Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE61817R1
Full Title:	Segmenting growth of endothelial cells in 6-well plates on an orbital shaker for mechanobiological studies
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TITLE:

Segmenting growth of endothelial cells in 6-well plates on an orbital shaker for mechanobiological studies

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KEYWORDS:

Segmenting Growth, Endothelial, Shear Stress, Orbital Shaker, Mechanobiology, Low Magnitude Multidirectional Flow, High Magnitude Uniaxial Flow, PDMS, Pluronic F-127, transverse wall shear stress

SUMMARY:

This protocol describes a coating method to restrict endothelial cell growth to a specific region of a 6-well plate for shear stress application using the orbital shaker model.

ABSTRACT:

Shear stress imposed on the arterial wall by the flow of blood affects endothelial cell morphology and function. Low magnitude, oscillatory and multidirectional shear stresses have all been postulated to stimulate a pro-atherosclerotic phenotype in endothelial cells, whereas high magnitude and unidirectional or uniaxial shear are thought to promote endothelial homeostasis. These hypotheses require further investigation, but traditional in vitro techniques have limitations, and are particularly poor at imposing multidirectional shear stresses on cells.

One method that is gaining increasing use is to culture endothelial cells in standard multi-well plates on the platform of an orbital shaker; in this simple, low-cost, high-throughput and chronic

method, the swirling medium produces different patterns and magnitudes of shear, including multidirectional shear, in different parts of the well. However, it has a significant limitation: cells in one region, exposed to one type of flow, may release mediators into the medium that affect cells in other parts of the well, exposed to different flows, hence distorting the apparent relation between flow and phenotype.

Here we present an easy and affordable modification of the method that allows cells to be exposed only to specific shear stress characteristics. Cell seeding is restricted to a defined region of the well by coating the region of interest with fibronectin, followed by passivation using passivating solution. Subsequently, the plates can be swirled on the shaker, resulting in exposure of cells to well-defined shear profiles such as low magnitude multidirectional shear or high magnitude uniaxial shear, depending on their location. As before, the use of standard cell-culture plasticware allows straightforward further analysis of the cells. The modification has already allowed the demonstration of soluble mediators, released from endothelium under defined shear stress characteristics, that affect cells located elsewhere in the well.

INTRODUCTION:

Responses of vascular cells to their mechanical environment are important in the normal function of blood vessels and in the development of disease[1]. The mechanobiology of the endothelial cells (ECs) that line the interior surface of all blood vessels has been a particular focus of mechanobiological research because ECs directly experience the shear stress generated by blood flow over them. Various phenotypic changes such as inflammatory responses, altered stiffness and morphology, the release of vasoactive substances, and the localization and expression of junctional proteins depend on EC exposure to shear stress[2–4]. Shear-dependent endothelial properties may also account for the patchy development of diseases such as atherosclerosis[5–7].

It is useful to study the effect of shear on ECs in culture, where stresses can be controlled, and ECs can be isolated from other cell types. Commonly used in vitro devices for applying shear stress to ECs include the parallel-plate flow chamber and the cone-and-plate viscometer, but only uniaxial steady, oscillatory, and pulsatile flow can be applied[8,9]. Although modified flow chambers with tapered or branching geometries and microfluidic chips that mimic a stenotic geometry have been developed, their low-throughput and the relatively short culture duration that is possible pose a challenge[10, 11].

The orbital shaker (or swirling well) method for the study of endothelial mechanotransduction, in which cells are grown in standard cell culture plasticware placed on the platform of an orbital shaker, is gaining increasing attention because it is capable of chronically imposing complex, spatially varying shear stress patterns on ECs with high throughput (see review by Warboys et al.[12]). Computational Fluid Dynamics (CFD) simulations have been employed to characterize the spatial and temporal variation of shear stress in a swirling well. 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 center and High Magnitude Uniaxial Flow (HMUF, or putatively atheroprotective flow) at the edge of the wells of a 6-well plate. For example, time-averaged wall shear stress (TAWSS) is approximately

0.3 Pa at the center and 0.7 Pa at the edge of a 6-well plate swirled at 150 rpm with a 5 mm orbital radius[13]. The method requires only commercially available plasticware and the orbital shaker itself.

There is, however, a drawback to the method (and to other methods of imposing flows in vitro): ECs release soluble mediators and microparticles in a shear-dependent manner[14–16] and this secretome may affect ECs in regions of the well other than the one in which they were released, due to the mixing in the swirling medium. This may mask the actual effects of shear stress on EC phenotype. For example, Ghim et al. have speculated that this accounts for the apparently identical influence of different shear profiles on transcellular transport of large particles[17].

