UNIVERSITY OF TWENTE.

Creating a multiplexed Gut-on-Chip with individually addressable chambers

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1 Samenvatting

Een Darm-op-Chip is een microfluïdisch apparaat dat darmcellen bevat en dat erop gericht is om de fysiology van de darm beter na te bootsen dan dat een conventionele celkweek op een kweekplaat dat kan. Het maakt het bijvoorbeeld mogelijk om de opname van voedingsstoffen en medicijnen te onderzoeken. Er is al een poly(dimethylsiloxaan) (PDMS) chip ontworpen die acht kweekkamers bevat die afzonderlijk adresseerbaar zijn door middel van Quake kleppen in stroomkanalen ervoor en erna. Het doel van dit onderzoek is om deze chip te gebruiken om een gemultiplexte Darm-op-Chip te creëren met uniforme cellagen in individueel adresseerbare kweekkamers. Er is gevonden dat een collageen type I coating goed werkt om celadhesie in de kweekkamers te bevorderen. Een poly(L-lysine) poly(ethylene glycol) (PLL-g-PEG) coating is getest omdat die celadhesie tegen zou moeten gaan, maar er bleek dat geen coating zorgde voor minder celadhesie dus in de stroomkanalen is geen coating gebruikt. Verder is in dit onderzoek het fabricageprotocol voor de Quake kleppen geoptimaliseerd voor een goede sluiting van de kleppen. Twee van de lagen in de chip zijn dunner gemaakt, zodat er minder druk nodig is om de kanalen af te sluiten. De chip heeft nog verbeteringen nodig om een meer consistente dikte van de controlelaag te krijgen en om de levensduur van de chip te verbeteren. Om langdurige celkweek experimenten uit te voeren op de chip moet de binding tussen de controlelaag en de onderlaag worden verbeterd. Er is al wel eerste initieel celkweek experiment van 18 uur uitgevoerd op de chip. Caco-2 cellen werden succesvol in de chip gezaaid waarbij de cellen alleen op de gewenste plekken terecht kwamen. De kamers waren individueel addresseerbaar en met enkele verbeteringen van het kweekprotocol heeft de chip potentie om te worden gebruikt als gemultiplexte Darm-op-Chip.

2 Abstract

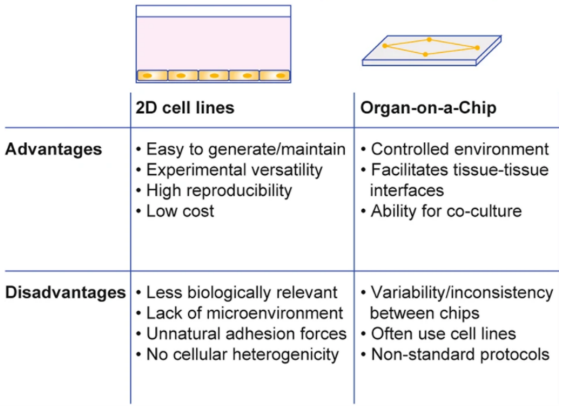
A Gut-on-Chip is a microfluidic device containing intestinal cells that aims to better resemble the physiology of the intestine that a conventional cell culture on a wells plate can. For example, it allows to examine the absorption of nutrients and medicine. A Gut-on-Chip design was already made for a poly(dimethylsiloxaan) (PDMS) chip which contained eight culture chambers which are individually addressable by actuating Quake valves in flow channels before and after them. The aim of this research was to create a multiplexed Gut-on-Chip containing uniform cell layers in the individually addressable culture chambers of this chip. It was found that a collagen type I coating should be applied in the culture chambers to promote cell adhesion. A poly(L-lysine) poly(ethylene glycol) (PLL-g-PEG) coating was tested because it should counteract cell adhesion, but no coating caused fewer cells to bond so no coating should be used in the flow channels. Furthermore the provided fabrication protocol for the Quake valves needed to be optimized for proper closure of the valves. Two layers were made thinner, so less pressure would be needed to close off the channels. The control layer which contains the valves also differs in thickness in a chip and between multiple chips and the chip did not have a lifespan longer than a few hours when pressure was put on it. The chip therefore still has problems when used for long term experiments. The bond between the control layer and layer underneath needs to be improved, to be able to perform these long term cell culture experiments. An initial culture experiment of 18 hours was however performed on the multiplexed chip. Caco-2 cells were cultured in the culture chambers and only adhered in the desired places. The chambers were individually addressable and with some improvements of the culture protocol the chip has great potential to be used as a multiplexed Gut-on-Chip.

3 Introduction

3.1 Organ-on-Chip

Organ-on-Chip (OoC) is a popular subject in current research. An OoC is a microfluidic device in which cells are cultured. The cells are being grown and maintained in a controlled environment to study them on for example cell differentiation, gene expression and the influence of medicine on the cells. Conventionally, cells are cultured and studied in a 2D structure in a well plate or a culture flask. A 2D culture is highly reproducible and it has low costs. However, this method also has some shortcomings and disadvantages, as can be seen in figure 1. In the human body, cells grow in a more complex 3D structure. Therefore, culturing in a 2D structure does not mimic the normal cell behavior optimally. Furthermore, the effect of other organs, tissues and the microenvironment on the cells is not taken into account [1]. In the future, OoCs may solve these problems because these can better mimic the physiological environment of cells [2, 3].

Some example structures of OoCs can be seen in figure 2. In most cases, cells are cultured in one or more culture chambers. More channels, chambers and other elements are added to influence the environment of the cells. An OoC can for example be used to create a Heart-on-Chip. In a human heart, mechanical and electrical stimuli are present which influence the formation of heart tissue. An OoC has been made in which these stimuli are simulated to create a tissue that better resembled an in vivo heart. Furthermore some OoC models of kidneys are being made, in which the many different cell types of the kidney are co-cultured in an organized manner. They need to be cultured together to fully grasp the cooperation of these parts [4]. Also some Gut-on-Chip models are shown. Here, cells are cultured on a porous membrane which divides two chambers. This enables the researcher to monitor the absorption of nutrients or medicine from one chamber to the other, as would normally be done in the small intestine. Fluid streams have been applied to the chambers to resemble the human small intestine [5].



	2D cell lines	Organ-on-a-Chip
Advantages	• Easy to generate/maintain • Experimental versatility • High reproducibility • Low cost	• Controlled environment • Facilitates tissue-tissue interfaces • Ability for co-culture
Disadvantages	• Less biologically relevant • Lack of microenvironment • Unnatural adhesion forces • No cellular heterogenicity	• Variability/inconsistency between chips • Often use cell lines • Non-standard protocols

Figure 1: Advantages and disadvantages of 2D cell culture and OoCs [1].

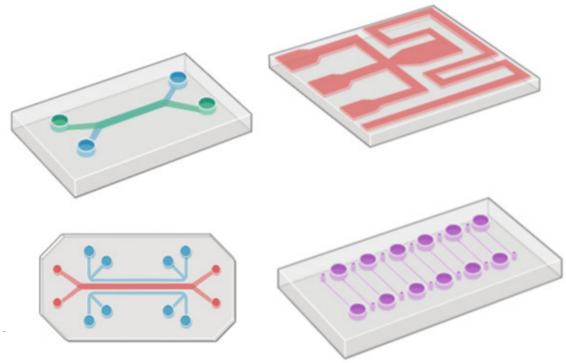


Figure 2: Examples of structures in OoCs [6].

An advantage of OoCs is that the flow of fluids through the micro sized channels can be controlled precisely. In a macro sized channel, the flow would be turbulent while in a micro sized channel the flow is laminar, causing a diffusion and shear stress that can be calculated [4]. Furthermore, an OoC can better resemble the extracellular matrix (ECM). The extracellular matrix is a combination of a scaffold surrounding the cells and biochemical and -mechanical cues which steer cells to differentiate and to gain the right morphology [7]. An OoC can contain structures alike the ECM providing the mechanical cues. In a 2D culture, most of the time a rigid surface is used to culture the cells on, which resembles the soft tissues in the body worse than an OoC can. Therefore an OoC provides a better model for the cultured tissue, making studying the cells more effective [2]. Besides that, an OoC creates the possibility for co-culture and thus the effect of different tissue types on each other can be better resembled [1]. Lastly, microfluidic devices use few of the reagents and they are and easy to transport [3].

3.2 Gut-on-Chip

As said, one application of the OoC is the Gut-on-Chip. The most important function of the small intestine is the absorption of nutrients. The inner surface of the small intestine is adjusted to do so as optimal as possible, as can be seen in figure 3. First of all, the intestinal wall is rimpled into circular folds. Those then contain villi on top of them which again contain microvilli. These are all meant to increase the surface area, to be able to take in more nutrients. The nutrients are taken in by enterocytes, which are one of the five cell types in the small intestine wall. Enterocytes absorb nutrients and electrolytes into the blood and lymphatic capillaries. The other four cell types in the small intestine are goblet cells, which secrete mucus, enteroendocrine cells, which secrete enterogastrones, paneth cells, which release antimicrobial agents and stem cells, which divide and differentiate into all of the other cell types [8].

