**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.

*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 lf 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. “*n 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.*