Monitoring endothelial cells in microfluidic systems
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Chapter 4

Effects of channel width combined with flow on endothelial cell culture in microchannels

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Abstract

In the past decade, the study of cellular behavior in microfluidic devices has rapidly developed and started to contribute to numerous research areas. Microfluidic devices have been applied to study the endothelium, in which their use allows the application of flow and the emulation of blood vessels. The endothelium is involved in many physiological events such as molecular trafficking between the blood stream and surrounding tissues, blood coagulation, and immunological response to pathological conditions. However, studies of the endothelium are often limited by the dimensions of in vitro systems, which poorly represent the (diverse) dimensions of blood vessels and thus flow conditions. In previous work (Chapter 3), we optimized microchannel geometry to allow the study of endothelial cells in channels with dimensions of 60 - 360 µm. The next step is to introduce shear stress in these channels, because endothelial cells in the body experience shear stress from flowing blood. The phenotype of the endothelial cells is influenced by such factors, as the cells align themselves with flow. These morphological rearrangements are executed by the cytoskeleton, while intercellular interactions are coordinated by membrane proteins. The aim of this work was to identify whether we can reproduce these changes in microchannels. In order to achieve this we applied flow (0 – 10 dyn/cm²) to microchannels of varying widths (80 – 360 µm) using a customized setup. We specifically looked at changes in the actin cytoskeleton, and the expression and distribution of several membrane proteins in human umbilical vein endothelial cells (HUVEC). We used confocal fluorescent microscopy to obtain high-resolution images for inspection. The molecules that were selected for this study have a proven responsiveness towards different shear stress levels. We could thus inspect both the influence of shear stress level and microchannel width on cellular structure. The results revealed differences in HUVEC behavior dependent both on applied shear stress and microchannel dimension.

Keywords: Microfluidic cell culture, Microvasculature, Human Umbilical Vein Endothelial Cells (HUVEC), Shear stress, Confocal imaging
4.1. Introduction

The endothelium is the one-cell thick layer barrier between the blood and the rest of the body. It constitutes a total surface of 7 m² [1] which is spread out over blood vessels ranging in size from as small as 8 µm (in capillaries) to as large as 3 cm in diameter (in aorta) [1], [2]. Endothelial cells exhibit natural heterogeneity dependent on where blood vessels are located in the human body [3]. Endothelial cells also respond to cues from surrounding cells [4], local partial oxygen pressure (pO₂) changes [5], [6], oxidative stress [7], [8], and pH alterations [9] which contribute to constant cellular adaptation to environmental conditions. The endothelium is important in different pathologies, such as coronary artery disease (aorta) [10] and diabetes-associated renal dysfunction (capillaries) [11]. In all cases, however, in vitro tests are generally performed with cells being cultured on the bottom of standardized wells. The dimensions of these wells, as well as the absence of flow, poorly represent the conditions in human blood vessels. The lack of these in vivo occurring stimuli might lead to differences between data obtained from in vitro endothelial cell culture research and in vivo studies on the endothelium [7], [8], [12]–[14].

In order to address this discrepancy between in vitro and in vivo studies, there is a need for a versatile cell culture platform which allows modification of the cell culture environment. Recent research by Varma and co-workers has shown that the use of microchannels is in fact strongly correlated to cellular behavior, due to their operation in the laminar flow regime. However, in this work little attention was paid to the possible influence of the diameter of the channels on cellular behavior [15]. In Chapter 3 we have introduced an optimized channel geometry that allows the successful seeding of HUVEC in channels down to 60 µm, whereas other available microfluidic cell culture devices offer channels with a minimum width of 400 µm. Those are useful for studying large-vessel phenomena, but from a dimensional standpoint less suited to the study of human microvasculature (dimensions <100 µm) [14]. The latter is regarded as a physiologically dynamic structure involved in molecular trafficking, inflammatory response, and blood coagulation [16]–[18]. In this follow-up study we exposed HUVEC in microchannels (80 – 360 µm wide) to shear stress (0 – 10 dyn/cm²) to further approximate the in vivo situation in blood vessels. Previous research on the effect of shear stress on endothelial cells indicated that these cells are less prone to inflammation, which indicates the importance of this parameter. However, this research was carried out in devices with channel widths above 400 µm, thus those results are not immediately applicable to microvasculature [12], [14].

In this study, the aim was to investigate the influence of (i) channel width and (ii) shear stress in different combinations on HUVEC. In the blood vessels these factors affect the phenotype of endothelial cells as well as the interaction between the cells. These phenotypical changes were visualized with immuno-histology and observed with confocal fluorescent microscopy. The cell morphology under different experimental conditions was visualized by staining of actin cytoskeleton. The intercellular interaction between neighboring endothelial cells was visualized by staining several membrane proteins (Vascular Endothelial Cadherin (VE-Cadherin), Zonula occludens-1 (ZO-1), and Platelet Endothelial Cell Adhesion Molecule-1 (PECAM)[19]–[25]). The function of these molecules and their relation to the blood vessels conditions are described in the Theory section below.

4.2. Theory

The conditions in human blood vessels have an influence on endothelium. These effects can be seen within the cells and in the interaction between them. Therefore both types of effects need to be visualized when trying to assess the influence of in vitro parameters on endothelial cells in newly
developed culture systems. Cellular changes can be related to cytoskeletal rearrangement, whereas intercellular effects are reflected in distribution of membrane and junctional proteins. This section addresses how the actin cytoskeleton and several membrane proteins are influenced by shear stress, which explains why they were chosen for this study.

