliability of this method;

Author Response: We have revised our figures to show three repeated experiments. Our initial intent was also to demonstrate inter-sample variability in the efficiency of RNA extraction/cDNA synthesis by using an exogenous reference RNA - the luciferase efficiencies (experimental/theoretical luciferase copy numbers x 100). We realize that our results, as previously presented, did not clearly present these concepts. Therefore, we have revised our figures to show: 1) three repeated experiments with multiple flow chambers per experiment; 2) Examples of inter-sample variability in the efficiency of RNA extraction/cDNA synthesis - Luciferase efficiencies (expected/theoretical luciferase copy numbers x 100). We have also revised the text of our representative results to reflect these changes.

4. Why did not connect two parallel-plate flow chamber into one flow loop system instead of connecting two chambers into two circuits in Figure 2? If so, it will save one peristaltic pump and other materials;

Author Response: Using two flow chambers in series is a possibility with the appropriate complement of materials and hardware. In our experience and those of colleagues, we

have found differences in the heights of our flow chambers such that each chamber requires a different flow rate to achieve the equivalent shear stress conditions. As a result, we have adopted our system of measuring flow rate to each chamber in parallel so that we can achieve equivalent shear stress conditions.

5. How the different heights of the PPFCs in Table 1 were accurately measured?

Author Response: The heights of the PPFCs were measured using a depth micometer. The measurement was determined in thousandths of an inch and multiplied by 25.4 μm/inch to obtain values in Table 1.

Minor Concerns:
1. In this manuscript, some paragraphs are aligned on both ends, while some are left aligned, please unify the format of paragraphs;

Author Response: We have reviewed the paragraph alignment and unified the format.

2. The format of the references should also be the same.

Author Response: We have amended the References using the JoVE endnote file.

Reviewer #2:

Manuscript Summary:
The authors describe a method to cultivate endothelial cells under laminar flow in a chamber providing temperature and moisture suitable for cell culture. With this setup, it is possible to cultivate endothelial cells under various conditions, either different flow rates or different treatment options, in one experiment. In addition, they suggest the use of a spike-in reference RNA to avoid inter-experimental differences in PCR analyses. Overall, the manuscript is well-written and the protocol is clearly described. The need for cultivation of EC under laminar flow is reasonably explained.

Minor Concerns: Some aspects could be added, see below:

Introduction:
-more information about the EC used should be inserted (are they microvascular or from bigger vessels? Influence on shear stress that should be applied) line 78-79

Author Response: We have added a paragraph line #s 110-120 to include the types of ECs used in our protocol and our intent to model arterial shear stress. We have also included some examples of shear stress conditions in arteries and veins.

- it should be noted somewhere what the maximum sample size is for the setup (the authors say "multiple", but a number would be useful)

Author Response: We have now added a statement that describes the maximum sample size for our current setup. "h our current setup, we use a single monitoring system that can simultaneously monitor 4 separate flow loops. *line #s "For labs that need more flow loops, there is space in the dedicated environment for an additional monitoring system*."

Protocol:
-while the protocol in general is very detailed, it is missing how much plasmid DNA is used (line 143-146)

Author Response: We have added the quantity of plasmid DNA used. Line # "1.1.2.1 Perform restriction enzyme (RE) digestion of 1 µg of full-length plasmid (pSP-luc+, 4100 bp) using single-cutter RE (XhoI) in 1.5 mL microfuge tubes. "

-chapter 4/ Flow chamber assembly: how much medium is needed for flushing in total? Can this be reused/ does it go back to the reservoir or is it discarded? Should be noted, in case the extra medium needs to be prepared in advance. (line 418-430)

Author Response: We have amended the protocol to include the volume of medium needed for flushing. Line # 466 Line # 471 We have also included a step to discard the medium used for flushing.

-chapter "10" (Extraction of cells....) should be chapter 5 (line 447)

Author Reponse: We have amended our numbering system and corrected this section number.

Representative Results:
-line 518 and 529: correct citations/ citation style

Author Response: The citations in these lines have been corrected. Line # 562, Line # 580.

-regarding the explanation of data from Figure 3 and 4, it is not quite clear what the difference is between the two data sets- Fig 3 shows sample 1 and Fig 4 sample 2. Why not compare them in one figure? It would be easier for the reader to compare them.

Author Response: Thank you for your comment. Our intention was to show that the exogenous reference RNA sometimes shows relative stability of intersample efficiency of RNA extraction/cDNA synthesis (original Figure 3) and sometimes shows variability of intersample efficiency of RNA extraction/cDNA synthesis (original Figure 4). We realize that our data presentation lacked clarity. As per your suggestion, we have revised and simplified our figures and amalgamated into one figure to show: 1) three repeated experiments with multiple flow chambers per experiment suggested by the other reviewer (Figure 4B); 2)

Examples of inter-sample variability in the efficiency of RNA extraction/cDNA synthesis - Luciferase efficiencies (expected/theoretical luciferase copy numbers x 100). We have also revised the text of our representative results to reflect these changes (Figure 4A).

-Line 534- 537: is the "greater induction" referring to sample 1 in Fig 3? Or compared to what?

Author Response: Thank you for your comment. Our wording was unclear. Our intention was to compare the results within the same sample when normalized to an internal reference RNA, an exogenous reference RNA, or to both an internal reference RNA and an exogenous reference RNA. We have revised the text of our representative results to improve clarity.

-Figure 1 and 2 are quite redundant; Fig. 1 is just a more detailed depiction of one flow circuit. Maybe this could be integrated for a better overview?

Author Response: Thank you for your comment. We have amended the figures so that Figure 2 reflects the set-up of the overall dedicated environment, the "beach". Figure 3 now includes additional details and labelling to support the text in the protocol that describes each stage of the flow loop assembly.

-Figure legend to Fig. 3 and 4: please add explanations for the abbreviations (e.g. FC, CycA) used in the graphs

Author Response: In our new amalgamated data figure (Figure 4), we have now added explanations for the abbreviations in the graphs.

Some general comments:
-line 227: "is" is missing after RNA concentration

Author Response: Thank you. We have revised this line.

-line 257: "24" -> "h" is missing

Author Response: Thank you. We have revised this line.

-use consistent naming for the cell types used

Author Response: We have now indicated that the human endothelial cells we use are human umbilical vein endothelial cells (HUVEC) to model arterial flow. Within the remainder of the manuscript, we now use the term "human endothelial cells".

-the description of Flow Loop Assembly is hard to read and follow (chapter 3); could this be improved, maybe with a picture instead of words?

Author Response: Thank you. We have amended the protocol to clarify each sub-assembly of the Flow Loop Assembly and to clarify the action sequences for each sub-assembly. We have revised Figure 3 with additional detail and labelling so that it can be more helpful in following the description of Flow Loop Assembly.

-what are the advantages of this system compared to already available systems using multiple parallel-plate flow chamber systems like ibidi or others? The authors should state the benefits or uniqueness of their proposed system more clearly.

Author Response: Thank you for the comment. We have adapted our system to address experimental designs we required for our experiments that required multiple flow chambers with real-time monitoring. These are outline in Line # 106-108 and Line # 664-680. We have also amended our protocol to better address differences between the system in this protocol and other systems in Line # 96-97 and Line # 736-741.

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None of the figures as shown are re-used from the PNAS paper. Some data from Figure 4 of this JoVE manuscript overlap with the data in the PNAS paper and are cited line 415 of the manuscript. However, the figure is new.