Here we describe a method for promoting human umbilical vein endothelial cell (HUVEC) 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. The method resolves the limitation described above because, by segmenting cell growth, ECs experience only one kind of shear profile, and are not influenced by secretomes from ECs exposed to other profiles elsewhere in the well.

PROTOCOL:

1. Fabrication of devices and preparation of reagents

1.1 Fabrication of stainless-steel module

1.1.1 Fabricate the stainless-steel module from a grade 316 stainless-steel using a CNC milling machine according to the engineering drawing provided (**Figure 1**).

1.2 3D printing of a polydimethylsiloxane (PDMS) mold

1.2.1 Prepare a 3D computer aided design (CAD) model of the PDMS mold using SolidWorks according to the engineering drawing provided (**Figure 2Error! Not a valid bookmark self-reference.**).

1.2.2 Export the CAD model to an STL file and import the STL file to Cura 2.6.2.

1.2.3 Slice the model into layers with a print speed of 50 mm/s and infill density of 60%.

1.2.4 Export the file as a G-code and upload it to an Ultimaker^2 3D printer for printing. Use polylactic acid (PLA) as the printing material.

1.3 Casting of PDMS ring

1.3.1 Mix the PDMS base and curing agent (both from a silicone elastomer kit) with the ratio of 90.9% base and 9.1% curing agent.

1.3.2 Pour approximately 2.6 mL of the well-mixed solution into the 3D printed mold.

1.3.3 Remove bubbles in a vacuum degassing chamber.

1.3.4 Cure it for 1 h in an 80 °C furnace.

1.3.5 Allow the PDMS ring to cool to room temperature, then remove the cured PDMS ring carefully from the mold. The engineering drawing of PDMS ring is shown in **Figure 3**.

1.4 Preparation of 1% Pluronic F-127

1.4.1 Weigh out 5 g of Pluronic F-127, pour it into a glass bottle, then add 100 mL of sterile water into the glass bottle. This gives a 5% Pluronic F-127 solution.

1.4.2 Ensure that all Pluronic F-127 powder is submerged in the water, close the cap and autoclave it using a liquid sterilisation cycle program.

1.4.3 After autoclave, let the solution cool to room temperature before use.

1.4.4 Add 10 mL of 5% Pluronic F-127 solution to 40 mL of autoclaved sterile water to make 1% Pluronic F-127 solution. Perform the dilution in a biosafety cabinet (BSC) hood.

1.4.5 Store both 1% and 5% Pluronic F-127 at room temperature.

1.5 Preparation of 4% paraformaldehyde (PFA)

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).

1.5.2 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.

1.5.3 Add 1 M sodium hydroxide (NaOH) slowly dropwise into the PFA solution until the solutions turns clear.

1.5.4 Adjust the pH of the PFA solution to approximately 7.4 with 1 M of hydrochloric acid (HCl).

1.5.5 Top up the solution to 1 L with 1X PBS. This gives 1 L of 4% PFA.

1.5.6 Filter the PFA solution with 0.2 µm filters to remove any particulates, aliquot and freeze in a -20 °C freezer.

1.6 Preparation of 0.1% Triton-X

1.6.1 Add 50 µL of pure Triton-X into 50 mL of PBS to make 0.1% Triton-X solution.

1.7 Preparation of 1% bovine serum albumin (BSA)

1.7.1 Weigh 0.5 g of BSA, pour it into a 50 mL centrifuge tube, and then add 50 mL of PBS into the tube.

1.7.2 Let it roll for 1 h at room temperature on a roller to dissolve.

1.7.3 Store 1% BSA at 4 °C for up to two weeks.

2. Coating of a 6-well plate

2.1 Autoclave stainless-steel module, PDMS ring, and tweezers before use. Perform all the subsequent procedures in a BSC hood and observe aseptic techniques to ensure sterility.

2.2 Place the PDMS ring in a 6-well using tweezers. Use the external rim of the PDMS ring to align the PDMS ring concentrically with the well.

NOTE: Only non-tissue culture treated well plates should be used.

2.3 Place the stainless-steel module on top of the PDMS ring using tweezers.

2.4 Insert the tips of the internal retaining ring pliers into the grip holes of the retaining ring, squeeze the holder to reduce the diameter of the retaining ring. Fit it into the 6-well, press it firmly on the stainless-steel module and release the pliers to secure the PDMS ring in the well.