It was demonstrated that the endothelium is able to sense and discriminate between hemodynamic forces. Thus, the endothelial cell reaction to exerted forces influences both the cell layer integrity and permeability [25]. In vivo the endothelium has a certain permeability, which depends on the location and function of the blood vessel. Permeability of the endothelium is important for transport of molecules from the blood stream to underlying tissue. Therefore, high permeability of the endothelium is found blood vessels located in the organs, whereas the main arteries are less permeable. Permeability of the endothelium is regulated by cell the junctional proteins located in the membrane such as ZO-1 or VE-cadherin. Tight junctions are reduced in number and transcellular electrical resistance decreases when endothelial cells are isolated and cultured [19]. In static culture conditions, the permeability is lower than in in vivo and only upon the application of the shear stress does the permeability increases. While this is already evidence that shear stress in in fact important in in vitro cultures it does not take the localized and dimensional nature of in vivo endothelium into account.

4.2.1. Actin

Filamentous actin (microfilaments), as a part of the cytoskeleton, is sensitive to shear stress and responsible for morphological changes. The actin network connects internal cell compartments with each other and to the outer environment via adherent and tight junctions. It thus allows the cell to adjust its shape in response to environmental stimuli (e. g. shear stress) and to communicate with neighboring cells [26]. Most of the microfilaments in static in vitro cell cultures are localized just beneath the plasma membrane, where they provide mechanical support to the cell and enables cellular motion. In the presence of the shear stress, actin filaments elongate and distributed evenly throughout the cell, yet aligned with the direction of the flow.. In case of inflammatory stimulation in vitro, actin filaments can be transformed into contractile stress fibers which are distributed throughout, which resembles the situation after application of the shear stress in in vitro cell cultures [27]. Such adjustments to different physiological conditions demonstrate the adaptability of the cytoskeleton to the cell culture status. It is therefore a valuable parameter to monitor in the context of this work.

4.2.2. VE-Cadherin

The adherens junction protein selected for this study was Vascular Endothelial Cadherin (VE-cadherin). This protein is located basally between two endothelial cells (Figure 1); an extracellular segment of the cadherin molecule extends into the intercellular space to seek contact with cadherin molecule from a neighboring endothelial cell [28]–[30]. The VE-cadherin connections are homophilic and regulated by calcium ions (Ca^{2+}), the presence of which is necessary for the stability of the adhesive junction between neighboring cells [30]. Additionally, formed VE-cadherin junctions are required for the proper organization of tight junctions, however the molecular basis of this interaction is not completely understood [32]. The VE-cadherin junction is supported from the cell interior by the actin cytoskeleton via catenin proteins (Figure 1) [33]. Due to its junctional function, VE-cadherin is, involved in regulation of microvasculature permeability, in which the higher presence of VE-cadherin-catenin complexes in the adherent junction is related to decreased permeability [28]. Furthermore, VE-cadherin is part of a junctional mechanosensory complex which mediates endothelial cell response to shear stress[34].
4.2.3. ZO-1

Endothelial-cell tight-junction proteins are required to create physical connections between adjacent cells. The groups of molecules responsible for the close connections between cells and composing tight junctions are claudins, occludins and junctional adhesion molecules (JAMs). These molecules are localized in the proximity of the apical side of endothelium (Figure 1) [23], [35], [36]. This group of proteins, orchestrates transport of molecules through the cell layer by tightening and loosening cell junctions depending on physiological stimuli [36]. Occludins undergo homotypic adhesion when linked to ZO-1 and link to the actin cytoskeleton either directly or indirectly through the proteins ZO-2 and cingulin. [19]. Shear stress regulates the expression, organization, and membrane positioning of occludin and ZO-1 [37].

4.2.4. PECAM-1

The endothelium is responsible for guiding transport of white blood cells, and one family of the molecular entities taking part in this process in blood capillaries are adhesion molecules (Figure 1), which enable contact between the endothelium and the white blood cell. Three major proteins involved in the process of cell adhesion to the endothelium are vascular cell adhesion molecule (VCAM-1), and the constitutively expressed intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule (PECAM-1) [38]. Same as VE-cadherin, PECAM-1 is part of a mechanosensory complex in the junctions that senses shear stress exerted on the endothelium[34].

![Figure 1. A schematic diagram representing an interstitial region between adjacent endothelial cells with tight and adherent junctions. Extracellular domains of tight junctional proteins like occludins, claudins and junctional adhesion molecules (JAM) make contact with tight junction proteins of neighboring cells. Their cytosolic parts are connected to Zonula occludens-1 (ZO-1), which is through a catenin molecule tethered to filamentous actin. Similarly, transmembrane VE-cadherin participates in homophilic interactions and is linked to F-actin through catenin. PECAM-1 is located both intracellular and in the cellular junction where it is a part of mechanosensory complex sensing shear stress.](image-url)
4.3. Material and Methods

4.3.1. Microchannel design and fabrication

The detailed description of the microchannel design used in this study is presented in Chapter 3. The microfluidic chip consists of a 10-mm cell culture chamber and two 5-mm-long fluidic resistors located between the chamber and the inlet/outlet (Ø 1 mm) (Figure 2). The height of the channels is 100 µm, and widths of the culture chamber in this work were 360 µm, 120 µm, and 80 µm. The associated inlet and outlet channels had widths of 180 µm (360 µm wide culture chamber), 60 µm (120 µm wide culture chamber), and 40 µm (80 µm wide culture chamber).