In a Gut-on-Chip model these functions of the small intestine can be monitored. In 2D culture, the physiology of the gut can not be recreated well because they are not able to recapitulate the fluid flow and peristaltic motion of the small intestine [4]. This can however be done in an OoC model. A commonly used cell line to resemble intestinal epithelial cells in vitro is Caco-2. This cell line is derived from a human colorectal adenocarcinoma. In cell culture it forms a single cell layer, in which the cells are connected by tight junctions. This enables the cell layer to selectively let through nutrients and medicine molecules [9].

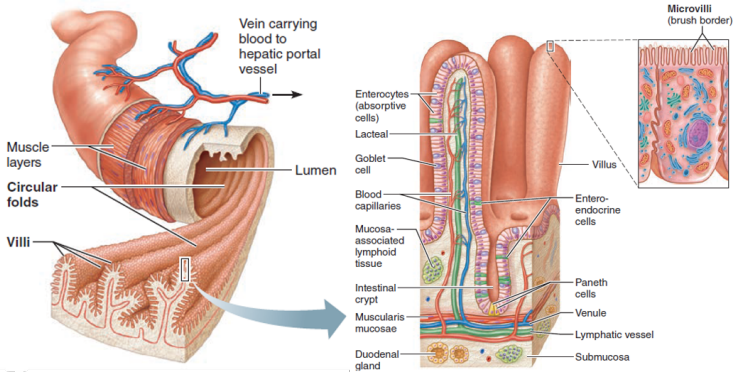


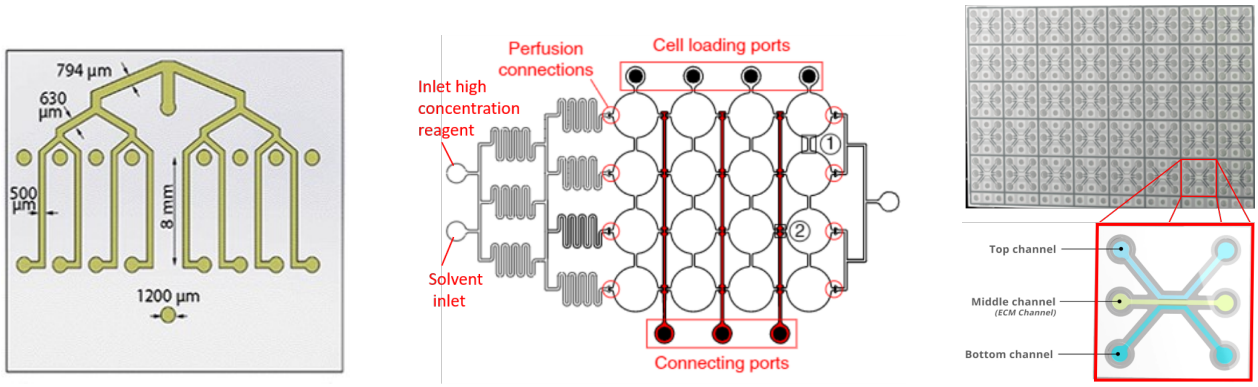
Figure 3: Structure of the small intestine [8].

3.3 Multiplexing

To achieve a higher throughput, a multiplexed OoC can be used. This means that the chip contains more than one culture chamber, which can be controlled individually [10]. There are multiple ways to create a multiplexed OoC. Some examples are shown in figure 4. Figure 4(a) shows a multiplexed chip with eight parallel chambers in which cells can be cultured. They all originate from one common inlet, which can be found on the top of the chip. This inlet can be used to fill all chambers at the same time with the same fluids. The chambers however all have separate outlets, which can be found on the bottom of the chip. These give the opportunity to individually address each chamber to create different conditions in each chamber [11]. However, this method requires a lot of manual handling when e.g. changing medium.

A second method can be seen in figure 4(b). Here, a multiplexed chip is shown in which cells are cultured in a 4x4 array. Each white circle is a spot on which a droplet of culture medium with cells can hang. These droplets are created by flowing the medium with cells from the cell loading ports on top. Each column has its own cell loading port, so four different cell types or cell seeding conditions can be used. Then, another fluid can be added to the hanging droplets via a gradient generator which can be seen on the left of the hanging droplets. In the top inlet, a high concentration of a reagent is put in and in the other a solvent to dilute this reagent. The highest concentration of the reagent flows into the upper row of hanging droplets. The lower the row, the lower the concentration of the reagent gets. This means that with this chip, four different cell conditions can be seeded which can all be cultured in four different concentrations of a reagent [12].

Another recently created multiplexed chip design is made by Mimetas [13]. Forty OoCs are placed on one chip as can be seen in figure 4(c). Each OoC consists of two channels, separated by an ECM channel. The chips can for example be used to model angiogenesis. This can be done by adding a blood vessel cell suspension in the top channel, a gel in the ECM channel and culturing medium in the bottom channel. The chip is placed on a plate which slowly tilts the chip to its sides one by one to create a fluid flow in the channels. This resembles the fluid flow of blood in a blood vessel, causing the cells to arrange in a tubular form covering the channel walls. From here, the formed vessel can create new vessels into the ECM gel. Because forty of these OoCs are placed on one chip, a high throughput is created [13].

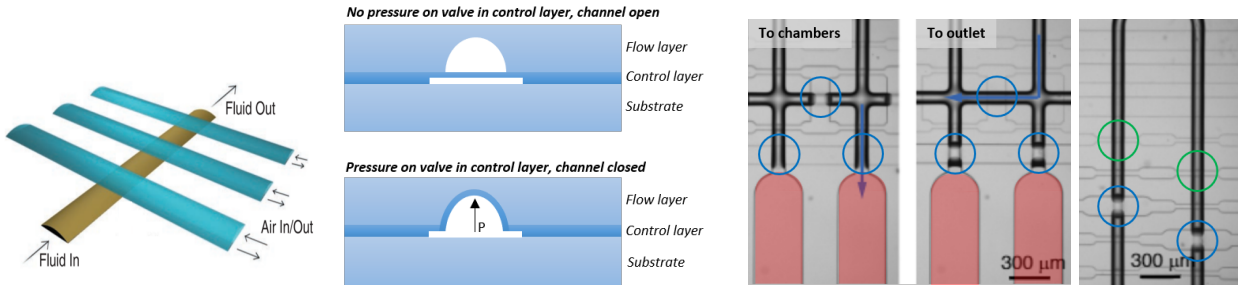


(a) (b) (c)

Figure 4: Three ways to create a multiplexed chip with individually addressable culture chambers, a) by giving each chamber its own outlet [11], b) with the use of hanging droplets in a 4x4 array [12] and c) with forty OoCs on one chip [13]

3.4 Quake valves

Another way to create a multiplexed chip, is with the use of Quake valves [14]. A Quake valve is formed by placing control channels perpendicular over a flow channel, as can be seen in figure 5(a). All channels are made of an elastomer and the flow channels are separated from the control channels by a thin layer of the elastomer. The fluid stream in the flow channel can be stopped by actuating a control channel with air pressure. The control channel expands into the flow channel, closing it off as can be seen in figure 5(b). The flow channels are rounded, because the valves can better close rounded channels. This principle can be applied to flow channels surrounding culture chambers, to be able to individually address culture chambers. This has been done for example by Vollertsen et al. [15] as can be seen in figure 5(c) and figure 5(d). Figure 5(c) shows that the valves circled in blue can be actuated to control the fluid stream direction. If the top valve is actuated but the bottom two are not, the fluid flows to the chambers to address all chambers at the same time. If the top valve is not actuated and the bottom two are, the fluid flows to an outlet. Figure 5(d) shows that with the use of valves and bridges in control channels, flow channels and therefor culture chambers can be individually addressed. Bridges are thin parts in the control channels which do not close off a flow channel when actuated, so a flow channel can be passed without closing it. The control channels can be automatically actuated by a programmed pressure pump, which allows for automated and highly parallelized cell culture [15]. Quake valves are thus of great use in an OoC and therefor they are used in the chip design of this research too.



