Monitoring endothelial cells in microfluidic systems
Grajewski, Maciej

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Chapter 3

Geometry optimization of microfluidic channels for endothelial cell cultures

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Abstract

The growing interest in microfluidic endothelial cell culture has resulted in a number of devices facilitating the in vitro investigation of the behavior of these vascular cells. With microchannels generally on the order of hundreds of micrometers in diameter, these devices are not optimal for mimicking the microvasculature, in which the endothelium is highly responsive to inflammatory stimuli. Channels with sufficiently small width are needed, and perhaps more importantly, robust strategies and protocols on how to reproducibly work with endothelial cells in such challenging geometries. In this report, we describe the optimization of microchannel geometry and protocols for endothelial cell cultures in narrow microchannels for the use in the context of cardiovascular research.

The goal of this work was to develop a channel geometry for efficient and reproducible cell seeding in microchannels as narrow as 60 µm. Decreased channel widths, as compared to previously published approaches, allow better recreation of the in vivo dimensions of human microvasculature. Three different channel geometries to optimize the cell seeding process were considered in this study. The first channel design consisted of a straight channel with fluid reservoirs; the second design was shorter and had smaller reservoirs than the first channel design; in the third channel design, narrow channels serving as fluidic resistors were introduced between the cultivation channel and fluid reservoirs, but otherwise had the same dimensions as the second channel design. The third channel design led to the most consistent cultivation parameters (good cell density and even cell distribution, both of which are determined during the cell seeding process). The resistors in this design lead to improved retention of the introduced cell suspensions in the microchannels during cell seeding, and therefore to more consistent cell seeding and culture. Robust protocols were then established for coating, controlled seeding, cultivation, fixation and staining of Human Umbilical Vein Endothelial Cells (HUVEC) in these microchannels. The HUVEC concentration used was 5000 cell/µL. Volumes seeded ranged from 3 µL for 60 µm wide channels to 15 µL for 360 µm wide channels.

This work provides a complete description of chip fabrication and preparation, as well as cell culture protocols. Finally, we discuss the impact of channel geometry on cell seeding in microchannels.

Keywords: Microfluidic cell culture, Channel geometry, Primary endothelial cells
3.1. Introduction

The endothelium is of great scientific interest, because of its involvement in numerous (patho-)physiological processes, including macro- and micromolecular transportation to and from organs, blood coagulation, and blood pressure regulation in the human body [1]. Conventional \textit{in vitro} culture has generally involved culturing endothelial cells in 2D layers on the bottom of well plates. Physiological conditions in blood vessels are vastly different from those offered by standard flasks and well plates in cell culture laboratories. Currently, we observe progress in the adaptation of endothelial cell culture conditions towards better resemblance of the \textit{in vivo} status [2]–[4]. Human vasculature is heterogeneous with different functions and dimensions [1], [5]–[7]; the use of \textit{in vitro} systems tailored to a specific vasculature region might benefit the study of endothelial cells from that region [2]. Microfluidics offers a solution in this regard by enabling the fabrication of channels and channel networks with the desired dimensions, branches, and curvatures, and by allowing the application of shear stress, to which endothelial cells are exposed naturally, in a controlled fashion [2], [8], [9].

Over the past two decades, numerous microfluidic cell culture systems have been developed for biomedical research [3], [8]–[11]. This interest has resulted in the application of microfluidic devices in cardiovascular research has led to a discussion about whether and how channel geometries and dimensions affect cellular behavior both \textit{in vitro} and \textit{in vivo} [2], [4]. Since the vascular bed consists of vessels with lumen dimensions varying from centimeters (in the aorta) to micrometers (in the organs), its adequate representation in \textit{in vitro} cultures requires a variety of culture devices with dimensions corresponding to those occurring in \textit{in vivo}. In reality, however, we find that to date, most of the (commercially) available microfluidic devices are straight channels with widths ranging from 5 mm to 400 µm. Moreover, many of these \textit{in vitro} models employ larger channel dimensions to facilitate translational and fundamental studies, because they are easier to work with and result in a larger amount of material for analysis [2], [9], [12]. Until now, little research has been dedicated to the \textit{in vitro} study of endothelial cells in microchannels that actually mimic the microvasculature from a geometric perspective.