![Figure 2](A) Schematic design of the microfluidic channel used in this experiment. Fluids are introduced in the inlet, and are guided through a fluidic resistor. The resistor then opens up into a cultivation channel where the endothelial cells are cultured. The narrow outlet-channel serves to create back pressure. As a result, cells are retained in the culture channel for improved seeding efficiency. (B) Photograph of a microfluidic chip (5 parallel channels) with 3D-printed reservoirs (green) mounted on top the of inlets and outlets to the microchannels.

4.3.1.1. Master mold fabrication

The master mold fabrication procedure was performed in a cleanroom. Microchannels with rectangular cross-sectional profiles were fabricated with SU-8 50 photoresist resin (MicroChem Corp., MA, USA) on borofloat glass wafers (Borofloat 33 wafers: diameter 100 mm; thickness 0.7 mm; Handelsagentur Helmut Teller, Germany). SU-8 50 photoresist resin was spin-coated to a thickness of 100 µm with a spincoater CEE™ (Brewer Science; MO, USA). The soft-bake step for the photoresist layer was performed on a hot plate (precision hotplate & program controller PR 5-3T: Harry Gestigkeit GmbH, Germany) with the following temperature program: (i) heating with a ramp of 1°C per minute from 20°C to 65°C, (ii) followed by 10 min heating at 65°C, (iii) then the photoresist was heated at a ramp of 1°C per minute from 65°C to 95°C, and (iv) exposed for 30 min to 95°C. After cooling down of photoresist to room temperature (RT = 20°C), the coated wafers were exposed with photoresist layer facing collimated ultraviolet light (365 nm, power: 250 mJ/cm²) (collimated UV light source, OAI, CA, USA) for ~25 seconds. The post-UV-light-exposure baking step was performed as follows: (i) heating with a ramp of 1°C per minute from 20°C to 65°C, (ii) 1 min incubation at 65°C; (iii) the photoresist was then heated with a ramp of 1°C per minute from 65°C to 95°C, and (iv) heated for 10 min at 95°C. After cooling down the wafers to RT, they were developed for 15 min. in SU-8 developer (MicroChem Corp., MA, USA) until uncurved SU-8 50 residues were washed away. To extend the lifespan of the master molds, developed wafers were silanized with hexamethyldisilazane (HMDS; Sigma-Aldrich Co Ltd, UK) for 30 min. under the vacuum. For more details, see Chapter 3.
4.3.1.2. Microchannel replica molding in poly(dimethylsiloxane) (PDMS)

Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning Corp., USA), a pliable silicone rubber, was employed for replica molding of microchannels, based on its optical transparency, solvent compatibility and the biocompatibility with selected biological model and solutions [39], [40]. Microchannels were obtained by casting PDMS monomer mixed with PDMS curing agent (Dow Corning Corp., USA) (PDMS elastomer : curing agent 10:1 (w/w)) on the SU-8 50 master mold and curing it at 62°C for 2h (approximately 70 g of PDMS per wafer, final thickness of the obtained PDMS chip was approximately 3 mm). 3D-printed frames were placed on the master, around the protruding SU-8 structures, prior to casting. This was done to aid in peeling off the PDMS rubber after curing and thereby extend the lifespan of the SU-8 50 master molds. The temperature for curing PDMS was lower than recommended [40], because the 3D-printed frames were made from polylactic acid, which becomes soft above 62°C. Holes for inlets and outlets were punched with biopsy punchers (Kai punchers; D-Care B.V., The Netherlands) with a diameter of 1 mm. The microchannels in PDMS were sealed irreversibly to glass coverslips (24 x 24 x 0.17 mm; Fisherbrand™ Borosilicate Glass Square Coverslips, Thermo Fisher Scientific) by exposure of both parts to oxygen plasma (30W; 350-380 mTorr; 20 s) (Harrick Plasma Cleaner; Harrick Plasma, USA). Directly after plasma treatment, the microchannel structures were brought into contact with the glass coverslip and pressed against it. Irreversible bonding occurred after a few minutes. 3D-printed liquid reservoirs (polylactic acid, more details in Chapter 3) were attached to the chip over the punched reservoirs with silicon glue (Silicon Rubber Compound – Flowable Fluid, RS 692-542; The Netherlands). All steps performed after oxygen plasma treatment were performed in a flow cabinet to ensure chips were sterile for experiments. For more details on microchannel fabrication see Chapter 3.

4.3.1.3. Gelatin coating protocol

After PDMS bonding to the cover slips, a sterile solution of 1% porcine gelatin (Sigma code G9382; Sigma Aldrich Co Ltd, UK) diluted in a sterile and filtered phosphate-buffered saline (PBS; pH=7.4) (Sigma-Aldrich Co Ltd, UK) was introduced to the channels and incubated at RT for 45 min. Afterwards, cross-linking with 0.5% glutaraldehyde solution (Polysciences Europe GmbH, Germany) was performed in the microchannels. The reaction was carried out at RT for 15 (360-µm-wide channels), 10 (120-µm-wide channels), and 6 min. (80-µm-wide channels) (for more detail see Chapter 3). Next, the microchannels were rinsed three times (3x) with sterile PBS to remove possible residues of glutaraldehyde with 10 min. incubation at RT in between consecutive rinses. The last preparation step was chip conditioning with endothelial cell medium (EC medium; composition below) in a cell incubator (37°C, 95% air, 5% CO₂) (Thermo, model 3111, USA) for approximately 1h before cell seeding. During all the described preparation steps it is important to protect inlet/outlet of the channel from drying by depositing an extra fluid on top of them, otherwise air bubbles could be introduced in the channels. All the described steps are performed with a 1 mL syringe (Omnifix-F B Braun, The Netherlands). For more details see Chapter 3.