2.5 Add 1 mL of 5 µg/mL fibronectin into the center or the edge of the well (depending on the region of interest) through the opening of PDMS ring and stainless-steel module.

2.6 Swirl the plate to ensure that the fibronectin solution covers all the region of interest.

2.7 Incubate for 30 min at 37 °C in a humidified incubator under 95% air/5% CO_2.

2.8 Remove the fibronectin solution from the well and wash twice with PBS. Completely remove the PBS from the well.

2.9 Remove the retaining ring, stainless-steel module, and PDMS ring from the well.

2.10 Add 1.5 mL of 1% Pluronic F-127 into the center or the edge of the well (uncoated surface) and incubate for 1 h at room temperature to passivate the uncoated surface.

2.11 Remove the Pluronic F-127 solution from the well and wash three times with PBS.

2.12 Use the coated well immediately or store it at 4 °C for up to two weeks with a layer of PBS in the coated well.

3. Seeding of HUVECs

3.1 Use HUVECs below passage 5 in the experiment.

3.2 Remove all culture medium and wash cells once with PBS.

3.3 Add 3 mL of 0.05% trypsin and incubate for 3 min at 37 °C in a humidified incubator under 95% air/5% CO_2. Gently tap the flask to dislodge the cells.

NOTE: This protocol has been tested using HUVECs and the concentration of trypsin and its incubation time may be different for other type of ECs.

3.4 Transfer the solution to a 15 mL centrifuge tube and neutralize the trypsin using 6 mL of culture medium (e.g., Lonza EGM-2) prewarmed to 37 °C.

3.5 Centrifuge at 200 x g for 5 min. Remove the neutralized trypsin solution and resuspend cells with 1 mL of prewarmed culture medium.

3.6 Count the cells using a haemocytometer and seed 180k cells into a coated 6-well plate in 1.5 mL of prewarmed culture medium.

3.7 Shake the well plate laterally to ensure that the cells distribute evenly in the well.

3.8 Leave it in a 37 °C humidified incubator under 95% air/5% CO_2 overnight.

3.9 Remove the unattached cells and culture medium and replace with 2mL of prewarmed culture medium.

NOTE: many unattached cells floating in the medium are expected.

4. Shear stress application using an orbital shaker

4.1 HUVECs should reach confluence after 3 days of growth. Replace the medium with 1.9 mL of prewarmed culture medium (to achieve a height of 2 mm).

4.2 Place the plate on the platform of an orbital shaker in a humidified incubator under 95% air/5% CO_2 and swirl it at 150 rpm for 3 days.

NOTE: Wipe down the exterior surface of the orbital shaker using 70% ethanol before placing it into the incubator.

4.3 (Optional) After 2 days of shear, cytokines can be added to the culture medium to investigate the interplay between cytokines and shear stress. After treatment, shear the cells for another day. In this study, TNF-α was used to activate the cells.

4.4 Perform analyses after 3 days of shear stress application.

5. Staining and imaging of cells

5.1 After 3 days of shear, remove the plate from incubator and wash cells twice with PBS.

5.2 Fix the cells by adding 1.5 mL of 4% PFA into the well and incubate for 10 min at room temperature.

5.3 Remove the 4% PFA from the well and wash twice with PBS.

5.4 Permeabilize the cells by adding 0.1% Triton-X into the well and incubate for 5 min at room temperature.

5.5 Remove the 0.1% Triton-X solution from the well and add 1.5 mL of 1% BSA into the well for blocking. Incubate the cells with 1% BSA for 1 h at room temperature.

5.6 Dilute rabbit anti-human ZO-1 antibody at a 1:200 dilution in 1% BSA. Add 1.5 mL of diluted antibody into the well and incubate it with the cells overnight at 4 °C.

5.7 After overnight incubation, remove the diluted antibody and wash cells three times with PBS.

5.8 Dilute Alexa Fluor 488-labelled goat anti-rabbit IgG secondary antibody at a 1:300 dilution in PBS. Add 1.5 mL of diluted secondary antibody into the well and incubate it with the cells for 1 h at room temperature.

5.9 Remove the diluted secondary antibody and wash cells twice with PBS.

5.10 Dilute DRAQ5 at a dilution of 1:1000 in PBS. Add 1.5 mL of diluted DRAQ5 into the well and incubate it with the cells for 15 min at room temperature to stain the cell nuclei.