A second important parameter for reproducing \textit{in vivo}-like \textit{in vitro} systems is controlled perfusion of endothelial cell layers, as demonstrated for the first time by Fernandez \textit{et al.} [13]. Significant discrepancies between static and perfused systems were demonstrated by checking gene expression profiles of endothelial cell markers from cells cultured in both types of systems. Moreover, research performed with perfusable microfluidic devices for endothelial cell cultures have helped address many questions related to the role shear stress in controlling cell morphology, cell alignment, cytoskeletal regulation, gene and protein expression [12], [14]–[16].

However, there is a lack of standardized channel geometry which allows closer scrutiny of the human microvasculature (100 - 500 µm) \textit{in vitro} [3]. Interesting work attempting to culture endothelial cells in narrow channels was performed by Esch \textit{et al.}, in which the influence of microchannel shape (rectangular vs. circular) was assessed for endothelial cells (HUVEC) [17]. Esch \textit{et al.} managed to seed HUVEC in channels as narrow as 50 µm, their protocol, however, did not describe the seeding procedures and injected cell suspension volumes in full detail [17].

In this work, we pursue the development of a channel geometry which allows endothelial cell cultures to be set up and maintained in channels significantly narrower than 400 µm. Additionally, we improve the seeding efficiency in our microchannels, while avoiding the injection of large numbers of cells. In doing so, we address the issue of uneven cell distribution along the length of the microchannels, which is a result of the seeding procedures and, as observed in this study, causes problems in obtaining
a confluent endothelial cell layer. The above-mentioned issues result in a loss of cells, time, and money. This can be especially problematic when only limited numbers of cells are available, as is the case when experimenting with primary cells from e.g. patients’ biopsies or transgenic cells harvested from animals [18].

In this report, we present an optimized channel geometry which allows reproducible seeding of a primary endothelial cell type obtained from umbilical cord veins (HUVEC) in microchannels with dimensions ranging from 360 µm down to 60 µm using a single experimental protocol. Additionally, we optimized cell concentrations and the cell suspension volumes required to obtain even seeding along microfluidic channels having lengths in the order of 1 to 2.5 cm.

3.2. Materials and Methods

3.2.1. Microchannel/chip design and experimental setup

All microchannel designs were drawn in CleWin, a mask layout editor (MESA Research Institute, University of Twente and Deltamask, The Netherlands), and printed as negative masks by ProArt BV (The Netherlands). The negative masks used in this work contain the inverse (or photographic "negative") of the pattern to be transferred to a wafer covered with negative photoresist (procedure below). The channel diagrams and tested dimensions are presented in Table 1, with total channel volumes in Table 2. Three channel designs were tested: (i) Design 1 is the initial design and comprises a straight microchannel of uniform cross-section and a length of 25 mm; (ii) Design 2 differs from Design 1 in that it has a shorter channel length of 10 mm. Additionally, the diameter of inlets/outlets was decreased from 2.5 mm to 1 mm; (iii) Design 3 consists of a cultivation channel with inlet and outlet channels (fluidic resistors, function explained below) having widths half that of the cell cultivation channel. Every fluidic resistor is 0.5 cm long, while the cell cultivation channel is 1 cm long. Additionally, the third design (Design 3) of the microchannels has 3D-printed reservoirs made in polylactic acid (PLA) (Figure 1), which facilitate coupling of syringe pumps to the device, as well as ensure that cells are in continuous contact with cell medium during the experiments. All channels, independent of design, have a depth of 100 µm determined by the velocity used to spin coat a UV-curable resin during the fabrication of device masters (described below). Additional parts like liquid reservoirs and chip holders were designed in Solid Works (Waltham, MA, USA) and 3D-printed in polylactic acid (PLA) (filament thread diameter = 1.75 mm) (EasyFil, Formfutura, The Netherlands) with a Felix v3.0 (nozzle diameter = 0.35 mm) (Felix printers, the Netherlands) (Figure 1).
Table 1. Overview of the different microfluidic devices made and tested.