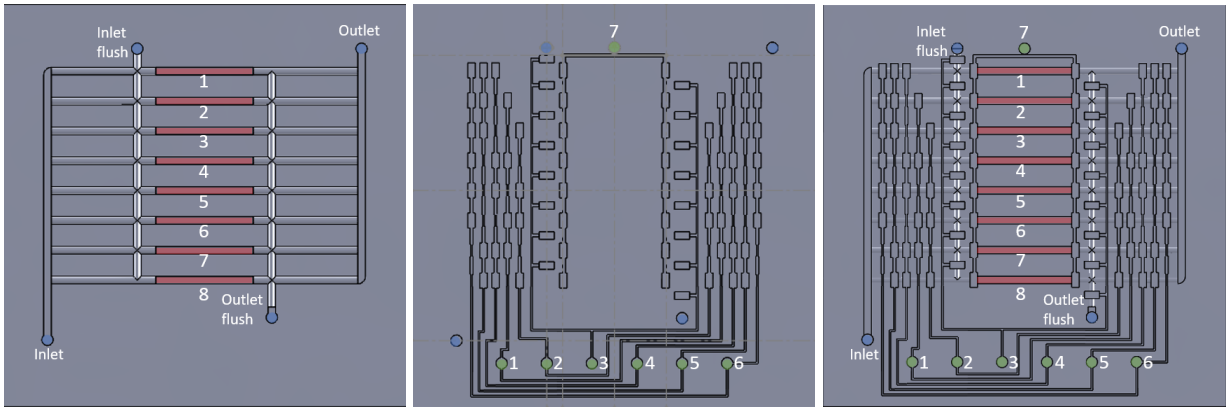
(a) (b) (c) (d)

Figure 5: Working principle of a Quake valve. a) Control channels are placed perpendicular over a fluid channel [14]. b) Cross-section of a Quake valve. When pressure is applied on a control channel, it expands into the fluid channel closing it off. c) An example of an application of Quake valves [15]. Actuation of the valves, circled in blue, cause the fluid to flow either into the cell culture chambers, highlighted in red, or to an outlet. d) Quake valves and brigdes in the design of Vollertsen et al. [15] which allow for individually addressable chambers.

3.5 Chip design

The multiplexed OoC used in this research was already designed by E.G.B.M Bossink. The final device consists of four layers; a glass slide, a plain layer, a control layer and a flow layer, all three made of poly(dimethylsiloxaan) (PDMS), see figure 7. The designs of the two molds for the control and flow layer can be seen in figure 6. PDMS is casted or spincoated on these molds, as further explained in section 5.1. The mold for the flow layer contains protruding structures for eight culture chambers with a width of 0.5 mm, a height of 0.2 mm and a length of 6.5 mm in which the cells can grow, highlighted in red in the mold in figure 6(a). Flow channels connect these culture chambers with an inlet and an outlet. A flush inlet and outlet are added to be able to flush either only the culture chambers or only the flow channels. The mold of the control layer seen in figure 6(b) contains structures to create control channels with a width of 0.2 mm and a height of 0.1 mm with valves with a width of 0.5 mm and bridges with a width of 0.1 mm. The control layer contains seven different control channels, each with their own pressure inlet. By applying pressure on the right inlets, the user can choose which flow channels and culture chambers in the flow layer will be open and which will be closed. If for example control channel 6 is pressurized, culture chambers 1 to 6 are closed off. This means that all culture chambers can be controlled separately. The pressures can be applied on the control channels by a programmed pump.

A simplified version of the flow layer, see figure 8, was used to test coatings, cell seeding densities and stainings before applying them on the multiplexed chip. This flow layer is placed directly on a plain PDMS layer on a glass slide, without a control layer in between. It consists of eighth culture chambers which all have their own direct inlets and outlets.



(a) Flow layer (b) Control layer (c) Layers combined

Figure 6: Molds of the multiplexed OoC that was used in this research. The culture chambers are highlighted in red, the inlets and outlets of the flow layer in blue and the inlets of the control layer in green.

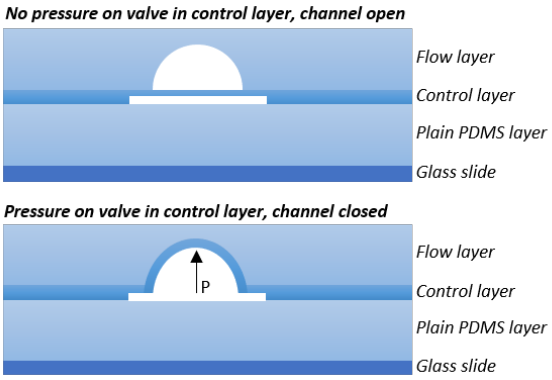


Figure 7: Cross section of che chip, with a flow channel that is opened and closed off by the Quake valve.

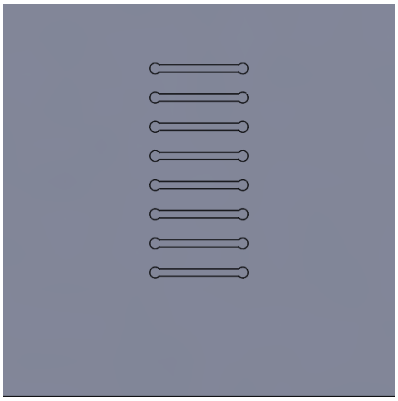


Figure 8: Mold of simplified flow layer, containing only the culture chambers.

3.6 Cell adhesion

PDMS is a material with a low wettability. If it is untreated it typically has a water contact angle of 109°[16]. This means that PDMS is hydrophilic, because it's water contact angle is bigger than 90°[16]. This is however not ideal for cell adhesion. Cells usually adhere better to a surface with moderate hydrophilicity, with a water contact angle of 20-40°[17]. Therefore, the culture chambers of the OoC have to be treated to promote cell adhesion. A widely used method is to coat PDMS with ECM proteins to which the cells can adhere. Two proteins are used most; fibronectin and collagen type I. Fibronectin is a protein which binds to proteins on the cell surface, and therefore it enhances cell adhesion and spreading in in vitro models [18]. Collagen type I binds to cells via receptors of the cell membrane and therefore enhances cell adhesion too [19]. It was found that coating PDMS with fibronectin decreases the water contact angle to 77°and that with collagen type I it decreases to 90°[17], but that both improve attachment of Caco-2 on PDMS cells equally. They cause the cells to attach in the same way as they do to a conventional culture plates [17]. Collagen type I is widely used [15, 20, 21], and therefore it will be used in this thesis to promote cell adhesion in the culture chambers.

Although cell adhesion is desired in the culture chambers, the cells should not adhere in the surrounding flow channels. Cells will not adhere well to the PDMS in the channels, but these can be coated to even further counteract cell adhesion. This can be done by using poly(L-lysine) poly(ethylene glycol) (PLL-g-PEG) [15, 23]. This copolymer forms a brush-like structure on a negatively charged surface as can be seen in figure 9. The PLL part of the polymer chains point towards the surface, while the PEG part points outwards. The polymer physically blocks the PDMS surface, so no proteins and cells can adhere to the surface. Furthermore, the PEG parts are terminated with a methoxy group, which is unable to bind a protein. Cell adhesion happens via protein binding, and therefor cells can not bind to a PLL-g-PEG coated surface [22]. The PLL-g-PEG coating could be applied to the flow channels surrounding the culture chambers of the chip.

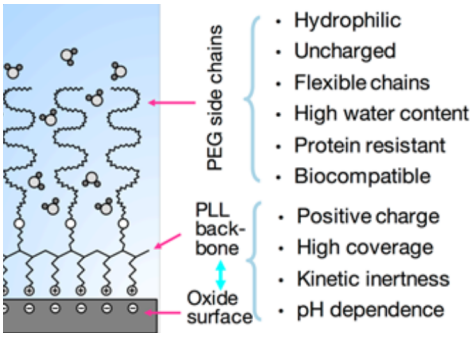


Figure 9: Working principle of a PLL-g-PEG coating [22]

4 Aim

A multiplexed Gut-on-Chip with Quake valves allows for highly parallelized and automated cell culture of gut tissues. Eight cell cultures can be created at ones which can all be used in e.g. medicine testing with eight different concentrations of medicine. In this thesis the multiplexed chip design will therefore be used to answer the research question "How can a multiplexed Gut-on-Chip containing uniform Caco-2 cell layers in individually addressable culture chambers successfully be created?". The research is split in four sub-questions:

1. Can we fabricate the multiplexed chip and individually address all chambers?
2. Can we coat the culture chambers to promote cell adhesion and the flow channels to counteract cell adhesion?
3. Can we successfully culture and stain Caco-2 cells off-chip?
4. Can we successfully culture and stain Caco-2 cells on-chip, with different treatments for different chambers on the chip?