5.11 Remove the diluted DRAQ5 and wash three times with PBS.

5.12 Perform a tile scan from the edge to the center of the well with a confocal microscope.

6. Quantification of shape index and cells number

6.1 Post process the images using MATLAB R2016a.

6.2 Read the LIF file from confocal microscope into MATLAB and convert the merged tile scan to a binary image, then threshold the image by area and intensity to distinguish nuclei from background.

6.3 Subdivide the binary tile scan into 1 mm radial segments.

6.4 Fit an ellipse to each individual nucleus.

6.5 Count the number of ellipses within each radial segment to give a cell number.

6.6 Define the shape index = as SI = 4π x Area / Perimeter^2. Calculate the shape index for each ellipse[18].

REPRESENTATIVE RESULTS:

Adhesion of HUVECs to regions of the well plate not coated with fibronectin was abrogated by Pluronic F-127 passivation; growth was confined to the region coated with fibronectin even after 72 h of culture, with and without shear stress application (**Figure 4A, C**). Without the Pluronic F-127 passivation, HUVECs attached to the surface without fibronectin and had proliferated further by 72 h of culture (**Figure 4B, D**).

Alignment and elongation of HUVECs are evident at the edge of a swirling well, which has HMUF, while the cells at the center of the well, which has LMMF, exhibited a cobblestone morphology and no alignment (**Figure 5A,B**). Elongation of HUVECs was quantified as shape index: 4π x Area/Perimeter^2. A shape index of 1 indicates a circle, whereas a value of 0 indicates a line. Shape index decreased with radial distance from the center, and there was no significant difference between segmented and full wells. TNF-α treatment increased elongation of HUVECs compared to untreated controls (**Figure 5C**). HMUF also increased the number of HUVECs per mm^2 compared to LMMF under both conditions. The number of HUVECs increased gradually with distance along the radius. No significant difference was observed in the number of HUVECs grown in segmented and full wells (**Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1 Engineering drawing of stainless-steel module.
Dimensions are in mm.

Figure 2 Engineering drawing of PDMS mold.
Dimensions are in mm.

Figure 3 Engineering drawing of PDMS ring used to segment the wells.
Dimensions are in mm. From Ghim et al.[13].

Figure 4 Microscope images showing that Pluronic F-127 prevented human umbilical vein endothelial cells (HUVECs) adhesion to the region without fibronectin coating.

No HUVECs were attached to the part of the well surface that had not been pre-treated with fibronectin prior to passivation with Pluronic F-127, after 24 h (**A**) and 72 h (**C**) of growth. Without Pluronic F-127 passivation, HUVECs were attached to the surface without fibronectin 24 h after seeding (**B**) and had proliferated further by 72 h (**D**). (Scale bar = 500 μm). From Ghim et al.[13].

Figure 5 The morphology of sheared HUVECs in a segmented or full well.
Nuclear (red) stain shows the morphology of sheared HUVECs **(A)** in the center and **(B)** at the edge of a full well (scale bar = 100 μm). **A** and **B** also show cell outlines, delineated by immunostaining of ZO-1 (green). Note the alignment and elongation of cells at the edge but not at the center **(C)** No significant difference in nuclear shape index, indicating roundness, between HUVECs grown in full wells and segmented wells was seen for untreated or TNF-α treated HUVEC. Cells were more elongated near the edge of the well. A tendency for greater elongation in TNF-α-treated HUVECs was not consistently significant across locations. (Two-way ANOVA and Bonferroni's post hoc test; n = 3). This figure has been modified from Ghim et al.[13]

Figure 6 Number of HUVECs per mm^2 increased with radial distance in a swirling well plate.
No significant difference was observed between full and segmented wells in the density of **(A)** untreated and **(B)** TNF-α-treated HUVECs at different radial locations. In both cases, there were more cells per unit area at the edge than the center of the well. (Two-way ANOVA and Bonferroni's post hoc test; n = 3). This figure has been modified from Ghim et al[13].

DISCUSSION:
The swirling-well method is capable of generating complex flow profiles in a single well - Low Magnitude Multidirectional Flow (LMMF) in the center and High Magnitude Uniaxial Flow (HMUF) at the edge of the well. However, shear stress-mediated secretions of soluble mediator will be mixed in the swirling medium and affect cells in the whole well, potentially masking the true effect of a particular shear stress profile on the cells.