<table>
<thead>
<tr>
<th>Sketch</th>
<th>Design 1</th>
<th>Design 2</th>
<th>Design 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivation channel length [mm]</td>
<td>25</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Diameter of inlet/outlet holes [mm]</td>
<td>2.5</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Channel depth [µm]</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Tested widths [µm]</td>
<td></td>
<td>360, 120, 100, 80, 60</td>
<td></td>
</tr>
</tbody>
</table>

1. Inlet/outlet channel; 2. Cultivation channel

Table 2. Overview of the total volumes of the tested microchannels

<table>
<thead>
<tr>
<th>Channel width [µm]</th>
<th>Channel volumes [nL]</th>
<th>Design 1</th>
<th>Design 2</th>
<th>Design 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>360</td>
<td>900</td>
<td>360</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>300</td>
<td>120</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>250</td>
<td>100</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>200</td>
<td>80</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>150</td>
<td>60</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. (A) Chip Design 3 with 3D-printed reservoirs (green) mounted over the inlets/outlets to the microchannels; (B) Experimental setup mounted on a microscopic stage, where the original microscope table inset was replaced by a 3D-printed inset (pink) to provide stable positioning of the chip holder with microfluidic devices (green).
3.2.2. Microchannel fabrication

Microchannels with rectangular cross-sectional profiles were replicated from molds made using SU-8 50 photoresist (MicroChem Corp., MA, USA) on borofloat glass (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) maintained for 30 min at 95°C. After cooling down the photoresist to room temperature (20°C ), the photoresist coated wafers were exposed through the mask with ultraviolet light (365 nm, power: 250 mJ/cm²) (collimated UV light source, OAI, San Jose, CA, USA) for 25 seconds. The post-UV-light-exposure baking step was performed as follows: (i) wafers were heated with a ramp of 1°C per minute from 20°C to 65°C, (ii) followed by 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 room temperature, they were developed for 15 min in SU-8 developer (MicroChem Corp., MA, USA) until unexposed SU-8 50 residues were washed away. To extend the lifetime of the molds, developed wafers were silanized with hexamethyldisilazane (HMDS; Sigma-Aldrich Co Ltd, UK) for 30 min under vacuum. The silanization step covers SU-8-50 structures and borofloat glass with alkoxy-silane groups, resulting in a hydrophobic surface [19], [20], which allows cured PDMS to be peeled more easily from the master. It is thus less probable that SU-8 50 structures are dislodged from the glass surface during the peeling step. As a consequence it is possible to perform more replication cycles with one master and significantly reduce the number of borofloat glass wafers used.

3.2.3. Casting of PDMS and PDMS bonding to glass slides

For replica molding of microchannels, we used poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning Corp., USA), a pliable silicone rubber, based on its optical transparency, solvent compatibility, gas permeability and biocompatibility with the selected biological model and solutions [21], [22]. Biocompatibility is defined in this case as not harmful or toxic for cultured cells. Microchannels are obtained by casting a PDMS monomer mixed with a PDMS curing agent (Dow Corning Corp., USA) in a ratio of 10:1 (PDMS resin: curing agent) on an SU-8-50 master mold and curing at 62°C for 2h (approximately 70 g of PDMS per wafer; final thickness of the PDMS chip was approximately 3 mm). Before the bonding procedure, inlets/outlets were punched with biopsy punchers (Kai punchers; D-Care B.V., The Netherlands) having an appropriate diameter (2.5 mm or 1 mm). The microchannels replicated in PDMS were sealed irreversibly to glass slides (75 mm x 26 mm x 1 mm) (Thermo Fisher Scientific Gerhard Menzel & Co., Germany) by prior exposure of both parts to oxygen plasma (30W) (Harrick Plasma Cleaner; Harrick Plasma, USA). The chips used for cell observation with a confocal microscope were bonded to coverslips (21 x 26 x 0.17 mm) (Thermo Fisher Scientific Gerhard Menzel & Co., Germany). Oxygen plasma treatment involved a 20-second exposure to oxygen at a pressure of 350-380 mTorr. Directly after plasma treatment, the microchannel structures were brought into contact with the glass slides and pressed against each other. Irreversible bonding occurs after a few minutes. The same bonding procedure of PDMS to glass was applied for both glass slides and glass coverslips. Liquid reservoirs were mounted over the punched reservoirs using silicon glue (Silicon Rubber Compound – Flowable Fluid, RS 692-542; The Netherlands) in Design 3. Oxygen plasma exposure can also act as a chip sterilization step [23]. All fabrication steps
performed after oxygen plasma treatment were performed in a laminar flow hood to ensure a sterile environment for the experiments.

