

Promoting endothelial cell growth within microchannels

Modification of polydimethylsiloxane and microfabrication of circular microchannels

by

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Abstract

Polydimethylsiloxane (PDMS) microfluidic channels, fabricated using low cost and simple soft lithography methods, conventionally have rectangular cross-sections. Despite being often used for organs-on-a-chip and cardiovascular research, these devices do not mimic the circular cross-sections of blood vessels in the human body, creating potential inaccuracies in observed flow conditions and cell behaviours. The purpose of this thesis is to (i) compare and optimize fabrication techniques for microchannels with circular cross-sections, (ii) assess biocompatibility of different surface functionalization approaches for Human Umbilical Vein Endothelial Cell (HUVEC) adhesion and growth, (iii) culture HUVECs within circular microchannels to mimic blood vessel features, and (iv) compare gene expression of HUVECs cultured in 3D circular microchannels to those cultured on 2D surfaces. We show that wire molding is superior to the gas stream technique for producing circular cross-section microchannels with high aspect ratios, circularity, and channel geometry precision. Fibronectin (FN) and polydopamine (PD) surface coatings on PDMS, as well as alternative collagen substrates, were tested for biocompatibility with HUVECs in 2D cultures; fibronectin coated PDMS (PDMS-FN) substrates facilitated cell attachment, spreading and growth. We demonstrate the capability of growing HUVECs on the inner surface of circular PDMS microchannels created using the wire-mold method and treated with fibronectin. A syringe pump was used to induce shear stress on the HUVECs grown in circular microchannels. Relative to static growth conditions, longer cell culture growth periods were more feasible under flow and altered cell morphology was observed. Finally, Microarray analysis revealed significantly different gene expression profiles for HUVECs cultured within PDMS-FN circular cross-section microchannels as compared to HUVECs cultured on PDMS-FN in a 2D environment, thereby highlighting the critical importance of *in vitro* conditions for mimicking the *in vivo* reality.

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Table of Contents

1	Introduction	1
1.1	Motivation	1
1.2	Objectives	2
1.3	Background	3
1.3.1	Blood vessel structure and function.....	3
1.4	Literature review: state of the art	5
1.4.1	Surface functionalization approaches	5
1.4.2	Microfabrication techniques.....	9
1.4.3	Cell culture in 3D environments	14
1.4.4	Flow in microchannels.....	15
1.4.5	Shear stress	17
1.5	Conclusion.....	19
2	Methodology	20
2.1	Microfabrication experiments.....	20
2.1.1	Photolithography	20
2.1.2	Soft-lithography method to create rectangular channels	22
2.1.3	Wafer quality testing.....	23
2.1.4	Protocol modifications.....	23
2.1.5	Selected process modification.....	25
2.1.6	Gas stream fabrication method	26
2.1.7	Wire molding fabrication method.....	28
2.2	Characterization of substrates	32
2.3	Two-dimensional cell experiments.....	32
2.3.1	PDMS coating procedures	33
2.3.2	2D fixed cell imaging.....	35
2.3.3	2D fixed cell image processing	36
2.3.4	Cell motility.....	38
2.3.5	Live cell imaging	38
2.3.6	Live cell image processing.....	39
2.3.7	Statistical analyses for cell experiments	40
2.4	Three-dimensional cell experiments	40
2.4.1	Flow experiments	41
2.4.2	3D fixed cell imaging.....	41
2.4.3	Sample preparation for gene expression microarray	41
3	Results and Discussion.....	44
3.1	Microfabrication.....	44
3.1.1	Rectangular microchannels.....	44
3.1.2	Circular microchannels	45
3.2	Comparison of microfabrication methods.....	49
3.3	Substrate surface characterization	51
3.3.1	PDMS surface functionalization.....	51

3.3.2	Collagen substrates	51
3.4	Evaluation of substrate surface characterization.....	52
3.5	Substrate biocompatibility	54
3.5.1	Cell seeding efficiency and early proliferation	54
3.5.2	Cell proliferation	55
3.5.3	Cell spreading	56
3.5.4	Cell motility.....	58
3.6	Cellular response to substrates	61
3.7	Cell culture in microchannels	66
3.7.1	Microarray data	69
3.8	Evaluation of cell culture in microchannels	75
4	Conclusion	80
5	References	82
6	Appendix.....	95
6.1	Hemocytometer calculations	95
6.2	Spin coating PDMS on 35mm culture dishes	96
6.3	Fibronectin adsorption on PDMS.....	97
6.4	Shear stress and Reynolds number in circular microchannels	98
6.5	2D substrates from live imaging: Mean cell density sample calculations	99
6.6	Theoretical cell seeding density for live experiments.....	100
6.7	Cell spreading: Percent area coverage sample calculations	100
6.8	Microarray Data	101

List of Tables

Table 1.	Blood vessel mean diameters	4
Table 2	Changes made to photolithography procedure and resulting cross-sectional dimensions	25
Table 3	AWG equivalent diameter dimensions	28
Table 4	Summary of 2D sample conditions and sample size for nuclei count experiments.....	36
Table 5	Summary of 2D sample conditions and sample size for cell area coverage experiments	36
Table 6	Summary of 2D sample conditions and sample size for live cell experiments	38
Table 7	Summary of sample sizes and conditions for microarray experiment.....	43
Table 8	Experimental versus theoretical wire diameters	46
Table 9	Mean contact angle of water on PDMS and PDMS modified substrates	51

Table 10 Mean contact angle of water on Collagen gel over time	52
Table 11 Directness values for cells tracked live over 12 hours.....	60
Table 12 Summary of microchannels tested.....	67
Table 13 RNA concentrations for cell samples collected.....	69
Table 14 Functional grouping of several genes relevant to endothelial cell function	72
Table 15 PDMS thickness measurements from spin coated PDMS samples	96
Table 16 Area occupied by HUVECs on 2D Collagen-FN samples.....	100
Table 17 3D vs. 2D comparison of differentially regulated genes.....	101
Table 18 3D vs. 2D comparison of differentially regulated genes of specific interest.....	102

List of Figures

Figure 1 Blood vessel structures	4
Figure 2 Continuous endothelial cell capillary structure]	5
Figure 3 The ECM components	7
Figure 4 Simple single-layer photolithography.....	10
Figure 5 Reflow with positive photoresist to produce rounded features.....	11
Figure 6 Diagram of a glass capillary tube being used as a circular channel.....	11
Figure 7 Diagram of air pressure creating a circular cross-section in liquid PDMS.....	13
Figure 8 Varying cross-sectional channel geometries	17
Figure 9 Velocity profiles for flow in circular and rectangular cross-section channels.....	17
Figure 10 Photomask used in wafer fabrication	21
Figure 11 Photolithography and soft lithography processes.....	22
Figure 12 Bonded microchannel cross-sectional view and top view.....	22
Figure 13 Preliminary wafer fabrication from photolithography techniques	23

Figure 14 Resulting microchannel cross-sections from photolithography step modifications.....	24
Figure 15 Gas stream method PDMS with hexanes mixture	27
Figure 16 Blocked microchannels.....	27
Figure 17 Diagram of pressure system used to apply air stream to microchannel.....	28
Figure 18 Microchannel cross-sectional view.....	28
Figure 19 Wire mold design.....	30
Figure 20 Wire mold design, cross-sectional view.....	30
Figure 21 Wire molding chip fabrication process.....	31
Figure 22 Cell nuclei counting flowchart using ImageJ	37
Figure 23 Determining cell area coverage flowchart using ImageJ.....	37
Figure 24 Mean accumulated distance.....	39
Figure 25 Box plot of rectangular channel dimensions	45
Figure 26 Box plot of gas stream circular channel dimensions.....	45
Figure 27 Box plots of wire mold circular channel dimensions.....	47
Figure 28 Box plot of aspect ratios for microchannels.....	48
Figure 29 Box plot of circularity for microchannels	48
Figure 30 Water contact angles on untreated PDMS, PDMS _{PD} , and PDMS _{FN}	51
Figure 31 Water contact angles on Collagen.....	52
Figure 32 Cell seeding efficiency and cell proliferation (live imaging on 2D substrates)	55
Figure 33 Cell proliferation (fixed imaging on 2D substrates)	56
Figure 34 Cell spreading (live imaging on 2D substrates).....	57
Figure 35 Cell spreading (fixed imaging on 2D substrates)	57
Figure 36 Fluorescent images of fixed HUVECs at 48 hours after seeding.....	58
Figure 37 Migration plots showing distances travelled by HUVECs.	59

Figure 38 Mean velocity values for travelling HUVECs (live imaging on 2D substrates)	60
Figure 39 Mean accumulated distance travelled by HUVECs (live imaging on 2D substrates).....	60
Figure 40 Phase contrast images of live HUVECs on 2D substrates	61
Figure 41 Fixed fluorescent images of HUCVECs in 24 AWG microchannels.....	68
Figure 42 Fixed fluorescent images of HUVECs in 24 AWG microchannels, flow vs. no flow.....	69
Figure 43 Normalization of sample probe intensity.....	70
Figure 44 Relative gene expression comparison.....	71
Figure 45 Heat map comparing the most differentially expressed genes, 3D vs. 2D.....	73
Figure 46 Heat map comparing expression of specific genes of interest, 3D vs. 2D	74
Figure 47 PDMS thickness using spin coater.....	97
Figure 48 Fibronectin-rhodamine adsorption in microchannel	97

Acronyms

HUVEC	Human Umbilical Vein Endothelial Cell
EC	Endothelial Cell
RBC	Red Blood Cell
WBC	White Blood Cell
PBS	Phosphate Buffered Saline
FN	Fibronectin
PD	Polydopamine
Col	Collagen
HF	Hydrofluoric acid
ECM	Extra Cellular Matrix
UV	Ultraviolet: a range of electromagnetic radiation
PDMS	Polydimethylsiloxane: Silicon based organic polymer
AWG	American Wire Gauge: unit of measurement for microwire
PDMS-FN	PDMS substrate treated with fibronectin solution, also denoted by PDMS _{FN}
PDMS-PD	PDMS substrate treated with polydopamine, also denoted by PDMS _{PD}
Collagen-FN	Collagen substrate treated with fibronectin solution, also denoted by Collagen _{FN}

Chapter 1

1 Introduction

1.1 Motivation

The blood vessel networks of the microcirculation system comprise arterioles, venules and capillaries, with diameters of approximately 100 μm or less. The inner lumen of these vessels is lined with endothelial cells. Vascular endothelial cells can alter their morphology based on cell-to-cell interactions and in response to blood flow [1]. Microfluidic systems are important tools that can be used for various biomedical applications (i.e. organs-on-a-chip). Conventional microfluidics often use microchannels with rectangular cross-sections. These rectangular microfluidic channels, created using soft lithography methods and used to study blood flow, do not accurately mimic the circular cross-sections of blood vessels in the human body. These differences can lead to abnormal cell behavior in microfluidic systems, especially for the margination of circulating cells in vessels [2]. Rectangular channels may also be a poor model for analyzing blood flow dynamics and cell-cell communication and interactions *in vitro*. This geometry could potentially cause inaccuracies when attempting to study cell interactions and mimic blood vessel disease conditions. Furthermore, it has also been shown that the hematocrit, the volume percentage of red blood cells (RBC) in blood, is locally influenced by vessel geometry [3] and junctions [4].

In this work, we compare microfabrication methods for producing microchannels with circular cross-sections. Furthermore, we examine PDMS functionalization procedures and the use of alternative collagen substrates to improve microchannel biocompatibility, aiming at long term endothelial cell cultures within the microchannels and maintaining a cellular monolayer under flow.

The experimental techniques described in this thesis will aid in the development of models that can more accurately mimic blood vessels. This will allow us to gain a greater understanding of cell-cell interactions and the effects of flow on cellular functions within a microcirculation system. Furthermore, such models may help contribute to the study of cardiovascular diseases and allow for preliminary drug testing *in vitro*.

1.2 Objectives

The primary objectives of this study are:

- Optimize the microfabrication process of microchannels with circular cross-sections
- Promote endothelial cell adhesion and proliferation in 2 and 3D
- Analyze cell growth and survival in microchannels
- Compare gene expression for HUVECs cultured in 3D circular microchannels versus 2D environments

These objectives will be accomplished by first creating microchannels with circular cross-sections using varying microfabrication techniques and comparing the efficacy of the techniques. Second, the biocompatibility of rationally functionalized PDMS and collagen substrates will be assessed.

Examining several factors such as: substrate surface wettability, and cell proliferation, spreading and motility on two-dimensional substrates can help give an indication of how cells may respond when cultured within the microchannels. Evaluation of surface wettability of a surface can help to predict the ability of the tested substrates to promote cellular adhesion. Surface wettability is reportedly an important factor directing cell adhesion, proliferation and migration [5]. Creating an environment to promote a high degree of cell proliferation and spreading (characterized by the surface area occupied by the cells) is an important aspect for attempting to colonize the inner surface of a synthetic

microchannel and create a monolayer of endothelial cells, to most accurately simulate a blood vessel. For anchorage dependent cell types, high cell motility may be associated with reduced cell spreading [6], thus, lower cell motility would be more desirable for promoting the creation of a HUVEC monolayer within a synthetic microchannel, to model a blood vessel.

Growth of endothelial cells on the inner surface of the circular cross-section microchannels will be examined and the effects of flow on the endothelial monolayer will be studied. Finally, a microarray will be used to assess gene expression of HUVECs grown in 3D and 2D settings to elucidate the effects of the cell microenvironment on cellular functions.

1.3 Background

1.3.1 Blood vessel structure and function

In the human body, blood is transported through the circulatory system, which comprises pulmonary and systemic circulation [7]. Body tissues are supplied with blood by arteries in the systemic circulation and deoxygenated blood is returned to the heart through systemic veins. Pulmonary circuit arteries transport deoxygenated blood to the lungs, where gas-exchange occurs between capillaries and air-filled alveoli. Pulmonary veins carry oxygenated blood from the lungs to the heart, where it is distributed to the rest of the body. Gas, nutrient and waste exchange occurs in the microcirculation system, where arterioles branch into capillaries that surround tissues, providing oxygen. Capillaries exit to venules, returning deoxygenated blood to the veins, which lead back to the heart [7].

Blood vessels are generally composed of three anatomical layers: the intima, media and externa layers; each with varying morphology [8]. The intima consists of the endothelium, basal lamina and a cell free sub-endothelial space. The internal elastic lamina separates the intima from the media and plays a role in vessel wall resilience to maintain blood pressures. Smooth muscle cells (SMC) and elastic tissues

make up the middle, media layer. This tissue layer is highly contractile, allowing for contraction and dilation of the vessel. The external elastic lamina separates the media from the adventitia. The adventitia is the outermost layer of the vascular wall and consists of elastin, collagen and fibroblasts. The main function of the adventitia is to limit excessive extension and recoil of the vessel [8]. Vascular endothelial cells form the endothelium in the tunica intima of blood vessels (Figure 1). The endothelium is a semi-selective barrier (Figure 2) to the constituents of blood and a tissue that helps to regulate the migration of white blood cells (WBCs) and proteins in and out of the blood stream, therefore influencing immune function. Endothelial cells synthesize and release various agents that control the integrity of the vascular wall, altering wall permeability, vasoconstriction, vasodilation and blood pressure. As a result, endothelial cells have been shown to play a critical role in the pathogenesis of vascular disease [9].

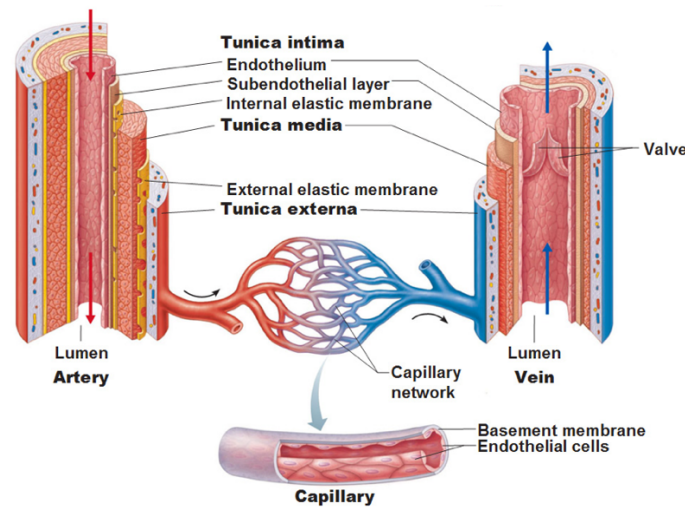


Figure 1 Blood vessel structures, reproduced from [7]

Table 1. Blood vessel mean diameters [7]

Vessel Type	Mean Diameter (um)
Artery	4000
Vein	5000
Arteriole	30
Venule	20
Capillary	8

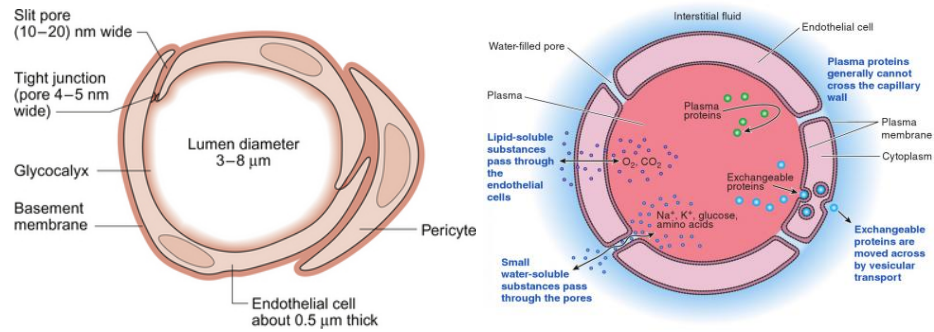


Figure 2 Continuous endothelial cell capillary structure (left); the semi-permeable nature of the endothelium (right), reproduced from [10],[11]

1.4 Literature review: state of the art

1.4.1 Surface functionalization approaches

Surface functionalization of substrates is commonly used to improve cell functions (i.e. adhesion and spreading) and allow for 2D cell culture. Using protein constituents of the extracellular matrix (ECM), glass or polystyrene culture dishes can be coated to promote cell attachment and growth for adherent cells, such as HUVECs, *in vitro* [12]. These proteins can be immobilized onto a material surface via direct adsorption of protein from solution, or through the chemical modification of surface molecular characteristics [13]. In 3D environments, hydrogels containing ECM proteins are often used to mimic the natural extracellular environment. These proteins self-assemble into nanofibrous structures that replicate *in vivo* conditions [14],[15]. 3D Collagen hydrogels can be used to investigate endothelial cell behaviours, in some cases allowing for cell migration in three dimensions [14],[16],[17],[18]. For example, several studies examined the mechanisms by which transendothelial flow promotes angiogenesis in collagen hydrogels [19],[20]. While there are numerous ECM proteins that can be used to promote cell adhesion, proliferation and spreading in cell culture [21], fibronectin and collagen have been shown to provide particularly optimal growth conditions for HUVECs compared to other ECM proteins [22].

Additional surface functionalization methods exist, including the use of other chemical coatings, as well as creating physical modifications to the material surface through chemical processes, changing their inherent structure and improving biocompatibility (i.e. nanotubes and nanopatterned surfaces) [23], [24]. This study will focus on the comparison of fibronectin coated PDMS, collagen substrates, and polydopamine modified PDMS.

1.4.1.1 Fibronectin and Collagen

The ECM is made up of three types of molecules: structural proteins (collagens and elastins), protein-polysaccharide complexes that surround the structural proteins (proteoglycans), and adhesive glycoproteins, such as fibronectin and lamin [25]. These molecules create a woven network outside the cell. Fibronectin and collagen treatments of polystyrene culture dishes are conventional methods used to promote attachment and growth of adherent cells, such as HUVECs [26]. Type 1 collagen, a triple helical molecule found in the ECM, comprises one $\alpha 2(I)$ chain and two identical $\alpha 1(I)$ chains, which contain a fibronectin binding site [27], [28]. Fibronectin has a rod-like configuration with type I, II, and III homologous repeating modules and several domains which can bind to ECM components. The collagen binding sites on fibronectin have been identified as the amino acid sequence that links the type I and adjacent type II modules [28]. The ECM provides structural support for tissues, separates tissues and plays an important role in cell signaling and regulation. Cell-glycoprotein interactions mediate cellular adhesion, differentiation, growth, spreading and migration of adherent cells [29]. Integrins, proteins located in the phospholipid bilayer of the cellular membrane (Figure 3), attach to specific cell binding sites on the collagen, fibronectin and other proteins of the ECM, linking microfilaments of the cytoskeleton to the ECM. The integrins are critical to achieving cellular adhesion, as well as migration [15],[30], and also act as receptors, receiving signals from the

ECM and modulating cell signaling pathways. *In vitro* studies make use of the integrins in the cell to link the cytoskeleton to ECM proteins (Figure 3) that are deposited on non-native material surfaces [29]. In several instances in literature, fibronectin functionalized collagen substrates have been shown to provide optimal conditions for endothelial cell growth [31], [32]. Thus, this protein combination as well as the proteins individually, were selected for investigation in the study.

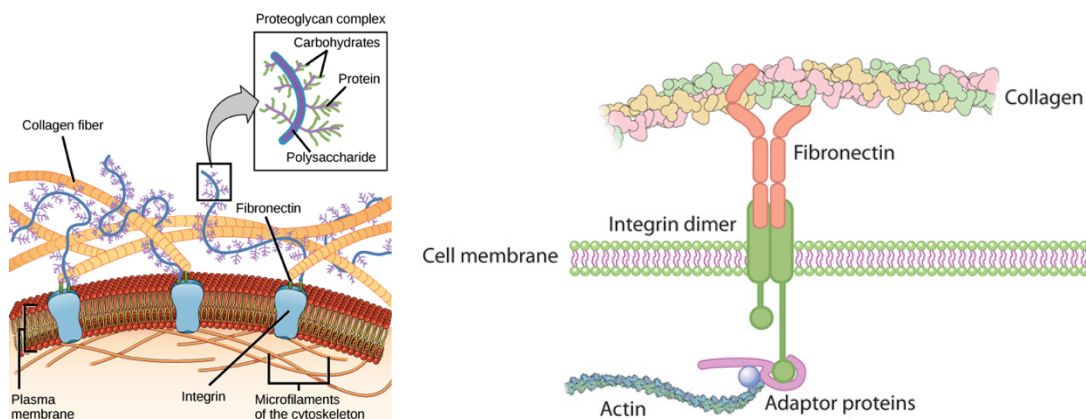


Figure 3 The ECM components(left), Integrin-collagen-fibronectin interaction in the ECM (right), reproduced from[33]

1.4.1.2 Polydopamine

Mussel adhesive proteins (MAPs) are secreted by marine mussels as they form secure attachments to surfaces in their environments. The adhesive properties of these proteins are attributed to their high concentration of the amino acids lysine and 3,4-dihydroxyphenyl-L-alanine (L-DOPA). Dopamine has a similar chemical structure to that of the combined lysine and DOPA, and its adhesive potential makes it of particular interest to researchers [34].

Evidence in literature suggests that polydopamine can create bioactive surfaces and increase biocompatibility of substrates; this made polydopamine of particular interest for our study in order to create comparisons with traditional ECM protein coatings used to promote cell growth. Ku *et al.* demonstrated that polydopamine is effective in encouraging cell proliferation and adhesion on

substrates such as glass, silicone rubber, polyethylene, polytetrafluoroethylene, and PDMS. When untreated, these substrates are fairly resistant to cellular adhesion. The authors showed that the addition of a polydopamine coating significantly improved substrate adhesive characteristics for HUVECs, making substrates more comparable to gelatin-coated substrates. Enhanced initial HUVEC attachment, cytoskeleton structure stability, and increased proliferation was observed on polydopamine-coated substrates [35]. Likewise, Zhong *et al.* demonstrated improved EC attachment, proliferation, migration and release of nitric oxide on polydopamine modified titanium dioxide nanotube surfaces [36].

Oxidative self-polymerization of dopamine occurs at alkaline conditions, forming a polymerized layer that can uniformly coat ceramics, polymers and metals, independent of their surface properties [37], [38]. The polymerized layer is bioactive, containing catecholamine functional groups that alter the material surface properties and have been shown to modulate cell responses. In addition, the catechol groups can be used to immobilize other biomolecules on the biomaterial surface to further improve surface bioactivity [34].

The polydopamine polymerization coating process occurs at room temperature in pH 8.5 Tris-HCl buffer. Temperature, coating time, Tris-HCl buffer pH and the initial dopamine concentration are the main factors that influence polymerization of the dopamine [34]. Researchers suggest that the thickness of the polydopamine coating is dependent on the duration of the polymerization reaction, and independent of substrate type. A thickness of approximately 50nm of polydopamine was obtained for a reaction time of 24 hours [37], [34].

1.4.2 Microfabrication techniques

1.4.2.1 Photolithography

Microfluidic devices are widely used to study fluid behaviour on a microscale, and as tools to perform a wide variety of tasks such as biochemical detection for diagnostic purposes. This is accomplished by selecting a microfluidic chip material and designing the chip to carry out specific functions. For example, Khan *et al* observed the effect of disturbed flow patterns on endothelial cell phenotype using a type I collagen microfluidic module [39]. The low input volume and small footprint of these Lab-on-a-chip devices makes them highly advantageous.

Photolithographic techniques, originating from the semiconductor industry, are conventionally used to create microfluidic devices, and ordinarily result in microchannels with rectangular cross-sections. This process involves the creation of a master mold, often from silicon coated with a photoresist, UV light, and a photomask [40]. Simple single layer photolithography involves spin coating a SU-8 negative photoresist onto a silicon wafer, heating to cure and then placing a photomask containing the design features on top of the photoresist. When exposed to UV light, the transparent region of the photomask allows the light to pass through, curing the SU-8 beneath. Uncured regions of the photoresist are dissolved, leaving behind only the raised features of the master mold on the silicon wafer. Soft lithography involves pouring uncured PDMS onto the master mold, heating it to cure, and then removing the flexible polymer from the mold to obtain the inverse feature. Inlet and outlet holes are punched into the PDMS; the chip is then bonded to another substrate to seal the bottom of the channel (Figure 4) [41].

Multi-step photolithography is a more complex method that allows for the creation of multiple layers and features in the master mold by application of several photoresist coatings to achieve different

aspect ratios and feature depths, as well as use of multiple photomasks. Several UV exposures and position markers to align the photomasks are required for this method [42].