The coating method demonstrated here overcomes this issue by restricting the growth of cells to a specific region of the well. Cells typically attach to hydrophilic surfaces rather than hydrophobic ones. For this reason, polystyrene culture ware is pre-treated with plasma oxidation. Alternatively, hydrophobic surfaces can be coated with extracellular matrix proteins such as fibronectin, as demonstrated in this protocol; non-fibronectin coated regions were passivated with Pluronic F-127 to prevent any residual adhesion to the hydrophobic surface.

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.

This method is simple yet effective in allowing the application of a specific type of shear stress (HMUF or LMMF) to cells. It is also convenient to set up as most of the consumables, reagents, and equipment are commercially available. Using this method not only allows the examination or harvesting of cells exposed to well-defined flows but allows the collection of medium conditioned by those cells. The method provides a new avenue investigating endothelial mechanobiology.

ACKNOWLEDGMENTS:

The authors gratefully acknowledge a British Heart Foundation project grant (to PDW), a National Medical Research Council Singapore TAAP and DYNAMO Grant (to XW, NMRC/OFLCG/004/2018, NMRC/OFLCG/001/2017), an A*STAR Graduate Scholarship (to KTP), and a British Heart Foundation Center of Research Excellence studentship (to MA).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

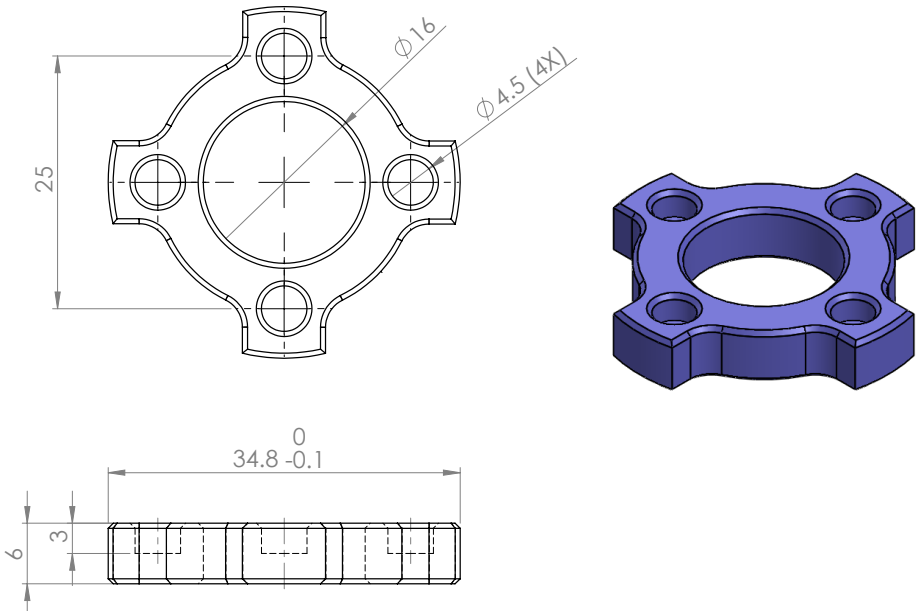


Figure 2

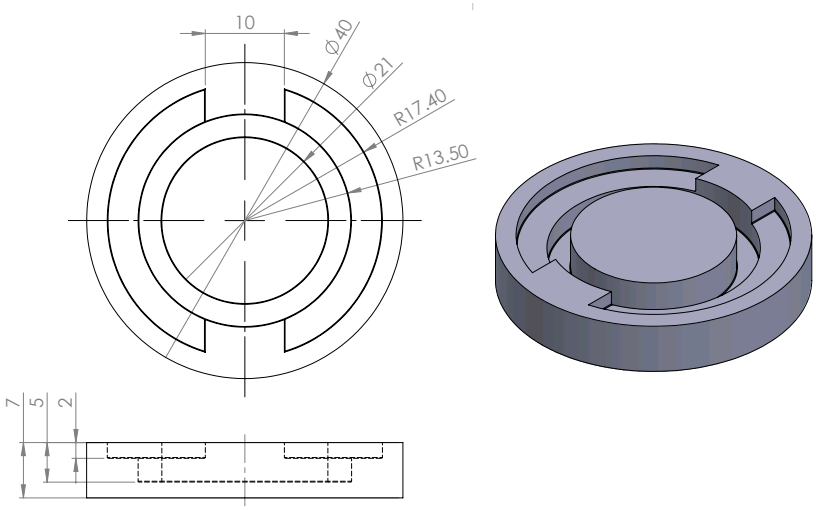
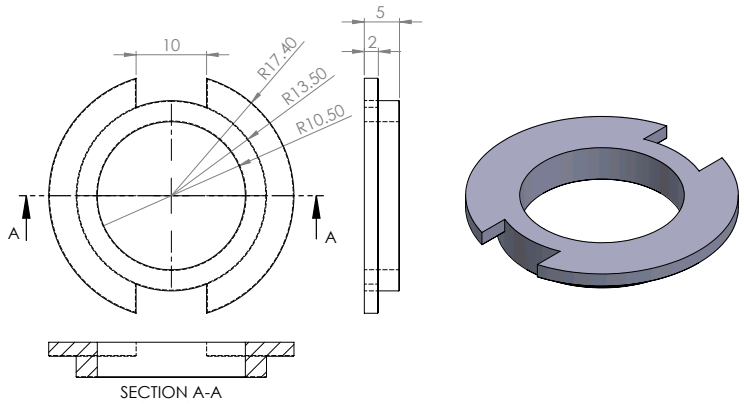