3.2.4. Gelatin coating protocol

After PDMS bonding to glass slides, a sterile solution of 1% porcine gelatin (Sigma code G9382; Sigma-Aldrich Co Ltd, UK) diluted in sterile and filtered phosphate-buffered saline (PBS; pH=7.4) (Sigma-Aldrich Co Ltd, UK) was introduced to the channels and incubated at room temperature (RT) for 45 min. The gelatin acts as a substitute for extracellular matrix (ECM), which encourages HUVEC (or potentially other adherent cell types) to adhere to a surface. After incubation with gelatin solution, 0.5% glutaraldehyde solution (20 µL of 25% glutaraldehyde stock solution diluted in 980 µL of sterile PBS; Polysciences Europe GmbH, Germany) was introduced to the microchannels to cross-link the gelatin layer.. The crosslinking reaction was carried out at RT, and the duration of the process was experimentally optimized for every channel width (Table 3) and was independent of channel design. The optimization of cross-linking time was dictated by differences in microchannel volumes, and aimed to avoid clogging channels with cross-linked gelatin, which occurred when cross-linking times were too long. The purpose of the cross-linking step is to ensure that deposited gelatin remains in the channel upon flow application introduced by consequent rinsing steps and cell injection). Glutaraldehyde is toxic for cells, thus three rinsing steps with sterile PBS were performed to remove possible residues of glutaraldehyde with 10 min incubation at RT in between rinses. Afterwards, the channel was rinsed with PBS to remove unbound gelatin and glutaraldehyde solution from its interior. 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 the inlet and outlet of the channel from drying by deposition of extra solution in the reservoirs. Otherwise undesirable effects might occur, like the appearance of air bubbles in the channels. All the described steps were performed with a 1-mL syringe (Omnifix-F B Braun, The Netherlands).

Table 3. Overview of the cross-linking times with 0.5% glutaraldehyde solution.

<table>
<thead>
<tr>
<th>Channel width [µm]</th>
<th>360 µm</th>
<th>120 µm</th>
<th>100 µm</th>
<th>80 µm</th>
<th>60 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-linking time [min.]</td>
<td>15</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

3.2.5. Cell harvesting protocol

HUVEC were isolated from healthy umbilical cords in Endothelial Cell Facility of the University Medical Center Groningen and cultured to confluence in T25 flasks for at least 2 passages before being seeded in microfluidic channels. For seeding efficiency tests only HUVEC from between passages 2 and 5 were used for the experiments [5]. HUVEC were removed from the T25 bottles by trypsinization (0.05% trypsin solution in sterile PBS; 20 µL of trypsin solution per cm²of HUVEC) at 37°C for approximately 2 min. Afterwards, cells were counted in a Neubauer chamber using microscope, centrifuged (1800 rpm, 5 min.) (Rotina 48S, Hettich GmbH, Germany) and re-suspended in cold (4°C) EC medium at a concentration of 5000 cells/µL. After harvesting, HUVEC were kept on ice (4°C) for no longer than 60 min. The cell suspension was gently shaken in the Eppendorf tube to resuspend cells directly before injection to the channels. In this way, we ensured the same cell concentration in all injected suspension volumes to the channels. 

3.2.6. Endothelial cell medium

Endothelial cell medium (EC medium) consists of RPMI 1640 (Lonza Benelux BV, Breda, The Netherlands); endothelial cell growth factor (ECGF) (50 µg/mL isolated from bovine brain);
L-glutamine (2 mM; Gibco-BRL, Paisley, Scotland); heparin (5 U; Leo Pharm. Prod., Weesp, The Netherlands); K-penicillin G (100 IE/mL; Astellas Pharma Europe B.V., Leiderdorp, The Netherlands); streptomycin (100 µg/mL; Fisiopharm, Italy); Fetal Calf Serum (FCS) (20% v/v; Hyclone, Perbio Science, Etten-Leur, The Netherlands).