4.3.2. Flow system development

4.3.2.1. Components of the system

The system for medium perfusion through the microchannels consisted of a sterile syringe with Luer-Lock (syringe volume: 20 mL; Omnipix F Braun B.V.; The Netherlands) filled with cell medium and installed on a syringe pump (Prosense Ne-1000; Prosense B.V., The Netherlands). A needle with luer lock (outer diameter 0.8 mm, Henke Sass Wolf GmbH, Germany) was connected to the syringe and its sharp end was inserted into piece of Teflon tubing (PTFE-tubing 1/16”OD; 0.8 x 1.6 mm [internal diameter x outer diameter]) (Polyfluor Plastics B.V., The Netherlands) (Figure 3) A metal cylinder
was attached to the other end of the tubing (outer diameter: 0.8 mm) was attached in order to facilitate connection of the tubing to the PDMS chip. The metal cylinders were obtained by cutting off 6 mm long pieces from needles (outer diameter: 0.8 mm) (Henke Sass Wolf GmbH, Germany) and polishing them with a sand paper prior the attachment in the Teflon tubes. Finally, the metal cylinders were inserted into the inlets/outlets of the microchannels.

Figure 3. (A) An overview of the flow system applied in the experiments which consists of a syringe pump, tubing connecting the microfluidic chip with a syringe filled with cell culture medium and a waste container. (B) Enlarged view of a microfluidic chip connected to the system with tubing. The chip is mounted in 3D-printed holders to improve stability of the setup.

4.3.2.2. Teflon tubing sterilization

Teflon tubing was sterilized before and after every experiment with 70% ethanol (Sigma Aldrich B.V.; The Netherlands) for 30 min. Afterwards, three consequent rinses with a sterile PBS solution were performed. Sterilized Teflon tubing was stored in a sterile box prior to the application in the experiments.

4.3.2.3. Flow setup

Sterile syringes (20 mL) were filled with Lonza cell medium and pre-warmed in an incubator (95% air and 5 % CO₂ at 37°C) (Thermo, model 3111, USA) for approximately one hour (1h) to equilibrate the temperature and dissolved gases. Afterwards, the Teflon tubes were connected to pre-warmed syringes with cell medium and mounted in the syringe pumps (Figure 4). The syringe pumps were programmed and set to apply flow with the desired shear stress in the microchannels and started before the tubing was connected to the chips. Prior to connecting tubing to the microchannels, it was ensured that cell medium had completely filled the tubing and all air bubbles were removed from the tubing. When the drop of excess medium was formed at the end of metal cylinder, the tubing was immersed in the 3D-printed reservoir with cell medium and positioned in the channel inlet. PDMS sealed around the metal cylinder to ensure leakage-proof connection, thus perfused medium travelled through the channel instead of upwards to the inlet reservoir (Figure 4). Afterwards, the outlet tubing filled with medium was attached to the chip and the free end was immersed in a beaker filled with a couple of milliliters of cell medium, which acts as a waste container for used medium. Disconnection of the system components was performed in reversed order (from the last step to the first) (Figure 4).
4.3.3. HUVEC culture
4.3.3.1. Culture conditions
Cells, during all experiments, were cultured in Lonza cell medium (the composition is described below) in an incubator under 95% air and 5% CO\textsubscript{2} at 37°C (Thermo, model 3111, USA). In parallel to all performed experiments in microchannels, cell culture in a standard 96-well-plate was carried out as a reference experimental setup.

4.3.3.2. Cell medium
Lonza EGM-2 MV medium (CC-3202) (Lonza Group Ltd., Switzerland) supplemented with BulletKit from Lonza (CC-3156 & CC-4147) (Lonza Group Ltd., Switzerland) was used for HUVEC culture.

4.3.3.3. Cell culture in T25 bottles and cell harvest
Lonza HUVEC (CC-2519) were cultured to confluence in T25 flasks for at least two passages prior to being seeded in the microchannels at UMCG Endothelial Cell Facility. For this work HUVEC between passages 2 and 5 were used [41]. HUVEC were removed from the T25 bottles by
trypsinization (0.05% trypsin solution in sterile PBS, 20 µL of trypsin solution per cm² of cell culture), for approximately 2 min at 37°C, and collection in an Eppendorf tube. Next, the collected cells were counted in a Neubauer chamber, centrifuged (1800 rpm, 5 min.) (Rotina 48S, Hettich GmbH, Germany) and re-suspended in cold (4°C) Lonza medium to a concentration of 5000 cells/µL. HUVEC were kept on ice (4°C) until the seeding procedure. Before every cell injection into the microchannel, the cell suspension was gently shaken in the Eppendorf tube to re-suspend cells. This action ensured an even cell concentration in all injected suspension volumes.