Figure 3



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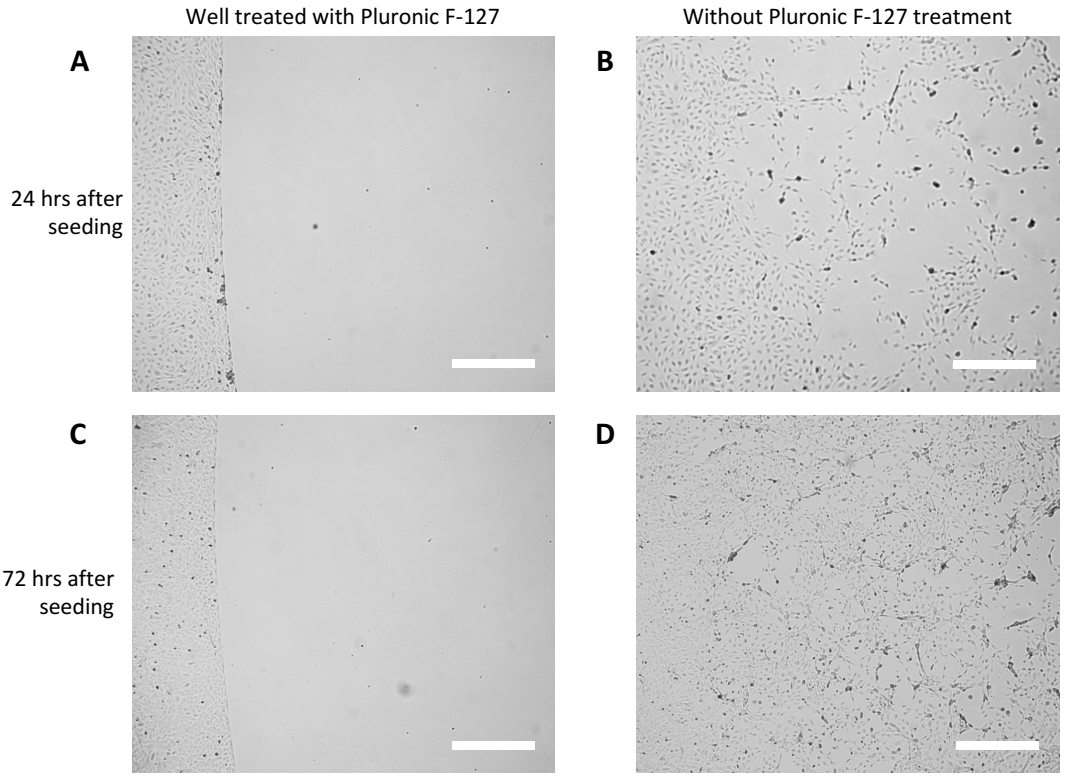