### 3.2.7. HUVEC injection to the microchannels and culture protocol

HUVEC were injected with an Eppendorf Research plus pipette (0.5 – 10 µL) (ref. number 3120000020; Eppendorf, Germany) into pre-warmed microchannels at a concentration of 5000 cells/µL. Suspension volumes were selected to obtain sufficient cell loading to allow HUVEC to grow to confluence in less than 24h, without overcrowding and clogging the microchannels (especially the fluidic resistors in the third channel design). These volumes were adjusted for the channel width and did not vary between channel designs (Table 4). For Design 1, HUVEC injection was performed by introduction of the cell suspension volume in an inlet reservoir (diameter of 2.5 mm) and the removal of an equal volume of fluid from an outlet reservoir. Hydrostatic pressure was thus employed for cell distribution along the microchannels. Cell suspension injection for Designs 2 and 3 was performed by inserting the pipette tip (1 - 20 µL) (reference number: 7320 02; Brand GmbH, Germany) into the inlet (diameter of 1 mm) of the channel until the PDMS tightly sealed around the tip. It was important to avoid touching the bottom glass surface of the device with the pipette tip during this procedure, as the cell suspension might have been pushed upwards and leaked from the chip instead of entering the channel. The injection was performed gradually until the pipette tip was empty, at a rate which slowed as microchannel widths shrunk. This reduced the shear stress during cell injection in microchannels to a minimum. Therefore, cell injection times varied from approximately 3 seconds for 360-µm-wide channels to approximately 15 seconds for 60-µm-wide channels. After the injection of the cell suspension, chips were left for 3 min, after which they were moved to the cell incubator. All three channel designs were tested simultaneously with the same cell batch to avoid differences between cell cultures originating from donor variation. Chips were placed in the incubator for 60 min to allow HUVEC to attach in the microchannel. Afterwards, unattached HUVEC were removed by rinsing with a 1-mL syringe containing pre-warmed EC medium, after which chips were placed back into the incubator for 120 min. For successful culture of HUVEC in microchannels, every reservoir should be filled with a few microliters of EC medium to at all times prevent the culture channel from drying out under cell culture conditions (37⁰C). Furthermore, the presence of a meniscus on top of the inlet reservoir prevents the introduction of air into the channel upon injecting fresh medium with a 1-mL syringe. The latter could lead to detachment of cells from the channel during medium exchange. As a positive control, cell culture in a standard 96-well-plate was carried out in parallel to each tested width of microchannels (well-plate culture protocol based on reference [24]).

Table 4. Overview of the conditions for HUVEC seeding and cultivation in the microfluidic devices tested. These conditions were used for cell cultures in all three designs. Injected cell suspension volumes were experimentally adjusted to channel widths.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Human Umbilical Vein Endothelial Cells (HUVEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell concentration</td>
<td>Human Umbilical Vein Endothelial Cells (HUVEC)</td>
</tr>
<tr>
<td>Injected volume of cell suspension in single channel</td>
<td>360µm</td>
</tr>
<tr>
<td></td>
<td>8 µL</td>
</tr>
</tbody>
</table>
3.2.8. Microscope setup and 3D-printed chip holders

The microscope setup for cell inspection and characterization consisted of a phase-contrast microscope with a fluorescent light source (DM-IL, Leica Microsystems, Germany) with a Leica DFC300FX camera (Leica Microsystems, Germany). For visualization of nuclei staining, the CFP ET (ex/em 430-445/460-500 nm) filter cube from Leica Microsystems was applied. We noted that visual inspection of cultured cells is time consuming (~5 min.) and possibly harmful for cells, because of heat dissipation in the microchannels during inspection. The temporary change of external temperature from 37°C to RT is not a problem for well plate-cultured cells, since wells contain significantly more warm medium than microfluidic cell cultures (mL vs nL volumes), resulting in much slower temperature decreases than in microfluidic cell cultures. To solve this issue, we adapted the microscope table by inserting a 3D-printed holder (made in PLA) (Figure 1B) for precise positioning of the chip under the microscope, thus eliminating the need to position the chip manually under the microscope. Furthermore, this reduced the risk of contamination to the cell culture, because the culture spent less time outside of the sterile incubation environment. The microscope stage adaptation reduced the number of components/materials between the microscope objective and a sample, which made it easier and faster to focus on the cell layer and acquire an image. The microfluidic chips were inserted into the 3D-printed holder (made in PLA), which was in turn positioned in the adapted microscope table (Figure 1B).

3.2.9. Cell fixation protocol

Autoclaved PBS was additionally filtered in order to remove PBS crystals, which could perturb good-quality image acquisition in microchannels 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.

HUVEC were fixed in the microchannels approximately 3h after cell seeding with 4% formalin (Sigma Aldrich Co Ltd, UK) in autoclaved and filtered PBS (Sigma Aldrich Co Ltd, UK). The fixative solution was introduced to the microchannels for 3 min while chips were kept at RT. Afterwards, microchannels were rinsed three times with PBS, with 10 min incubation steps between consecutive rinses.