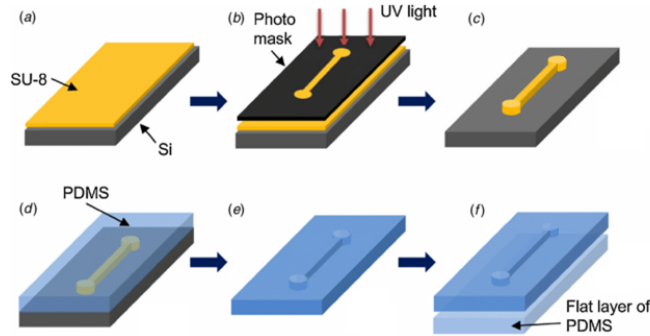


Figure 4 Simple single-layer photolithography, reproduced from [43]

1.4.2.2 Circular microchannel fabrication and 3D cell cultures

To obtain microchannels with circular cross-sections, adaptations to the standard photolithography methods are required. A number of methods for developing circular microchannels have been reported in the literature, and four commonly used procedures (the semi-circular microchannel method, the glass capillary method, gas stream method and wire-molding) are described here.

Silicon wafers with semi-circular cross-sections can be produced using a positive photoresist and a clear-field photomask, exposing everything on the wafer, except the channel to UV (Figure 5)[44]. When the positive photoresist is baked at a high temperature, reflow of the photoresist will occur, rounding the corners of the features. Soft lithography processes can then be employed to create semi-circular PDMS microchannels. To create a full circular channel, two semi-circular PDMS channel halves must be bonded together. Difficulties arise in ensuring the correct alignment of the channels at the microscale.

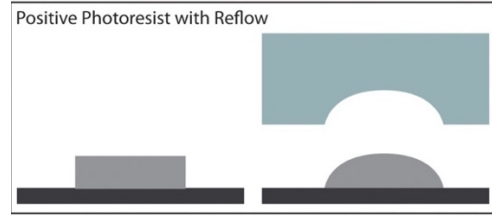


Figure 5 Reflow with positive photoresist to produce rounded features, adapted from [44]

The glass capillary method is a fast and simple method to create circular channels from premade glass capillary tubes; a variety of tube diameters are available on the market. Using this technique, the capillary tube is often supported and the inlet and outlet ports are connected to tubing (Figure 6).

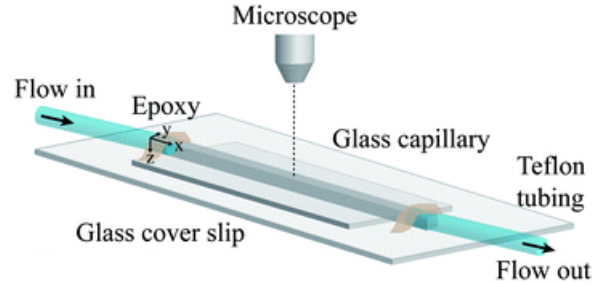


Figure 6 Diagram of a glass capillary tube being used as a circular channel, reproduced from [45]

The application of glass capillary tube microfluidics to the study of blood flow is limited as red blood cells may undergo echinocytosis, forming thorny cell membrane projections after interacting with the glass surface [46] and thereby interfering with experimental findings. In contrast, adherent cells have satisfactory growth on unmodified glass, which is often improved with protein coatings.

Several studies demonstrate the successful culture of endothelial cells within glass capillary microchannels. Ye *et al.* separately cultured Human brain microvascular endothelial cells (HBMECs) and HUVECs in glass capillary tubes, treated with 150 $\mu\text{g}/\text{ml}$ of type I collagen, with diameters from 10–500 μm , and compared the growth of the two cell types. Cell culture medium was changed every day, and cells were cultured for 3–4 days before. For shear stress studies, 200 μm tubes were perfused

with a flow rate gradually increasing to 640 ml/min over 6 hours, and a total exposure time of 24 hours. Ye *et al.* observed that for the smallest tube diameters, the HBMECs wrapped around forming junctions with themselves, while the HUVECs did not [47].

Compared to unmodified glass, untreated PDMS substrates have been found to have poorer biocompatibility for several adherent cell lines [48]. However, PDMS is a low cost useful material for microfluidics, and unlike glass, it can be easily manipulated to have different dimensions, shapes and material stiffness, all of which give it more potential to create complex devices.

The gas stream process, which has been employed in several studies [49] [50], employs the polymerization of PDMS around a nitrogen or air stream. Liquid PDMS is injected into a rectangular microchannel, fabricated using conventional photo and soft lithography. The pressurized gas stream is then introduced and displaces uncured PDMS, resulting in a circular channel cross-section (Figure 7). Nitrogen gas pressures between 5 and 15 psi have been shown to create circular cross-section channels that had controllable diameters between 40–100 μm : decreasing gas pressures resulted in reduced circular channel diameters. Pressures larger than 15 psi resulted in square cross-sections with rounded corners, and pressures less than 5 psi created circular cross-sections with inconsistent diameters [49]. Using a 1mm x 1mm x 5cm channel, Fiddes *et al.* demonstrated the ability to culture primary porcine aortic endothelial cells (PAECs) in a fabricated circular PDMS microchannel treated with tetraethyl orthosilicate to form a SiO_2 layer, promoting cell adhesion. Fiddes *et al.* used a minimal flow of less than 0.1 ml/hour during the cell seeding process and otherwise maintained static conditions for a 24 hour growth period [49].

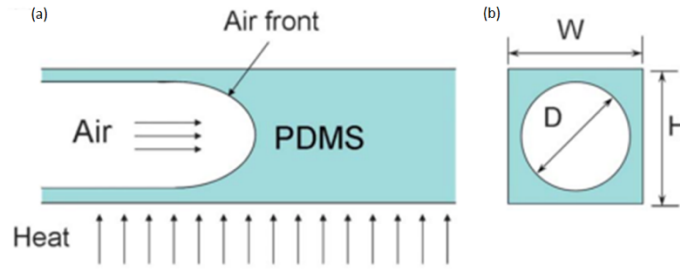


Figure 7 Diagram of air pressure creating a circular cross-section in liquid PDMS, reproduced from [50]

Stainless steel microwires, 24-80 μm in diameter [51] and silica glass rods, 57-250 μm [52] have been used to produce circular cross-section microchannels in PDMS via the wire-molding method. In this technique, microwires are aligned in an array and PDMS is poured on the configuration. The PDMS is cured and becomes solid. Finally, the microwires are removed, leaving behind circular channels. A disadvantage of this technique is that inlets and outlets formed by microwire removal are not easily accessible for attachment to tubing. Inlets and outlets are therefore often added after the channel has been formed, and exit holes from microwire removal can be sealed with PDMS [52]. An additional limitation of the wire-molding method is the risk of channel blockages from small pieces of PDMS dislodged by microwire removal. The flexibility of microwires allows for the production complex geometries such as curving and crossed channels [51], however, there can be difficulties removing wires of greater length due to friction in the PDMS, which can result in damage to the microchip. Using ethanol to swell PDMS prior to wire removal can reduce friction [51], however removing absorbed ethanol from the PDMS can be challenging, leading to a potentially cytotoxic environment that might not be suitable cell culture [53]. To overcome these limitations and to increase design complexity, solder wires and ABS plastics can be used as a mold, and then melted with heat or dissolved with a solvent, without damaging the PDMS. Minimum achievable channel diameters are dependent on commercial availability of materials [50].

In a technique similar to the wire-molding method, Dolega *et al.* used glass capillary tubes as molds to form circular microchannels within PDMS. Glass capillaries were assembled with needles on the ends and positioned on supports. PDMS was poured onto the assembly. After PDMS solidification, needles and glass capillaries were removed, leaving behind a circular PDMS channel, and achieving diameters of 100-400 μm [54]. Dolega *et al.* coated PDMS microchannels with either polyelectrolytes or Matrigel to improve cell attachment. Epithelial prostate (RWPE-1 and PC3) and breast (MCF10A) cell lines were cultured in the microchannels in conditions that were static except for cell culture medium replacement, for up to 5 days. The authors found that these cells could form a confluent layer in channels of 150 μm diameter or greater, while increased cellular death was observed in smaller sized channels [54].

1.4.3 Cell culture in 3D environments

Conventional two-dimensional (2D) cell culture involves growing cells on planar dishes, typically made from a rigid plastic material such as polystyrene. Polystyrene dishes are often coated with fibronectin and collagen to improve cell adhesion and spreading [55]. Although 2D culture techniques are relatively straightforward and easy to use, growth conditions and cell-cell interactions differ vastly from the 3D environment of tissues in the human body. In a 2D culture, cells are limited to one plane and experience little resistance to migration from structural confines that they would normally encounter in a 3D setting [25]. As a result, 2D culture studies of cell growth, behavior, and response to stimuli are often not representative of *in vivo* conditions [56],[57]. Three-dimensional (3D) cell cultures are artificially created microenvironments with architectures that more accurately simulate physiological geometries [12]. The cells growing in these structures can interact in three dimensions with each other and their surroundings, providing a closer representation of what would be observed in the human body. A number of studies demonstrate significant differences in the behavior and

characteristics of 2D and 3D cultured cells. Peterson *et al.* demonstrated that human breast epithelial cells developed like tumor cells under 2D culture conditions, but returned to regular growth behavior in the setting of 3D culture conditions that approximated their physiological environment [58]. Furthermore, cells cultured in 2D and 3D environments have been shown to exhibit altered gene expression [56]. Luca *et al.* found that colorectal cancer cells showed significant differential regulation of more than 200 genes when cultured in 3D spheroid cultures compared to 2D environments [59], while Ferreira *et al.* observed differential expression for Human Alveolar Bone Osteoblastic Cells on varying nanopatterned titanium surfaces [60]. Various techniques including spheroid cultures and hydrogels exist for the creation of 3D cell cultures; these methods have been optimized and are increasingly becoming more feasible [61]. Microfluidics represent an important method for 3D cell culture and can be effectively utilized to mimic a number of physiologic conditions, including microvascular circulation.

1.4.4 Flow in microchannels

Rectangular microchannels are most often used in microfluidics research given their cost-effectiveness and ease of fabrication relative to circular microchannels. However, rectangular microchannels do not accurately represent blood vessel geometry and may therefore be poor models for blood flow dynamics and cell-cell interactions in microvascular systems. In 2011, Yang *et al.* showed that blood hematocrit is locally influenced by vessel geometry [3]. Furthermore, rheological properties are different in microchannels with circular and rectangular cross-sections. The velocity profiles (Figure 9), flow rates, and shear stress at the channel wall are significantly influenced by channel geometry [3],[62]. Channel geometry also affects margination (the outward movement of particles in a fluid flow toward channel walls), a property that white blood cells and platelets make use of in order to migrate to the vessel wall and perform various functions [3],[62]. Cell interactions and blood flow

characteristics within rectangular microchannels may therefore differ significantly from physiological conditions [3]; circular microchannels provide a means of more accurately approximating the *in vivo* microvascular environment.

Assuming a straight and inelastic microchannel, where the fluid in the microchannel is Newtonian with constant viscosity, and steady, laminar flow, the velocity field for the Hagen-Poiseuille flow in a rectangular channel is given by [62]:

$$v_{x_{rectangle}}(y, z) = \frac{4h^2 \Delta p}{\pi^3 \mu L} \sum_{n \text{ odd}} \frac{1}{n^3} \left(1 - \frac{\cosh\left(n\pi \frac{y}{h}\right)}{\cosh\left(n\pi \frac{w}{2h}\right)} \right) \sin\left(n\pi \frac{z}{h}\right)$$

And, for a flat and very wide rectangular channel, flow rate is approximated as [62]:

$$Q_{rectangle} \approx \frac{h^3 w \Delta p}{12 \mu L} \left(1 - 0.630 \frac{h}{w} \right)$$

where μ is the viscosity, L is the channel length, ΔP is the pressure drop, w is the channel width, h is the channel height for a rectangular cross-section (Figure 8) and n is an odd integer, and $w > h$.

For elliptical or circular channel cross-sections, velocity field and flow rate equations are given by [62]:

$$v_{x_{circle}}(y, z) = \frac{\Delta p}{2 \mu L} \frac{a^2 b^2}{a^2 + b^2} \left(1 - \frac{y^2}{a^2} - \frac{z^2}{b^2} \right)$$

And,

$$Q_{circle} = \frac{\pi \Delta p}{4 \mu L} \frac{a^3 b^3}{a^2 + b^2}$$

where a is the major axis length and b is the minor axis length for an ovoid cross-section (Figure 8), and $a=b$ for circular channel cross-sections.

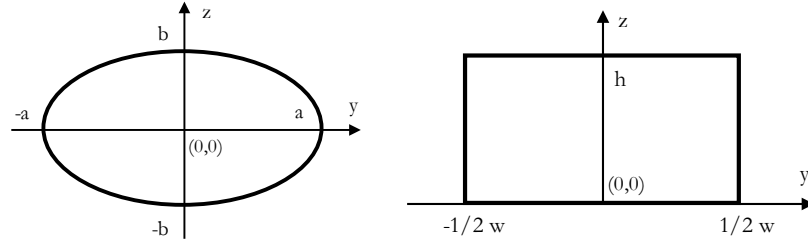


Figure 8 Varying cross-sectional channel geometries, elliptical (left), rectangular (right)

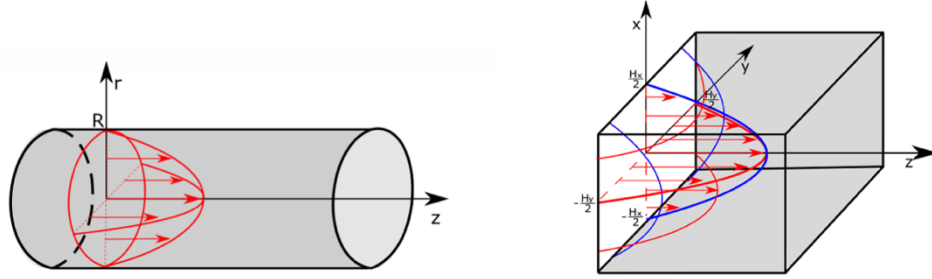


Figure 9 Velocity profiles for flow in circular and rectangular cross-section channels, reproduced from [63]

1.4.5 Shear stress

In the blood vessels of the human body, vascular endothelial cells are affected by shear stress, a friction force caused by both blood flow and cyclical strain from vessel wall distention by transmural pressure, which also deforms vessel wall components. Shear stress and cyclical strain have a major influence on endothelial cell morphology. Shear stress acts tangentially to the endothelial cell surface, deforming cells in the direction of blood flow, while wall distention tends to pull cells in many directions. Acute shear stress (exposure from seconds to hours) has been shown to cause rapid cytoskeleton remodeling and signal cascade activation in endothelial cells [64]. In response to acute shear stress, endothelial cells release a number of signaling factors, including nitric oxide and prostacyclin, which cause smooth muscle relaxation in the vessel wall leading to vasodilation [65]. Under acute shear stress, endothelial cells also activate transcription of genes such as ICAM-1 and MCP-1 (which are involved in intercellular adhesion and help to regulate migration of monocytes and macrophages), and platelet-derived growth factor-B (PDGF-B) (involved in wound healing and the regulation of angiogenesis, cell proliferation and differentiation) [1]. Endothelial cells respond to inflammatory cytokines in a

similar way [1]. Alternatively, when exposed to chronic shear stress over days to weeks, endothelial cells undergo differentiation and structural remodeling, flattening in order to minimize shear stress, and changing integrin distributions to increase cellular focal adhesions, and increase endothelial cell (EC) adhesion at locations of highest stress [1].

In our study, channels were perfused with culture medium rather than whole blood. We may therefore take a Newtonian modeling approach, where shear stress is linearly proportional to the applied shear rate, and viscosity remains constant with varying shear rate. For future studies where whole blood is incorporated into the model, it would be important to consider its non-Newtonian properties. In a non-Newtonian fluid, viscosity is dependent on shear rate. Blood is a shear thinning fluid, meaning that as the shear rate increases, fluid viscosity decreases [66]. Shear stress is dependent on channel geometry given its relation to the shear rate, which is directly dependent on the flow rate. For a Newtonian fluid flow within an inelastic, cylindrical and straight vessel with circular cross-section, the shear stress at the wall from the Hagen-Poiseuille equation is given by [1],[66]:

$$\tau = 32\mu \frac{Q}{\pi \cdot d^3}$$

where Q is the flow rate, μ is the fluid viscosity, and d is the channel diameter. The shear stress at the wall (boundary) for a parallel plate configuration can be calculated using the following formula derived from Poiseuille's law [67]:

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

where Q is the flow rate, μ is the fluid viscosity, w is the channel width, and h is the channel height.

This equation assumes a Newtonian fluid, with steady and laminar flow.

1.5 Conclusion

From literature, it is evident that there is a strong relationship between channel cross-sectional geometry and the resulting flow behaviors. Because of these conditions, endothelial cells and blood constituents including RBCs, WBCs and platelets may behave differently when situated in a microchannel with a rectangular cross-section compared to a circular one. As gene expression is often altered in 3D environments for various cell types [56],[59], HUVECs cultured in a 3D microchannel may additionally express genes differently than when cultured under standard 2D conditions. Both rectangular microchannels and 2D endothelial cell culture may inaccurately model microvasculature *in vivo*. To create a model more representative of physiological conditions, we propose constructing circular cross-section microchannels in which endothelial cells coat channel walls. In order to optimize channel biocompatibility, several substrates will be tested. The use of glass capillary tubes for cell culture was excluded from this study, based on limitations as described in the literature review.

We hypothesize that, under acute shear stress conditions, we will visualize increased cytoskeleton alignment of the HUVECs in the direction of flow. We also hypothesize that, relative to 2D culture conditions, we will observe significant changes in the gene expression of HUVECs grown in 3D channels.

Chapter 2

2 Methodology

2.1 Microfabrication experiments

Several microfabrication techniques described in literature were tested to develop channels with rectangular and circular cross-sections. These microfabrication techniques were experimentally compared.

2.1.1 Photolithography

The master mold fabrication process for the development of rectangular microchannels begins with standard photolithography methods described in MicroChem product information [68]. After determining microchannel dimensions, a transparency photomask is printed, containing the desired configuration (Figure 10), which appears as the transparent regions of the mask. This geometry is eventually transferred onto a silicon wafer to create the inverse mold. The photomask was designed in Solidworks for a 4-inch diameter silicon wafer. The design was sent to CAD/Art Services, Inc. (Bandon, OR), where it was printed onto a clear cellulose acetate sheet as a high precision photomask.

The silicon wafer was cleaned in a 1% v/v hydrofluoric acid (HF) solution (Fisher Scientific, Canada), allowing for the removal of built up silicon oxide in order to improve surface bonding capability with the photoresist. SU-8-50 (MicroChem) epoxy-based negative photoresist was spin coated on a spread cycle of 500 rpm for 10 seconds, and then 1000 rpm for 30 seconds. This creates the desired thickness of 100 μm , according to the manufacturer's instructions. The silicon, SU-8-50 coated wafer was soft baked at 65 °C for 10 minutes, and hard baked at 95 °C for 30 minutes.

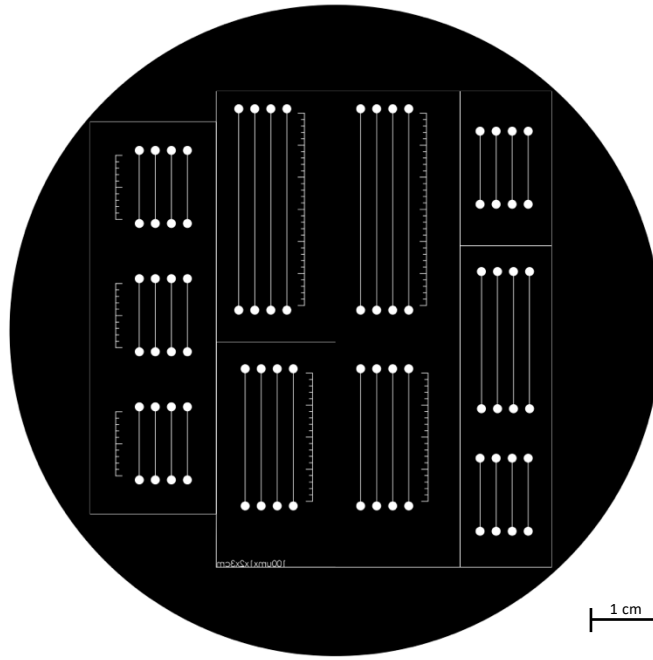


Figure 10 Photomask used in wafer fabrication

The photo-mask was placed on top on the coated wafer and the assembly was exposed to of 350 mJ/cm² of UV light for 2 second in a UV box (Intelli-Ray 600W: UVitron International, West Springfield, USA). The transparent regions on the photomask expose the photoresist to the UV light causing it to cure, while the opaque regions block the light. A soft bake at 65 °C for 3 minutes and then a hard bake at 95 °C for 10 minutes was completed to cross-link the exposed regions of the photoresist. After, the assembly was placed in SU-8 50 developer solution (MicroChem) for 10-15 minutes with agitation. This allows uncured regions to dissolve, leaving the elevated cured regions, and forming the inverse master mold for the microfluidic chip (Figure 11). The silicon wafer was rinsed with isopropyl alcohol and dried with a nitrogen gas stream for storage.

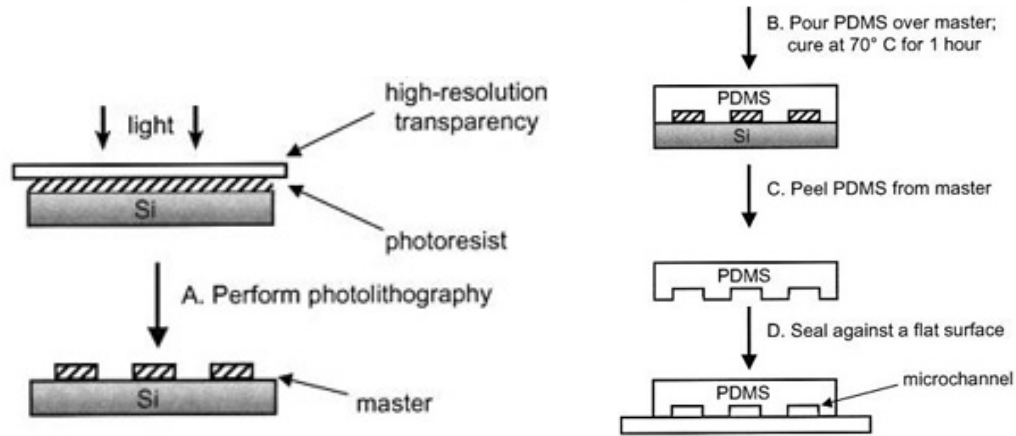


Figure 11 Photolithography and soft lithography processes, adapted from [69]

2.1.2 Soft-lithography method to create rectangular channels

A 10:1 ratio mixture of PDMS elastomer base and curing agent (Sylgard 184, Dow Corning Ltd.) was prepared, degassed in a vacuum desiccator, poured on a master mold placed in a Pyrex dish, and cured on a hot plate for 12-15 min at 100 °C. PDMS channels were then peeled from the master mold (Figure 11), cut out, and inlet and outlet holes were punched using 2 mm diameter punches (Ted Pella, Inc).

To seal the bottom of the microchannel, bonding of the PDMS microchips to unpatterned PDMS substrates, approximately 1-5 mm thick, was completed using a portable BD20-AC corona treater (Electro-Technic Products Inc) as described by Haubert *et al.* [70]. PDMS pieces were treated, pressed together and allowed to bind for 24 hours at room temperature, creating a non-reversible bonding of the two surfaces (Figure 12).

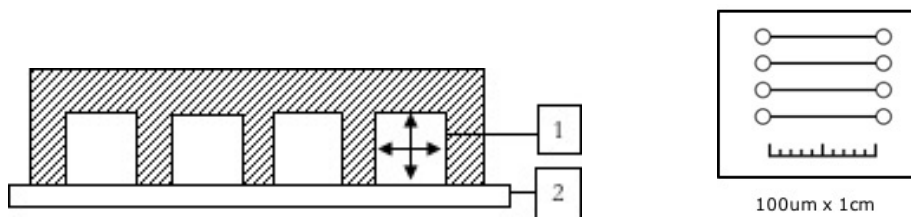


Figure 12 Bonded microchannel cross-sectional view 1) rectangular channel cross section, 2) unpatterned PDMS substrate (left); and top view of 1cm length microchannel chips (right)

2.1.3 Wafer quality testing

The quality of each wafer produced was determined by assessing the inverse mold (PDMS microchannel geometry) from soft lithography techniques. After pouring uncured PDMS over the manufactured wafer, heat curing, and removing the wafer, a negative copy of the wafer was obtained. PDMS microchannels were bonded, as described above. A thin cross-sectional piece of the microchannel was cut with a razor and placed on a glass slide to be examined under a brightfield ZEISS Axio Lab A1. Microscope with 10x A-Plan 0.25 NA objective. An image was captured using a ZEISS AxioCam 150 camera and analyzed using Zen software. The first two wafer fabrication tests resulted in microchannels with poor dimensions and geometry (Figure 13). Some of the SU-8 channel features also lifted off the wafer after PDMS was poured, indicating inadequate annealing of the SU-8 to the wafer during fabrication. To improve wafer geometry, several different modifications to the protocol were tested (see below). Based on the results from these modifications, an optimized protocol was developed to create microchannels with a rectangular cross-section (Results).

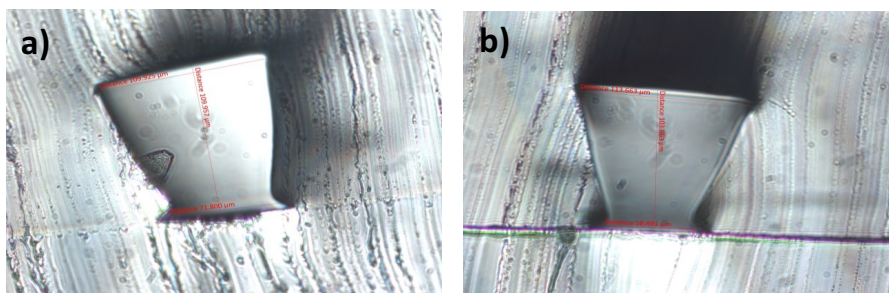


Figure 13 Preliminary wafer fabrication from photolithography techniques, a) microchannel from wafer 1 production, b) microchannel from wafer 2 production

2.1.4 Protocol modifications

Five alternate protocol steps were identified from troubleshooting literature for photolithography techniques and used to modify the original procedure in an attempt to improve the quality of the microchannel. Each step was tested independently and added to the original protocol. A total of six

silicon wafers were used, one to test each protocol modification, and one control wafer where no modifications were made. Individual modifications to the original protocol are as follows:

1. Additional bake at 120 °C for 10 minutes following etching of the wafer with hydrofluoric acid, and prior to adding the SU-8, in order to ensure that the wafer is completely dried after washing [71].
2. Bake for 100 minutes at 95 °C (one minute for every μm thickness) during the first soft baking step, as opposed to the original 30 minutes [72].
3. Relaxation for 10 minutes after the first hard bake, and 10 minutes relaxation after the UV exposure [72].
4. Coating the SU-8 covered silicon wafer with glycerol prior to UV exposure, to ensure that the photomask is positioned as close as possible to the SU-8 coating. This has the potential of eliminating any air gaps between the surfaces, and reducing light diffraction through the air after UV passes through the photomask [42].
5. UV exposure time increased to 4 seconds as opposed to 2 seconds; potential to create more perpendicular channel walls [73].