Figure 5

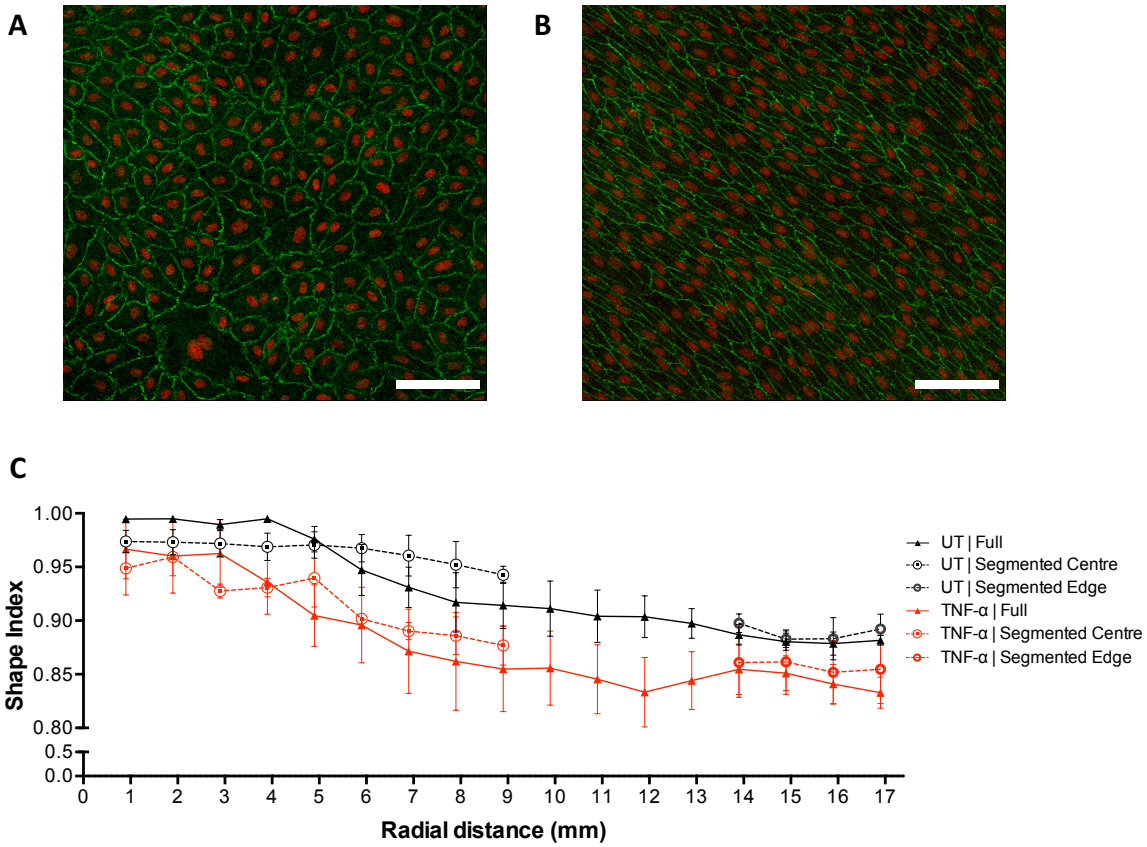
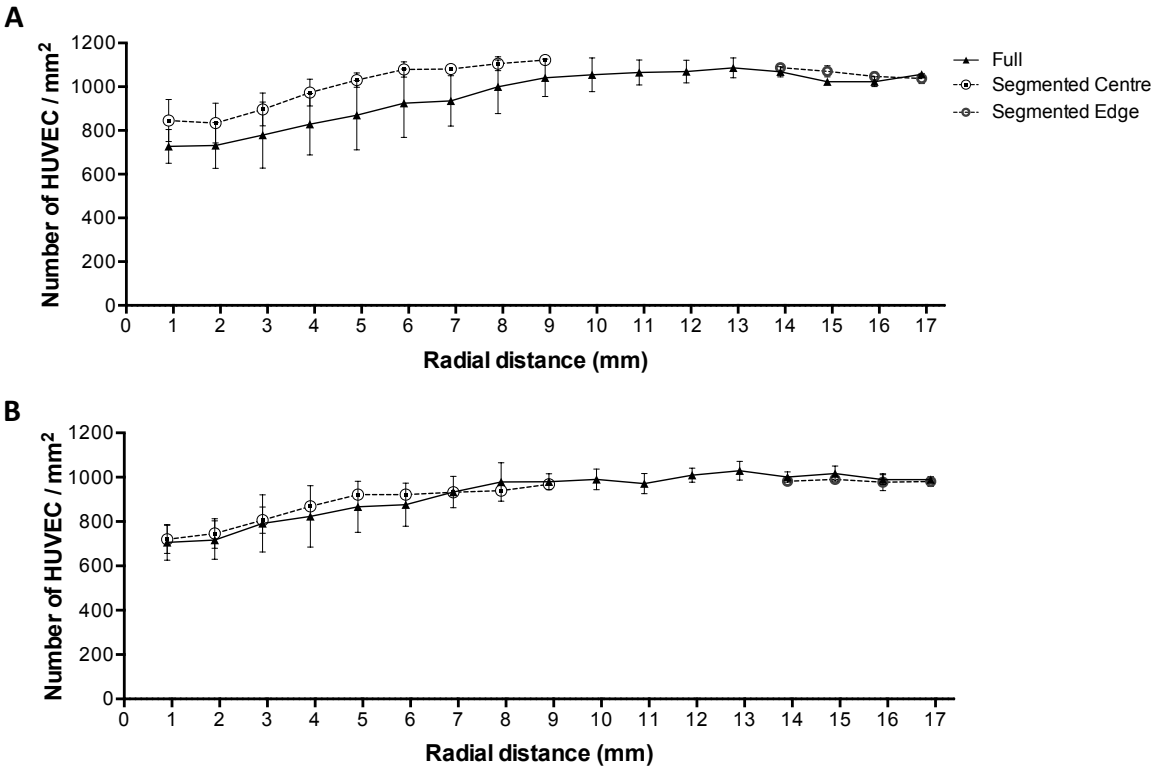