3.2.10. Staining protocols

3.2.10.1. Nucleus staining protocol

To assess the number of seeded HUVEC, cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, stock solution 2 mg/mL of DMSO) (Sigma Aldrich Co Ltd, UK) solution in PBS (1:150) (Sigma Aldrich Co Ltd, UK). DAPI selectively binds to deoxyribonucleic acid (DNA) by intercalation in DNA chains in the cell nucleus. After a 10-min incubation with DAPI, three consecutive rinses with PBS were performed for every microchannel with a 1-mL syringe to remove the excess of DAPI.

3.2.10.2. Vascular Endothelial Cadherin (VE-cadherin) staining protocol

VE-cadherin (CD-144) is an adhesion protein specifically expressed by endothelial cells that is located in the membranes and is involved in creating intracellular connections [25]. The distribution of this protein in HUVEC cultured in microchannels provides information about the quality of the cell culture. Additionally, we wanted to confirm confocal imaging compatibility with the latest chip design (Design 3). HUVEC fixation was performed after 24h cell culture in the cell incubator with the protocol described above. Confluent cell cultures were incubated with primary VE-cadherin antibody (Concentration: 5 µg/mL; labelled with AlexaFluor488; excitation: 488 nm emission: 519
nm); (Thermo Fisher Scientific Inc., MA, USA) diluted forty times in PBS (1:40; 50 µL per channel). This solution was then added to microchannels and incubated for 90 min at RT in the presence of fixed HUVEC culture. Afterwards, stained cell cultures were rinsed three times with PBS with 10 min incubation steps between consecutive rinses. After the VE-cadherin staining, DAPI staining was performed according to the protocol described above. The results were visualized with a confocal fluorescence microscope (Confocal Laser Scanning Platform Leica TCS SP8; Leica Microsystems, Germany). All the described staining steps were performed in Design-3 microchannels bonded to 0.17-mm-thick coverslips, and 1-mL syringes were used in all described actions.

### 3.2.11. Cell counting protocol and data analysis

HUVEC seeded in microchannels of different widths in the three chip designs were fixed, and their nuclei were stained with DAPI according to the protocol described above before imaging. Photos of designated microchannel sections were taken (objective magnitude 10x). The spots chosen for imaging were as follows: (i) For Design 1, pictures were taken 5 mm, 15 mm, and 20 mm from the inlet. (ii) For Design 2, pictures were taken 3 mm, 5 mm and 7 mm from the inlet. (iii) For Design 3, pictures were taken 3 mm, 5 mm and 7 mm from the entrance to the cultivation channel. All chips incorporated a molded “ruler” next to the cultivation channels, to be able to monitor changes in cell culture at defined distances along the channel. Afterwards, the photos were transferred to ImageJ software [26] and cells were counted in the field of view (n=5 per experimental condition), which was dependent on the channel width (Table 5). Results were inserted to Microsoft Excel calculation sheet (Microsoft Inc.; USA) and two parameters were analyzed: cell density and cell distribution. Cell density was calculated by adding the cell counts from all three observation windows and relating them to the total area of those windows (n = 5 per condition). Cell distribution was obtained by calculating the relative distribution per channel first, and averaging these normalized values per condition (n = 5).

<table>
<thead>
<tr>
<th>Channel width [µm]</th>
<th>Imaged channel length [µm]</th>
<th>Area [µm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>360</td>
<td></td>
<td>286740</td>
</tr>
<tr>
<td>120</td>
<td></td>
<td>95580</td>
</tr>
<tr>
<td>100</td>
<td>796.5</td>
<td>79650</td>
</tr>
<tr>
<td>80</td>
<td></td>
<td>63720</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>47790</td>
</tr>
</tbody>
</table>
3.3. Results and Discussion

3.3.1. Testing of three different channel geometries

HUVEC exhibit differences in morphology depending on their location in the body, as this dictates to which physical and physiological factors they are exposed and to what extent [2]. In our initial experiments, endothelial cells were cultured in well plates and exhibited a cobblestone morphology, but when cultured in narrow channels without flow, they tended to align along the channel walls (Figure 2) [27]. Based on the observation made in this simple experiment, we hypothesize that the elongated morphology of endothelial cells in microchannels is dependent on both channel geometry and applied flow. When using a straight channel of uniform cross-section (Design 1), our initial culture experiments showed imperfections in terms of seeding efficiency and experimental reproducibility. This originated from difficult, irreproducible control over cell distribution along the channels during the cell seeding procedure. This is particularly problematic when the number of cells available is limited, which is often the case in work with primary cells. Moreover, control over cell transport through microchannels diminished as channel widths shrunk (Design 1). Therefore, we designed a channel geometry which allows for efficient and reproducible cell seeding in the microchannels, independent of the channel width.

