Manuscript reference number: JoVE61817

Dear Dr Nam Nguyen,

We thank you for giving us the opportunity to submit a revised draft of the manuscript titled "Segmenting growth of endothelial cells in 6-well plates on an orbital shaker for mechanobiological studies" for publication in *JoVE*. We appreciate the time and effort that the editorial team and the reviewers dedicated to providing feedback on our manuscript and are grateful for the insightful comments that would improve our paper.

We have incorporated suggestions made by the editorial team and reviewers. Those changes are highlighted within the manuscript. Please see below, in blue, for a point-by-point response to the reviewers' comments and concerns.

Thank you.

Best Regards,

p.p.

Peter D. Weinberg
Professor of Cardiovascular Mechanics
Department of Bioengineering
Imperial College London

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
The manuscript has been vetted to ensure that there are no spelling or grammar mistakes.

2. Are stl files available for Figures 4, 5, 6?
STL file is only available for PDMS mold (Figure 2). Figure 1 and 3 are available in SLDPRT format. Files are attached as supplements. (Please take note that figures have been renumbered as requested)

3. Please number the figures in order of appearance in the manuscript. Currently, Figure 4 appears first.
The figures are now numbered in order of appearance in the manuscript. Figure files have been renamed and are attached with the manuscript.

4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
More details have been added to protocol step 1.1.1, 5.1, 5.6, 5.7, 5.8, 5.9, 5.10, and 5.11. All changes are tracked in the manuscript.

5. 1.3.1: How much is prepared?
The volume of PDMS to prepare will depend on the number of molds being produced. PDMS is very viscous and therefore it is difficult to handle. We therefore advise the user to prepare more than the required volume per mold. The volume of PDMS required per mold can be seen below.

6. 1.3.2: How much is poured?
Volume of PDMS required to fill the mold is approximately 2.6 mL. This information has been added to 1.3.2.

7. Please specify all volumes and concentrations throughout.
Volumes and concentrations have been added to step 3.4, 3.5, 5.6, 5.8, and 5.10.

8. 3.4: How much medium is used to resuspend? What is the medium prewarmed to?
Endothelial cells are resuspended in 1 mL of medium. Culture medium (Lonza EGM-2) are prewarmed to 37 °C. This information has been added to section 3.4 and 3.5, respectively.
'3.4 Transfer the solution to a 15mL centrifuge tube and neutralise the trypsin using prewarmed (37 °C) culture medium (Lonza EGM-2).'
'3.5 Centrifuge at 200 g for 5 min. Remove the neutralised trypsin solution and resuspend ECs with 1 mL of prewarmed culture medium.'

9. 3.5: How is used for resuspension?
Lonza EGM-2 was used to resuspend endothelial cells.

10. Please reference Figure 3 in the manuscript.
Figure 6 (the numbering is changed according to order of appearance in the manuscript) is referenced in 'Representative results' (Line 278).
'The number of ECs increased gradually with distance along the radius. No significant difference was observed in the number of ECs grown in segmented and full wells (Figure 6).'

11. Please provide titles for all of the figures.
Titles have been added to all figures in bold text.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Explicit permission to reuse any figures from Ghim et al (2018) has been obtained from Springer Nature (publisher of Journal of Biological Engineering). The letter is attached with this submission.
All figures adopted or modified from Ghim et al (2018) are cited in the figure legends (Figure 3-6).

13. Please discuss some limitations and critical steps of the protocol in the discussion.
We have added a paragraph to discuss the limitations of the protocol in the discussion. (Line 331)

'This protocol is dependent on the accuracy of the printed mold. Depending on the 3D printer, there may be variation in the exact dimensions of the mold. This will affect the final PDMS construct, which will in turn result in the cells adhering in an incorrect location within the well. The cells would therefore experience a shear stress profile other than the one modelled by CFD. Another drawback to using a 3D printer is that the mold may not be flat, due to warping during the printing. This will result in the final PDMS construct allowing Pluronic F-127 to leak underneath, preventing cells from adhering in the desired locations. Therefore it is crucial to check for leaks and measure the dimension of the PDMS construct before use.'