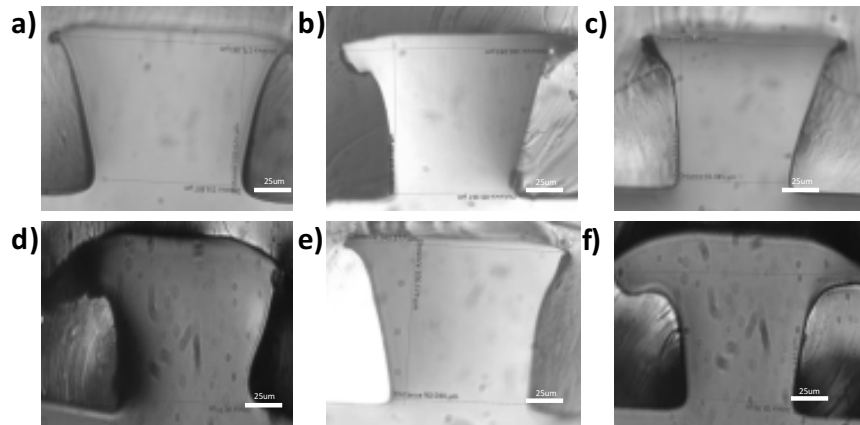


Figure 14 Resulting microchannel cross-sections from photolithography step modifications, a) control, b) step 1, c) step 2, d) step 3, e) step 4, f) step 5, scale bar 25um.

Cross-sections were examined using a brightfield ZEISS Axio Lab A1. Microscope with 10x A-Plan 0.25 NA objective. Dimensions were obtained using Zen 2 Lite (Blue Edition) Software. The width of the channel at the widest (top width) and most narrow location (base width) were measured (Table 2). The height of the channel is a function of speed during the spin coating step of the fabrication process and should therefore remain relatively constant. The expected width is 100 μm , given the photomask design. Preliminary results used in microfabrication optimization are presented in Table 2. Optimal channel geometry is defined as channels with ratio of top and base width closest to 1. The measurements and procedures for this protocol modification were completed in collaboration with laboratory colleagues, Mark Stroobach and Niko LeeYow.

Table 2 Changes made to photolithography procedure and resulting cross-sectional dimensions obtained, adapted from [74]

Updated procedure	Dimensions	Base width to top width ratio
No change (original procedure)	Top Width: 171 μm Base Width: 117 μm Height: 104 μm	0.68
1) Bake at 120°C for 10 minutes after HF etching [71]	Top Width: 146 μm Base Width: 89 μm Height: 118 μm	0.61
2) Baked for 100 minutes at 95°C, after spin coating [72]	Top Width: 141 μm Base Width: 81 μm Height: 104 μm	0.57
3) 10 minutes relaxation time after 1 st hard bake and 10 minutes after UV [72]	Top Width: 241 μm Base Width: 141 μm Height: 100 μm	0.59
4) Glycerol coating [42]	Top Width: 143 μm Base Width: 91 μm Height: 106 μm	0.64
5) 4 second UV exposure time [73]	Top Width: 254 μm Base Width: 105 μm Height: 105 μm	0.41

2.1.5 Selected process modification

Of the modifications tested, updated procedure #4 (the application of a thin layer of glycerol to the photomask prior to placement on the SU-8 coated silicon wafer) and no modifications (the control), produced the optimal results for channel geometry. It was observed that the glycerol layer eliminated

the air gap between the mask and wafer, helping to reduce UV light diffraction and ensuring even curing of the SU-8, resulting in channel walls that were more perpendicular. Additionally, with this modification, SU-8 tended to adhere more strongly to the silicon wafer, as less SU-8 was found to lift off the wafer following repeated use with PDMS molding. As such, the updated procedure #4 modification was ultimately added to the original protocol.

2.1.6 Gas stream fabrication method

PDMS microchannels with circular cross-sections were developed from 1cm long, rectangular cross-section microchannels created with conventional photo and soft lithography and using the gas stream fabrication method adapted from Fiddes *et al.* and Abdelgawad *et al.* A 10:1 PDMS solution was dissolved in hexanes (Sigma–Aldrich, Canada) in a 1:1 ratio, to reduce PDMS viscosity. The PDMS-hexane solution was then injected by syringe into rectangular cross-section microchannels. An air stream with pressure ranging from 5-20psi (produced with a pressure control device, MFCS™-EZ: Fluigent, Villejuif, France) (Figure 17) was introduced into the channels for varying amounts of time while channels were heat-cured on a hot plate set to 100 °C. Channel cross-sections were examined under a brightfield ZEISS Axio Lab A1. microscope with 10x A-Plan 0.25 NA objective. Initial results showed insufficient residual PDMS in the rectangular microchannels to create a fully circular, or even elliptical cross-section (Figure 15). Thus, undiluted 10:1 PDMS was used for subsequent gas-stream channel fabrication, despite increased difficulties injecting the solution into the microchannel due to its high viscosity.

Initially, a two-minute curing time at 100 °C simultaneously with an air stream applied at 5 psi was tested. This resulted in blocked channels (Figure 16). Following this, curing time was increased to 20 minutes on the hot plate at 100 °C with continuous air flow at 5 psi, prior to curing. These modifications resulted in an optimized protocol: 1cm length, rectangular cross-section microchannels,

produced from the standard lithography techniques, were filled by syringe with liquid 10:1 ratio of PDMS. A stream of compressed air was applied using a pressure control device (MFCS™-EZ: Fluigent, Villejuif, France) (Figure 17) with constant pressure of 5 psi to first clear the PDMS-filled microchannels. When the channels showed visible signs of clearing, the chips were heated on a hot plate for 20 minutes at 100 °C, with continued air flow. Residual PDMS cured in the corners, leaving behind near circular cross-sections (Figure 18 and Figure 26). Cross-sections of microfabricated channels were imaged using a bright field ZEISS Axio Lab A1. Microscope with 10x A-Plan 0.25 NA objective. Dimensions were obtained using Zen 2 Lite (Blue Edition) Software. Aspect ratios and circularity values were determined and are displayed in the Results section.

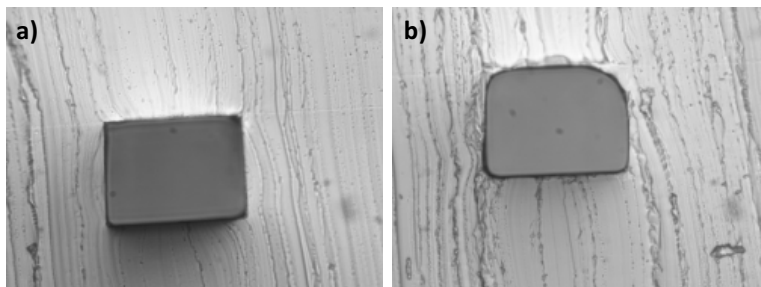


Figure 15 Gas stream method PDMS with hexanes mixture, a) 20 psi b) 5psi

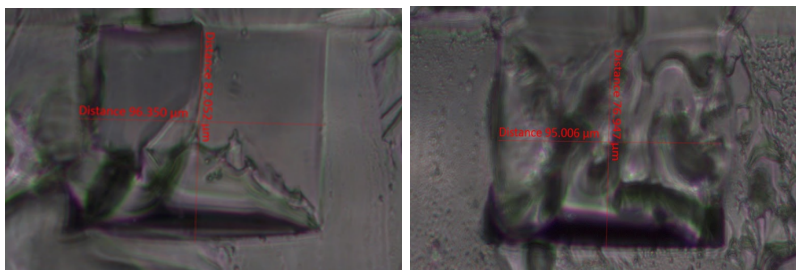


Figure 16 Blocked microchannels, 2 minutes on heat with air

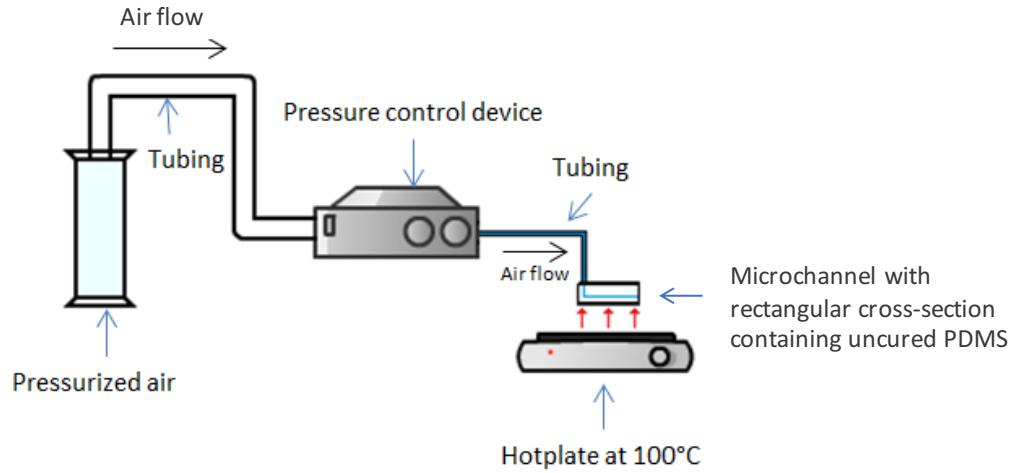


Figure 17 Diagram of pressure system used to apply air stream to microchannel, adapted [74]

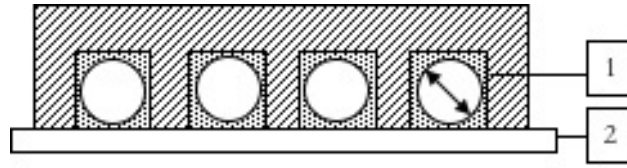


Figure 18 Microchannel cross-sectional view, circular cross-section produced from rectangular channels 1) cured residual PDMS in rectangular channel corners 2) unpatterned PDMS substrate

2.1.7 Wire molding fabrication method

A wire molding technique (adapted from Jia *et al.* [51]) was achieved by 3D printing a plastic mold (Figure 19 and Figure 20) that would enable the alignment of microwires. The SS316L stainless steel microwires (RBA Depot) with the following American Wire Gauge (AWG) and equivalent theoretical diameters are presented (Table 3).

Table 3 AWG equivalent diameter dimensions [75]

Microwire American Wire Gauge (AWG)	Diameter (mm)
24	0.5106
26	0.4049
32	0.2019
38	0.1007
40	0.0799

The mold was designed to align microwires horizontally and maintain an equal vertical distance once wires were suspended in PDMS. The mold was designed in Tinkercad, an open source, online 3D designing and printing application. The file was then imported to Cura, a 3D printing and slicing software, and then printed using a Ultimaker 2+ 3D printer. A thermoplastic polymer, PLA, with a melting temperature of approximately 178 °C was used as the material for the mold. Two molds were attached per glass slide, uncured PDMS was applied along the perimeter of the molds, which were then heated at 100 °C. PVC plastic tubing (Saint-Gobain Performance Plastics, USA) with a 1.6 mm inner diameter was cut to approximately 3 cm in length and inserted into the inlet and outlet holes of the mold (Figure 21). Microwires of varying diameters were then inserted into the mold through the plastic tubing, leaving some wire extending past the tubing. Liquid PDMS was poured into the mold, submerging the microwires. The assembly was heated at 100 °C for 12-15 minutes to allow for solidification of the chip. After curing, tweezers were used to extract the microwires from the chip, leaving behind circular channels of varying diameters, with the embedded PVC plastic tubing to create accessible inlet and outlet ports.

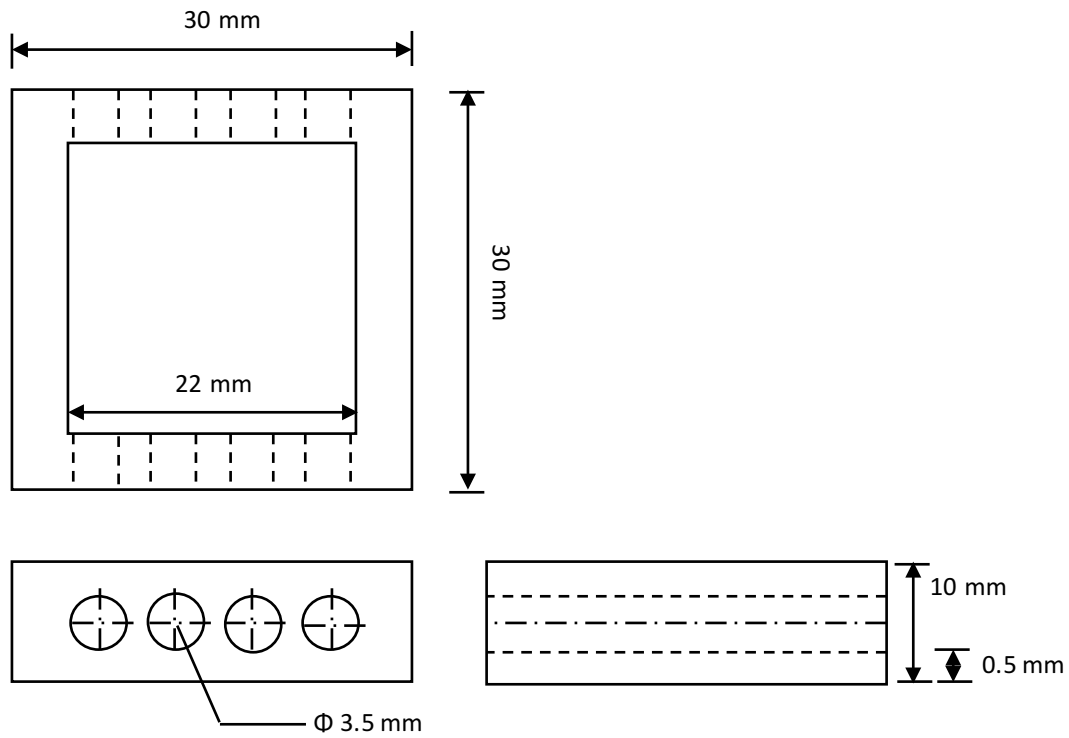


Figure 19 Wire mold design a) top, b) front and c) side views (not to scale)

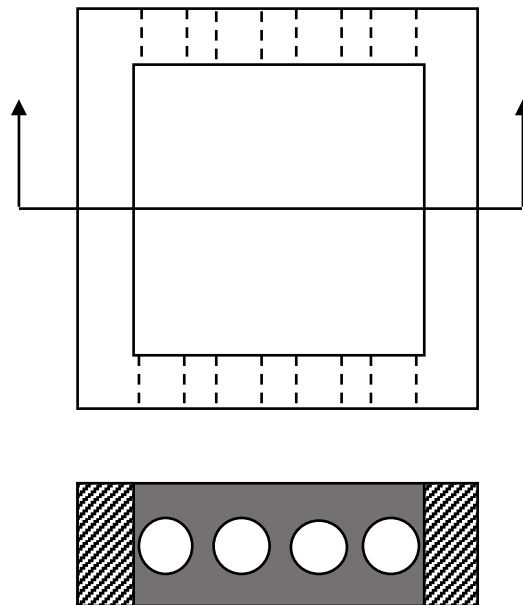


Figure 20 Wire mold design, cross-sectional view

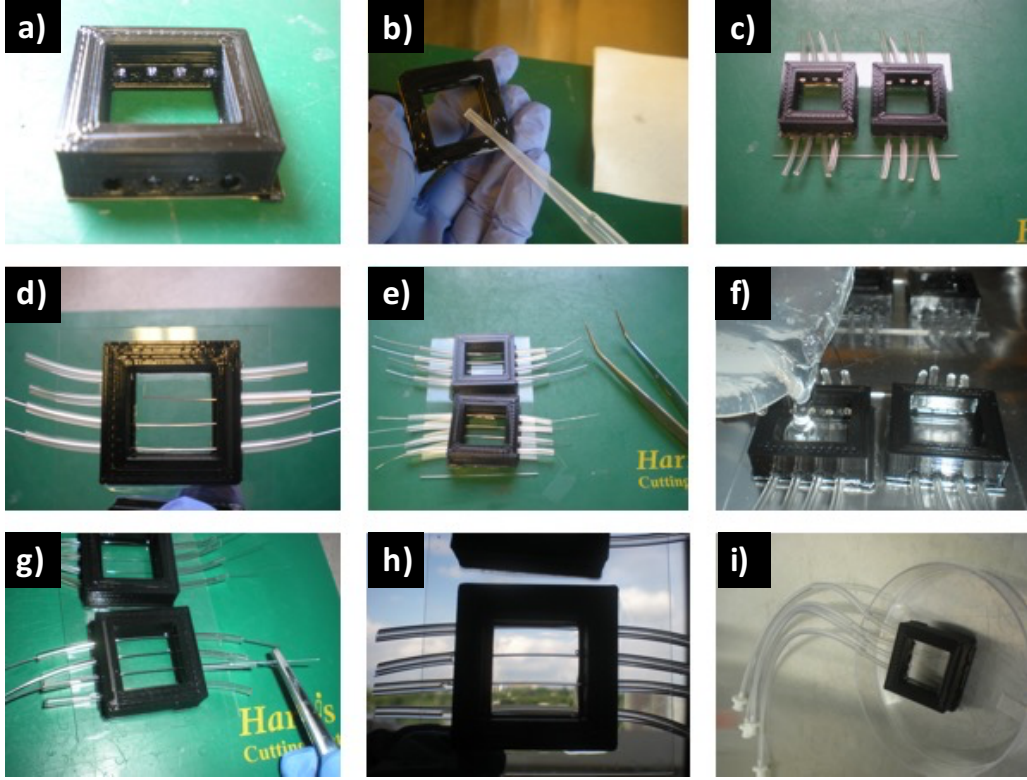


Figure 21 Wire molding chip fabrication process a) 3D printed mold, b) application of uncured PDMS to underside of 3D printed mold, c) molds with uncured PDMS placed on glass slide, heated to cure, PVC tubing inserted into ports, d)-e) microwire inserted through PVC tubing, f) mold is placed on hot plate and uncured PDMS poured into the mold g)-h) microwires removed with pliers after PDMS solidification, i) addition of tubing connectors for cell culture

Cross-sections of microfabricated channels were imaged using a brightfield ZEISS Axio Lab A1. Microscope with 10x A-Plan 0.25 NA objective. Diameter measurements were completed using ImageJ software. Comparisons to theoretical wire dimensions, aspect ratios and circularity values are displayed in the Results section of this report. For all microchannels, created using the different fabrication methods described, the aspect ratio and circularity were calculated as follows:

$$\text{Aspect Ratio} = \frac{\text{major axis length}}{\text{minor axis length}}$$

And,

$$\text{Circularity} = 4\pi \frac{\text{Area}}{\text{Perimeter}^2}$$

An aspect ratio of 1 indicates an equiaxed geometry. A circularity value of 0 indicates an infinitely elongated polygon, a value of 1 signifies a perfect circle [76], and a circularity value of approximately 0.785 indicates a perfect square.

2.2 Characterization of substrates

Contact angles were measured using static drops of distilled water using a VCA Optima contact angle measurement system (AST Products Inc, USA) to control drop advancement. A fixed drop volume of 1 μ l was applied to each surface, and images were captured using VCA Optima software. Three measurements were recorded at different locations on each sample tested (N=3) for PDMS, PDMS_{FN} and PDMS_{PD} conditions. Two to three measurements were taken at different locations on the collagen substrate (N=1) at each of the different time points. Data are presented in the Results section (Table 9 and Table 10). The samples tested were flat pieces, cut to dimensions of approximately 0.5x0.5x0.2cm (LxWxH).

2.3 Two-dimensional cell experiments

Primary Human Umbilical Vein Endothelial Cells, HUVECs, (ATCC, USA) were grown in VasculLife EnGS Endothelial Medium supplemented with ascorbic acid (50 μ g/mL), hydrocortisone hemisuccinate (1 μ g/mL), 2% fetal bovine serum (FBS), L-glutamine (10 mM), rh EGF (5 ng/mL), heparin sulfate (0.75 U/mL), EnGs (0.2%) and antimicrobial supplements: gentamicin (30 mg/mL) and amphotericin B (15 μ g/mL) from Life Line Cell Technology, USA. Cells were grown in a humidified incubator with 5% CO₂ at 37 °C. Cells were subcultured by washing in 1x phosphate-buffered saline (PBS) pH 7.4 (Corning, USA), and a 0.25% trypsin–EDTA solution (ThermoFisher Scientific, USA) was added to the cell dishes and incubated at 37 °C for 2 minutes to facilitate cell detachment [26]. The cell and trypsin solution was neutralized with an equal volume of fresh media,

and the cell solution was collected and centrifuged at 200 g for 5 minutes. The cell pellet was resuspended in fresh medium, and a 10-15 μ L sample of the cell suspension was applied to the hemocytometer to determine the cell concentration (Appendix). The volume of cells (determined from hemocytometer calculations) corresponding to the desired seeding amount of 20,000 cells per well was removed from the suspension and added to wells in a 24-well plate (VWR, USA) containing substrate samples. Primary cultures between passage three and six were used in all experiments.

2.3.1 PDMS coating procedures

Two dimensional PDMS substrates were prepared by mixing a 10:1 ratio of PDMS prepolymer (Sylgard 184, Dow Corning) to curing agent, mixing vigorously for 5 minutes and then placing the solution in a vacuum desiccator chamber for 30 minutes to degas, removing bubbles. A drop of liquid PDMS was placed on a #1.5 glass coverslip (ThermoFisher Scientific, USA), which was heated on a hot plate at 100 °C for approximately 10 minutes allowing the PDMS to cure and form a thin layer (approximately 1-3 mm thick) over the glass substrate. PDMS covered glass substrates were placed in 24-well plates for further cell culture experiments.

2.3.1.1 Fibronectin-functionalized PDMS surfaces

PDMS covered glass substrates were washed with 1x PBS (Corning, USA). A solution of fibronectin was prepared by diluting a 1 mg/mL Human Plasma Fibronectin Purified Protein solution (Millipore, USA) into 1xPBS to obtain a 5 μ g/mL solution. The fibronectin solution was added to the PDMS covered glass substrates, contained in 24-well plates, and remained in contact with the substrates for 1 hour at room temperature to allow for fibronectin adsorption on the PDMS surfaces. Substrates were washed in 1xPBS (Corning, USA) prior to cell seeding. Fibronectin adherence in microchannels was assessed using a 5 μ g/ml fluorescent rhodamine-fibronectin solution (Cytoskeleton Inc, USA);

fibronectin was present for at least 4 hours after microchannels (that had been incubated for 1 hour at room temperature with fibronectin solution) were rinsed with 1x PBS solution (Appendix).

2.3.1.2 Polydopamine-functionalized PDMS surfaces

PDMS samples were coated with a poly(dopamine) film by immersion in a solution of 2 mg of 3-hydroxytyramine-HCl (Dopamine hydrochloride) (Sigma Aldrich, USA) dissolved in 1 mL of 10mM Tris buffer (Sigma Aldrich, USA) at pH 8.5, for 24 hours. After removal of the polydopamine solution, the samples were washed twice with 1x PBS (Corning, USA) and incubated in a 70% ethanol solution to sterilize. Substrates were washed again in 1xPBS prior to cell seeding.

2.3.1.3 Collagen substrates

A neutralized Type I collagen solution was prepared following a protocol developed by Gavrielle M. Price and Joe Tien, Department of Biomedical Engineering, Boston University [77]. PBS 10x, 7.5 % w/v NaHCO₃, 0.2 M NaOH (Sigma, USA) and 8 to 10 mg/mL Type I collagen from rat tail (ThermoFisher Scientific, USA), stored at 4 °C was aseptically transferred to the biosafety cabinet. Working quickly, and keeping all components at approximately 4 °C [77], the following ratios were mixed to obtain a collagen concentration of approximately 6.35 mg/mL; higher concentrations have shown less invasion of cells into the collagen layer when comparing concentrations of 3-6.5 mg/ml[78].

- 12.5 parts PBS 10x
- 1 part NaHCO₃
- 12.5 parts NaOH
- 100 parts Collagen Type I

The pH of the solution was tested by pipetting a small amount of solution onto a pH-indicator strip. It was ensured that the pH was approximately 7.0 – 7.5 near physiological levels, to be suitable for cell

culture. The solution was quickly transferred into 24-well plates to form a thin coating over the well surface. After approximately 1 hour at room temperature, gelation of the neutralized collagen solution was complete. Following gelation, samples were stored at 4 °C for no more than 1-2 days prior to cell seeding.

2.3.1.4 Fibronectin-functionalized collagen substrates

Following the protocol above, the collagen solution was prepared. After gelation, a 5 µg/mL fibronectin solution was added to the surface of the collagen substrate in the wells and allowed to incubate for 1 hour at room temperature. The fibronectin solution was removed carefully to avoid disturbing the collagen gel. Samples were stored at 4 °C for no more than 1-2 days prior to cell seeding.