![Figure 2. Comparison of HUVEC morphology in a 96 well plate (A) and in microfluidic channels (B, C, D) after 24h culture without application of flow. These results were obtained with channels corresponding to Design 1 (straight, uniform width, 25-mm-long channels). Results showed that HUVEC exhibited increased alignment with respect to the channel walls as channel widths decreased. The cobblestone morphology of HUVEC in the (A) 96 well plate and (B) 400-µm-wide microchannel is different from HUVEC cultured in channels with widths of (C) 120 µm and (D) 80 µm which are elongated and aligned with the channel walls. Magnification: 10x; all scale bars (lower right): 100 µm](image)

Initially tested devices (Design 1) consisted of channels having a height of 100 µm, a length of 25 mm, and different widths ranging from 360 µm to 60 µm (Figure 3). Reproducible seeding of HUVEC in these channels proved to be difficult, as cells tended to flow unhindered through the channels without settling. Therefore, cultures in Design 1 exhibit a large variation in cell distribution along the microchannels, especially in microchannels narrower than 360 µm (Figure 3 and Figure 4). A solution to this problem is the application of a slight backpressure by plugging the outlet reservoir with a syringe filled with cell medium, but this approach was difficult to implement both manually and with syringe pumps. Moreover, the level of difficulty in seeding cells into Design 1 chips increased in narrower channels due to decreasing channel volumes, making fluid and cell manipulation operations in the channel with a syringe or a pipette impossible to control.
All three channel geometries were compared in terms of cell density (per mm²) in different channel widths (Figure 5) and reproducibility of the cell seeding protocol. Design 1 showed good results only for channels with a width of 360 µm and it was not possible to reproducibly seed HUVEC in channels narrower than 360 µm. Design 2 showed significant improvement in the number and distribution of seeded HUVEC in microchannels narrower than 360 µm. This was achieved by decreasing inlet/outlet diameter and channel length (from 25 mm to 10 mm). However, for channels narrower than 100 µm we noticed that Design 2 is suboptimal for cell cultures due to insufficient number of seeded cells in the microfluidic channels. Therefore, we developed Design 3 in which an additional improvement of cell density, especially for the smallest channel widths, is observed, with an equally good cell distribution compared to Design 2 (Figures 3 - 5). Furthermore, we did not observe gaps between seeded HUVEC in Design 3, which was the case in other two designs.

The reason for enhanced cell seeding results in Design 3 lies in the incorporation of inlet and outlet channels having a width which is half that of the cultivation channel. These additional structures act during cell injection to force the fluid containing cells to slow down when it reaches the cultivation channel. Additionally, the outlet channels act as fluidic resistors, presenting a backpressure at the end of the cultivation channel. These effects promote cell retention in the cultivation channel in comparison to Design 1 and 2. Moreover, even cell distribution throughout the microchannels was achieved for Design 3 in all tested channel widths, which was not the case for Design 1 and 2.

![Figure 3. An overview of HUVEC distribution after seeding in channels of designs 1, 2, and 3 with widths of 360 µm, 120 µm and 60 µm, using the developed seeding protocol. Photos were taken 3 h after cell seeding at 5 mm from the inlet reservoir for Design 1, or 3 mm from the inlet to the cultivation channel for Design 2 and 3. Magnification: 10x; scale bar in the bottom right corner applies to all presented images: 200 µm]
Figure 4. The top panel presents an overview of evenly distributed HUVEC in an 80-μm-wide Design 3 microchannel. Photos were taken 3h after cell seeding into the device. Magnification: 10x; scale bar: 200 μm. The chart presents an overview of HUVEC distribution 3h after seeding into 80-μm-wide channels for all tested designs. Each bar (n = 5) represents a separate cell-counting spot within the tested channel designs. When working with Design 1 we obtained an insufficient cell number in the microfluidic channel to achieve a confluent HUVEC culture, which is reflected by the uneven cell distribution. Data obtained for Designs 2 and 3 shows an even cell distribution along the microchannel.