Figure 6



Name of Material/ Equipment	Company	Catalog Number
Cell and Media		
Endothelial Growth Medium (EGM-2)	Lonza	cc-3162
Human Umbilical Vein Endothelial Cells	NA	NA
Reagents and Materials		
Alexa Fuor 488-labelled goat anti-rabbit IgG	Thermofisher Scientific	A11008
Bovine Serum Albumin	Sigma-Aldrich	A9418-50G
Falcon 6 Well Clear Flat Bottom Not Treated	Scientific Laboratory Supplies Ltd	351146
Fibronectin from Bovine Plasma	Sigma-Aldrich	F1141-5MG
Paraformaldehyde	Sigma-Aldrich	158127-500G
Phosphate-Buffered Saline	Sigma-Aldrich	D8537-6X500ML
Pluronic F-127	Sigma-Aldrich	P2443
Recombinant Human TNF-a	Peprotech	300-01A
RS PRO 2.85 mm Black PLA 3D Printer Filament, 1 kg	RS	832-0264
Stainless Steel 316	Metal Supermarket	NA
Sylgard184 Silicone Elastomer kit	Farnell	101697
Triton X-100	Sigma-Aldrich	X100-100ML
Trypsin-EDTA solution	Sigma-Aldrich	T4049-100ML
Zonula Occludens-1 (ZO-1) antibody	Cell Signaling Technology	13663
DRAQ5 (5mM)	Bio Status	DR50200
Equipments		
Grant Orbital Shaker PSU-10i	Scientific Laboratory Supplies Ltd	SHA7930
Leica TCS SP5 Confocal Microscope	Leica	NA
Retaining Ring Pliers	Misumi	RTWP32-58
Retaining Rings/Internal/C-Type	Misumi	RTWS35

Ultimaker 2+3-D printer	Ultimaker	NA
Softwares		
Cura 2.6.2	Ultimaker	NA
MATLAB	The MathWorks	NA
Solidworks 2016	Dassault Systemes	NA

Comments/Description
Isolated from cords obtained from donors with uncomplicated labour at the Hammersmith Hospital

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.

Re: Permission to reproduce figures- Journal of Biological Engineering 2018 12:15 [Ticket ID #75651]

Open Research Support <orsupport@springernature.com>
Fri 5/7/2019 2:03 PM

To: Pang, Kuin <k.pang15@imperial.ac.uk>

Dear Dr. Pang,

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With kind regards,

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> On Fri, 5 Jul at 3:50 PM , Pang, Kuin <k.pang15@imperial.ac.uk> wrote:
> Dear Sir/Madam
>
> I am writing with regards to the article that I have co-authored and published in the Journal of Biological Engineering (Ghim, M., Pang, K. T., et al. A novel method for segmenting growth of cells in sheared endothelial culture reveals the secretion of an anti-inflammatory mediator. *J. Biol. Eng.***12:15**, (2018))

We have been approached by the Journal of Visualized Experiments (JoVE)'s editorial team to publish a video protocol on the method published. We will adopt some of the figures in the published article in the JoVE manuscript. We understand that BMC's Journal of Biological Engineering is an open access journal and the data can be reproduced provided the figure is original. But we would still like to seek formal approval from BMC before we reproduce it in our JoVE paper. Please advice.

Best Regards,
Kuin Tian Pang

CAD file-PDMS ring

CAD file-Stainless-steel module

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Stainless-steel Module.SLDPRT

CAD file-PDMS mold