5 Materials and methods

5.1 PDMS chip production

The fabrication process for the PDMS device was provided to me and is based on Unger et al. [24]. The molds for the flow and control layers were designed in SOLIDWORKS® and they were micromilled in polymehtylmethactylate (PMMA)(Datron Neo). 2.1 grams of PDMS (RTV615) (1:20 w/w) for the control layer and 4 grams (1:7 w/w) for the flow layer were mixed. Both were degassed for 2 hours. The 1:7 w/w PDMS was cast onto the flow layer mold. The 1:20 w/w PDMS was spincoated (SPIN150) on the control layer mold at 750 rpm for 60 seconds, resulting in a 70 µm PDMS layer atop the valve site[1]. The control layer was placed on a flat surface for 20 minutes at room temperature (RT). Both layers were pre-cured for 45 minutes at 60°C. The flow layer was peeled out of the mold and the inlets for the flow channels were punched with a 0.75 mm puncher. The layers were aligned, gently pressed together to bond and cured overnight (ON) at 60°C. The fully cured chip was peeled off the control mold and the inlets for the culture channels were punched with a 0.75 mm puncher. 1 gram of PDMS (Sylgard) (10:1 w/w) was cast on a large glass slide, resulting in an approximately 400 µm thick layer[2]. The layer was pre-cured for 20 minutes[3] at 60 °C. The control layer and the PDMS on the glass slide were treated with oxygen plasma (Femto Science Cute) and bonded. The chip was placed at 60 °C ON to promote the bond and fully cure the 10:1 PDMS.

5.2 Testing valve closure and lifespan

The chip was tested on valve closure and lifespan. The set-up to actuate the chip can be seen in figure 10. To be able to actuate the control channels, a pressure regulator (Festo LRP-1/4-2,5) was connected to an instrument air supply and set on a pressure between 1 to 2 bar. The regulator was connected to a Convergence Inspector Systems (model Plate Mini) which can be programmed to apply pressure (ON/OFF) at its 8 outlets. Seven of these were connected to the inlets of the control channels on the chip. The chip was placed under a microscope to be able to see the valve closure. The pressure was put on 1 bar and risen to the pressure at which all the valves of the chip closed. The pressure was never put higher than 2 bar. At this pressure, the chip was tested on lifespan by pressurizing the control chambers with the programmed Convergence Inspector Systems in such a manner that the culture chambers were actuated one by one for 10 seconds and by checking for leaks or breaks in the chip.

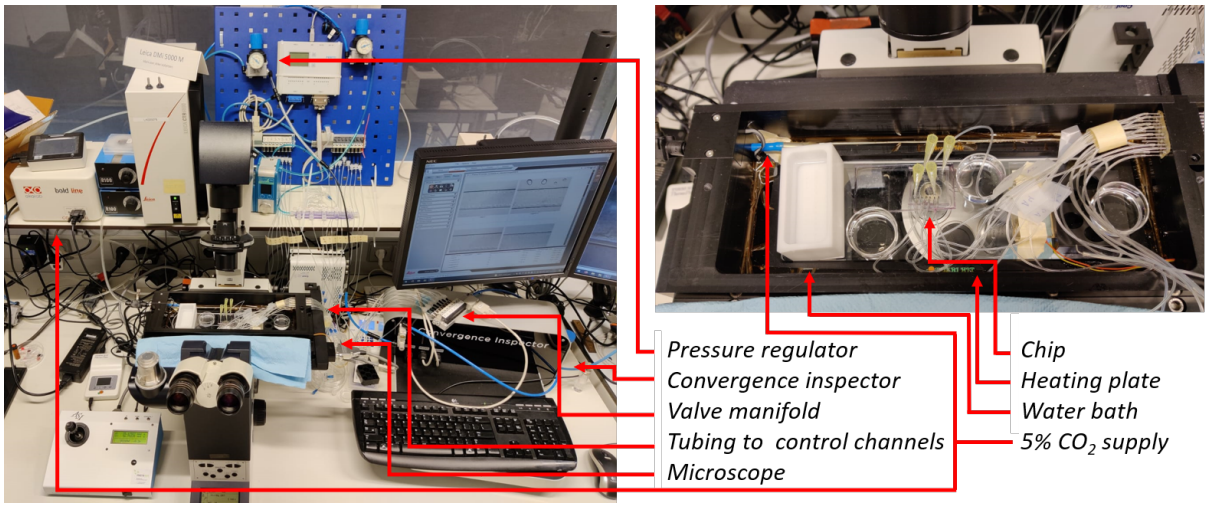


Figure 10: Set-up of culturing experiments on the multiplexed chip. An incubator setting, containing a heating plate, water bath and 5% CO2 supply was build in a microscope. The pressure regulator, convergence inspector and valve manifold and tubing can apply a pressure to the control channels while the chip lays in the incubator.

[1] Improved in this thesis. Initially spincoated at 600 rpm for 60 sec, resulting in a 100 µm PDMS layer atop the valve site.
[2] Improved in this thesis. Initially no specific amount was used and a thicker plain PDMS layer was formed.
[3] Improved in this thesis. Initially was 30 minutes.

5.3 Coating chips

5.3.1 Simplified chip

For practical reasons, the coatings were tested on the simplfied chip, before applying them to the multiplexed chip. Four coatings were tested: no coating, coating with PLL-g-PEG, coating with collagen type I and coating with PLL-g-PEG and then collagen type I, based on [15]. The chip was first treated with oxygen plasma. Then all channels were flushed with 70% ethanol and with phosphate-buffered saline (PBS). The channels were filled with a 100 µg/mL PLL-g-PEG in PBS solution and the chip was incubated for 30 minutes at room RT. Some of the PLL-g-PEG coated channels were filled with PBS and some with a 0.1 mg/mL collagen type I in PBS solution. Some uncoated channels were also filled with the collagen type I solution and the chip was incubated for 60 minutes at 37 °C. All channels were flushed with PBS. The coated chips were used for cell culture immediately.

5.3.2 Multiplexed chip

The culture chambers of the multiplexed chip were coated with collagen type I. The flow channels were not coated. To do this, first, the chip was treated with oxygen plasma. The flow channels and culture chambers were than filled with 70% ethanol which was flushed out with PBS. All control channels were pressurized, except for channels 3 and 7, and a 0.1 mg/mL collagen type I in PBS solution was flushed through the culture chambers via the flush inlet and outlet. Pipette tips with the collagen type I solution were placed in this inlet and outlet and the chip was incubated for 60 minutes at RT. The culture chambers were flushed with PBS and the control channels were depressurized. The coated chips were used for cell culture immediately.

5.4 Cell culture

5.4.1 Off-chip

Caco-2 cells were maintained in T25 and T75 culture flasks throughout the research. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 100 units/mL penicillin-streptomycin (pen/strep), further referred to as DMEM+. Every two to three days, the medium was renewed. Every four to five days, when the cells reached about 80% confluency, the cells were passaged. The medium was aspirated, the cells were washed with PBS, 10% trypsin in PBS was added and the cells were incubated for 3-5 minutes. Then DMEM+ was added to deactivate the trypsin, the cells were centrifuged (Beckman Coulter Allegra X-12R, 130 g for 6 min), medium and trypsin were aspirated and the cells were resuspended in DMEM+. The cells were seeded in a new flask at approximately 10.000 cells/cm^2. As a control experiment, Caco-2 cells were cultured on a 12-wells plate. Before seeding, the cells were counted with an automated cell counter (Luna automated cell counter model L1001) using 50% trypan blue. They were resuspended in DMEM+ and seeded at a seeding density of 10.000 cells/cm^2. They were cultured for three days and on the second day they were washed with PBS and new medium was added.

5.4.2 Simplified chip

The culture chambers of a coated chip were filled with DMEM+ and the chip was incubated for two hours at 37 °C. Cells were trypsinized and centrifuged as described in section 5.4.1. They were counted and resuspended in the right amount of DMEM+ to create a cell suspension with which a seeding density of 100.000 cells/cm^2 would be reached. The cell suspension was flushed through the culture chambers, pipette tips with the cell suspension were placed in all inlets and outlets and the chip was incubated for 1 hour at 37 °C to let the cells attach. The chambers were flushed with DMEM+ to remove non-adhered cells and pipette tips with DMEM+ were placed in the inlets and outlets as medium reservoirs. The cells were incubated for three days and the medium was replaced two times a day.

5.4.3 Multiplexed chip

The culture chambers and flow channels of the coated chip were filled with DMEM+ and the chip was incubated for two hours at 37 °C. The cells were trypsinized and centrifuged as described in section 5.4.1. They were counted and resuspended in the right amount of DMEM+ to create a cell suspension with which a seeding density of 200.000 cells/cm^2 would be reached. All control channels except 3 and 7 were pressurized and the cell suspension was flushed through the culture chambers via the flush inlet and outlet. Pipette tips

with the cell suspension were placed in the flush inlet and outlet as reservoirs. A stage top incubator has been created under the microscope so the chip can be monitored continuously as can be seen in figure 10. The heating plate (Tokai Hit TPi-SX) was set on 37 °C, the 5% CO_2 supply (Okolab Bold Line CO_2 unit 3L) was turned on, the water bath was filled and a clear lid was placed on top. The chip was incubated for 1 hour at 37 °C to let the cells attach. The chambers were flushed with DMEM+ and pipette tips with DMEM+ were placed in the inlets and outlets as medium reservoirs. All control channels were depressurized. The chip was incubated for 18 hours.