4.3.3.4. Cell seeding

The chips were pre-warmed in the cell incubator and the channels were conditioned with medium for at least 1h. Suspended HUVEC were injected with an Eppendorf Research plus pipette (0.5 – 10 µL) (ref. number 3120000020; Eppendorf, Hamburg, Germany) into the chips at a concentration of 5000 cells/µL. Injected cell volumes were experimentally optimized in Chapter 3 (Table 1). Approximately one hour (1h) after cell seeding, the channel was washed by flowing a volume of approximately 50 µL of cell culture medium through the channel with a 1-mL-syringe to remove unattached cells, and devices were placed into the incubator for another two hours, in order to allow cells to firmly adhere to the surface and create cell-cell connections.

<table>
<thead>
<tr>
<th>Channel width</th>
<th>µm</th>
<th>360</th>
<th>120</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell concentration</td>
<td>cells/ µL</td>
<td>5000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injected volume of cell suspension per channel</td>
<td>µL</td>
<td>8</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

4.3.3.5. Flow application to the cell culture (programs and shear stress calculations)

Approximately three hours (3h) after seeding the cells in the microchannels, the microfluidic chips were inspected under the microscope (DM-IL, Leica Microsystems, Wetzlar, Germany) and a confluency level was estimated. For the flow experiments, only microchannels exhibiting cell culture confluency above 80% were used. This level of confluency is demanded for flow application in microchannels to avoid damaging the cell culture [42]. After channel selection, flow was applied by connection of the syringe pump to the chip. For calculation of the flow rates required to obtain selected shear stresses during the experiments, Equation 1 was used. The obtained values of shear stresses are presented in the Table 2.

Equation 1 [43]:

\[
\tau = \frac{6 \cdot \eta \cdot Q}{h^2 \cdot w}
\]

\[
Q = \text{flow rate in cm}^3/\text{s}
\]

\[
\eta = \text{viscosity of Lonza EC Medium (ca. 0.0075 dyn * s/cm}^2\]

\[
h = \text{channel height (cm)}
\]

\[
w = \text{channel width (cm)}
\]

\[
\tau = \text{wall shear stress (dyn/cm}^2\)
Table 2. An overview of flow rates and corresponding shear stresses applied in the experiments.

<table>
<thead>
<tr>
<th>Shear stress (τ) (dyn/cm²)</th>
<th>Applied flow rate (µL/min) in channel with the width of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80 µm</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>9.02</td>
</tr>
<tr>
<td>5</td>
<td>23.04</td>
</tr>
<tr>
<td>10</td>
<td>46.11</td>
</tr>
</tbody>
</table>

In the experiments presented, HUVEC were cultured in microchannels under the shear stress of interest (0, 2, 5, 10 dyn/cm²) for a period of 20h. Afterwards, the flow was stopped, tubes were disconnected from the chips, cells were inspected under the microscope, and fixed in the microchannels before further experimental steps (protocols below).

4.3.4. Imaging

4.3.4.1. Cell fixation protocol

PBS solution was autoclaved and filtered afterwards in order to ensure the removal of PBS crystals, the presence of which in microchannels would disturb good-quality image acquisition by scattering light and blurring the picture. For PBS filtration, the Millex-GP syringe filter unit (0.22 µm) from Merck Millipore (USA) was used. All the described steps were performed with a 1 mL syringe (Omnifix B Braun B.V.; The Netherlands).

After 20h of HUVEC culture under shear stress, the cells in microchannels were washed with pre-warmed to 37⁰C PBS. Immediately after, HUVEC were fixed in the microchannels by incubation with a solution of 4% paraformaldehyde (Sigma Aldrich Co Ltd, UK) and 4% sucrose (Sigma Aldrich Co Ltd, UK) in PBS at 37⁰C for 3 min. The paraformaldehyde was used to fixate HUVEC by creation of cross-links between all encountered proteins. It thus inactivates the enzymes involved in energy metabolism, which results in cell death. The purpose of sucrose addition to the fixative solution is to ensure sufficient osmolality to fixed cells to prevent them from collapsing and membrane disruption during multiple rinses with different solutions. Afterwards, cell culture microchannels were washed three times (3x) with PBS with 5 min. incubations steps at 37⁰C between consecutive washes. To facilitate the staining of intracellular molecules, the cell membrane was next perforated (permeabilized) with 0.3% Triton X-100 (Sigma Aldrich Co Ltd, UK) for 5 min at RT. The cells were then rinsed three times (3x) with PBS with 5 min. incubations steps at RT between consecutive washes. Next, a 5% bovine serum albumin solution (5% BSA) (Sigma Aldrich, UK) in PBS was introduced to the microchannels in order to block channel regions not covered by cultured HUVEC. This prevents non-specific antibody attachment to unoccupied surfaces, which will appear under the microscope as a false positive signal. Cell culture blocking with 5% BSA solution was performed at RT for a period of one hour (1h). Blocked microchannels were rinsed three times (3x) with PBS with 5 min. incubations steps at RT between consecutive washes. HUVEC cultures fixed using this protocol were stored before start of the staining procedure for a period of up to two weeks at 4⁰C in PBS solution.
4.3.4.2. Cell staining protocol

HUVEC cultures in microchannels were stained for selected membrane molecules (VE-cadherin, PECAM, and ZO-1) or actin cytoskeleton for a period of ninety minutes (90 min.) at RT. The list of staining reagents and dilutions is presented below (Table 3). Afterwards, three washing steps with PBS were performed with 10 min incubation periods between consecutive washes. To visualize cell nuclei, the 4',6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich Co Ltd, UK) solution in PBS (1:150) was introduced to the microchannels. After a 10 min incubation with DAPI, three rinses with PBS were performed for every microchannel with a 1 mL syringe to remove the excess of DAPI solution. All the described steps were performed with 1 mL syringe (Omnifix B Braun B.V.; The Netherlands). The PBS volume used for every rinse was approximately 50 µL.