2.3.2 2D fixed cell imaging

After a 48 hours growth period, cell medium was removed from 24-well plates, and cells on the substrate surfaces were fixed with 4% (v/v) paraformaldehyde (PFA) for 15 minutes and then washed once with 1x PBS (Corning, USA). Cells were subsequently permeabilized with 0.5% Triton X-100 (ThermoFisher Scientific, USA) for 10 minutes and washed once with 1x PBS. Cells were stained for filamentous (F)-actin using AlexaFluor555 Phalloidin (Sigma, USA) in a 1:20 dilution [79]. Cell nuclei were stained using NucBlue Fixed Cell Ready Probes (ThermoFisher Scientific, USA) in a 2 drop/mL concentration [80]. PDMS-FN and PDMS-PD samples were inverted and mounted on #1.5 glass coverslips using mounting media (DAKO). Collagen and Collagen-FN samples were imaged directly in the 24-well plates. Fluorescently labeled cells were imaged with an inverted epifluorescence Zeiss D1 AxioObserver microscope with a monochrome AxioCam MRm CCD detector and a 20x, 0.40 NA, Air, LD Plan NeoFluar objective. For each substrate, nine fluorescent images were captured in a grid formation (Figure 22). Fixed imaging experiments were completed for determining HUVEC proliferation (Table 4) and spreading on the different substrates (Table 5).

Table 4 Summary of 2D sample conditions and sample size for nuclei count (cell proliferation) experiments (fixed cell imaging)

2D Substrate condition and abbreviations	Sample size (N)	Images acquired/sample
Untreated PDMS	20	9
PDMS treated with fibronectin (PDMS _{FN} /-FN)	20	9
PDMS treated with polydopamine (PDMS _{PD} /-PD)	19	9
Collagen gel (Collagen)	27	9
Collagen gel treated with fibronectin (Collagen _{FN} /-FN)	26	9

Table 5 Summary of 2D sample conditions and sample size for cell area coverage (cell spreading) experiments (fixed cell imaging)

2D Substrate condition and abbreviations	Sample size (N)	Images acquired/sample
Untreated PDMS	8	9
PDMS treated with fibronectin (PDMS _{FN} /-FN)	8	9
PDMS treated with polydopamine (PDMS _{PD} /-PD)	8	9
Collagen gel (Collagen)	8	9
Collagen gel treated with fibronectin (Collagen _{FN} /-FN)	8	9

2.3.3 2D fixed cell image processing

Each image covered an area of 684.25 μm x 512.69 μm (frame area), and a total of nine images/frames were acquired per 2D sample. Thus, a total area of approximately 3.16mm² was imaged. Cell nuclei were counted on each image (Figure 22) using an image analysis routine in ImageJ software (<http://rsb.info.nih.gov/ij/>). Cell area coverage was determined by creating thresholds for each image analyzed and measuring the area covered by that threshold value in routine ImageJ processing (Figure 23). Cell area was represented as a fraction of the total area in each frame. The mean percent area coverage was then plotted using Prism 7 (GraphPad Software Inc.) (Figure 35). Cell density was calculated using the nuclei count in each image acquired and dividing by the frame area. The mean cell density from all the samples in each condition tested was determined and plotted in Prism 7 (Figure 33).

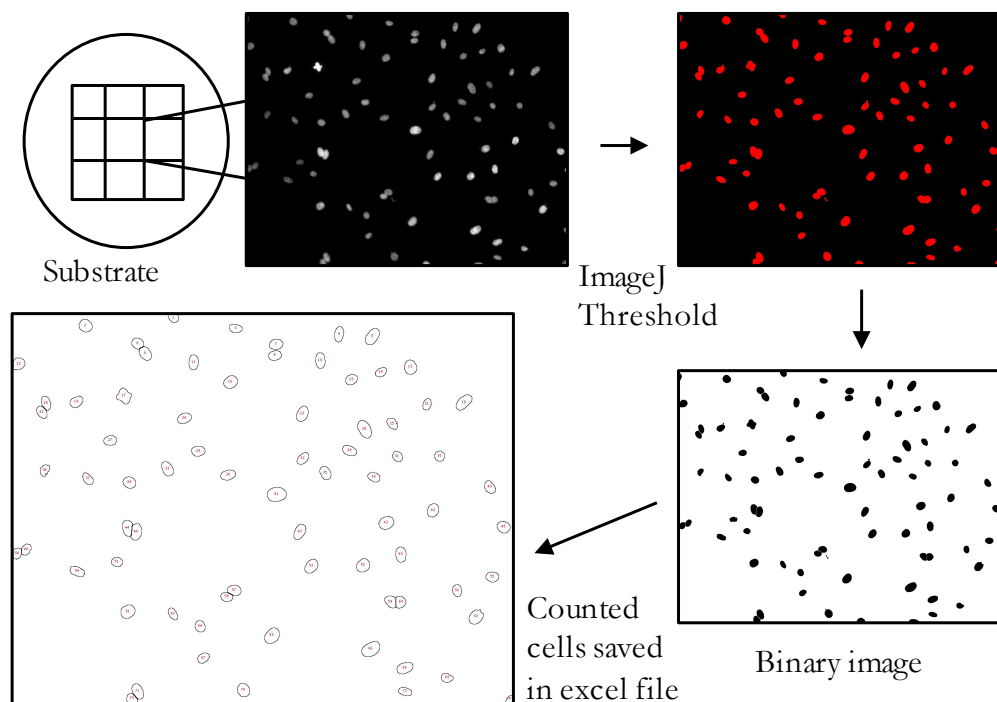


Figure 22 Cell nuclei counting flowchart using ImageJ

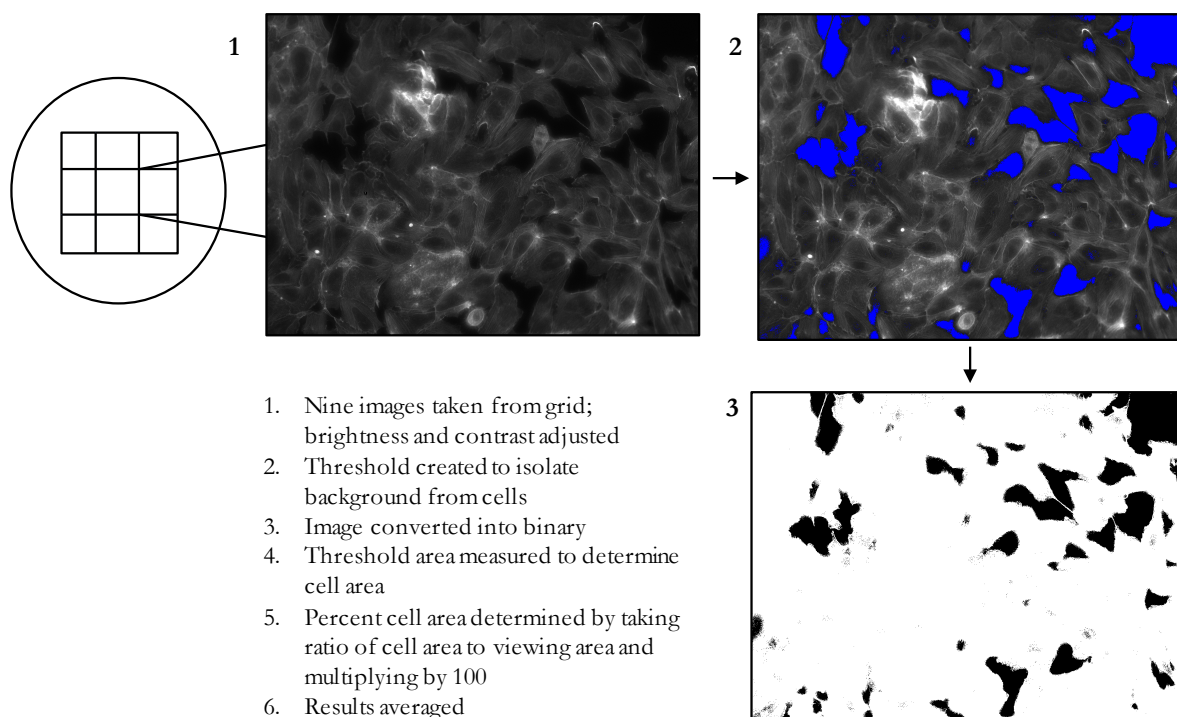


Figure 23 Determining cell area coverage flowchart using ImageJ

2.3.4 Cell motility

To prepare 2D substrates for cell migration tests, one drop of liquid PDMS was spin coated on 35 mm glass bottom culture dishes at 2000 rpm for 2 minutes (WS-650-23B: Laurell Technologies Corporation, North Wales, USA). The PDMS coated culture dish was heated on a hot plate at 100 °C for 10 minutes to allow for curing. The layer formed was approximately 48.8 µm thick (Appendix). Substrates were treated according to conditions previously stated, in order to obtain PDMS-PD (PDMS_{PD}), PDMS-FN (PDMS_{FN}) and untreated PDMS surfaces. For collagen substrate testing, 2 ml of the collagen solution was transferred to the 35-mm dish prior to gelation, and the dish was rotated to promote an even coating. Cells were cultured as previously stated and 20,000 cells were deposited in the 35 mm culture dishes with 2 mL of warmed Vasculife EnGS Endothelial Complete Medium (Life Line Cell Technology, USA). All culture dishes were set up in the environmental chamber for live imaging within 10 minutes after cell seeding.

Table 6 Summary of 2D sample conditions and sample size for live cell experiments

2D Substrate condition and abbreviations	Sample size (N)	Images acquired/sample
Untreated PDMS	1	15
PDMS treated with fibronectin (PDMS _{FN} /-FN)	1	15
PDMS treated with polydopamine (PDMS _{PD} /-PD)	1	10
Collagen gel (Collagen)	1	15

*For cell motility analysis (accumulated distance and velocity values) individual cells were treated independently of one another, and so sample sizes for untreated PDMS, PDMS_{FN}, PDMS_{PD}, and Collagen were 24, 53, 53, and 53 cells, respectively.

2.3.5 Live cell imaging

Phase contrast imaging was completed using an inverted Zeiss AxioVert 200 microscope with a monochrome AxioCam CCD detector, and a 10x 0.3 NA EC Plan-Neofluar objective. To facilitate live imaging, the microscope was equipped with an environmental stage adapter to maintain a humidified environment at 37 °C and 5% CO₂. Imaging of the sample commenced within 10 minutes of cell seeding. Exposure was adjusted to obtain a clear image of the cells on the substrate and focus

was adjusted during the first hour of acquiring the images because of changes in cell morphology, as cells began to adhere and spread on the substrates, and as a result of thermal drift. A grid was established to capture an image at 10-15 different locations on the samples (Table 6) at each image capture interval. Images were captured at intervals of 10 minutes for a period of 12 hours.

2.3.6 Live cell image processing

After acquisition, images were analyzed using a manual cell tracking routine from ImageJ. These data were then analyzed using the Chemotaxis and Migration Tool, a cell tracking software developed by ibidi (USA). From this, the mean accumulated distance travelled by individual cells (Figure 24) was obtained and the paths of individual cells were determined for the various substrates that were tested. Mean velocity of the cell and Directness are two other parameters calculated by the ibidi software. Directness (D) signifies a measurement of the directness of cell trajectories and is the ratio of the euclidian distance to the accumulated distance (Figure 24). $D=1$ represents a completely linear trajectory, whereas $D \rightarrow 0$ signifies random motion [81].

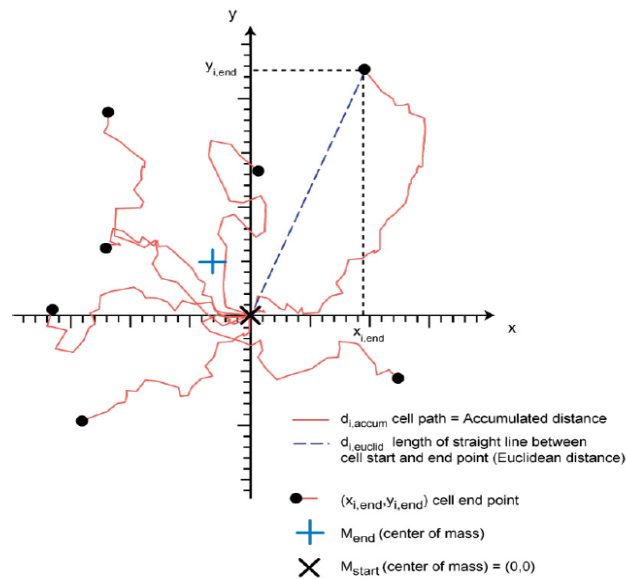


Figure 24 Mean accumulated distance, reproduced from [81]

2.3.7 Statistical analyses for cell experiments

Brown–Forsythe tests were used to assess the equality of group variances. Experimental groups were found to have unequal variance; therefore, comparisons were made using a Kruskal-Wallis test by ranks, with Dunn’s post hoc multiple comparisons tests. The Kruskal-Wallis test is the nonparametric alternative to the one-way ANOVA and used for comparing two or more independent samples, where sample variance is not assumed to be equal and sample sizes may vary [82]. For all tests, P-values of less than 0.05 were considered significant. Statistical analyses were performed in Prism 7 (GraphPad Software Inc.)

2.4 Three-dimensional cell experiments

Primary Human Umbilical Vein Endothelial Cells, HUVECs (ATCC, USA) were grown as previously described. The cell solution was adjusted to obtain a final concentration of 1×10^6 cells/mL, comparable concentrations were used for cell seeding in microchannels with a diameter of a similar order of magnitude in literature [47], [49]. The channels were filled with the cell suspension using a 50 μ l pipette. The channels were placed in a humidified incubator at 37 °C with 5% CO₂ under static, no flow conditions. After 20 minutes, channels were re-seeded with the same cell concentration and the channels were rotated 180 degrees in the incubator; this was repeated another two times. HUVECs were maintained in microchannels with no flow or media exchange for periods of 4 to 12 hours. With media exchange, HUVECs were maintained for 20 hours (for microarray experiments) and up to 48 hours with a flow rate of 0.1ml/h. Cells in the channels were fixed at the varying time points using 4% PFA, permeablized with 0.5% Triton and stained with NucBlue Fixed Cell Ready Probes and AlexaFluor555 Phalloidin, for nuclei and F-actin staining, following the same protocols stated in previous sections.

2.4.1 Flow experiments

After culturing and seeding channels according to the protocols stated above, HUVECs were provided a period of 4 hours to allow for initial cellular attachment, after which a 5 mL Luer-Lok tip syringe (BD, USA) was connected to each microchannel PVC tubing using a 1.6 mm inner diameter connector (Cole-Parmer, Canada). Syringes were set up in the Nexus 3000 syringe pump (Chemyx, USA) to provide a constant flow rate to the microchannels for a period of 48 hours. Syringes were filled with 5ml of HUVEC media and a low flow rate of 0.1 ml/h was applied to the channels to reduce the likelihood of cell detachment [49]. Characteristics of HUVECs that underwent exposure to a constant flow rate were then compared to those that were maintained in static conditions.

2.4.2 3D fixed cell imaging

Prolong Gold mounting media (Thermofisher Scientific, USA) was injected into the channels to preserve the fluorescent dyes. Samples were imaged immediately or stored at 4 °C. Images were obtained using an inverted epi fluorescent Zeiss D1 AxioObserver microscope with a monochrome AxioCam MRm CCD detector and a 10x, 0.25 NA, Air, N-AchroPlan Plan objective. Images were captured at top, middle, and bottom focal planes of the circular microchannels to help indicate the level of cell coverage.

2.4.3 Sample preparation for gene expression microarray

To detect differences in gene expression of HUVECs grown in 2D and 3D environments, HUVECs were cultured according to protocols described previously. 2D substrates were prepared by coating an equal volume of liquid PDMS onto three 10-cm culture dishes and then curing at 100 °C for 10-15 minutes (2-5mm approximate PDMS thickness). 2D substrates were then treated with a 5 µg/ml solution of fibronectin for 1 hour at room temperature. 3D microchannel environments of approximately 565 µm in diameter (Table 8) were produced using the wire mold fabrication technique

outlined previously. Twelve chips, each containing four channels, were treated with a 5 µg/ml solution of fibronectin for 1 hour at room temperature. 2D substrates were seeded with 250,000 cells per dish, and 3D microchannels were seeded at a concentration of 1×10^6 cells/ml. For 3D microchannels, HUVECs were allowed to settle for 20 minutes prior to re-seeding of the HUVECs, which was repeated another three times. HUVECs were grown on 2D and 3D substrates for approximately 20 hours under predominantly static conditions, however media was exchanged at approximately 8 hours for both 2D and 3D substrates, to allow for prolonged culture in the microchannels. At this point, some cell wash out was noted in the microchannels. To lift cells from the surface after 20 hours, a 0.25% trypsin solution was added to the 10cm dishes for 2 min at 37 °C with 5% CO₂. After, the solution was diluted with fresh HUVEC media and the suspension was collected in a 50-ml tube and centrifuged at 200 g for 5 minutes, the cell pellet was re-suspended in fresh media. To lift the cells from the inner surface of the 3D microchannels, individual channels were flushed twice with alternating solutions of 0.25% trypsin solution and fresh HUVEC media. The solution was collected in a 50-ml tube and centrifuged at 200 g for 5 minutes; the cell pellet was re-suspended in fresh media. A 10-15 µl sample was taken for each collection to determine the cell concentration using the hemocytometer (Table 7). Cell solutions were transferred to 1.5ml eppendorf tubes and 300 µl of an RNA protecting solution was added to the sample to prevent deterioration of HUVEC RNA. Samples were sent to Genome Quebec (University of McGill), for RNA extraction and completion of a microarray to determine gene expression.

2.4.3.1 RNA protect solution preparation

The RNA protecting solution was made with the following reagents and quantities, according to protocols from literature [83], [84].

- 4ml of 0.5 M EDTA
- 2.5 ml of 1 M Sodium citrate
- 70 g Ammonium sulfate
- 93.5 ml H₂O

Reagents were mixed in a 500ml glass beaker on a hot plate at 100 °C, until dissolved. After the solution cooled, the pH was adjusted to 5.2 using H₂SO₄. Measurements of pH were completed using a glass pH electrode.

Table 7 Summary of sample sizes and conditions for microarray experiment

Condition	2D polystyrene dish	3D wire mold microchannel
Samples tested	3 dishes	48 channels
Approximate number of cells harvested/sample	~50,000	~20,000
Total samples collected	3	3
Microchannel inner surface treatment	FN	FN
Flow conditions	static with media change	static with media change

2.4.3.2 Microarray Analysis

RNA extraction and gene expression microarray procedures were completed by Genome Quebec at the University of McGill, Montreal, Quebec, Canada. Since sample RNA concentrations of four of the samples were below 35 ng/μL (Table 13), microarray analysis was conducted by using a Clariom-S Pico Assay (Human) kit (ThermoFisher Scientific, USA). Final microarray results were provided as .CEL files by Genome Quebec, and data was analyzed using the open access Transcriptome Analysis Console (TAC) software (ThermoFisher Scientific, USA).

Chapter 3

3 Results and Discussion

3.1 Microfabrication

Microchannels were made using various fabrication methods and the accuracy and precision of channel dimensions were assessed. After measurements were completed, dimensions were separated into larger lengths (major) and smaller lengths (minor); descriptive statistics for the major and minor dimensions are depicted graphically in box plots (Figure 25-Figure 27). The accuracy of each fabrication method in producing a perfect square or circular cross-section was assessed using aspect ratio and circularity shape factors. The wire molding technique produced channels with significantly higher aspect ratios compared to those obtained from the gas stream fabrication technique (Figure 28).

3.1.1 Rectangular microchannels

For microchannels with a rectangular cross-section, the mean length obtained from all measurements (whether major or minor) was determined to be $L_{avg} = 113.8 \mu\text{m} \pm 1$ (SEM), $N=24$. According to the MicroChem protocol that was followed and the wafer design that was printed, the theoretical width and depth of the microchannel is $100 \mu\text{m}$. This indicates that through the standard photolithography process to create a microchannel with a rectangular cross-section, there was a percent error of approximately 13.8%.

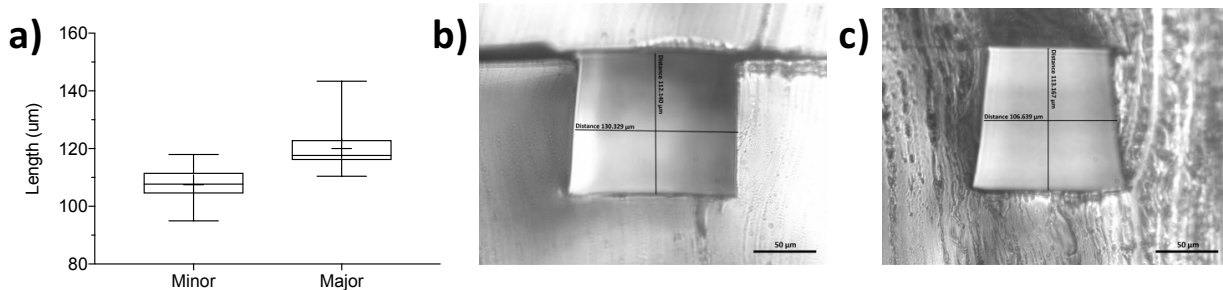


Figure 25 Box plot of rectangular channel dimensions measured along major and minor axes. Box plots represent the 25th-75th percentiles (box), median (long horizontal line within box), mean (crosshairs), and 1.5 times the interquartile range (whiskers) (a). Brightfield images of two PDMS microchannel cross-sections obtained from two channels from different silicon wafers (b,c), scale bar 50μm

3.1.2 Circular microchannels

Circular microchannels were produced using two different fabrication methods (gas stream and wire molding). As with rectangular microchannels, cross-sections of circular microchannels were measured in both x and y directions, and major and minor diameters were determined.

3.1.2.1 Gas Stream

Diameters measured from circular channels produced by gas stream fabrication are represented in box plots (Figure 26). The mean diameter obtained using the gas stream fabrication method was $D_{avg}=103.8 \mu\text{m} \pm 3$ (SEM), $N=10$.

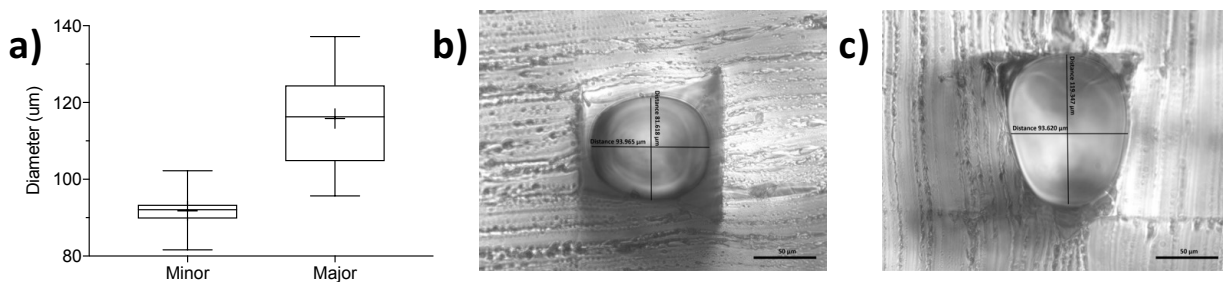


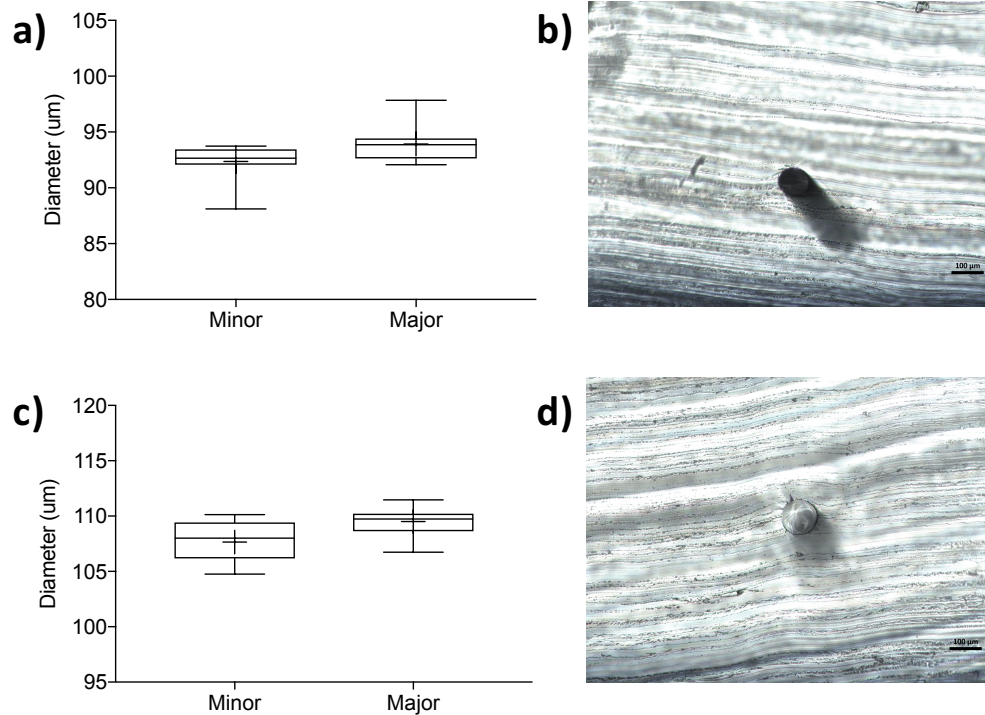
Figure 26 Box plot of gas stream circular channel dimensions measured along major and minor axes. Box plots represent the 25th-75th percentiles (box), median (long horizontal line within box), mean (crosshairs), and 1.5 times the interquartile range (whiskers) (a). Brightfield images of two different PDMS microchannel cross-sections obtained from the gas stream technique (b,c), scale bar 50μm.

3.1.2.2 Wire molding

Varying wire dimensions were used to produce microchannels with circular cross-sections using the wire mold fabrication method. The diameters of the cross-sections were measured, and the total mean diameters for each sample set are presented in Table 8, along with the sample size used for wire measurements, and the percent errors calculated between the theoretical and experimental values. Boxplots representing major and minor dimensions for the wire-molded channels are presented in Figure 27.