5.5 Cell staining

Cells were fixed and stained with an actin filaments and a nucleus staining on the wells plate, simplified chips and multiplexed chips. To show that the chambers in the multiplexed chip are individually addressable, a 10% trypsin in PBS solution was flushed into chamber 2, 4, 6 and 8 and the chip was incubated for 10 minutes before staining. Then, all channels were flushed through with PBS thoroughly. In all three situations the cells were washed 3x with PBS and a 4% formaldehyde in PBS solution was added to the cells to fix the cells. The cells were incubated for 20 minutes at RT. The cells were washed 3x with PBS. A 0.3% TritonX in PBS solution was added to the cells to permeabilize them and they were incubated for 30 minutes at RT. They were washed 3x with PBS. An ActinGreen 488 ReadyProbes Reagent (Invitrogen) in PBS solution was added at the concentrations given in table 1 and the cells were incubated for 30 minutes at RT, protected from light. The cells were washed 3x with PBS. A NucBlue Live ReadyProbes Reagent (Invitrogen) in PBS solution was added at the concentrations given in table 1 and the cells were incubated for 20 minutes at RT, protected from light. The cells were washed 3x with PBS. The cells were observed with a fluorescent microscope (EVOS M5000) with a GFP filter for ActinGreen and a DAPI filter for NucBlue.

Table 1: Seeding densities and staining solutions for the culture flasks, well plate and both chips.

	Seeding density	*ActinGreen concentration*	*NucBlue concentration*
T25/T75 flask	10.000 cells/cm2	-	-
Well plate	10.000 cells/cm2	2 drops per mL	2 drops per mL
Simplified chip	100.000 cells/cm2	2 drops per 250 µL	2 drops per 250 µL
Multiplexed chip	200.000 cells/cm2	2 drops per 250 µL	2 drops per 250 µL

6 Results and discussion

6.1 Chip testing

6.1.1 Valve closure

First of all, the valves on-chip were tested on valve closure. It was found that with the existing protocol, the valves in the chip would not entirely close below 2 bar. When only one chamber was meant to be addressed, the fluid would leak into the other chambers too. In figure 11 an example of a leaking valve and a fully closed valve can be seen. In a fully closed valve, it can be seen that the valve presses up into the full width of the flow channel because it creates a full white bar across the channel. A leaking channel does show a white closed part in the middle of the flow channel, but it can be seen that the blue food dye still flows past this on the sides of the channel. Increasing the pressure on the control channels, starting with a pressure of 1 bar, would cause the leakage to become less. It was however not preferred to use a pressure higher than 2 bar, because this would either cause some valves to break or it would cause the control layer to release from the plain PDMS layer. With a pressure below 2 bar, the valves would not entirely close off the flow channels. Therefore, the fabrication protocol was optimized. First of all, the plain PDMS layer was made thinner. Because PDMS is a flexible material, the pressure that was put on the control channels presumably did not only push up the PDMS layer atop the valves into the flow channel but it also pushed a dent into the plain PDMS layer. The pressure was thus not entirely utilized to close of the channel. In the provided protocol, no specific thickness for this plain PDMS layer was given. In this research it was found that with casting 1 gram of PDMS onto the glass slide, resulting in an approximately 400 µm thick layer, the valves would close off the flow channels better. Secondly, the control layer was made thinner. The same mold was used, so the height of the control channels and valves was kept the same, but the layer of PDMS atop the valves became thinner, so less pressure would be needed to stretch the PDMS into the flow channel. This was done by using a higher spinning speed while spincoating the control layer. The initial spinning speed was 600 rpm, which resulted in an approximately 100 µm thick layer. It was found that increasing it to 750 rpm, resulting in an approximately 70 µm thick layer, would result in valves which fully closed off below 2 bar. The change in thickness of the control layer can be seen in figure 12.

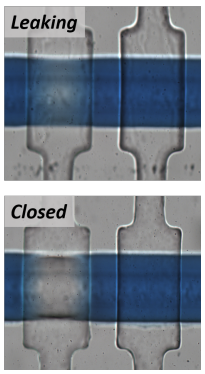


Figure 11: Valve closure.

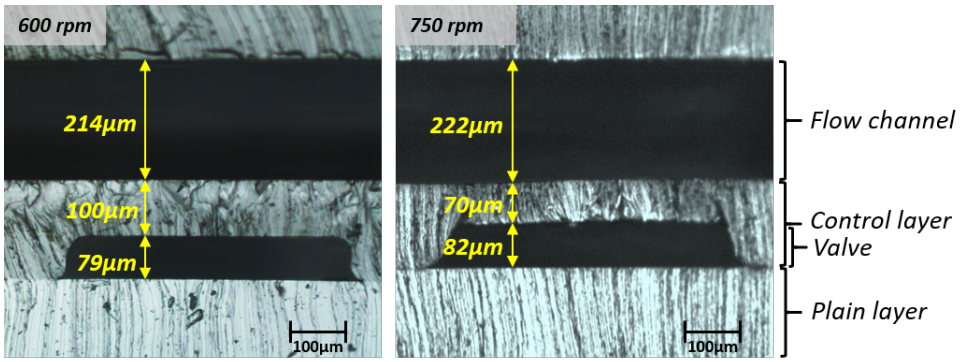


Figure 12: Cross sections of chips off which the control layers were made at 600 and 750 rpm.

After making these two changes to the production protocol, the channels were individually addressable as can be seen in figure 13. Here, it can be seen that by closing the right valves, the chambers can be filled one by one. Furthermore, in figure 14 it can be seen that if all horizontal flow channels are closed off and the flush in- and outlet are used, all chambers can be filled without the fluid leaking into the flow channels. This is essential for coating the cell chamber and cell seeding. However, it can be seen that while filling the chambers in this way, the flow rate is not constant in all chambers. The food dye flows fastest through the top chambers, then through the bottom chambers and slowest through the middle chambers. This means that when this manner of filling is used during cell seeding, the top and bottom channels might get a higher seeding density than the middle channels. When fluids are flushed through the chip in this way during cell culture, enough fluid needs to be used to make sure that it does not only fill the top and bottom channels, but also the middle channels.

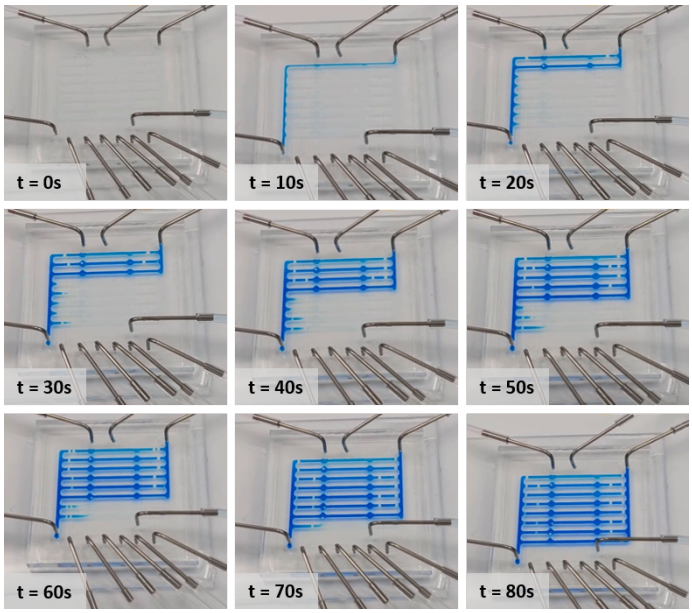


Figure 13: Filling the culture chambers one by one.

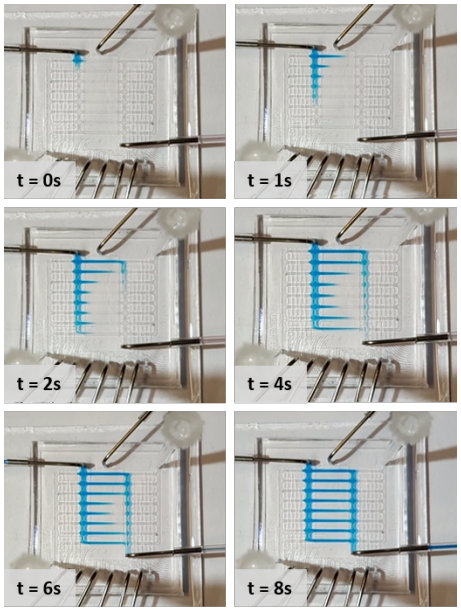


Figure 14: Filling the culture chambers all at ones via the flush inlet and outlet.