For staining membrane proteins and the actin cytoskeleton, fluorescent staining methods were employed. Molecules with high selectivity for the biological targets were combined with a fluorophore. In this work, staining reagents with Alexa Fluor Alexa Fluor 488 or Alexa Fluor 594 were used, which emit green or red fluorescent light respectively. All the applied staining reagents were directly combined with a fluorescent probe (Table 3), thus the staining reaction did not require secondary antibodies to visualize binding to cell molecules. By the application of the single-step staining, we reduced the number of operations performed in the microchannels. The photobleaching of fluorophores was reduced by injection of Prolong Gold Antifade Mountant (Thermo Fisher Scientific Inc., USA) into the microchannels before inspection under a confocal microscope.

Table 3. Staining molecules with fluorophores combined with them.

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Phalloidin</td>
<td>AF488; AF594</td>
<td>490; 590</td>
<td>525; 617</td>
<td>1:100</td>
<td>Thermo Fisher Scientific A12379; A12381</td>
</tr>
<tr>
<td>VE-cadherin Antibody</td>
<td>AF488</td>
<td>490</td>
<td>525</td>
<td>1:40</td>
<td>Thermo Fisher Scientific 53-1449-42</td>
</tr>
<tr>
<td>PECAM-1 Antibody</td>
<td>AF488</td>
<td>490</td>
<td>525</td>
<td>1:40</td>
<td>Thermo Fisher Scientific MA5-18135</td>
</tr>
<tr>
<td>ZO-1 Antibody</td>
<td>AF594</td>
<td>590</td>
<td>617</td>
<td>1:40</td>
<td>Thermo Fisher Scientific 339188</td>
</tr>
<tr>
<td>DAPI</td>
<td>DAPI</td>
<td>350</td>
<td>470</td>
<td>1:150</td>
<td>Sigma Aldrich D9542</td>
</tr>
</tbody>
</table>

AF – Alexa Fluor
4.3.4.3. Imaging of the microfluidic cell cultures with confocal microscopy

Imaging of stained HUVEC in microchannels was performed with the Confocal Laser Scanning Platform Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany). For the acquisition of the images presented in this work, an immersion objective with magnification of 63 times was used [44]. Prior to sample positioning in the microscope holder, a drop of immersion oil (refractive index 1.4811 at 23⁰C and 546 nm) (Cargille immersion oil Type FF, Cargille Labs, USA) was deposited on the microscope objective. Afterwards, the microfluidic chip was mounted in the holder and brought into contact with the drop of immersion oil on the objective. Images were acquired with Leica Application Suite X (LAS X) software (Leica Microsystems, Wetzlar, Germany). To acquire images, caption spots were selected inside the microchannels, and scans of samples approximately 5 µm thick (±3 µm) were acquired with a Z-step resolution of 0.5 µm from the bottom to the top of the channel. Therefore, every presented image is a composition of approximately 10 overlaid images (±6 images). Prior to each image acquisition LAS X software was used to automatically adjust signal saturation. Laser diodes with wavelengths of 405 nm; 488 nm, and 552 nm were used for sample excitation. The applied procedure was based on reference [45].

4.3.5. Image processing

The acquired microscopy data was processed using Imaris software v 7.6.4 (Bitplane AG, Zurich Switzerland). The recorded data was uploaded to the Imaris program and displayed with the Easy 3D option. Afterwards, color saturation was checked for every color channel present in the image and adjusted with the purpose of removing over-saturated regions from the image. After the color adjustment, snapshots were taken of acquired images and saved in a Tagged Image File Format (TIFF).
4.4. Results and Discussion

4.4.1. Flow system development

We have developed a system which allows HUVEC to be cultured in channels as narrow as 60 µm (Chapter 3). The decreased size of the cultivation channel width means that a lower number of cells is required to obtain a confluent cell layer in the device. In turn, this means that there is less biological material available for the chemical analysis during or after the experiment. Therefore, in *in vitro* microvasculature research analysis is mostly limited to optical analysis. One advantage that planar transparent glass or silicone rubber devices offer is easy visualization of the micrometer-sized channels and their content by (fluorescence) microscopy. Moreover, microscopy encompasses a popular analytical methods for *in vitro* determination of induced changes in cells such as morphological adaptations, composition of cell membranes and their surface molecules (glycocalyx) or cellular junctions.

4.4.2. Filamentous actin

Figure 5 shows a reference experiment, in which HUVEC were cultured in a well plate, absent of flow, and Figure 6 shows HUVEC in our microchannels. It demonstrates how actin fibers respond to channel geometry and applied flows. The main observation is that the microfilaments align in the direction of flow. Additionally, threshold for actin responsiveness of the HUVEC to the shear stress, seems to be independent of the channels width. We found a shear stress of minimally 5 dyn/cm² is required to induce actin fiber alignment. Actin fibers of HUVEC cultured on the well plate (Figure. 5) look similar to the results obtained for the static condition (0 dyn/cm²) and the shear stress of 2 dyn/cm² (Figure 6), regardless of channel dimension. Our observations are in agreement with existing data about actin changes in HUVEC cultured under flow [46]–[48].