Table 8 Experimental versus theoretical wire diameters corresponding to Figure 27

Theoretical Wire Diameter (μm)	Mean Channel Diameter Measurement (μm)	Percent Error (%)	Sample size (N)
79.9	$93.1 \mu\text{m} \pm 0.4$ (SEM)	16.5	11
100.7	$108.6 \mu\text{m} \pm 0.4$ (SEM)	7.8	12
201.9	$220.0 \mu\text{m} \pm 0.5$ (SEM)	8.9	10
404.9	$446.3 \mu\text{m} \pm 1$ (SEM)	10.2	10
510.6	$565.4 \mu\text{m} \pm 1$ (SEM)	10.7	9



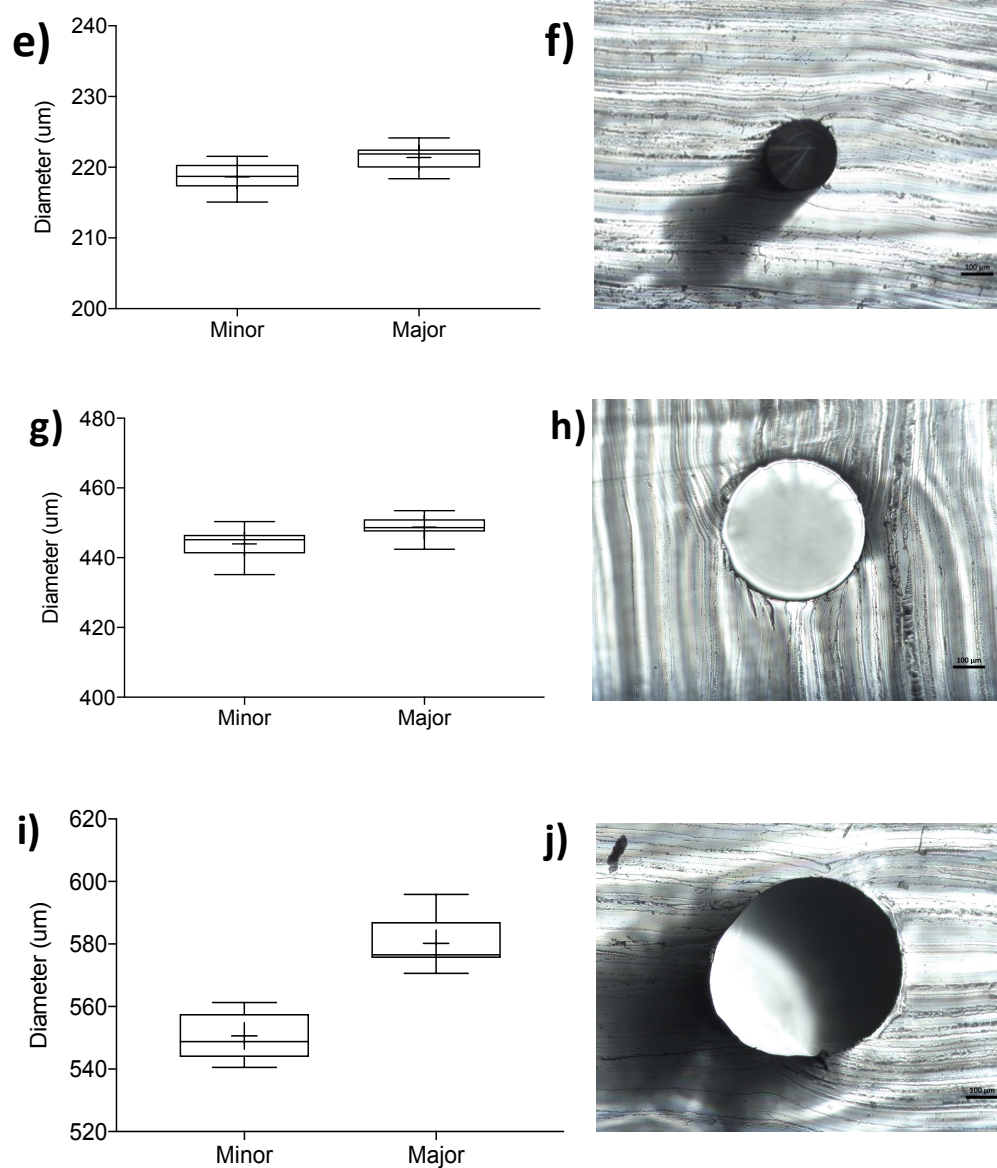


Figure 27 Box plots of wire mold circular channel dimensions measured along major and minor axes. Box plots represent the 25th-75th percentiles (box), median (long horizontal line within box), mean (crosshairs), and 1.5 times the interquartile range (whiskers) (left), averages presented in Table 8 (a,c,e,g,i), brightfield cross-section of PDMS microchannel (right), scale bar 100μm (b,d,f,h,j). Each row corresponds to wire mold circular channels made using wires of a different diameter: 79.9 μm (a,b); 100.7 μm (c,d), 201.9 μm (e,f), 404.9 μm (g,h), 510.6 μm (i,j).

Fabricated rectangular microchannels resulted in a mean aspect ratio of 0.90 ± 0.01 (SEM), N=24. The wire-molding microfabrication method produced circular microchannels with a mean aspect ratio of 0.98 ± 0.003 (SEM), N=52, which was significantly greater than that of the gas stream fabrication method (0.80 ± 0.03 (SEM), N= 10) (Figure 28). Mean circularity values were found to be 0.77 ± 0.006 (SEM), N=24

for the rectangular microchannels, 0.93 ± 0.01 (SEM), $N=10$ for gas-stream microchannels, and 0.99 ± 0.0007 (SEM), $N=52$ for wire-molding microchannels. When comparing the gas-stream and wire-molding fabrication methods, microchannel cross-sectional geometry produced from the wire-molding method had a significantly greater mean circularity (Figure 29).

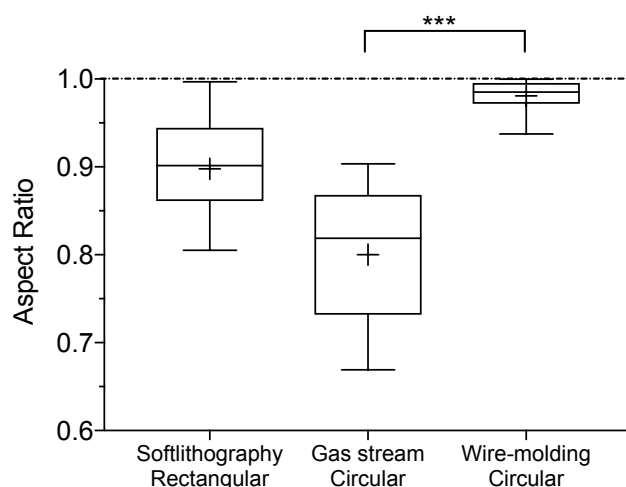


Figure 28 Box plot of aspect ratios for microchannels produced using each fabrication method. Box plots represent the 25th-75th percentiles (box), median (long horizontal line within box), mean (crosshairs), and 1.5 times the interquartile range (whiskers). $N=24$ for rectangular channels, $N=10$ for gas stream circular channels, and $N=52$ for wire-molding circular channels. Comparison between gas stream and wire-molding circular channels made using Welch's t-Test for unequal variance; *** $p<0.001$

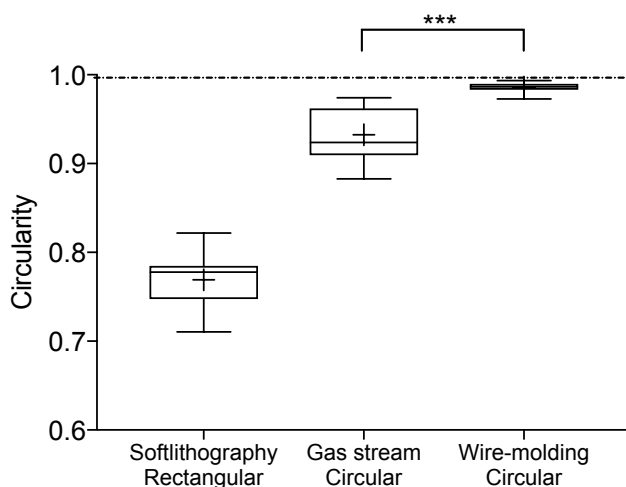


Figure 29 Box plot of circularity for microchannels produced using each fabrication method. Box plots represent the 25th-75th percentiles (box), median (long horizontal line within box), mean (crosshairs), and 1.5 times the interquartile range (whiskers). $N=24$ for rectangular channels, $N=10$ for gas stream circular channels, and $N=52$ for wire-molding circular channels. Comparison between gas stream and wire-molding circular channels made using Welch's t-Test for unequal variance; *** $p<0.001$

3.2 Comparison of microfabrication methods

Relative to the gas-stream technique, the wire molding technique was found to be a faster and simpler microfabrication method. We found that the wire-molding microfabrication method produced microchannels with aspect ratios that were both closer to 1 and had better precision than aspect ratios of channels produced by the gas stream method (Figure 28). For microchannels produced with a 24-gauge wire, which were ultimately selected for the majority of cell culturing experiments, the percent error between the theoretical and mean experimental wire diameter was 10.7% (Table 8). This percent error may have been influenced by the tolerance of the wire, which could affect the degree to which microchannel diameters matched the theoretical wire size. The use of wires manufactured with a smaller tolerance may minimize percent error in future studies.

The diameter of gas-stream channels depends on several factors, including the air stream pressure, the time and temperature used for PDMS curing, and the dimensions of the rectangular cross-section microchannel from which the gas-stream channel was made. Channel depth is determined by the photoresist height obtained from spin coating, which remains constant within one wafer. Therefore, to develop gas-stream microchannels with a large range of dimensions, multiple silicon wafers and different chemical photoresist types (needed to achieve certain aspect ratios) would be required.

The aspect ratios obtained from the circular microchannels produced using the gas stream method were highly dependent on the initial geometry of the rectangular microchannels that they were created from. Using techniques to create perfectly square cross-sections (aspect ratio of 1 and circularity of 0.785) would result in an increased likelihood of obtaining circular microchannels from the gas stream method with higher aspect ratios and circularity [50]. Reducing light scattering in the photolithography microfabrication process would have assisted in creating rectangular channels with more perpendicular

walls, and improved symmetry. Further reducing light scattering could be accomplished by using a high-quality collimator in the optical path length to ensure that the UV light arrived in parallel rays, vertical to the photoresist surface. Additionally, using a vacuum to create greater contact between the photomask and the wafer, eliminating the air gap, would have significantly minimized light scattering [85]. Future investigation of the parameters influencing microchannel cross-sectional geometry would aid further optimizing the gas-stream method.

Compared to gas-stream channels, the factors influencing wire-mold channel parameters are far more straightforward as they depend only on the diameter and geometry of commercially available microwires. The technique of submersing a wire in PDMS and removing it when the PDMS has cured allows for the creation of a simple microfluidic layout with great ease. For the purposes of this study, a simple straight microchannel was the desired result. Given the higher aspect ratios, circularity, and higher precision of channel geometry obtained using the wire-mold method (Figure 28 and Figure 29), this method for creating circular microchannels was selected for our application. For microfluidic systems with greater complexity, the gas-stream technique may prove to be more useful. Abdelgawad *et al.* showed how air streams can conform to changing geometries [50], allowing for the creation of branching networks of circular microchannels using the gas-stream method.

3.3 Substrate surface characterization

3.3.1 PDMS surface functionalization

Contact angle measurements were recorded for untreated PDMS, PDMS_{PD} and PDMS_{FN}. Average contact angle measurements are displayed in Table 9. Representative images for each substrate are shown in Figure 30.

Table 9 Mean contact angle of water on PDMS and PDMS modified substrates, N = 3 per condition

θ_{H_2O} (deg) $\pm$ SEM	Surface		
	PDMS	PDMS _{PD}	PDMS _{FN}
	119.2 $\pm$ 0.3	18.8 $\pm$ 6 ***	107.2 $\pm$ 1

A One-way ANOVA with post hoc Tukey's multiple comparisons test was used to compare the contact angle measurement obtained on different substrates. A significant difference (***) $p < 0.001$ was observed between PDMS_{PD} and PDMS, as well as PDMS_{PD} and PDMS_{FN}, N= 3 for each condition (Table 9). Multiple measurements were taken per sample.

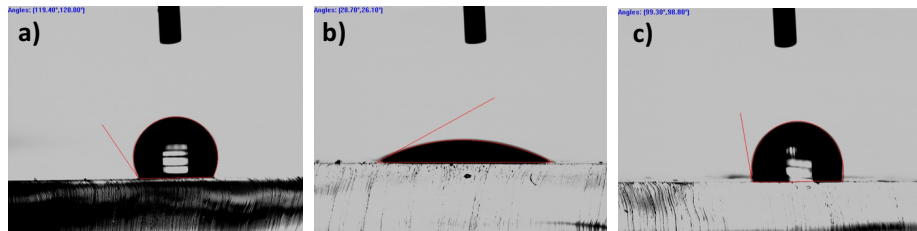


Figure 30 Water contact angles on a) untreated PDMS, b) PDMS_{PD}, c) PDMS_{FN}

3.3.2 Collagen substrates

Contact angle measurements were carried out for the Type 1 collagen gel substrate (referred to as Collagen). Contact angles on the collagen substrate were dynamic, and observed change was recorded over a period of approximately 4 minutes from the initial drop deposition. N=1, however, multiple measurements were recorded at each time point (Table 10, Figure 31).

Table 10 Mean contact angle of water on Collagen gel over time, N=1, intra experimental variability indicated by SEM

	Surface		
	Collagen		
	0 sec	40 sec	4 minutes
$\theta_{\text{H}_2\text{O}}$ on collagen(deg) $\pm$ SEM	40.8 ± 3	17.4 ± 1	17.3 ± 1

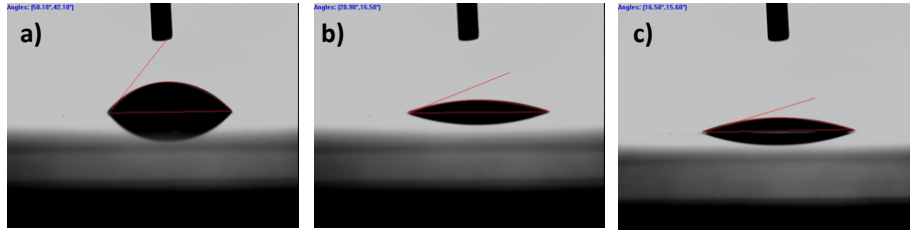


Figure 31 Water contact angles on Collagen a) initial contact, b) 40 seconds after contact, c) 4 minutes after contact

3.4 Evaluation of substrate surface characterization

Surface wettability can be an important factor used in assessing surfaces that are favorable for cell adhesion [5], [86]. Substrate surface characterization allowed the tested substrates to be quantitatively compared to literature values, allowing conclusions to be drawn about the effect of surface wettability on HUVEC adhesion and growth in this study. Surface wettability of each substrate was characterized by measuring the water contact angles. Both untreated PDMS ($119.2^\circ \pm 0.3$) and PDMS_{FN} ($107.2^\circ \pm 1$) surfaces exhibited hydrophobic surface properties ($\theta > 90^\circ$) and therefore low wettability, whereas, PDMS_{PD} ($18.8^\circ \pm 6$) has a significantly smaller mean water contact angle ($\theta < 90^\circ$), corresponding to a higher wettability [87], when compared to the other substrates tested (Table 9). Measured water contact angles for untreated PDMS were similar to literature values [70],[48], [88], [86]. Smaller water contact angles were reported on PDMS treated with FN and PD. Wang *et al.* observed a 72° contact angle for a PDMS sample treated with a $50 \mu\text{g}/\text{ml}$ concentration of fibronectin [86]. This fibronectin concentration was 10-fold greater than the concentration used in our study, likely contributing to the notably smaller contact angle these authors observed for PDMS-FN, relative to that observed in our

study. The larger surface hydrophobicity observed on our fibronectin surfaces relative to the literature values could also be an indication of incomplete protein coverage obtained on our substrates. Water contact angles on the collagen substrate observed in our study were variable, we noted a reduction in contact angle over a period of 4 minutes. This was a representative experiment, as there was a sample size of one (with replicate measurements) for the collagen condition. Upon initial deposition, the collagen gel exhibited hydrophilic properties ($40.8^{\circ} \pm 3$) and the mean angle decreased to $17.4^{\circ} \pm 1$ after 40 seconds, before becoming relatively constant at $17.3^{\circ} \pm 1$ after 4 minutes (Table 10), indicating a highly wettable surface. Upon contact with water collagen is known to swell [89], thus water may have absorbed into the collagen gel causing a further reduction in contact angle.

Cell behavior including proliferation, cellular adhesion, migration and cell pattern is directed by surface wettability [86], [5]. A study completed by van Wachem *et al.* showed that moderately wettable polymers allowed for optimal adhesion of human endothelial cells, while polymers with extreme hydrophobicity or hydrophilicity showed reduced cellular adhesion [90].

Several studies suggest that the inherent hydrophobicity of PDMS is one of the major factors that causes poor cellular adhesion on its surface [91],[92]. Chuah *et al.* showed that for a 24-hour deposition period, contact angle decreased with increasing PD concentration [93]. Interestingly, the authors found that PD coating of PDMS for 1-8 hours resulted in surface hydrophobicity similar to the untreated PDMS, yet still promoted Bone Marrow Stromal Cell (BMSC) adhesion and proliferation. As a result, the authors concluded that surface wettability may not play a critical role in influencing BMSC behavior [93]. This is consistent with what was demonstrated in our study: no significant difference was detected between the mean water contact angles measured on the untreated PDMS and PDMS-FN surface, yet PDMS-FN showed a high degree of biocompatibility (Figure 32-Figure 36). The presence of cytotoxic compounds released from the untreated PDMS [53] [94], and/or the

absence of proteins that promote cell survival [95] [31] may have played a larger role than low surface wettability in the poor biocompatibility of PDMS that was observed.

3.5 Substrate biocompatibility

Two-dimensional HUVEC culture was used to assess the cellular response to the following substrates: untreated PDMS, PDMS_{FN}, PDMS_{PD}, a collagen substrate (Collagen), and a collagen substrate with fibronectin functionalization (Collagen_{FN}). Cell seeding efficiency, cell proliferation, cell spreading, and cell motility were examined.

3.5.1 Cell seeding efficiency and early proliferation

Cell seeding efficiency was determined from live cell phase contrast imaging, by counting the number of HUVECs at 10-15 different locations per sample at 10-minute and 12-hour time intervals. The field of view for each imaged location using live imaging was 895.26 μm x 670.80 μm . Mean cell densities for each time-point and substrate are displayed in Figure 32.

Statistical analysis was not conducted for live imaging experiments as imaging was performed on a single sample of each substrate. However, means and SEMs could be calculated from cell densities observed at different locations on the same substrate sample. Initial theoretical cell seeding density for live experiments was calculated to be 20.8 cells/ mm^2 for each substrate (Appendix). Slight variation was observed between substrates for mean HUVEC density at 10 minutes post-seeding. The untreated PDMS substrate displayed a decrease in cell density at 12 hours relative to its 10 minutes post-seeding time point, whereas all other groups showed an increase in mean density after 12 hours incubation. The magnitude of the mean cell density at 12 hours was much smaller on native PDMS, compared to all other substrates. At 12 hours post-seeding, the greatest HUVEC density was observed on the PDMS_{FN} surface (Figure 32).

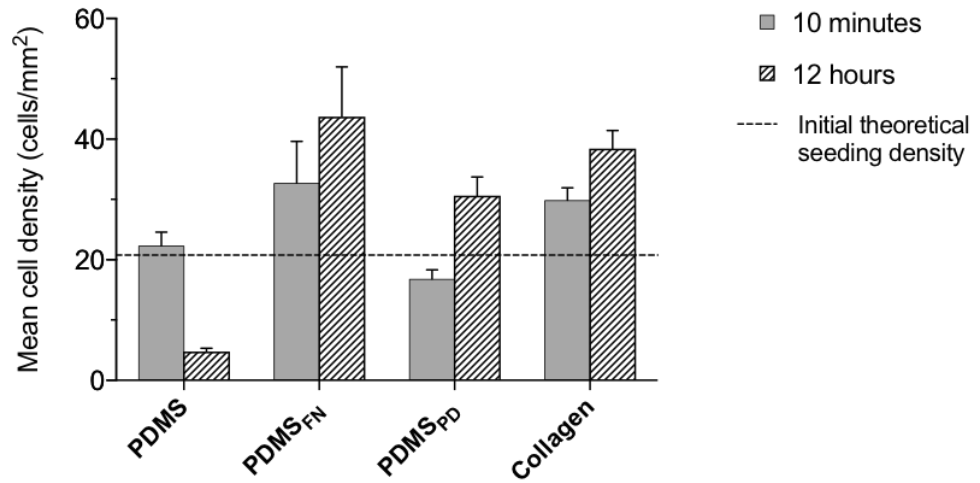


Figure 32 Cell seeding efficiency and cell proliferation. Mean $\pm$ SEM cell density (cells/mm²) from live imaging of 2D substrates at 10 minutes and 12 hours after seeding. N=1 for each substrate, with the same 10-15 locations imaged per sample at each time point of the experiment. Intra experimental variability is indicated by SEM error bars for this representative experiment. Dashed line indicates theoretical seeding density of 20.8 cells/mm².

3.5.2 Cell proliferation

Nuclei counts from fixed cell fluorescence experiments were used to estimate cellular proliferation on each 2D substrate. Initially, 20,000 cells per well were seeded on the substrate. For each substrate, multiple samples were assessed. Cells were grown for 48 hours prior to fixation, and mean HUVEC cell density was determined based on nuclei counts from 9 imaging locations on each sample (Figure 33). The field of view for each imaged location using fixed imaging was 684.25 μm x 512.69 μm .

After 48 hours, a significantly greater HUVEC density was observed on PDMS_{FN}, PDMS_{PD}, Collagen and Collagen_{FN} when compared to the native, untreated PDMS substrate. No other significant differences were noted between groups (Figure 33).

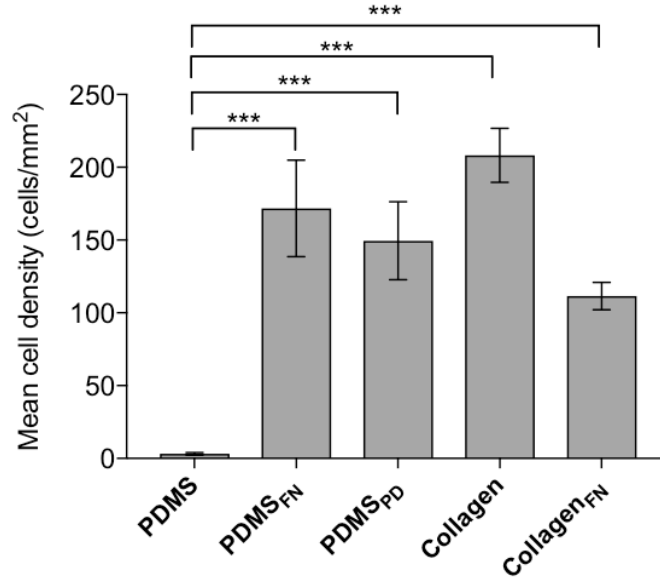


Figure 33 Cell proliferation. Mean $\pm$ SEM HUVEC densities (cells/mm²) from fixed imaging of 2D substrates after 48 hours of growth. PDMS N=20, PDMS_{FN} N=20, PDMS_{PD} N=19, Collagen N=27, Collagen_{FN} N=26. For each sample, 9 locations were imaged. Comparisons were made using a Kruskal-Wallis test with Dunn's post hoc multiple comparisons tests. ***p < 0.001

3.5.3 Cell spreading

Cell spreading was assessed by determining the percent-area coverage on each 2D substrate. Percent-area coverage was assessed both by live imaging (at 10-minutes, 4-hours, and 12-hours after seeding, Figure 34), and by fixed imaging (at 48 hours after seeding, Figure 35 and Figure 36). In both live and fixed imaging experiments, 20 000 cells per well/dish were initially seeded.

Live cell imaging revealed a consistently low percent area cell coverage over 12 hours on untreated PDMS. In contrast, there was an observed increase in the percent area coverage of cells on the PDMS_{PD} and PDMS_{FN} substrates over 12 hours. At 12 hours, cell area coverage was greatest on PDMS_{FN} compared to PDMS_{PD} or untreated PDMS (Figure 34).

A statistically significant decrease in percent area coverage of cells was observed on untreated PDMS as compared to PDMS_{FN} and PDMS_{PD} substrates in fixed imaging (Figure 35). A significantly greater percent cell area coverage on PDMS_{FN} relative to Collagen_{FN} was found. The general trend showed

the percent area coverage of HUVECs on PDMS_{FN} to be greater than all other substrate types from 48 hour fixed imaging cell spreading experiments. Percent area coverage on Collagen and Collagen_{FN} was slightly lower than that on PDMS_{PD} (Figure 35). Qualitatively, cells cultured on collagen substrates appeared to have reduced spreading, particularly at the sample edges, compared to cells cultured on PDMS_{FN} and PDMS_{PD} (Figure 36).

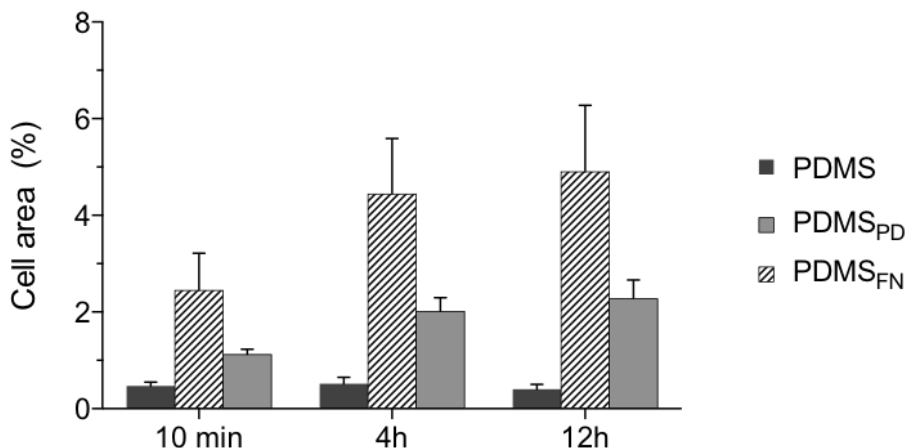


Figure 34 Cell spreading. Mean $\pm$ SEM cell percent-area coverage (%) from live imaging of 2D substrates at 10 minutes, 4 hours and 12 hours after seeding. N=1 for each substrate, with the same 10-15 locations imaged per substrate at each time point of the experiment. Intra experimental variability is indicated by SEM error bars for this representative experiment.