14. Please spell out journal titles in the references.
All journal titles are spelled out in references.

Changes to be made by the Author(s) regarding the video:
1. Please use American English instead of British English: mold instead of mould.
The spelling has been changed to 'mold' in the video and the manuscript.

2. Format & Content Standards
• The Results section exists but is not marked with a chapter title card, and there is no discrete Conclusion. Please add a discrete Conclusion after the Results. An easy way to achieve this is to move some commentary from the Introduction to the Conclusion.
A chapter title card for result section is added. A conclusion is added to the video.

• 10:32 This is where the Results would begin. A chapter title card is needed here to end the last protocol section and begin the Results section.

A chapter title card ‘Representative results’ has been added.

3. Chapter Title Cards & Graphics
• 01:44 "1.2 3D printing of Polydimethylsiloxane (PDMS) mould" Please adjust this chapter title card so that there are no black borders or margins in the video frame, so it matches the card @01:34
The black borders/margin in 1.2 title card has been removed.

• 04:01 "1.4 Preparation of 1% Pluronic F-127" Please adjust this chapter title card so that its resolution (sharpness) matches the previous chapter title cards that were well-placed and formatted, such as at 01:34 and 02:40. This card as-is is somewhat blurrier than the previous ones.
A high resolution title card has been used for title 1.4.

• 10:33 Please format the charts and figures to that their background colors fill the video frame area. There should be no black bars on the tops, bottoms, or sides of the video frame. (Do not simply stretch to fit the existing images, the easiest fix is to change the background layers to white from black.)
The background color of result figures have been changed to white.

4. Editing & Pacing:
There are several scenes in the video that show objects being put into processing appliances and then the object is shown being removed from the appliance once the processing is complete. This removal shot is probably unnecessary and indeed may slow down the pacing of the video unnecessarily, since the removal step is implied. I've included some time ranges that could be removed to help move the video along without losing any important information:
• 03:17-03:28 Removal from vacuum degasser
• 03:37-03:46 Removal from 80º furnace
• 04:30-04:40 Removal from autoclave
• 06:47-06:57 Removal from incubator
Suggested clips have been removed from the video.

• 10:12 There is a very quiet but audible double breath here. Sounds like an editing mistake that can be trimmed out
The ‘double breath’ has been trimmed out.

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/JnNTuVBaJvzxh3hLEjD8?oref=e

Reviewers' comments:
Reviewer #1:
A clear protocol describing how to apply shear stress separately to the center or to the edge of the well. This approach permits to avoid the influence of secretome, produced by endothelial cells growing in one area of the well (center or edge) on endothelial cells growing in another area of the well (center or edge). The protocol consists of six sections that fully cover the whole experimental workflow starting with the devices fabrication up to cell imaging and cell shapes characterization.

Video protocol is clear and accurate. Methods article is clear and well written.

Minor points about the manuscript:

1) Please mention in the Introduction and in Section 3 ("Seeding of endothelial cells EC") that this protocol is for HUVECs.
'ECs' has been replaced with 'HUVECs' in these sections- Introduction (line 105), section 3, section 4, and representative results.

2) Step 1.3.1: please mention that PDMS base and curing agent both come from Sylgard®184 Silicone Elastomer kit.
We have mentioned that both PDMS base and curing agent are from Sylgard®184 Silicone Elastomer kit in 1.3.1.

3) Section 1.5: it is not clear which steps in this section should be done while stirring, and which steps should be done in the fume hood.
Steps 1.5.1 to 1.5.5 should be performed while stirring. We have added this information to step 1.5.1.
'Add 800 mL of phosphate-buffered saline (PBS) to a glass beaker and heat it to 60 °C whilst stirring (keep stirring from step 1.5.1 to 1.5.5).'