Our results indicate that shear stress has a more profound influence on microfilament alignment than channel geometry, at least in the tested ranges. Interestingly, we observed no formation of focal adhesion points in HUVEC cultures in the microchannels in the absence of flow. Focal adhesion points are spots with high, local concentrations of adhesion protein, which are meant to anchor the cells to the ECM. Unlike in the microchannels, focal adhesion point were found in the cultures in well plates (Figure 5). This observation indicates a possible influence of channel geometry on the distribution of adhesion proteins, connected to actin. An uneven distribution of these proteins would lead to the development of focal adhesion points, which suggests that the distribution of anchoring proteins in the cell membrane is more even in the microchannels cell culture. In a study by Esch et al., vinculin, which is an adhesion protein, was monitored with confocal fluorescent microscopy in endothelial cell cultures in microchannels. They observed a higher expression of vinculin and a more even distribution in microchannels in comparison with a well plate culture [49], which supports our findings.
Figure 5. Image of HUVEC cultured for 24h on a 96 well plate (diameter of the well was 5 mm). Actin fibers were randomly distributed in all directions within cell borders. Brighter spots (indicated with red arrows) are defined as focal adhesion points, where individual cells gathered adhesion proteins anchoring them to the ECM on the surface of the well [50]. Actin fibers stained by phalloidin are shown in green and cell nuclei stained by DAPI in blue. The scale bar in the bottom left corner is 30 µm long.

Figure 6. Confocal images showing HUVEC cultures in channels with widths of 360 µm, 120 µm, and 80 µm under different shear stresses (0, 2, 5, and 10 dyn/cm²) for a time period of 20h and stained for actin (green) and cell nuclei (blue). There is a noticeable increase in actin fiber alignment in the direction of the flow (blue arrows) for HUVEC exposed to shear stresses of 5 and 10 dyn/cm². In the case of the first two flow conditions (0 and 2 dyn/cm²), microfilament distribution was similar to that observed in the static HUVEC culture in the well plate (Figure 5). Yellow bars show the microchannel walls, and the scale bar in the bottom left corner is 30 µm. Images of HUVEC cultures in 360-µm-wide channels were taken in the middle of the channel, thus side walls are not visible.
4.4.3. VE-cadherin

In our study, we compared VE-cadherin localization between HUVEC cultured on the well plates (Figure 7) with HUVEC cultured in microchannels with different widths exposed to a range of shear stresses (Figure 8). We observed preferred localization of VE-cadherin in cell membranes for all culture conditions. However, for HUVEC cultured in channels of 80- or 120-µm-wide under static conditions or shear stress of 2 dyn/cm², VE-cadherin was distributed unevenly across the cell rim (n=3). This effect was not observed in the well plate culture or channel of 360-µm-wide (n=3). For HUVEC in the microchannels of 120-µm-wide with shear stress of 5 or 10 dyn/cm² (n=3) the distribution of VE-cadherin in the membrane was even (Figure 8). Data for 80-µm-wide channels with shear stress of 5 and 10 dyn/cm² were not obtained. The reason for uneven VE-cadherin distribution is unclear, but might be related to a combination of the endothelial cell response to a lack of flow, and the microgeometry of the channel. However, the shear stress impact on cells in microchannels is visible and shows increased preference of VE-cadherin towards the membrane rim in narrower channels under higher shear stresses, whereas VE-cadherin distribution in HUVEC cultured in wider channels remained similar this from the well plate culture.

Figure 7. Image of HUVEC cultured for 24h on a 96 well plate (dimension of the well was 5 mm). VE-cadherin connections are showing cell membrane borders and connections between the cells. VE-cadherin is shown in green and cell nuclei in blue, scale bar in the bottom left corner is 30 µm.
Figure 8. Confocal images showing the HUVEC cultures in channels with widths of 360 µm, 120 µm, and 80 µm under different shear stresses (0, 2, 5, and 10 dyn/cm²) for a time period of 20h and stained for VE-cadherin (green) and cell nuclei (blue). For channels 120 µm and 80 µm wide and no shear stress or shear stress of 2 dyn/cm², we observed uneven distribution of VE-cadherin in HUVEC membranes (red arrows). An even VE-cadherin distribution in cell membranes was observed for channels 360 µm wide independent of shear stress condition and for 120 µm wide channel under the shear stress of 5 dyn/cm² and 10 dyn/cm². Data from cells cultured in 80-µm-wide channels under shear stresses of 5 and 10 dyn/cm² was not obtained. Yellow lines indicate the location of microchannel walls, and scale bar in the bottom left corner is 30 µm for all images. Images of HUVEC cultures in 360-µm-wide channels were taken in the middle of the channel, thus side walls are not visible.
4.4.4. PECAM-1