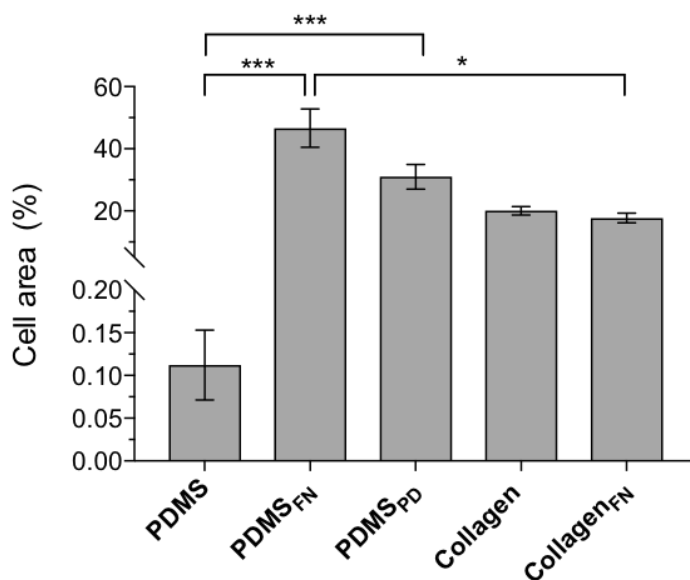


Figure 35 Cell spreading. Mean $\pm$ SEM cell percent-area coverage (%) from fixed imaging of 2D substrates at 48 hours after seeding. N=8 for each substrate and 9 locations were imaged on each sample. Comparisons were made using a Kruskal-Wallis test with Dunn's post hoc multiple comparisons tests. * $p < 0.05$ and *** $p < 0.001$.

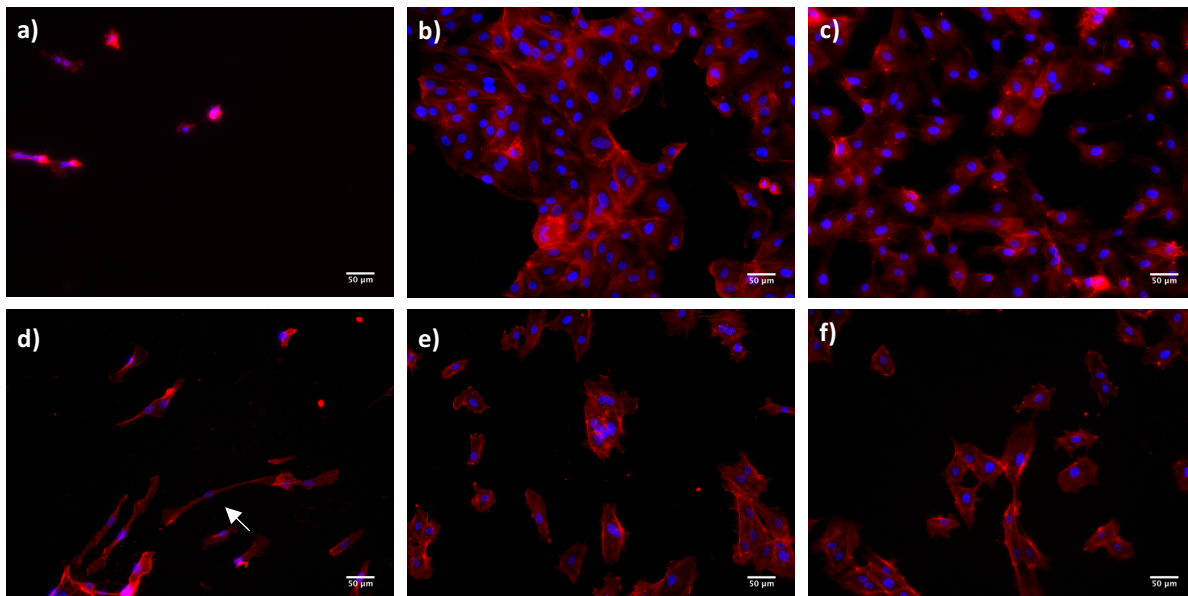


Figure 36 Fluorescent images of fixed HUVECs at 48 hours after seeding on a) untreated PDMS, b) PDMS_{FN}, c) PDMS_{PD}, d) Collagen at sample edge, e) Collagen, f) Collagen_{FN}. Elongated cells (white arrow). Scale bar 50 µm.

3.5.4 Cell motility

Cell motility was assessed using live imaging of cells on PDMS, Collagen, PDMS_{FN}, and PDMS_{PD} substrates. Motility was assessed on one sample for each condition. For each sample, 10-15 images arranged in a grid on the sample center, were taken every 10 minutes for 12 hours (Table 6). The cumulative data allowed 53 cells to be tracked on each test-substrate (PDMS_{FN}, PDMS_{PD}, and Collagen) over a period of 12 hours. Only 24 cells on PDMS could be tracked over a 12-hour period given the reduced viability of cells on this substrate. The x- and y-displacements of each tracked cell are shown in migration plots (Figure 37). Directness values were low for all samples (Table 11). Mean accumulated distances for each substrate were calculated by averaging the total distance traveled by each cell on a particular substrate. There was a significantly greater mean accumulated distance travelled on PDMS_{PD} when compared to untreated PDMS, PDMS_{FN} and collagen substrates (Figure 39). All conditions showed significantly greater mean accumulated distance travelled when compared to untreated PDMS (Figure 39). Mean cell velocity on each substrate was calculated by averaging the velocity of each cell (displacement/time) obtained from the Chemotaxis and Migration Tool (Figure

38). On PDMS_{PD}, mean cell velocity was significantly higher compared to that on the PDMS_{FN} and Collagen samples. Mean cell velocity was greatest on untreated PDMS compared to all other samples, however this was not statistically significant (Figure 38). Live images of HUVECs on different substrates at 10 minutes, 4-hours, and 12-hours post-seeding are shown (Figure 40). Filopodia - finger-like protrusions sprouting from the body of adhering cells [18] - are visible protruding from cells on PDMS_{FN} and PDMS_{PD}.

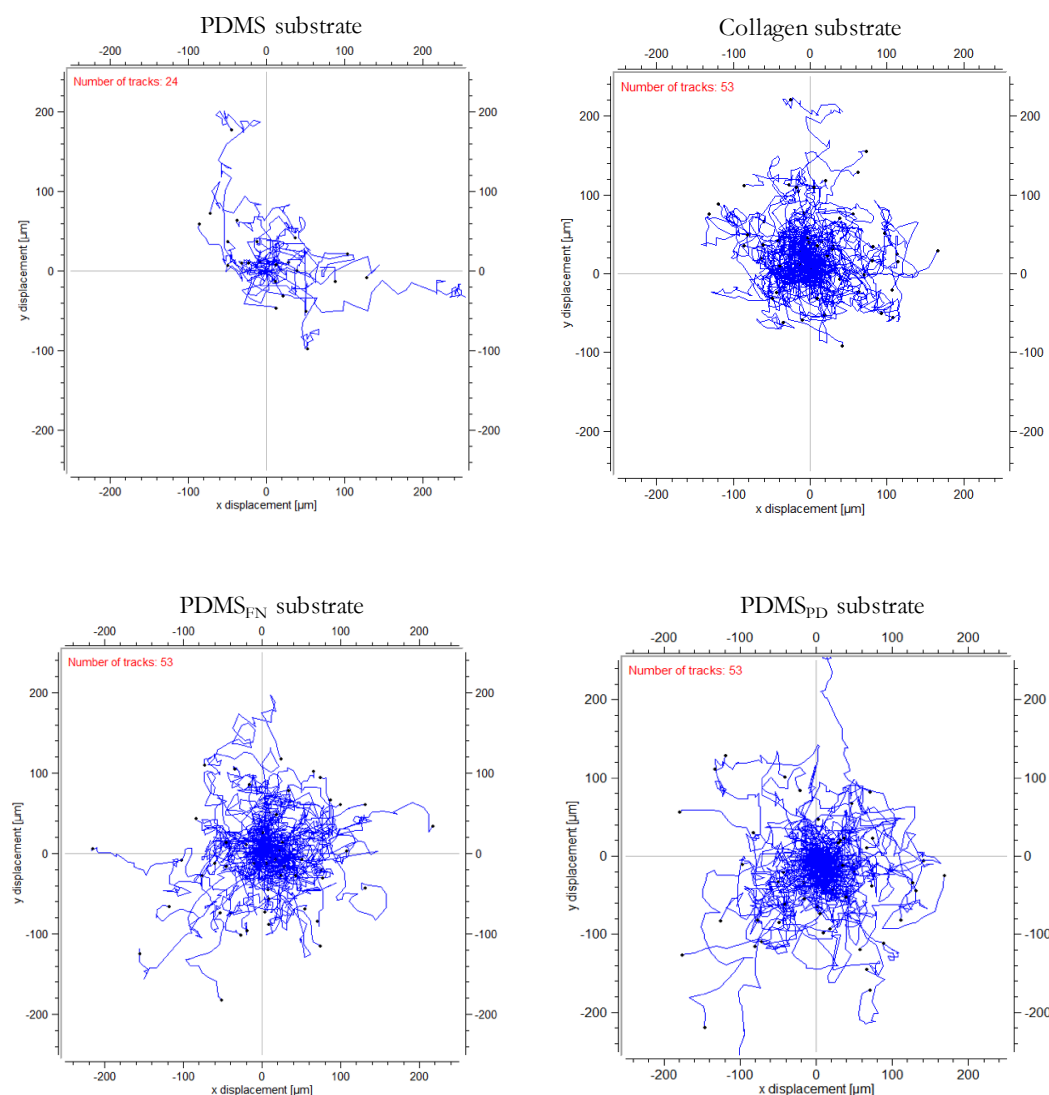


Figure 37 Migration plots showing distances travelled by HUVECs on 2D substrates over a 12 hour period, created using the ibidi Chemotaxis and Migration Tool. Images were captured every 10 minutes for 12 hours. Individual cell migration was tracked on PDMS, PDMS_{FN}, PDMS_{PD} and Collagen substrates. N=53 cells for Collagen, PDMS_{FN}, and PDMS_{PD}. N=24 cells for PDMS.

Table 11 Directness values from the ibidi Chemotaxis and Migration Tool, for cells tracked live over 12 hours.

Sample	PDMS	PDMS _{FN}	PDMS _{PD}	Collagen
Directness (D)	0.3	0.2	0.2	0.2
Sample size (N)	24	53	53	53

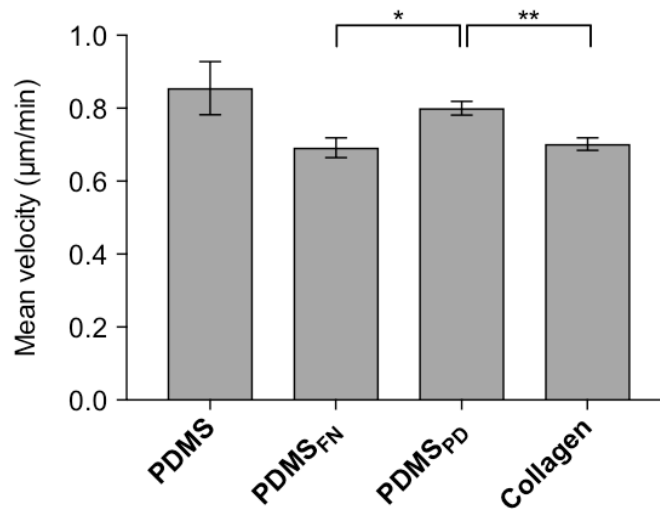


Figure 38 Mean velocity values $\pm$ SEM obtained from the ibidi Chemotaxis and Migration Tool, for HUVECs tracked live over 12 hours. A Kruskal-Wallis test with post hoc Dunn's multiple comparisons test was used to compare mean velocity data. * $p < 0.05$ and ** $p < 0.01$

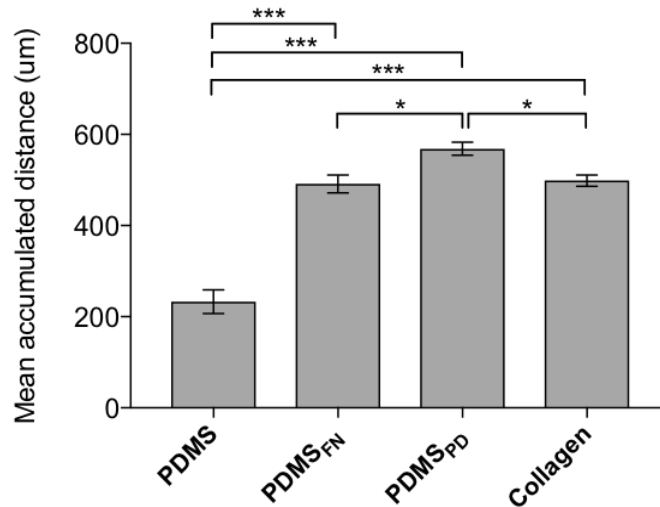


Figure 39 Mean accumulated distance $\pm$ SEM travelled by HUVECs on 2D substrates obtained from the ibidi Chemotaxis and Migration Tool. N=53 cells for Collagen, PDMS_{FN}, and PDMS_{PD}. N=24 cells for PDMS. A Kruskal-Wallis test with post hoc Dunn's multiple comparisons test was used to compare mean accumulated distance data. * $p < 0.05$ and *** $p < 0.001$.

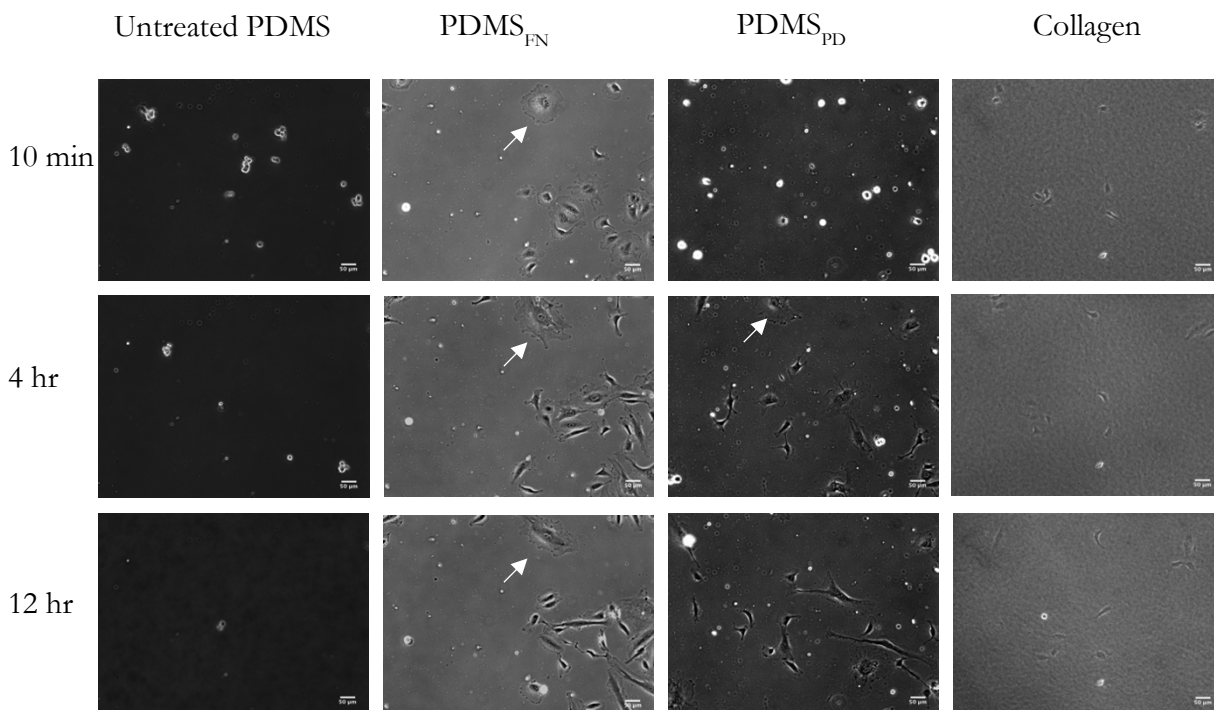


Figure 40 Phase contrast images of live HUVECs on several 2D substrates at 10 minutes, 4 hour and 12 hour intervals. Filopodia protrusions (white arrows). Scale bar 50um.

3.6 Cellular response to substrates

Cellular response on 2D substrates was assessed by quantifying cell seeding efficiency, cell proliferation, migration and cell area coverage. Both live cell and fixed cell imaging techniques were used. HUVEC density at 10 minutes post-seeding varied slightly between groups, but overall, was similar to the theoretical cell seeding density of 20.8 cells/mm² (Figure 32). These findings indicate a reasonable level of accuracy for the hemocytometer cell counting process used to determine cell density and seed a predetermined number of cells onto each substrate. While untreated PDMS showed a diminished cell density at 12 hours as compared to at 10 minutes post-seeding, all other substrates showed an increase in cell density over the 12-hour time period, suggesting good biocompatibility for all treated substrates (Figure 32). Our findings are similar to those of Chuah *et al.*, who demonstrated poor biocompatibility for untreated PDMS, but improved biocompatibility for PDMS treated with

PD when culturing bone marrow stromal cells [93]. Enhanced cellular response has also been shown for FN treated PDMS, when compared to native PDMS surfaces [86]. Although HUVEC density at 12 hours was observed to be greatest on PDMS_{FN} compared to other substrates, differences between 12-hour cell densities on the substrates were not tested for significance because the sample size was one. The trend toward higher cell density on PDMS-FN at 12-hours may have been due to the slightly higher cell density on PDMS-FN, relative to other substrates, at 10 minutes post-seeding (Figure 32). Although such differences in initial cell density may represent variability in the experimental setup, it is possible that the higher cell density on PDMS-FN at 10 minutes post-seeding reflects a role for the fibronectin coating of PDMS in promoting more rapid initial cell attachment. Kuddannaya *et al.* showed that adherent cell density after 90 min of initial Mesenchymal Stem Cell (MSC) seeding was significantly greater on PDMS-FN surfaces when compared to PDMS surfaces treated with type I collagen [96].

Biocompatibility trends from the live imaging correspond with the cell density results obtained from fixed cell imaging experiments, where a significantly greater mean HUVEC density was observed after 48 hours on PDMS_{FN}, PDMS_{PD}, Collagen and Collagen_{FN} when compared to untreated PDMS (Figure 33). Colombo *et al.* showed that for Endothelial Colony-Forming Cells (ECFCs) type I collagen coated polystyrene allowed for higher cell proliferation and longer cell survival, compared to fibronectin [79]. Similarly, we observed HUVEC density was greater on the collagen substrate compared to the other tested substrates, although the difference between cell density on collagen and PDMS_{FN} was not statistically significant. Interestingly, HUVEC density was greater on the collagen and PDMS-FN substrates compared to Collagen-FN (Figure 33). This finding is in contrast with several previous studies. Sgarioto *et al.* reported that, relative to FN alone, both collagen and collagen+FN coated polystyrene improved rates of HUVEC proliferation and migration, with a collagen+FN coating promoting further extracellular matrix remodeling [31]. Similarly, Cooper *et al.* found that for type I

collagen modules coated with FN, seeded with HUVECs, and then implanted into mice, there was increased long-term HUVEC survival, compared to collagen modules without FN [32]. Direct adsorption of a protein from a solution onto a surface can potentially result in protein denaturation[13]. It is possible that this was a contributing factor in the observed reduction of HUVEC density on the Collagen-FN surface. Unlike Sgarioto *et al.*, we used a high concentration collagen gel coated with FN, as opposed to lower concentration collagen coatings on a polystyrene surface. It is possible that differences in the methods used and relative compositions of collagen gel and FN, may have resulted in the reduced biocompatibility of collagen-FN that was observed in our study. Furthermore, Cooper *et al.* examined cell growth over a period of 21 days *in vivo*; it is possible that differences in study length contributed to the discrepancy between these findings and the observations of our study. Since the initial cell densities could not be accurately measured in fixed cell imaging experiments, it is possible that variability in cell seeding could influence the cell densities observed on the different substrates at 48 hours. However, the moderately large sample sizes used in fixed-imaging experiments (Table 4) help to make these findings more robust against initial variability.

Cell spreading was assessed by measuring the percent of total view area covered by cells in both live imaging (over a period of 12 hours) and fixed imaging (at 48 hours post-seeding). Live cell images showed a decrease in percent area cell coverage on untreated PDMS substrates over 12 hours, and lower percent area coverage on native PDMS compared to PDMS-FN and PDMS-PD (Figure 34). Similarly, fixed imaging revealed reduced cell area coverage on untreated PDMS when compared to all other tested substrates. In particular, the cell area coverage was significantly decreased for untreated PDMS relative to PDMS-FN and PDMS-PD substrates (Figure 35). These results were consistent with the cell proliferation data. Unlike healthy HUVECs, which appear flattened and spread out, HUVECs undergoing cell death may take on a more spherical geometry [97], losing their ability to adhere to surfaces and allowing the cells to be washed away. Thus, the reduced cell area coverage on

untreated PDMS, and the qualitatively observed balled-up appearance (Figure 36.a) is indicative of cell death, further supporting the finding of low biocompatibility for this substrate. In contrast, live imaging experiments showed an overall increase in the percent area coverage of cells on the PDMS-PD and PDMS-FN substrates over 12 hours. At 12 hours, cell area coverage on PDMS-FN was greater than both area coverage on PDMS-PD and on untreated PDMS. Although this difference may have been influenced by initial percent coverage, which was slightly greater for PDMS-FN than for PDMS-PD at 10-minutes post-seeding (Figure 34), the high initial percent area coverage for PDMS-FN, like the high initial cell density, may have biological significance. Area coverage of cells on collagen substrates could not be quantified by live phase contrast imaging because the low image contrast made it difficult to differentiate the cell from the background substrate (Figure 40). Fixed imaging at 48 hours showed greater percent area coverage of HUVECs on PDMS-FN relative to all other substrate types, and significantly greater percent area coverage when compared to the Collagen-FN substrates (Figure 35). The low stiffness of the collagen substrate may have contributed to the qualitative observation of elongated HUVECs with reduced spreading (Figure 36.d) at several locations on the edges of the collagen gel sample; similar findings have been reported in literature [98].

Migration patterns were assessed on PDMS, PDMS_{FN}, PDMS_{PD} and collagen substrates. As cell proliferation and area coverage on Collagen_{FN} was already demonstrated to be lower than that on the other treated substrates, cell migration on Collagen_{FN} was not assessed. On all four substrates, Directness values were low, indicating that cells moved randomly (Table 11). This finding is consistent with previous observations of random, non-directional movements of cells on homogenous 2D surfaces in the absence of physicochemical gradients, or flow [99]. As with our assessments of cell proliferation and spreading, fewer cells could be tracked on the untreated PDMS due to cell death and wash out, compared to the other surfaces. Furthermore, the mean accumulated distance travelled by the HUVECs on untreated PDMS was significantly lower than that on other substrates, which is

consistent with the low biocompatibility of untreated PDMS. Polydopamine-functionalized PDMS appeared to significantly promote cell motility compared to the other substrates (Figure 39). This finding is similar to a previous study, which found that the combination of PD functionalized titanium dioxide nanotube surfaces demonstrated synergistic effects in drastically increasing EC mobility [36].

Mean cell velocity on PDMS-PD was significantly greater than on PDMS-FN and Collagen substrates (Figure 38). The relatively higher velocity on the PDMS substrate, although not statistically significant, may be an indication of the HUVECs inability to form strong adhesions, leading to observed erratic movement and eventual cell wash away on this substrate. HUVECs on PDMS-PD had a significantly a greater degree of proliferation and spreading compared to untreated PDMS (Figure 35 and Figure 36). Therefore, unlike with PDMS, the high mean velocity observed on the PDMS-PD may represent the ability of polydopamine to provide chemical stimuli to promote HUVECs migration [36]. Polydopamine is foreign to HUVECs compared to fibronectin and collagen, which are derived from the natural environment of the ECM. The reduced similarity to the natural environment could be influencing cell signaling and cell behaviours. Although high cell motility on PDMS-PD may be useful for encouraging HUVECs migration in wound-healing applications, increased cell movement may not be optimal to obtain coverage within a microchannel.

While all treated substrates showed relatively higher biocompatibility compared to untreated PDMS, live and fixed cell imaging indicated that the collagen substrate and PDMS-FN were most amenable to cellular adhesion and proliferation. Furthermore, HUVECs cultured on PDMS-FN covered a greater percent area than HUVECs cultured on any other substrate tested in this study (Figure 34 and Figure 35). Finally, PDMS-PD was found to promote HUVEC migration significantly more than untreated PDMS, PDMS-FN and Collagen, and at a significantly greater mean velocity when compared to PDMS-FN and Collagen (Figure 38 and Figure 39). While increased cellular adhesion,

proliferation, and spreading are desirable characteristics for cell growth in microchannels, increased cell migration may reduce the possibility of obtaining a confluent monolayer of cells within a microchannel. Therefore, our findings suggest that of the materials tested, either the collagen or PDMS-FN substrate would be most suitable for our subsequent study of cell growth in microchannels. Two main factors were considered in determining the final material selection. First, greater cell area coverage was observed on PDMS-FN as compared to collagen substrates (Figure 35), favoring the former for use in microchannels. Second, the collagen substrate presented a greater challenge for microchannel fabrication, storage, and optical transparency. As a result, PDMS-FN was selected as the material to be used in subsequent microchannel experiments. Although it was beyond the scope of this study, PDMS-based microchannels may also be useful for future evaluation of the effect of channel wall stiffness on endothelial cell characteristics. Vascular diseases are often characterized by changes in blood vessel wall stiffness [100]. PDMS microchannels, such as those developed in this study, could be fabricated with a varying channel wall stiffness by mixing the PDMS base and curing agent in different ratios [91], [92] allowing different disease conditions to be modeled.

3.7 Cell culture in microchannels

Circular microchannels produced using the wire-mold method showed higher reproducibility of size and significantly greater aspect ratios and circularity compared to the microchannels produced using the gas-stream method (Figure 28 and Figure 29). From 2D experiments, PDMS_{FN} was found to be effective for promoting cell seeding, proliferation, and spreading, with acceptably low levels of migration. Therefore, 3D cell culture was performed in circular PDMS microchannels, made using the wire-mold method and coated with a 5 $\mu\text{g}/\text{ml}$ fibronectin solution. A 24 AWG ($\sim 565 \mu\text{m}$ experimentally) was selected for the majority of fabrication experiments, as cell death was found to increase with decreasing channel diameter (Table 12). This channel size corresponds with larger-sized

arterioles and venules in the human body (Table 1). HUVECs were seeded within channels and cultured under static and dynamic flow conditions. Cells were fixed, and fluorescent images were captured at several different time points. Because of difficulties culturing HUVECs in microchannels for long periods of time under no flow conditions, cells were grown for 6 and 12-hour time intervals under static conditions and for 48 hours under flow. With a 0.1 ml/h flow rate applied to the microchannel, resulting in a shear stress calculated to be approximately 0.0139 dynes/cm² (Appendix), HUVEC growth could be maintained for approximately 48 hours. Fluorescence images of fixed HUVECs in 3D microchannels under static and flow conditions at several time points after seeding are shown in Figure 42 and Figure 41.