It is worth mentioning that all experimental steps were accomplished using a 1-mL syringe or Eppendorf pipette (0.5-10 μL). No special tools were needed to seed cells into channels as narrow as 60 μm. Therefore, the presented experimental approach can be adapted in every biomedical laboratory interested in microfluidic cell cultures.
Figure 5. Overview of the number of cells attached in the channels with different widths 3h after seeding at a concentration of 3000 cells/μL. Working with design 3 led to a high cell density in all tested channel widths. Design 2 shows considerably more variation between different channel widths and on average a lower cell density than Design 3. Design 1 is unsuitable for cell cultivation in channels narrower than 360 µm; Each bar represents the average number of cells counted at three different microchannel locations (see Section 2 Material and methods 2.11 for details);[n=5].

3.3.2. Influence of channel geometry on HUVEC

The microchannels developed in this work have rectangular cross-sections, which might be regarded as insufficient for resembling natural blood vessels for certain experimental purposes [3]. This is supported by research performed by Forouzan et al., where a geometry dependence of leukocyte adhesion to endothelium was shown [28]. It was shown that in channels with rectangular cross-section, leukocytes tend to adhere in the corners of the channel, while in channels with circular cross-section, leukocyte adherence was random [28]. In work from Frame et al., it was shown that device geometry influences the number of newly formed actin stress fibers in endothelial cells cultured under flow [29]. It was estimated that endothelial cells cultured in curved vessels express only half of the actin stress fibers that endothelial cells growing on flat surfaces do. Additionally, exposure to the shear stress in circular channels decreases the number of stress fibers in endothelial cells, while no influence of shear stress on endothelial cells growing on flat surfaces was observed [29]. However, Esch et al. indicated that shear stress was a more important factor than channel geometry (semi-circular vs. rectangular channels) in terms of influence on endothelial cells [17]. Hence, the development of microchannels with rectangular cross-section was appropriate for our future experiments with microvascular endothelial cells under flow.

3.3.3. Characterization of HUVEC cultures with confocal microscopy

When designing cell culture systems for cell behavior studies, compatibility with analytical approach needs to be taken into consideration. One method used for cell culture inspection is microscopy. Our microfluidic chips were thus also designed and developed to be compatible with light, fluorescent, and confocal microscopy techniques.

We have tested the compatibility of the developed microfluidic devices with confocal microscopy techniques by staining HUVEC cultures with fluorescently labelled antibodies for VE-cadherin. VE-cadherin, an adherent junction protein, is found predominantly in the junctions between endothelial
cells [30]. Green staining in the image in Figure 6(B) confirms that cell junctions have been formed between HUVEC cultured in the microchannels. The presence of evenly distributed cell-cell connections between cultured HUVEC indicated their good health status in microfluidic culture, and thus confirmed the compatibility of our devices and protocols with HUVEC culture.

In future research, the channel geometry used in Design 3 could be implemented in more complex culture systems containing on-line sensors, to allow for the extraction of more information about HUVEC in a test cell culture. This optimal channel geometry allows cells to be cultured in channels as narrow as 60 µm, thus enabling closer examination of human microvasculature [2], [3].

![Figure 6](image)

**Figure 6.** (A) Confocal image of HUVEC in a 360-µm-wide channel stained with DAPI. Scale bar: 80 µm; (B) A uniform layer of HUVEC in a 360-µm-wide channel, stained with fluorescently labelled VE-cadherin antibodies. Green fluorescence signal is observed in cell membranes and confirms proper cell-cell interaction formation in the device. Scale bar: 100 µm

### 3.4. Conclusions

Our research approach complies with the need for flexible cell culture systems capable of maintaining multiple cell types as reported by Hospers *et al.* [5]. In addition, our technology will allow to integrate sensors regardless of exact channel geometry. Microchannel Design 3 facilitates the choice of channel widths which might be required for the cell culture needs, allowing for cell cultures in channels as narrow as 60 µm. This design is also suitable for numerous analytical approaches (e.g., microscopy). Protocols applied for this research have been optimized in terms of the ECM used (gelatin), cell type (HUVEC). Moreover, significant improvement of cell seeding reproducibility was achieved by introduction of fluidic resistors before and after the cell culture chamber in Design 3. As a result, retention of cells, during cell seeding in the culture chamber is prolonged, and a greater number of cells have time to adhere to the ECM. Therefore, the channel geometry presented in Design 3 is easy to use.

### 3.5. Acknowledgements

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3.6. References