The two improved steps were tested on multiple chips, and it was found that each chip needed a different pressure to fully close off the valves, but no chip needed a pressure higher than 1.6 bar. It was found that the thickness of the control layer also differed per chip, while they were made with the exact same fabrication protocol. This might be the cause of the difference in needed pressure. An overview of the mean thickness of the PDMS layer atop the valves and the pressure needed to close off the valves can be seen in figure 15. In this figure chips of which the control layer was made with 750 rpm and 800 rpm are shown, because these had shown the most promising results in closing off at a pressure below 2 bar. It seems that the chips with a thicker control layer, need a higher pressure to close off the valves but no clear conclusion can be drawn due to the high standard deviations. This is caused by the high variation in thickness of the PDMS atop the valves in each chip. It might be that the flat surface on which the control layer was placed after spincoating was not entirely level, resulting in an uneven layer. Furthermore, the data points at 1,25 and 1,3 bar fall out of line with the rest, so more research needs to be done to be sure if the difference in thickness between chips caused the difference in pressure needed to close all valves. Concluding, it was shown that all eight chambers can be individually addressed by actuation of the integrated valves. The valve closure varied between chips, but also between valves within one chip.

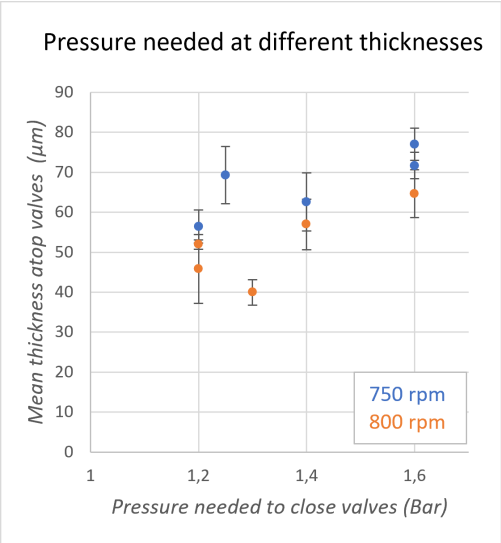


Figure 15: Relation between the mean thickness of the PDMS layer atop the valves in a chip and the pressure that is needed to close off the valves. Each datapoint corresponds to the thickness measured atop four valves at different parts of one chip.

6.1.2 Lifespan

The chip was also tested on lifespan, because for automated cell culture it should be able to endure pressure on the control channels for at least a week. However, most of the chips made with the provided protocol and the two adjustments described above did not last longer than a few hours. In most chips the chip failure was caused by the same issue: control channels 3 and 7 connected at their top left valves, as can be seen in figure 16. The control layer released from the plain PDMS layer because of the pressure on the valves. It was found that these two valves were closer to each other than all other valves, 200 µm with respect to 250 µm and

300 µm in the other valves. This can be seen in figure 17. If pressure was only put on the other five control channels and not on these two, the chips lasted a few hours longer. After this time, connections between all control channels were formed because the control layer released from the plain PDMS layer. Only one chip lasted for about 45 hours before it released from the plain PDMS layer.

To try to solve these problems, the mold for the control layer was changed to create more space between the control channels 3 and 7, as can be seen in figure 17. Furthermore, the bonding between the control layer and the plain PDMS layer was made stronger, to make it more durable. This was done by curing the plain PDMS layer for only 20 minutes instead of 30 minutes, so the plain PDMS layer was very sticky when bonding to the control layer on it. Due to limited time, only one of these new chips was tested on lifespan. Channels 3 and 7 also connected in this chip after 2 hours. They however connected at other valves this time, where the distance between the valves was 430 µm. Two other channels connected after 22 hours. Because this lifespan test was only done with one new chip, it is not clear whether this problem will occur for all chips made with this protocol, or if it was a problem with just this chip. It is possible that the plain PDMS layer and the control layer were not pressed together enough while bonding and that this made the bond weaker than expected. The layers can however not be pressed together too hard, because the plain PDMS layer is not fully cured at this moment and it easily clogs the control channels when too much pressure is applied during bonding. It is remarkable that still control channels 3 and 7 connected fastest, while these were now placed even further away from each other than the other valves are. It might be that the PDMS/PDMS bond can endure stretch in one way better than in two ways. The valves of channel 3 and 7 are placed perpendicular to each other, and therefore they cause the PDMS to stretch in two ways underneath the flow channel. It might also be that the pressure in the control channels is higher at ends of the channels. This would mean that the valves of control channel 3 pressurize the PDMS/PDMS bond most close to control channel 7. The other control channels do not have their end directed to other control channels, so there a higher pressure at the ends would not cause a connection between channels. To find out whether this difference in pressure is indeed present, a simulation of the pressure distribution in the control channels can be done.

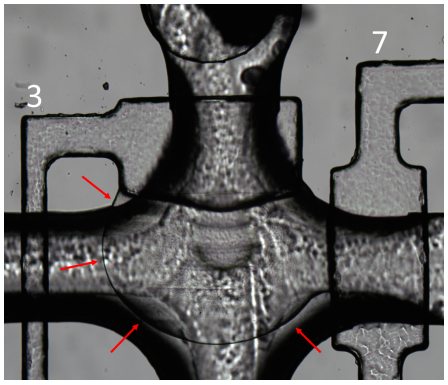


Figure 16: Connection between control channel 3 and 7. An air bubble can be seen underneath the flow channel which connects the two valves, indicated with the red arrows.

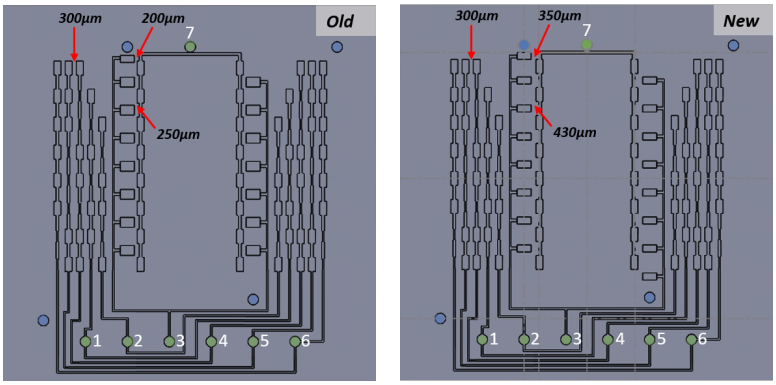


Figure 17: Distance between the valves in the old and new version of the control layer. The most middle control channel was moved to the distance from the valves of this channel were further away from the valves of the adjecent channel.

6.2 Test coatings on simplified chip

The coatings that were to be used in the multiplexed chip, were first tested on the simplified chip. In figure 18 it can be seen that without coating the channel, some islands of cells bind but no even cell layer forms and a mean confluence of 20% is reached after three days. When the channel is coated with collagen type I, the cells form a uniform layer with a confluence of 100% in the culture channel. Coating with PLL-g-PEG also caused a bit more cells to bind than no coating did, with a confluence of 28,3%. The cells did however still form islands an no confluent layer formed. It is remarkable that a PLL-g-PEG coating did not decrease cell adhesion, while literature shows that a PLL-g-PEG coating on PDMS should counteract cell adhesion. Oxygen plasma treatment causes the PDMS to become negatively charged, after which the PLL-g-PEG

coating should adhere due to electrostatic bonds [23, 25]. It might be that these electrostatic bonds are not strong enough to withstand the used fluid flow and that the PLL-g-PEG coating was washed away. The bond might also have become weaker over time, because an oxygen plasma treatment is temporary and usually quickly reverses in the first 12 hours [26]. Adding a collagen type I solution right after coating with PLL-g-PEG caused a confluence of 60%, which is higher than no coating did, but lower than only coating with collagen type I did. This indicates that the PLL-g-PEG coating did cover some of the surface right after coating, but that it did not cover the entire surface because some collagen type I is bound too. For the multiplexed chip a collagen type I coating will be used in the culture chambers to promote cell adhesion. The flow channels will not be coated, as this resulted in the lowest cell adhesion.