We have investigated the influence of channel width and shear stress on PECAM-1 distribution in HUVEC. This molecule is specifically responsible for white blood cells arrest during diapedesis, or leukocyte migration across the endothelium [51]. Additionally, PECAM-1 expression in HUVEC is upregulated by a shear stress application to cultured cells [52]. Under normal conditions in cell culture, PECAM-1 is constantly redistributed within the cell membrane (Figure 9) until the moment of leukocyte adhesion. In the case of white blood cell adherence to the endothelium, homophilic connections between PECAM-1 molecules on the endothelial cell and leukocyte membranes are created, and transmigration of leukocyte between endothelial cells occurs [53]. As already mentioned, PECAM-1 has been shown to be responsive to shear stress [22], [54]. In addition, it was interesting to observe PECAM-1 responses to different shear stresses in channels with different widths. Our results show that an increasing signal intensity coming from PECAM-1 molecule focused in cell-cell connecting region as the magnitude of the applied shear stress increased in the microchannels (n=3 per condition) (Figure 10). Application of the microscopy method does not allow a quantitative answer to be given to the question of whether the increased signal is a result of transcriptionally controlled PECAM-1 expression or protein gathering/accumulation in the junctional regions. Confirmation of this observation has to be obtained by independent quantitative methods (e.g. QRT-PCR, ELISA, Western Blotting).

![Image of HUVEC cultured for 24h on a 96 well plate (dimension of the well was 5 mm). PECAM is concentrated in the regions of cell-cell connections. PECAM is stained in green and cell nuclei in blue; scale bar in the bottom left corner is 30 µm.](image-url)
Figure 10. Confocal images showing HUVEC cultures in channels with widths of 360 µm, 120 µm, and 80 µm under different shear stresses (0, 2, 5, and 10 dyn/cm²) for a time period of 20h and stained for PECAM (green) and cell nuclei (blue). For the HUVEC culture in the channels we observed an increasing signal intensity coming from PECAM membrane localized in the cell-cell connecting regions with increasing shear stress applied to the culture. Yellow lines indicate the microchannel walls, and the scale bar in the bottom left corner is 30 µm. Images of HUVEC cultures in 360-µm-wide channels were taken in the middle of the channel, thus side walls are not visible.
4.4.5. ZO-1

In this work, we stained *Zonula occludens* (ZO-1) which plays a crucial role in tethering other junctional proteins with actin fibers in the cytoplasm (Figure 1) and ensuring stability of the junction complex [23]. Thus far, data has been obtained for 360-µm-wide channels. These preliminary results show a stronger preference of ZO-1 in membrane regions of HUVEC in the well plate and the microchannel under static conditions compared to microchannels with flow (Figure 11 and Figure 12). Data for 120- and 80-µm-wide channels were not obtained.

![Image of HUVEC cultured for 24h on a 96 well plate (dimension of the well was 5 mm). ZO-1 proteins is concentrated in the regions of cell-cell connections. ZO-1 is shown in red and cell nuclei in blue, scale bar in the bottom left corner is 30 µm.](image1)

![Image of HUVEC cultures in 360 µm wide channels under different shear stresses (0, 2, 5, and 10 dyn/cm²) for a time period of 20h and stained against ZO-1 (red) and cell nuclei (blue). Images of HUVEC cultures in 360-µm-wide channels were taken in the middle of the channel, thus side walls are not visible. Data from 120 µm, and 80 µm wide channels was not obtained, because of staining protocol incompatibility with the devices with given dimensions.](image2)
4.4.6. Conclusions and future direction of this research

In this work we developed a continuous flow system for microfluidic HUVEC cultures in channels with varying widths. We confirmed the applicability of these devices for HUVEC under flow conditions with confocal fluorescent microscopy. We looked at cellular responses in terms of morphology and cellular interactions to different levels of shear stress and channel widths. Actin fibers aligned at increasing intensities in the flow direction and at a higher level of fiber organization than under static condition. This effect was observed for all tested channel widths. This indicates that HUVEC exposed to a shear stress exhibit more in vivo-like phenotype with regards to the actin cytoskeleton. The adherens junction protein VE-cadherin, was located at increasing densities of VE-cadherin in cell-cell connections in HUVEC cultured under shear stresses above 5 dyn/cm². While, the results indicate that channel dimensions also have influence on the distribution of VE-cadherin, more research is required in order to understand the underlying mechanism. Additionally, PECAM-1, the adhesion molecule responsible for leukocyte arrest on endothelial surfaces, accumulated in cell junctional regions in HUVEC exposed to shear stresses of 5 and 10 dyn/cm². This was observed in all tested channel widths. Finally, preliminary results suggest that ZO-1, which is involved in the formation and the maintenance of tight junctions, shows decreased localization in the junctions upon an application of shear stress in channels of 360 µm wide. More experiments are required to verify this observation. These combined results show that especially a shear stress has a profound effect on the localization and distribution of proteins in cell junctions, additional research is required in order to understand this behavior.

For future work, it would be interesting to increase the level of applied shear stresses to simulate physiological situations in different types of blood vessels, as well as further decrease channel diameter. In addition to observations made with confocal fluorescent microscopy, gene expression studies (e. g. Q-RT-PCR) should be performed to obtain in depth information to understand HUVEC responses to different channel widths and applied shear stresses.

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4.6. References


Chapter 4


The image contains a reference list with bibliographic information for various scientific publications. Each entry includes authors, titles, journals, volumes, issue numbers, page ranges, and publication years. The references cover topics such as endothelial adherens junctions, cell-cell tension, angiogenesis, permeability, and other related fields in cell biology and physiology. The entries are formatted in a standard citation style, likely for an academic or research context. The references span a range of years from 2005 to 2015, indicating a contemporary focus on the subject matter.


PART III
MONITORING OF MICROMOTION WITH A NOVEL LABEL FREE APPROACH