Table 12 Summary of microchannels tested, sample sizes, and conditions for flow and no flow experiments

Mean cross-sectional dimension (μm)	113.8	103.8	93.1	108.6	220.0	446.3	565.4
Microchannel length (cm)	1	1	1	1	1	1	1
Microfabrication method	Photo lithography	Gas stream	Wire mold	Wire mold	Wire mold	Wire mold	Wire mold
Cross-section geometry	rectangular	circular	circular	circular	circular	circular	circular
Surface treatment	FN	FN	FN	FN	FN	FN	FN
Flow conditions	Static/gravity flow	Static/gravity flow	Static	Static	Static	Static/ $\frac{0.1 \text{ ml}}{\text{h}}$	Static/ $\frac{0.1 \text{ ml}}{\text{h}}$
Growth periods tested (hours)	4-48 hours						
Approx. number of microchannels fabricated and tested with/without flow	87/23	73/21	17	21	21	18/4	24 + 48 for gene expression experiments/8
Observations	High cell death/wash out	High cell death/channel blockages/wash out	High cell death/channel blockages/wash out	High cell death/channel blockages/wash out	Some cell death/wash out	Some cell death/wash out in flow	Some cell death/wash out in flow

It was qualitatively observed that increased rotations of the channel during the cell seeding process resulted in increased cell coverage of the microchannel. Regions of partial cell coverage were observed on several samples (Figure 41). After a 48-hour growth period where HUVECs had been exposed to a flow rate of 0.1 ml/hour, beginning 4 hours after seeding, the HUVECs appeared slightly more elongated in the flow direction compared to cells in static microchannels (Figure 42).

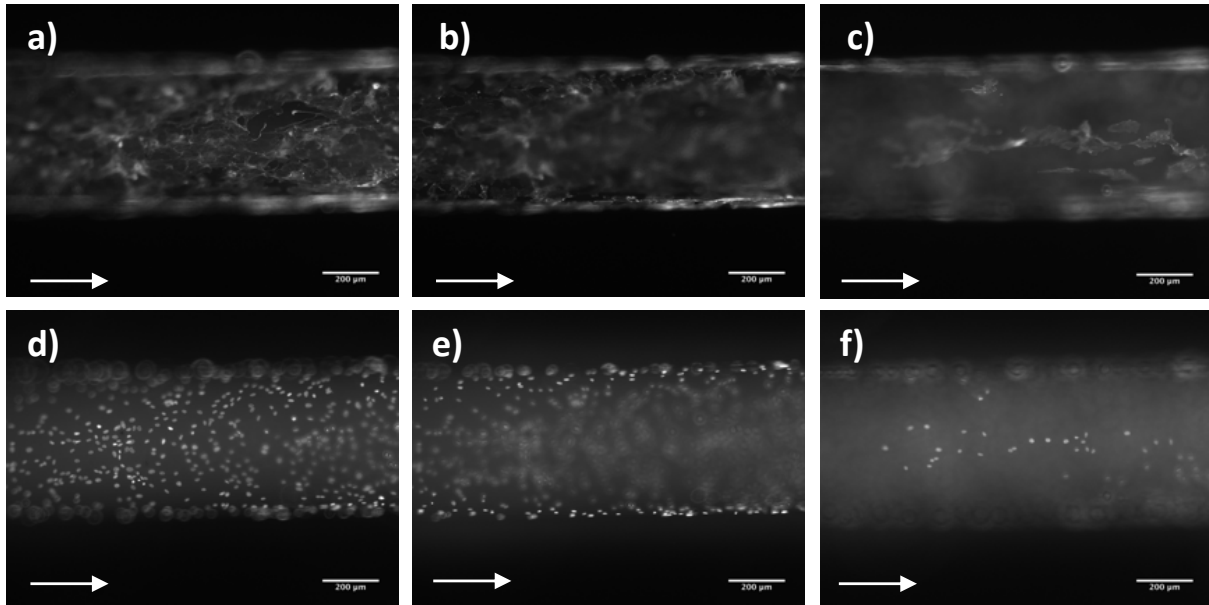


Figure 41 Fixed fluorescent images of HUVECs in 24 AWG microchannels for 48 hours under flow. Alexa555-phalloidin stain for actin cytoskeleton (a-c), DAPI nuclei stain (d-f) bottom, middle and top focal planes (left to right). Flow direction indicated by white arrows. Scale bar 200µm.

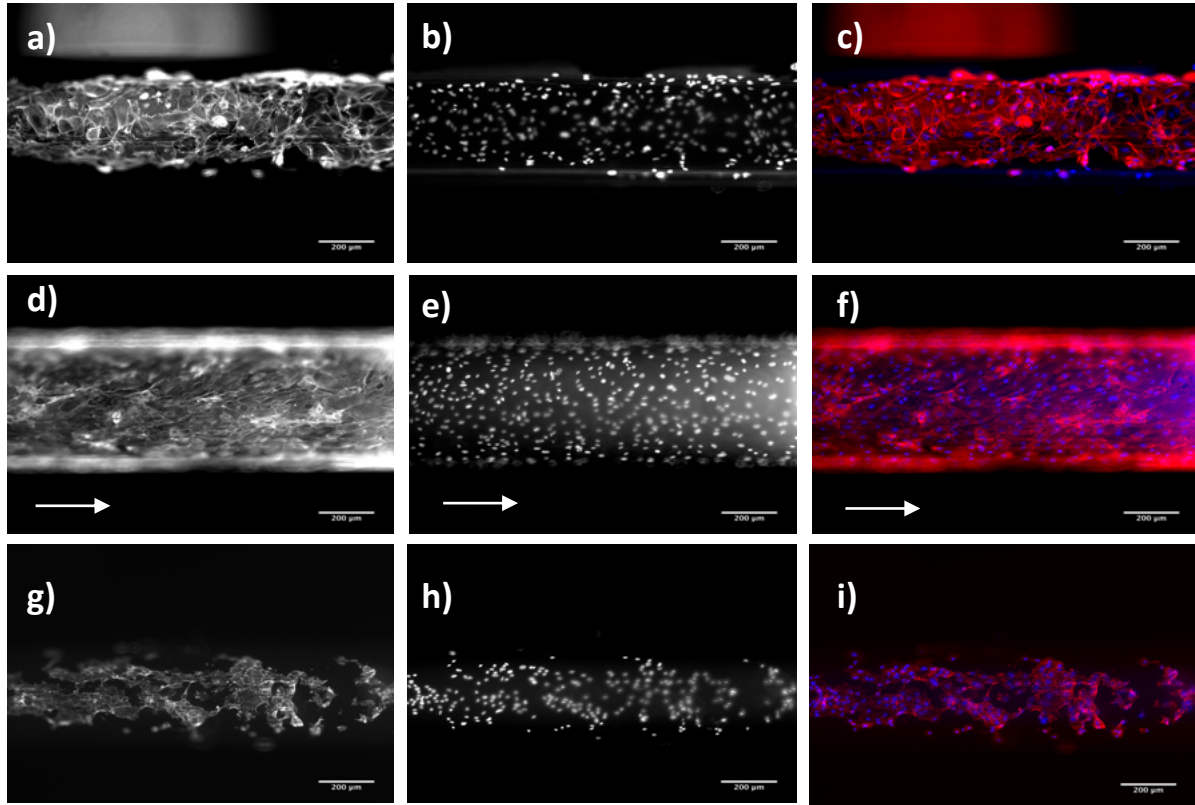


Figure 42 Fixed fluorescent images of HUVECs in 24 AWG microchannels at 6 hours with no flow (a-c), 24 AWG 48 hours with flow (flow direction indicated by white arrows) (d-f), 26 AWG 12 hours no flow (g-i). For all images (left to right) Alexa555-phalloidin stain for actin cytoskeleton, DAPI nuclei stain, Composite image nuclei (blue), actin (red). Scale bar 200um. Images were taken in the focal plane corresponding with the channel bottom.

3.7.1 Microarray data

RNA concentrations, sample conditions and quality are presented in Table 13.

Table 13 RNA concentrations for cell samples collected from 2D and 3D growth environments, provided by Genome Quebec (McGill University)

Sample label	Condition	Quality	RNA concentration
HUVEC 000-1	PDMSfn 3D microchannel	Good	2.89 ng/ul
HUVEC 000-2	PDMSfn 3D microchannel	Good	4.64 ng/ul
HUVEC 000-3	PDMSfn 3D microchannel	Good	3.79 ng/ul
HUVEC 000-4	PDMSfn 2D	Good	89.52 ng/ul
HUVEC 000-5	PDMSfn 2D	Good	157.02 ng/ul
HUVEC 000-6	PDMSfn 2D	Good	11.19 ng/ul

Signal values of probe intensity from each sample were normalized by TAC software (ThermoFisher Scientific, USA). Raw probe intensities appeared relatively consistent between the replicates in the 3D and 2D conditions (Figure 43).

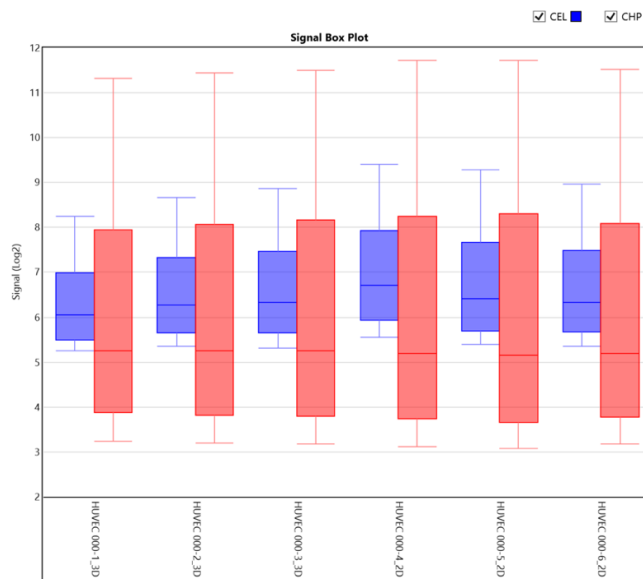


Figure 43 Normalization of sample probe intensity (signal). Raw un-normalized data (blue), normalized data (red). Each of the six samples are indicated on the x-axis. Samples 000-1, 000-2, and 000-3 represent cells cultured within microchannels. Samples 000-4, 000-5, and 000-6 represent HUVECs cultured on 2D surfaces.

HUVECs cultured in 3D PDMS circular cross-section microchannels treated with fibronectin had differential gene expression compared to 2D PDMS-FN substrates for 1662 genes (out of 21448), where a difference was defined as a fold change (FC) greater than 2 or less than -2 and a p-value less than 0.05 to obtain statistical and biological significance. Of the 1662 differentially expressed genes, 609 were up regulated in 3D microchannel growth conditions, and 1053 were down regulated.

The observed differences in gene expression patterns for 3D versus 2D growth environments are presented in a “Volcano plot” created in the TAC software, where log 2-transformed fold changes in gene expression are plotted against t-test p values. Genes plotted furthest away from the central axes have greatest fold changes and p-values (Figure 44).

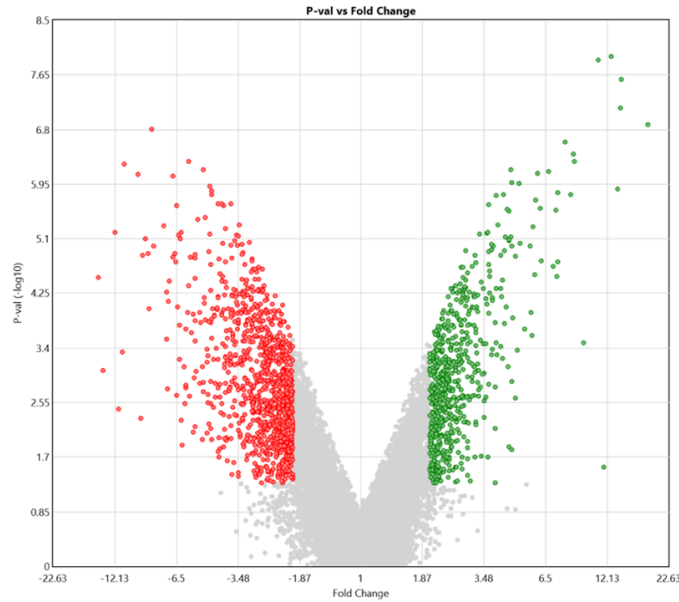


Figure 44 Relative expression comparison for 1662 differentially expressed genes in 3D versus 2D growth conditions. $p < 0.05$ with $FC > 2$ or < -2 . Upregulated genes are shown in green, downregulated genes are shown in red. The plot shows a log 2-fold change in gene expression on the x-axis and the negative log of t-test p values on the y-axis. Each gene is represented by a single point.

A heat map showing the top 55 (largest magnitude) differentially expressed genes with a fold change greater than 6 or less than -6 and a p-value less than 0.05 is displayed in Figure 45. Of these, 20 genes were found to be upregulated, and 35 were downregulated for HUVEC culture in a circular microchannel compared to the 2D conditions. Genes upregulated in 3D microchannel cultured HUVECs include JUNB and EGR1, TNFAIP3, PTGS2, ATF3, TNFSF15, NFKB1A, CXCL8 and CCL2. Downregulated genes include SPC25, CDK1, KIF20A, NCAPG, GMNN, FBXO5, CDCA2, MCM2, MCM5, MCM6, GINS1, GINS2, ACER3, LIPG, FABP3, and SERPINH. ADAMTS1 was the most upregulated gene with more than 18-fold changes in its expression, while HIST1H2AJ was the most downregulated gene with more than 14-fold changes in expression (Table 17).

Prior to microarray analysis, 32 genes of interest previously shown to be related to endothelial cell function in literature were identified for examination in this study [1], [101]. Among these, 21 genes displayed upregulation by at least 2-fold, while 11 genes were downregulated (Figure 46 and Table 18).

Specifically, VEGFC, VEGFA, VCAM1, ICAM1, SELE and NOS3 were upregulated in microchannel growth environments when compared to 2D conditions, whereas CASP6, ITGB1BP1 and PLGRKT were downregulated.

Table 14 Functional grouping of several genes relevant to endothelial cell function according to Moradipoor *et al.* and GeneCards, [101], [102]

Angiogenesis	Vasoconstriction & vasodilation	Inflammatory response	Apoptosis	Cell adhesion	Platelet activation
CCL2†	ICAM1	CCL2†	BCL2L11	ADAM23	IL6
ENG	NOS3	IL6	BCL2L1	ADAMTS9	ITGB8
FGFR1	PTGS2†	PTGS2†	CASP6	ADAMTS6	NOS3
IL6		SELE	CCL2†	ADAMTSL1	SERPINH1
ITGB1		TGFB2	IL6	ADAMTS1†	SERPINB8
NOS3		TNF*	TNFSF4	BCL2L1	TGFB2
PTGS2†		PLGRKT	TNFSF10	CDH5	VEGFA
VEGFA		ADAMTS1†	TNFAIP3†	ENG	THBS3
VEGFC		CXCL8	TNFRSF21	FGFR1	
			TRAF2	ICAM1	
			TNFSF15†	ITGB1	
			TNFSF13B	PTK2	
			TNFRSF12A	SELE	
			TNFSF9	SERPINH1	
			PTK2	SERPINB8	
			NFIL3	TGFB2	
				TNF*	
				VEGFA	
				THBS3	
				VCAM1	

*TNF super family see apoptosis column

†displayed a FC > 6, all others have a FC >2 or <-2

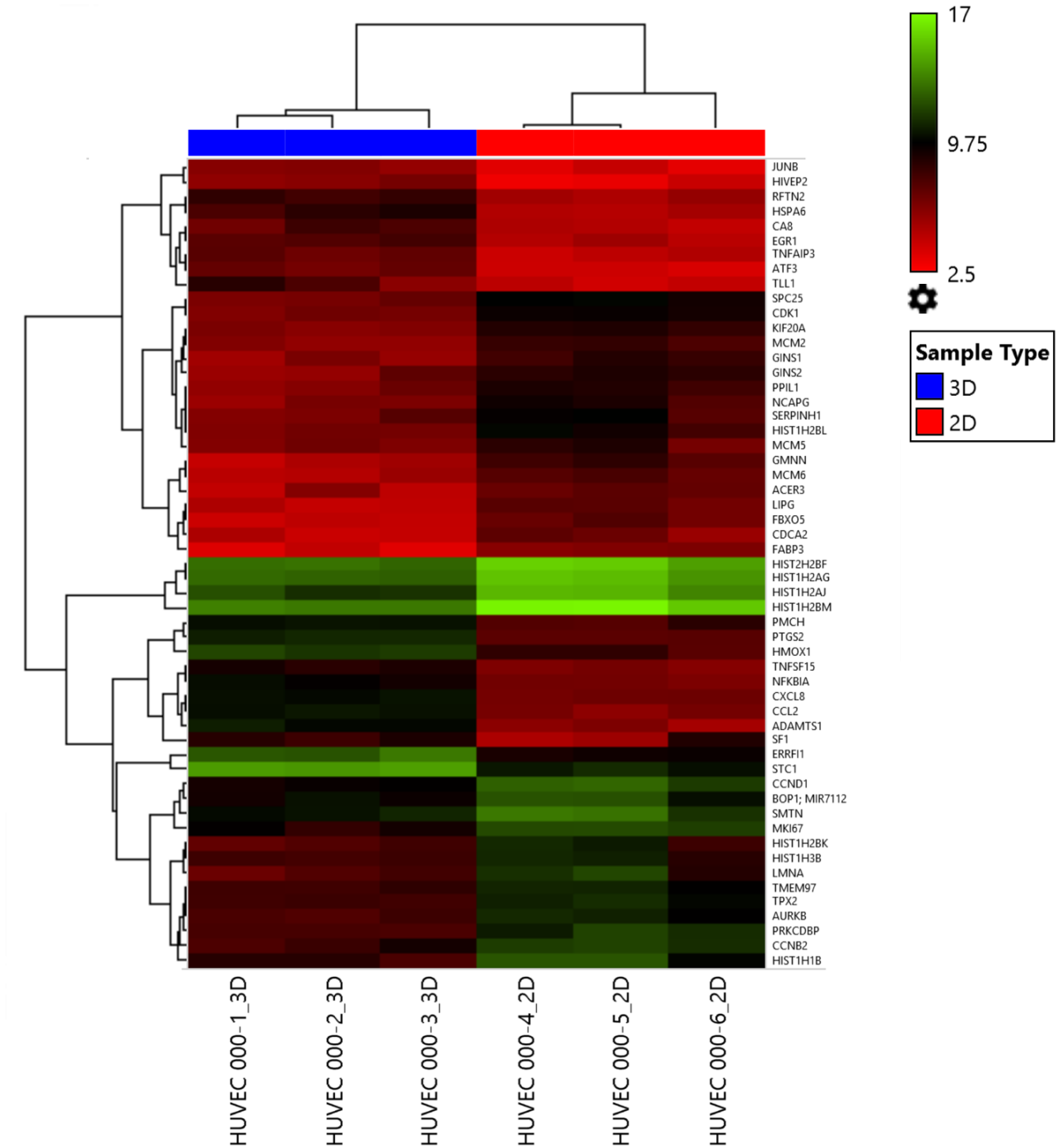


Figure 45 Heat map comparing expression of the most differentially expressed genes in HUVECs grown within 3D circular PDMS-FN microchannels to those grown in a 2D PDMS-FN culture. Only differentially expressed genes with $p < 0.05$ and $FC > 6$ or < -6 are included. Individual genes are represented in rows and samples are represented in columns. The color and intensity of the boxes represent changes in gene expression relative to the mean signal of all samples. Black represents no change in gene expression while green and red represent up and down-regulated genes respectively. Genes and samples are clustered based on the similarity of gene expression patterns, allowing commonly regulated genes to be identified [103]. Samples 000-1, 000-2, and 000-3 represent cells cultured within microchannels (3D). Samples 000-4, 000-5, and 000-6 represent cells cultured on 2D surfaces.

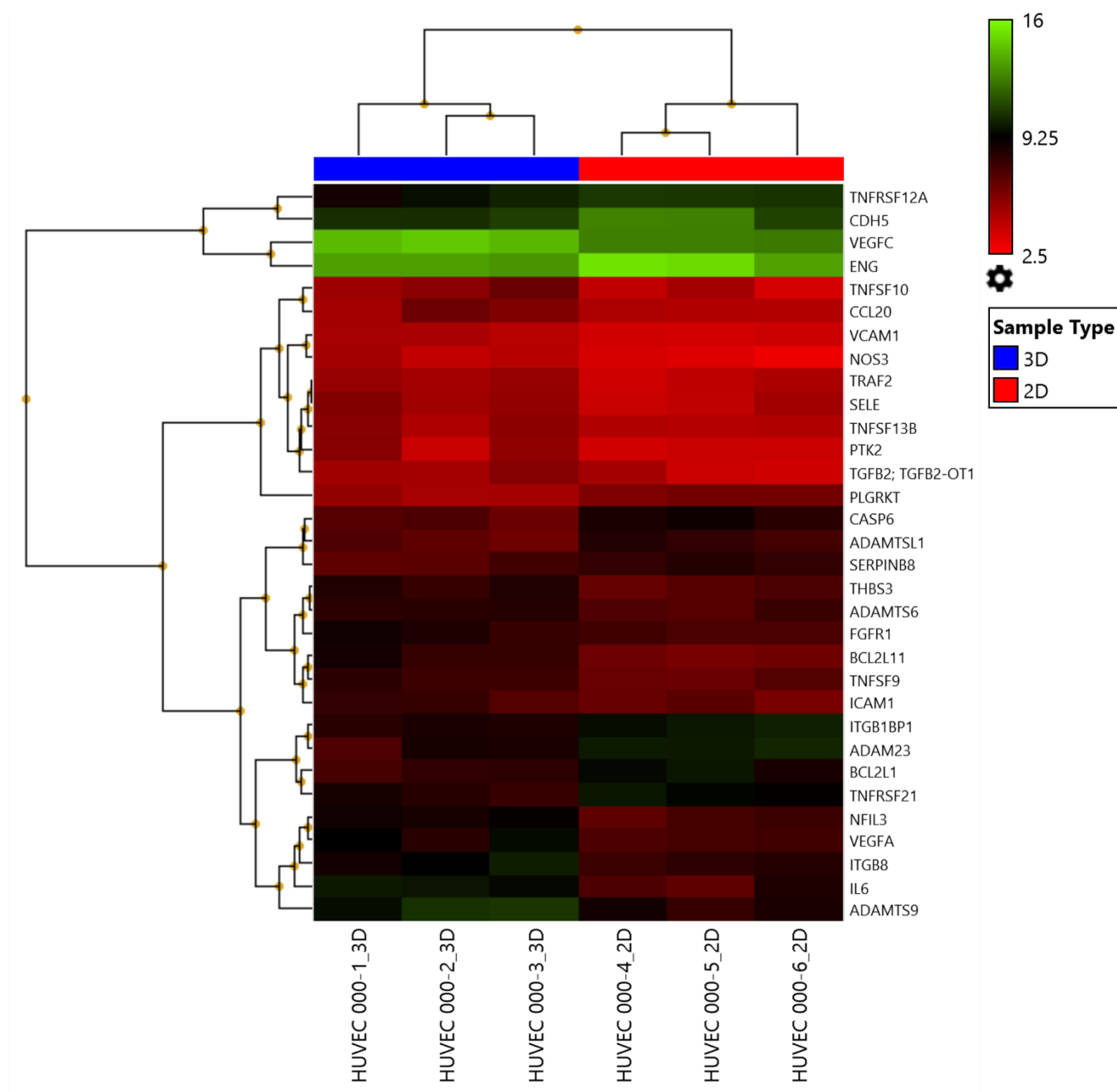


Figure 46 Heat map comparing expression of several genes specific to endothelial cell function obtained from literature, in HUVECs grown within 3D circular PDMS-FN microchannels to those grown in a 2D PDMS-FN culture. Only differentially expressed genes with a fold change greater than 2 or smaller than -2 are included. Individual genes are represented in rows and samples are represented in columns. The color and intensity of the boxes represent changes in gene expression relative to the mean of all samples. Black represents no change in gene expression while green and red represent up and down-regulated genes respectively. Genes and samples are clustered based on the similarity of gene expression patterns, allowing commonly regulated genes to be identified. Samples 000-1, 000-2, and 000-3 represent cells cultured within microchannels (3D). Samples 000-4, 000-5, and 000-6 represent cells cultured on 2D surfaces.

3.8 Evaluation of cell culture in microchannels

HUVECs were successfully grown for periods of approximately 6 to 48 hours in $\sim 565\ \mu\text{m}$ diameter channels, and for approximately 12 hours in channels $\sim 446.3\ \mu\text{m}$ in diameter. To maintain growth for 48 hours, it was necessary to perfuse the microchannels with fresh media at a low flow rate. Without an applied flow, we found that cultures could be maintained for approximately 12 hours, with no media exchange, prior to HUVEC death or detachment from the interior of the microchannel.

In this study, shear stress at the wall of the circular microchannel was minimized in an attempt to reduce rates of cell detachment. When a low flow rate of 0.1 ml/h was applied, corresponding to a wall shear stress estimate of $0.0139\ \text{dynes/cm}^2$ (Appendix), HUVEC detachment was still often observed, indicating a relatively low adhesion strength. This shear stress value is lower than physiological shear stress observed in blood vessels, which have a vast range from $1 - 6\ \text{dynes/cm}^2$ in the venous system and $10 - 70\ \text{dynes/cm}^2$ in arterial vessels [9].

We observed a decrease in cell viability corresponding with decreasing microchannel diameter (Table 12). This was also reported in previous studies that employed similar microfabrication techniques and produced circular microchannels with dimensions comparable to those in our study [54]. In some studies, researchers were able to maintain cell cultures in the microchannels for considerably longer periods (several days) compared to what was achieved in this study. This may be attributed to experimental set up, and an increased cell adhesion strength achieved in microchannels using type I collagen-coated glass and Matrigel-functionalized PDMS, [33] and [34] respectively.