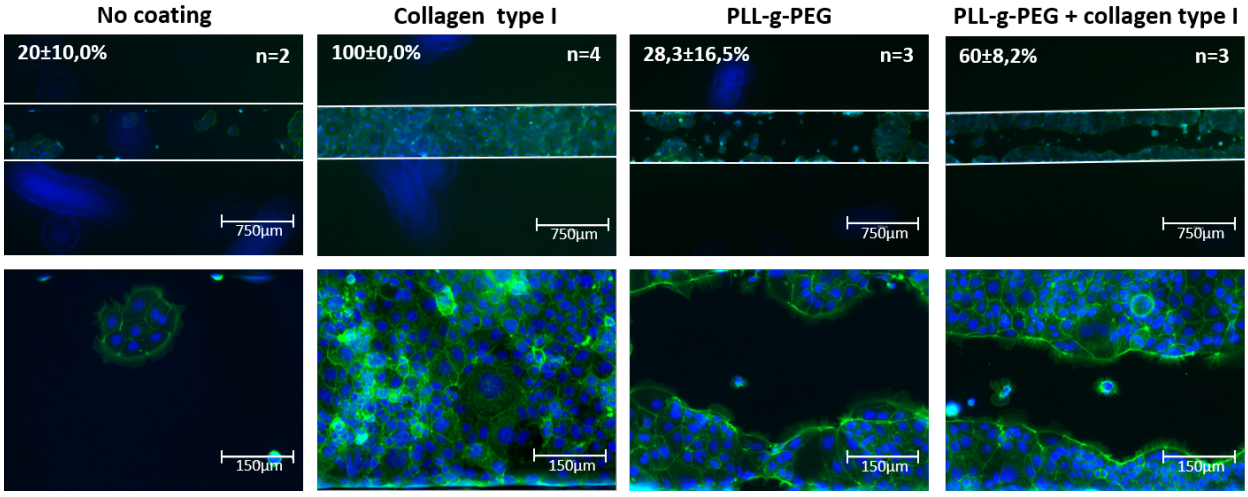


Figure 18: Test of the different coatings on the simplified chip. Caco-2 cells (p51 and p52) were cultured for three days and stained with NucBlue and ActinGreen. The mean confluence with standard deviation is given for each condition.

6.3 Cell culture on multiplexed chip

6.3.1 Cell seeding

Cells were seeded succesfully in the multiplexed chip. Figure 19 shows the transitions places of the horizontal flow channels with the vertical flow channels. It can be seen that after cells seeding, t = 0 h, the cells were stopped by the valves as desired, so no cells were present in the flow channels. Even when the flow channel contained first a bridge right next to the vertical flow channel, the cells do not flow into this channel.

6.3.2 Cell culture over night

After culturing the cells overnight, they were still only present in the desired places and not in the flow channels that were closed off during cell seeding, due to the good valve closure and the used coatings, see figure 19. The largest problem during overnight cell culture seemed to be air bubbles. On the first try, some pipette tips with DMEM+ fell out of the inlets overnight, and all chambers were filled with air in the morning. Therefore, the second time a big pool of DMEM+ was placed on the chip, so the inlets would not be connected to air directly if the pipette tips fell out. This caused the second experiment to succeed. After 18 hours of culture, some pipette tips fell out again, but now only chamber 7 and 8 were filled with air. Chamber 2 also contained some small air bubbles.

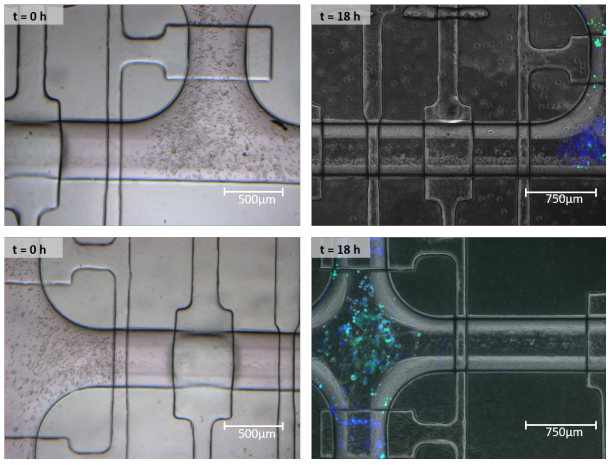


Figure 19: Transitions to flow channels right after cell seeding of Caco-2 cells and after 18 hours of culture and staining with NucBlue and ActinGreen, showing that cells seeded only in the culture chambers.

6.3.3 Trypsin treatment

The cells in chambers 2, 4, 6 and 8 were trypsinized after culturing overnight. Then the cells in all chambers were stained, of which the results can be seen in figure 20. It can be seen that in no chamber all cells were detached and washed away. However, chamber 2, 4 and 6 do seem to contain less cells than chambers 3, 5 and 7. Chamber 1 also contains few cells, while it should not have been trypsinized. Furthermore, chamber 8 still contains a quite even layer of cells throughout the middle of the channel. Trypsin was added to the cells via the normal inlet and outlet while addressing chamber 2, 4, 6 and 8 one by one. However, after this all control channels were depressurized. Trypsin from the pipette tips in the inlet and outlets was sucked into the chip and some of it came in the other chambers too. It is expected that most of this extra trypsin went through chamber 1, because this is the easiest route for the fluid to go from the inlet to the outlet. This might explain why chamber 1 also seems trypsinized. This problem could be solved by immediately closing off all chambers after trypsinizing some, so the trypsin will stay in the right chambers and does not flow into the wrong chambers.

Even though the trypsinized chambers seem to contain less cells than the non-trypsinized chambers, they still contain quite some cells. This might be caused by DMEM+ that was still present somewhere else in the chip while trypsinizing, which could deactivate the trypsin. Chambers 2, 4, 6 and 8 were washed with PBS before trypsinizing, but the other chambers still contained DMEM+. It might be that this medium flowed into the even number chambers, because the chambers were not closed off immediately after trypsinization. So immediately closing off the chambers after adding condition might also help with better trypsinization. A last remarkable result is that chamber 8 still contains a lot cells after trypsinization. This might be caused by the fact that this chamber was filled with air after 18 hours. It might be that trypsin does not work as well on died cells.

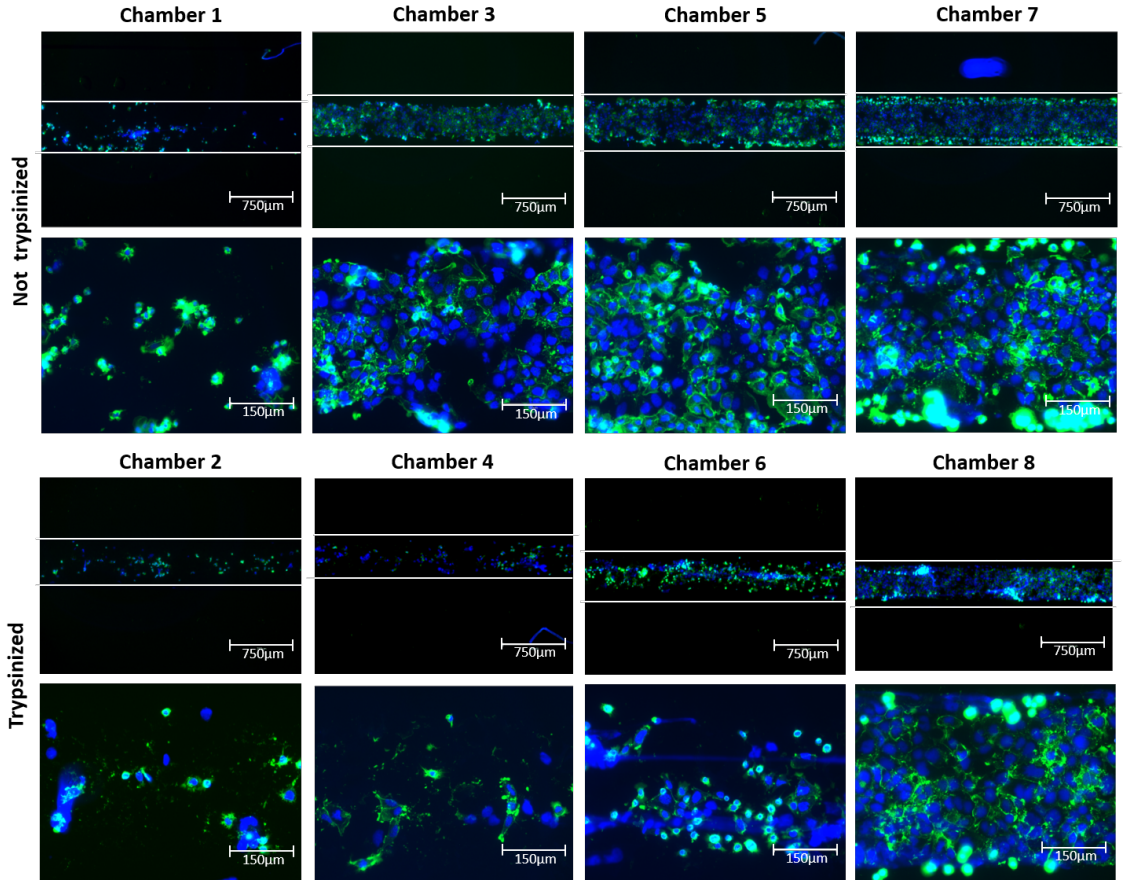


Figure 20: Cell culture of Caco-2 cells (p57) after 18 hours on the multiplexed chip. Nuclei are stained with NucBlue an actin filaments are stained with ActinGreen. Chambers 2, 4, 6 and 8 were flushed with PBS and trypsinized before staining, while the other chambers were stained directly after culturing. Chamber 7 and 8 were filled with air after 18 hours.