When preparing 4% PFA, it is vital that all steps are carried out in a fume hood due to the toxic nature of the compound. We have edited step 1.5.2 to emphasise the importance of conducting steps 1.5.2 to 1.5.6 in a fume hood.
'Weigh 40 g of PFA powder and add it to the warm PBS solution. CAUTION: PFA is hazardous, perform steps 1.5.2 to 1.5.6 in a fume hood.'

4) Step 1.5.3: please specify how many drops of 1M NaOH should be added (approximately).
Standard protocol to dissolve PFA powder is to add 1M NaOH dropwise until the PFA solution results in a clear solution. The number of drops will vary. The critical step is adjust the pH of the solution after the NaOH has been added with HCL to achieve a pH of 7.4. This has been stated in the methods.

5) Step 1.5.6: please specify pore sizes of filter used.
A filter of 0.2 µm pore size was used.

6) Section 3. Please mention that this trypsinisation protocol in for HUVECs and that it can be different for other types of ECs.
We have added a NOTE in 3.3 to specify that 'This protocol has been tested using HUVECs and the concentration of trypsin and its incubation time may be different for other type of ECs.'

7) What is the range of shear stress magnitudes that ECs will experience in the center and at the edge of the well? Please include this in Section 4 "Shear stress application using an orbital shaker".
Based on our previously published computational fluid dynamic simulation data, time-averaged wall shear stress (TAWSS) is approximately 0.3 Pa and 0.7 Pa at the centre and the edge of a swirling 6-well plate, respectively. Instead of including this statement in section 4, we included the statement in introduction (line 93).

8) Step 4.2: Do you disinfect an orbital shaker before placing it into the incubator?
The exterior surface of an orbital shaker is wiped down using 70% ethanol before placing it into the incubator. This step has been added in 4.2 as a NOTE.

9) Steps 5.6; 5.8; 5.9: please specify what PBS volume you dilute antibodies in is.
1.5 mL of PBS is used in each well. The information has been added to 5.6 and 5.8.

That's a minor point but please make sure to consistently use either "Pluronic" or "pluronic" throughout the manuscript. Currently, these two spellings are mixed.
The spelling of 'pluronic' has been edited- 'Pluronic' is used throughout the manuscript.

Reviewer #2:
Manuscript Summary:
Authors described a method for promoting EC adhesion in specific regions of a 6-well plate using fibronectin coating while using Pluronic F-127 to passivate the surface and prevent growth
elsewhere.

Major Concerns:
It looks that with this methodology EC can be influenced with only one kind of shear profile. More discussion are necessary for proposed method. For example, is possible to achieve similar effects by engineering method with separation of EC zones? Demonstration of computational fluid dynamics of proposed method could be also important in better understanding of different flow conditions and EC position with passivation using passivating solution.
It is possible to segment cell growth by engineering method (e.g. using a physical barrier), but that would alter the flow in a swirling 6-well plate. Moreover, the main advantages of the proposed method are that it uses cheap, readily available, off-the-shelf products; hence, using a custom-made well-plate would defeat that purpose.
Computational fluid dynamics of proposed method has been done in our previously published paper (Ghim et al., 2018) and it is cited in the manuscript (line 95). The following paragraph from line 89 discussed the flow characteristics of a swirling 6-well plate:

'The swirling motion of culture medium caused by the orbital motion of the shaker platform on which the plate is placed leads to Low Magnitude Multidirectional Flow (LMMF, or putatively pro-atherogenic flow) at the centre and High Magnitude Uniaxial Flow (HMUF, or putatively atheroprotective flow) at the edge of the wells of a 6-well plate. Time-averaged wall shear stress (TAWSS) is approximately 0.3 Pa and 0.7 Pa at the centre and the edge of a swirling 6-well plate, respectively.'

Minor Concerns:
Correct this bookmark error in line 113:
113 according to the engineering drawing provided (Figure 5Error! Not a valid bookmark self
This error has been rectified.