In this study, the $5\ \mu\text{g/ml}$ fibronectin was found to remain in microchannels for at least 4 hours, providing sufficient time for initial cell attachment (Appendix). A greater concentration of fibronectin for surface treatments of the PDMS microchannels may have helped to improve endothelial cell

attachment. However, Budd *et al.* found that EC attachment appears to plateau at concentrations above 20 $\mu\text{g}/\text{ml}$, when a range of fibronectin solution concentrations from 2-100 $\mu\text{g}/\text{ml}$ were tested for coating polytetrafluoroethylene vascular grafts [104].

In samples where sufficient cell growth took place, imaging of a lower, middle and upper focal plane of each microchannel allowed the uniformity of cell growth to be assessed. Rotation of the channel resulted in increased cell coverage of the channel sides, however the coverage was still often incomplete (Figure 41). Increased channel rotation after seeding, and increased seeding of cells into the channels after initial attachment, may help to improve cell coverage within channels. Additionally, increasing the viscosity of the cell culture medium during the seeding process could help reduce cell sedimentation velocity [105], and prevent cell accumulation on the bottom of the microchannel.

The major obstacles of cell death, poor cell adhesion, and lack of cell confluence within microchannels may have been influenced by the cytotoxicity of PDMS, which has previously been shown to have the potential to leach uncured oligomers into the cell culture environment [53]. To address this, methods to remove PDMS monomers could be examined. Several studies report using organic solvents to swell PDMS, extracting un-crosslinked oligomers, and then evaporating the solvent under vacuum [53], [106], [107].

Another influencing factor may have been the protein coating method used in this study. It can be difficult to control direct adsorption of a protein (such as fibronectin) from a solution onto a surface. Adsorption through this method can cause protein denaturation, reduced density of the protein on the surface and reduced protein functionality [13]. This may have contributed to non-uniform cell coverage observed in the microchannels. In the future, other methods to immobilize proteins on substrates, while limiting denaturation, could be used [13]. Additionally, plasma treatment of the substrate surfaces could potentially be used to increase cell adhesion and spreading [35], [54], [108].

Micro-environmental conditions within the PDMS microchannel, that could affect cell viability over time, such as pH, O₂ or CO₂ concentrations, might additionally be examined in future studies.

Although it was beyond the scope of our study, the optimization of purely collagen-based microchannels to promote cell adhesion and viability within microchannels represents an important area for further investigation. Alternatively, the coating of PDMS microchannels with collagen gels is a concept that has been explored in literature, where fibronectin has been used to promote adhesion of the collagen gel to the PDMS [78]. However, difficulties exist in ensuring a strong bond between the collagen construct and PDMS to prevent separation, particularly under fluid flow [109].

Based on qualitative observations, HUVECs cultured for 48 hours within channels under flow conditions appeared slightly elongated with the long axis more aligned in the direction of flow as compared to HUVECs cultured in channels under static conditions (Figure 42). Previous studies have reported a more pronounced effect on the morphology of endothelial cells with longer-term exposure to shear stress [47],[110]. Further investigation of the effect of duration of flow on cell morphology within our circular PDMS_{FN} microchannels would be an interesting area of future research.

The microarray analysis identified 55 differentially expressed genes, where a comparison was made between HUVECs cultured in 3D circular microchannels and 2D surfaces. Among these, 20 genes were significantly upregulated by greater than 6-fold in their expression and 35 genes were significantly downregulated by 6-fold in their expression (Table 17 and Figure 45). In particular, the downregulation of cell cycle gene CDK1, a large cell cycle promotor, was observed. Other downregulated genes included SPC25, KIF20A, NCAPG, GMNN, FBXO5 and CDCA2, which are involved in cell cycle regulation and mitosis; MCM2, MCM5, MCM6, GINS1 and GINS2 genes, associated with DNA replication; and ACER3, LIPG and FABP3 involved in lipid metabolism. HIST1H2AJ, found to have the greatest fold change decrease in expression, encodes for several variants of the H2A histone family,

which are involved in chromatin organization [102]. This grouping of downregulated genes may suggest that the cell cycle is slowed as a result of cells reaching confluence earlier within the microchannels.

The most dramatically up-regulated gene expression was observed in ADAMTS1, involved in various inflammatory processes and cellular adhesion. Other genes upregulated by greater than 6-fold in their expression included TNFAIP3, PTGS2, ATF3, TNFSF15, NFKB1A, CCL2 and CXCL8 as well as genes upregulated by at least 2-fold, such as IL6, SELE, TGFB2 and PLGRKT. These genes are mediators of inflammatory response, several associated with chemokine expression (Table 17 and Figure 45). Cytokine and chemokine genes are well known to be responsive to shear stress [111]. As a result, the upregulation of inflammatory genes may be an indication that the HUVECs experienced several seconds of acute shear stress when cell culture media in the microchannels was exchanged. Alternatively, reduced oxygen available to HUVECs in the microchannels during growth could have contributed to the release of inflammatory mediators [112].

Low shear stress or disturbed flow has reportedly resulted in the upregulation of the vascular cell adhesion molecule-1 (VCAM-1), E-selectin (SELE) [111] and the intercellular cell adhesion molecule-1 (ICAM-1) [1], [111] in endothelial cells. Our microarray data corroborates these results (Table 18). Another well-known HUVEC response to shear stress is the production of nitric oxide (NO)[113]. This potentially accounts for the upregulation of NOS3 (Nitric Oxide Synthase 3) observed in our data (Table 18).

Additional genes that were upregulated by at least 2-fold when HUVECs were cultured in microchannels, included genes involved with transcription regulation (JUNB and EGR1), angiogenesis (CCL2, FGFR1, IL6, NOS3, PTGS2 and VEGFA), vasodilation and vasoconstriction (ICAM1, NOS3 and PTGS2), cellular adhesion (ADAMTS1, ADAMTS9, ADAMTS6, FGFR1,

ICAM1, PTK2, SELE, TGFB2, THBS3, VEGFA and TNF family genes), apoptosis (BCL2L1, CCL2, IL6, PTK2, NFIL3, and TNF family genes), and platelet activation (ITGB8, NOS3, TGFB2, THBS3 and VEGFA).

For future work, Real-Time Quantitative PCR (qRT-PCR) experiments would be required to confirm microarray results, in addition to performing repeated microarray experiments to further solidify the results.

Chapter 4

4 Conclusion

The major aims of our research were to: (1) optimize and compare two different techniques used to make circular microchannels, (2) assess several substrates for biocompatibility with HUVECs, (3) culture HUVECS in circular microchannels coated with a biocompatible substrate under static and flow conditions, and (4) assess if differences in gene expression could be found between HUVECS cultured on 2D PDMS_{FN} surfaces versus 3D PDMS_{FN} microchannel environments.

We showed that the wire-mold method could be used to construct circular PDMS microchannels with superior channel dimension reproducibility, higher aspect ratio and circularity values, and ease of production compared to gas-stream circular microchannels. PDMS treated with polydopamine or fibronectin, as well as collagen and collagen+fibronectin substrates all had improved biocompatibility relative to untreated PDMS. Based on analysis of cell adhesion, proliferation, surface coverage, and motility, PDMS treated with fibronectin was selected as the optimal microchannel substrate. We demonstrated that HUVECs could be cultured in our fabricated microchannels, with viable cells being maintained cells in $\sim 565 \mu\text{m}$ diameter channels for a period of up to 48 hours with cell media perfusion from a syringe pump. Finally, we established that 1662 genes were differentially expressed when comparing HUVECs, cultured for approximately 20 hours, in 3D circular PDMS_{FN} microchannels versus 2D PDMS_{FN} substrates. Our findings demonstrate both the feasibility of circular microchannel production for 3D HUVEC culture as well as some of the remaining challenges. We show clear differences in the genetic characteristics of cultures grown in 2D and 3D conditions, supporting the need for physiologically representative models in cell culture. This microfluidic system has applications for future experiments where whole blood can be used as the as the working fluid. This would allow for the study of RBC- endothelial cell interactions, cellular adhesion strength and

cell response to varying blood flow conditions. Additionally, these microfluidic systems may aid in the study of circulating cells and cell margination in more physiologically representative conditions. Such investigations could provide greater insight into the characterization of vascular diseases, and responses to drug testing *in vitro*.

5 References

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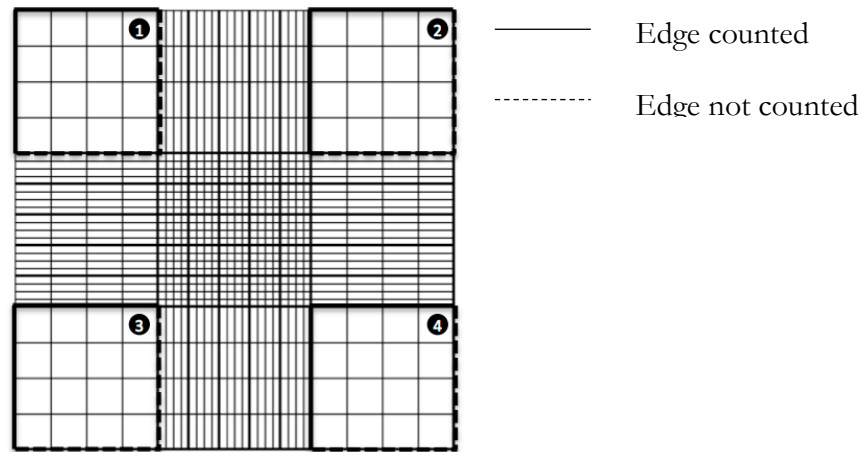
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6 Appendix

6.1 Hemocytometer calculations



In square regions 1-4 of the haemocytometer (image reproduced from [114]), the 16 squares used for counting mammalian cells represent a volume of 10^{-4} mL. According to standard practice, cells must be counted in a consistent manner. Cells were counted left to right, where cells on the bottom and left sides of the squares were left uncoun ted in each of the four square regions [114]. Cell counts in each of the four regions were then averaged and these values were multiplied by 10^4 to obtain the number of cells/mL in the suspension [114], [115]. To obtain a desired seeding amount, the seeding amount was divided by the calculated cell concentration, providing the volume of the cell suspension required for the desired seeding amount. The equations, from [114], are illustrated in the following example:

Cell counts:

Region 1: 100

Region 2: 109

Region 3: 94

Region 4: 106

Average cells per small square: 102.25

$$\text{Measured cell density} = \frac{\text{avg cells per small square} \cdot \text{Dilution factor}}{\text{Volume of a small square}}$$

$$\text{Volume of a small square} = W \cdot H \cdot D = 1 \cdot 1 \cdot 0.1 = 0.1 \text{ mm}^3 = 0.0001 \text{ mL}$$

Here the dilution factor is 1, so:

$$\text{Measured cell density} = \frac{102.25 \cdot (1)}{0.0001 \text{ mL}} = 1.0225(10^6) \text{ cells/mL}$$

$\therefore$ The concentration of the cell suspension is $1.0225(10^6) \text{ cells/mL}$

Desired seeding amount: 20,000 cells, then

$$\frac{20,000 \text{ cells}}{1.0225(10^6) \text{ cells/mL}} = 0.0195 \text{ mL} \cdot \frac{1000 \mu\text{L}}{\text{mL}} = 19.5 \mu\text{L}$$

$\therefore$ 19.5 μL of the cell suspension is removed to obtain 20,000 cells

6.2 Spin coating PDMS on 35mm culture dishes

After spin coating and curing, the layer of PDMS was carefully removed from the 35mm dish. A cross-sectional sample was cut and placed on a glass slide for observation. A bright field ZEISS Axio Lab A1. Microscope with 10x A-Plan 0.25 NA objective and ZEISS AxioCam 150 camera was used to capture images, which were analyzed using ImageJ. Thickness measurements were recorded at three different locations on four independent samples, and results were averaged to obtain a mean thickness (Table 15).

Table 15 PDMS thickness measurements from spin coated PDMS samples, N=4

	Sample			
	1	2	3	4
Mean length (μm)	37.99	64.16	45.96	46.98
Total mean length (μm) $\pm$ SEM	48.77 $\pm$ 5.51			

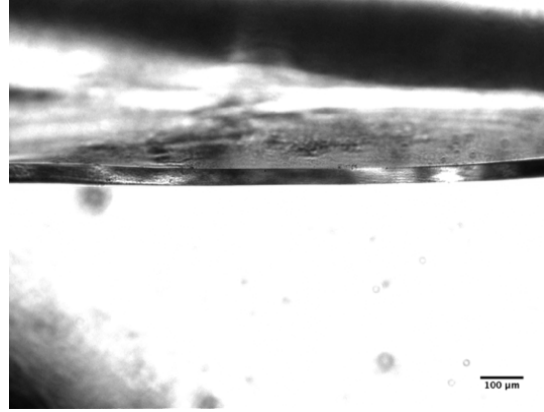


Figure 47 PDMS thickness using spin coater. Scale bar 100 μ m

6.3 Fibronectin adsorption on PDMS

A 5 μ g/ml fluorescent fibronectin-rhodamine solution was injected into a circular microchannel and incubated at room temperature for 1 hour. After 1 hour, the microchannel was flushed once with 1x PBS and then filled with 1x PBS. Fluorescent images were captured at the 1 hour mark, post PBS wash, and at 4 hours post PBS wash. The fibronectin solution was found to remain in the PDMS microchannels for a period of at least 4 hours after the initial 1 hour fibronectin solution incubation and PBS wash (Figure 48).



Figure 48 After 1 hour incubation with 5 μ g/ml fibronectin-rhodamine solution post PBS wash (left) and 4 hours post PBS wash (right). Scale bar 150 μ m.

6.4 Shear stress and Reynolds number in circular microchannels

Shear stress

The shear stress at the wall that the HUVECs were exposed to for approximately 48 hours, was calculated as follows, for an approximate circular geometry:

$$\tau = 32\mu \frac{Q}{\pi \cdot d^3}$$

If we assume the cell media viscosity to be equal to that of water at room temperature, 8.90×10^{-4} Pa.s, and the mean diameter for the 24AWG wire was approximately $565.4 \mu\text{m}$ (experimental results), then:

$$\tau = 32(8.9(10^{-4})Pa.s) \frac{0.1 \frac{ml}{h} (\frac{1h}{60min}) (\frac{1min}{60s})}{\pi \cdot \left(565.4um (\frac{1 \times 10^{-4}cm}{1um})\right)^3}$$

$$\tau = 1.39(10)^{-3} Pa$$

$$1 Pa = 10 \frac{dynes}{cm^2}$$

$$\therefore \tau = 0.0139 \frac{dynes}{cm^2}$$

Reynolds Number

$$Re = \frac{\rho V D}{\mu}$$

Where ρ is the fluid density, V is the fluid velocity, D is the diameter of the microchannel, and μ is the fluid viscosity.

Assumptions:

μ water at room temperature = 8.90×10^{-4} Pa.s

ρ water at room temperature = 999 kg/m^3

$D = 565.4 \mu\text{m} = 5.654 \times 10^{-4} \text{ m}$

$r = 2.827 \times 10^{-4} \text{ m}$

$Q = 0.1 \text{ ml/hour} \approx 2.78 \times 10^{-11} \text{ m}^3/\text{s}$

$V = V_{\text{avg}}$

Approximation for average velocity:

$$Q = V_{avg} \cdot A$$

$$\therefore V_{avg} = \frac{Q}{A}$$

Cross-sectional area:

$$A = \pi r^2$$

$$A = \pi(2.827(10)^{-4})^2 = 2.51(10^{-7}) m^2$$

$$V_{avg} = \frac{2.78(10^{-11}) \frac{m^3}{s}}{2.51(10^{-7}) m^2}$$

$$\therefore V_{avg} = 1.107(10^{-4}) \frac{m}{s}$$

Reynolds Number:

$$Re = \frac{\left(999 \frac{kg}{m^3}\right) \left(1.107(10^{-4}) \frac{m}{s}\right) (5.654(10)^{-4} m)}{(8.90(10)^{-4}) Pa \cdot s}$$

$$\therefore Re \approx 0.07$$

Re < 2300 is defined as Laminar flow

6.5 2D substrates from live imaging: Mean cell density sample calculations

Area of image frame captured for each location, from live imaging:

$$895.26 \mu m \times 670.80 \mu m (1388 \times 1040 \text{ px}) \approx 0.6005 \text{ mm}^2$$

Example PDMS substrate @10 minutes post seeding

Mean cell count from 15 different locations on substrate: 13.4

$$\therefore \text{sample density} = \frac{13.4 \text{ cells}}{0.6005 \text{ mm}^2} = 22.3 \frac{\text{cells}}{\text{mm}^2}$$

(See Figure 32)

6.6 Theoretical cell seeding density for live experiments

$$35 \text{ mm cell culture dish area} \approx \pi r^2 \approx \pi(35/2)^2 \approx 962.1 \text{ mm}^2$$

We desired that 20,000 cells be seeded on the 35 mm culture dishes for each condition, therefore the theoretical density was calculated to be $20,000 \text{ cells}/962.1 \text{ mm}^2 = 20.78 \text{ cells/mm}^2$

6.7 Cell spreading: Percent area coverage sample calculations

Area of image frame captured for each location, from fixed imaging 48 hour samples:

$$684.25\mu\text{m} \times 512.69\mu\text{m} \approx 350,808.1325\mu\text{m}^2$$

Sample calculations for 2DCollagen_{FN} sample where HUVECs were grown for 48 hours

Cell area coverage from nine image locations per sample were determined from ImageJ:

Table 16 Area (μm^2) occupied by HUVECs on 2D Collagen_{FN} samples after a 48-hour growth period, obtained from ImageJ analysis

i	Cell area coverage μm^2
1	77704.299
2	79708.743
3	54636.663
4	122507.325
5	153599.786
6	101509.992
7	101952.292
8	119015.586
9	59658.953

$$\text{mean area coverage} = \frac{1}{9} \sum_{i=1}^9 x_i$$

$$\therefore \text{mean area coverage for sample 1} \approx 96,699.29 \mu\text{m}^2$$

$$\% \text{ cell area coverage of total area for sample 1} \approx \frac{96,699.29 \mu\text{m}^2}{350,808.13 \mu\text{m}^2} \cdot 100$$

$$\% \text{ cell area coverage of total area for sample 1} \approx 27.56\%$$

% cell area coverage was determined for each of the samples (N=8 per condition) and the mean % cell area coverage was calculated. These mean values were plotted in Figure 35.

6.8 Microarray Data

Fold change from microarray data presented in Table 17 and Table 18. Information was obtained using TAC software.

Table 17 3D vs. 2D comparison of differentially regulated genes and associated p-values (FC > 6 or < -6)

No.	Gene	Fold Change	P-value	Description
1	HIST1H2AJ	-14.36	3.12E-05	histone cluster 1, H2aj
2	LMNA	-13.72	0.0009	lamin A/C
3	HIST1H2BM	-12.13	6.17E-06	histone cluster 1, H2bm
4	SERPINH1	-11.7	0.0035	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
5	HIST1H1B	-11.28	0.0005	histone cluster 1, H1b
6	SPC25	-11.03	5.44E-07	SPC25, NDC80 kinetochore complex component
7	PRKCDBP	-9.6	7.83E-07	protein kinase C, delta binding protein
8	HIST1H2BK	-9.3	0.005	histone cluster 1, H2bk
9	CCNB2	-9.12	1.42E-05	cyclin B2
10	GMNN	-8.86	7.77E-06	geminin, DNA replication inhibitor
11	CCND1	-8.67	1.34E-05	cyclin D1
12	GINS2	-8.56	9.56E-05	GINS complex subunit 2 (Psf2 homolog)
13	CDK1	-8.31	1.53E-07	cyclin-dependent kinase 1
14	AURKB	-8.16	1.03E-05	aurora kinase B
15	LIPG	-7.41	4.91E-06	lipase, endothelial
16	PPIL1	-7.22	5.39E-05	peptidylprolyl isomerase (cyclophilin)-like 1
17	NCAPG	-7.21	0.0003	non-SMC condensin I complex subunit G
18	SMTN	-7.09	0.0017	smoothelin
19	HIST2H2BF	-7.06	7.54E-05	histone cluster 2, H2bf
20	TMEM97	-6.96	3.61E-05	transmembrane protein 97
21	MCM6	-6.76	1.52E-05	minichromosome maintenance complex component 6
22	KIF20A	-6.73	8.37E-07	kinesin family member 20A
23	MKI67	-6.63	1.34E-05	marker of proliferation Ki-67
24	GINS1	-6.53	1.79E-05	GINS complex subunit 1 (Psf1 homolog)
25	HIST1H3B	-6.51	0.0022	histone cluster 1, H3b
26	FBXO5	-6.48	2.44E-06	F-box protein 5
27	HIST1H2AG	-6.4	8.93E-05	histone cluster 1, H2ag
28	FABP3	-6.35	6.86E-06	fatty acid binding protein 3, muscle and heart
29	ACER3	-6.3	0.0006	alkaline ceramidase 3
30	HIST1H2BL	-6.29	0.0002	histone cluster 1, H2bl
31	MCM2	-6.23	7.95E-06	minichromosome maintenance complex component 2

32	BOP1; MIR7112	-6.21	0.0052	block of proliferation 1; microRNA 7112
33	TPX2	-6.2	6.19E-06	TPX2, microtubule-associated
34	MCM5	-6.11	0.0128	minichromosome maintenance complex component 5
35	CDCA2	-6.04	0.0008	cell division cycle associated 2
36	TNFAIP3	6.12	2.64E-06	tumor necrosis factor, alpha-induced protein 3
37	JUNB	6.21	1.77E-05	jun B proto-oncogene
38	TNFSF15	6.68	6.99E-07	tumor necrosis factor (ligand) superfamily, member 15
39	CA8	6.99	2.10E-05	carbonic anhydrase VIII
40	EGR1	7.19	2.84E-06	early growth response 1
41	PMCH	7.26	3.02E-05	pro-melanin-concentrating hormone
42	HIVP2	7.32	1.80E-05	human immunodeficiency virus type I enhancer binding protein 2
43	ERRFI1	7.33	1.53E-06	ERBB receptor feedback inhibitor 1
44	ATF3	7.86	2.48E-07	activating transcription factor 3
45	HMOX1	8.35	1.62E-06	heme oxygenase 1
46	NFKBIA	8.62	3.77E-07	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
47	RFTN2	8.68	5.00E-07	raftlin family member 2
48	TLL1	9.52	0.0003	tolloid like 1
49	CXCL8	11.06	1.33E-08	chemokine (C-X-C motif) ligand 8
50	SF1	11.66	0.0283	Memczak2013 ALT_ACCEPTOR, ALT_DONOR, coding, INTERNAL, intronic best transcript NM_201998
51	PTGS2	12.62	1.14E-08	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
52	HSPA6	13.47	1.31E-06	heat shock 70kDa protein 6 (HSP70B)
53	STC1	13.77	7.37E-08	stanniocalcin 1
54	CCL2	13.97	2.60E-08	chemokine (C-C motif) ligand 2
55	ADAMTS1	18.28	1.30E-07	ADAM metalloproteinase with thrombospondin type 1 motif 1

Table 18 3D vs. 2D comparison of differentially regulated genes (FC > 2 or < -2) of specific interest relevant to endothelial cell function according to TAC software, Moradipoor *et al.* and GeneCards, [101], [102], items indicated by ⊥ symbol are repeated in Table 17.

No.	Gene	Fold Change	P-value	Description
1⊥	SERPINH1	-11.7	0.0035	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
2	CDH5	-4.7	0.0059	cadherin 5, type 2 (vascular endothelium)
3	ENG	-3.3	0.0125	endoglin
4	CASP6	-2.8	0.0002	caspase 6
5	ITGB1BP1	-2.64	7.18E-05	integrin beta 1 binding protein 1
6	PLGRKT	-2.57	0.0008	plasminogen receptor, C-terminal lysine transmembrane protein
7	BCL2L1	-2.57	0.0013	BCL2-like 1
8	ADAM23	-2.5	0.0005	ADAM metalloproteinase domain 23
9	ADAMTSL1	-2.3	0.0025	ADAMTS like 1
10	TNFRSF21	-2.26	0.0014	tumor necrosis factor receptor superfamily, member 21

11	SERPINB8	-2.22	0.0031	serpin peptidase inhibitor, clade B (ovalbumin), member 8
12	TNFRSF12A	-2.19	0.0056	tumor necrosis factor receptor superfamily, member 12A
13	TNFSF13B	2.01	0.0315	tumor necrosis factor (ligand) superfamily, member 13b
14	TRAF2	2.03	0.0069	TNF receptor-associated factor 2
15	TGFB2; TGFB2-OT1	2.12	0.0155	transforming growth factor beta 2; TGFB2 overlapping transcript 1
16	ADAMTS6	2.14	0.0042	ADAM metallopeptidase with thrombospondin type 1 motif 6
17	VCAM1	2.14	0.0012	vascular cell adhesion molecule 1
18	NOS3	2.17	0.0015	Nitric Oxide Synthase 3
19	FGFR1	2.18	0.0042	fibroblast growth factor receptor 1
20	CCL20	2.25	0.0089	chemokine (C-C motif) ligand 20
21	TNFSF9	2.27	0.001	tumor necrosis factor (ligand) superfamily, member 9
22	SELE	2.43	0.0086	selectin E
23	ITGB8	2.45	0.0026	integrin beta 8
24	ICAM1	2.59	0.0048	intercellular adhesion molecule 1
25	TNFSF10	2.73	0.0041	tumor necrosis factor (ligand) superfamily, member 10
26	THBS3	2.78	0.0006	thrombospondin 3
27	NFIL3	2.78	0.0002	nuclear factor, interleukin 3 regulated
28	BCL2L11	2.8	5.50E-05	BCL2-like 11 (apoptosis facilitator)
29	VEGFC	2.89	1.22E-05	vascular endothelial growth factor C
30	PTK2	3.16	0.0198	Transcript Identified by AceView, Entrez Gene ID(s) 5747
31	VEGFA	3.46	0.001	vascular endothelial growth factor A
32	ADAMTS9	4.12	0.0004	ADAM metallopeptidase with thrombospondin type 1 motif 9
33	IL6	5.66	0.0003	interleukin 6
34⌞	TNFAIP3	6.12	2.64E-06	tumor necrosis factor, alpha-induced protein 3
35⌞	TNFSF15	6.68	6.99E-07	tumor necrosis factor (ligand) superfamily, member 15
36⌞	PTGS2	12.62	1.14E-08	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
37⌞	CCL2	13.97	2.60E-08	chemokine (C-C motif) ligand 2
38⌞	ADAMTS1	18.28	1.30E-07	ADAM metallopeptidase with thrombospondin type 1 motif 1