7 Recommendations

7.1 Valve closure

For proper valve closure over the entire chip, the PDMS control layer should have the same thickness within one chip. Variation in thickness of the PDMS layer atop the valves, causes a higher pressure to be needed to close off all channels. The pressure should close all valves, and therefor it is put as high as needed to close the thickest valve. Furthermore, the thickness of the layer needs to be made more consistent for every chip, because now the thickness differs for all chips. This could also be part of why some chips can withstand pressure for a longer time than others. To make the control layer more even and consistent, a new mold could be used. The mold should contain walls on the sided with a height of 150 µm. The mold should be completely filled with the 1:20 (w/w) PDMS, and the excess of PDMS should be wiped off. This could result in an even layer in which all valves contain a 70 µm layer of PDMS on top of them. If this does not solve the full problem, it might be that the plain PDMS layer also needs to be optimized. It might be that the small variation in thickness of this layer also influenced the difference in valve closure. This layer could be spincoated after spreading the PDMS onto the glass slide to create a more even layer.

7.2 Solutions lifespan problem

Creating more consistent valves will hopefully result in a lower pressure that is needed on the valves and therefore a longer lifespan on the chip. It is however not expected that this will elongate the lifespan enough for a long cell culture experiment. It must first be tested on more chips if the lifespan was elongated by curing the plain PDMS layer less, because now the results come from only one chip. If the other chips show the same results, the bonding between the control layer and the plain PDMS layer needs to be improved more. This might be done for example with a PDMS/toluene mortar. A mixture of PDMS an toluene can be spincoated on a glass slide to create a thin layer. This layer can than be placed in between two PDMS layers to create a leakage-free bond [27], which could be used in this case in between the plain PMDS layer and the control layer. Furthermore, the lifespan might be increased by using an even higher spinning speed to fabricate thinner layers of PDMS on the valves in the control layer. It is expected that less pressure will than be needed to close the valves, which means that less pressure is put on the PDMS/PDMS bond and the control layer is less likely to release from the plain PDMS layer. If this layer is made too thin however, the valves might brake quickly when the control channels are pressurized.

7.3 Continue cell culture on chip

Even though cells were cultured in the multiplexed chip, some improvements need to be made to culture a healthy, even cell layer. First of all, the lifespan problem of the chip should be solved as described above, so the cells can be cultured for a few days or weeks. Furthermore, a way needs to be found to keep the inlets and outlets filled so no air will come into the chip. This might be done by filling the chip with blunt needles connected to tubing in stead of using pipette tips reservoirs. The protocol of culturing cells on the multiplexed chip contains quite some steps and for each step now new pipette tips were placed into the inlets and outlets. This might have caused them to wear out. When using blunt needles and tubing, the needles can stay in the inlets at all times so there is a smaller change that they wear out and start leaking. Furthermore, these are less high and heavy than pipette tips so they are less likely to fall out. To be able to better individually address each culture chamber, the chambers should be closed off right after a condition is added to them. This will make sure no fluids flow from one to the other chamber. If trypsin is used on some chambers, first all chambers and channels should be flushed with PBS so no DMEM+ is left to deactivate the trypsin. Lastly, a variation in seeding densities in the culture chambers can arise because the fluid flows faster through the first and last channels than through the middle ones while seeding from the flush inlet. If this problem occurs, the cells could be seeded via the normal inlet and outlet using the valves to address the chambers one by one. The flow channels should then be trypsinized afterwards.

8 Conclusions

In this thesis, the fabrication of a multiplexed chip with individually addressable culture chambers is described. The proper coating for cell adhesion and repulsion was examined and it was found that only the culture chambers should be coated, with collagen type I. The chip can successfully be coated and cells can be seeded in the chip. Initial cell culture experiments are preformed in the multiplexed chip, in which cells adhered only in the desired places. These however will need some more optimization. Furthermore, the chip fabrication process is improved by making two layers thinner for optimal valve closure. It was found that the chip still needs improvement of on it's lifespan. The plain PDMS layer was cured less to try and create a stronger bond, but this will need more testing. With the given recommendations to better the chip and the cell culture, the chip has great potential to be used as a multiplexed Gut-on-Chip with an even layer of cells in individually addressable chambers.

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References

[1] Ross J. Porter, Graeme I. Murray, and Mairi H. McLean. Current concepts in tumour-derived organoids. *British Journal of Cancer*, 123(8):1209–1218, oct 2020. ISSN 0007-0920. doi: 10.1038/s41416-020-0993-5.

[2] Qirui Wu, Jinfeng Liu, Xiaohong Wang, Lingyan Feng, Jinbo Wu, Xiaoli Zhu, Weijia Wen, and Xiuqing Gong. Organ-on-a-chip: recent breakthroughs and future prospects. *BioMedical Engineering OnLine*, 19(1):9, dec 2020. ISSN 1475-925X. doi: 10.1186/s12938-020-0752-0. URL `https://doi.org/10.1186/s12938-020-0752-0https://biomedical-engineering-online.biomedcentral.com/articles/10.1186/s12938-020-0752-0`.

[3] Maria Laura Coluccio, Gerardo Perozziello, Natalia Malara, Elvira Parrotta, Peng Zhang, Francesco Gentile, Tania Limongi, Pushparani Michael Raj, Gianni Cuda, Patrizio Candeloro, and Enzo Di Fabrizio. Microfluidic platforms for cell cultures and investigations. *Microelectronic Engineering*, 208:14–28, mar 2019. ISSN 01679317. doi: 10.1016/j.mee.2019.01.004.

[4] Juan Eduardo Sosa-Hernández, Angel M. Villalba-Rodríguez, Kenya D. Romero-Castillo, Mauricio A. Aguilar-Aguila-Isaías, Isaac E. García-Reyes, Arturo Hernández-Antonio, Ishtiaq Ahmed, Ashutosh Sharma, Roberto Parra-Saldívar, and Hafiz M. N. Iqbal. Organs-on-a-Chip Module: A Review from the Development and Applications Perspective. *Micromachines*, 9(10):536, oct 2018. ISSN 2072-666X. doi: 10.3390/mi9100536.

[5] Kornphimol Kulthong, Loes Duivenvoorde, Huiyi Sun, Samuel Confederat, Jiaqing Wu, Bert Spenkelink, Laura de Haan, Victor Marin, Meike van der Zande, and Hans Bouwmeester. Microfluidic chip for culturing intestinal epithelial cell layers: Characterization and comparison of drug transport between dynamic and static models. *Toxicology in Vitro*, 65:104815, jun 2020. ISSN 08872333. doi: 10.1016/j.tiv.2020.104815.

[6] Chao Ma, Yansong Peng, Hongtong Li, and Weiqiang Chen. Organ-on-a-Chip: A New Paradigm for Drug Development. *Trends in Pharmacological Sciences*, 42(2):119–133, feb 2021. ISSN 01656147. doi: 10.1016/j.tips.2020.11.009.

[7] Christian Frantz, Kathleen M. Stewart, and Valerie M. Weaver. The extracellular matrix at a glance. *Journal of Cell Science*, 123(24):4195–4200, dec 2010. ISSN 1477-9137. doi: 10.1242/jcs.023820.

[8] Elaine N Marieb and Katja N Hoehn. *Human Anatomy and Physiology*. Pearson, 10th edition, 2016. ISBN 9781292096971.

[9] Yuki Imura, Yasuyuki Asano, Kiichi Sato, and Etsuro Yoshimura. A microfluidic system to evaluate intestinal absorption. *Analytical Sciences*, 25(12):1403–1407, 2009. ISSN 13482246. doi: 10.2116/analsci.25.1403.

[10] Masoomeh Tehranirokh, Abbas Z. Kouzani, Paul S. Francis, and Jagat R. Kanwar. Microfluidic devices for cell cultivation and proliferation. *Biomicrofluidics*, 7(5):051502, sep 2013. ISSN 1932-1058. doi: 10.1063/1.4826935.

[11] M. Zakharova, M. A. Palma do Carmo, M. W. van der Helm, H. Le-The, M. N. S. de Graaf, V. Orlova, A. van den Berg, A. D. van der Meer, K. Broersen, and L. I. Segerink. Multiplexed blood–brain barrier organ-on-chip. *Lab on a Chip*, 20(17):3132–3143, sep 2020. ISSN 1473-0197. doi: 10.1039/D0LC00399A.

[12] Olivier Frey, Patrick M Misun, David A Fluri, Jan G Hengstler, and Andreas Hierlemann. Reconfigurable microfluidic hanging drop network for multi-tissue interaction and analysis. *Nature Communications*, 5(1):4250, sep 2014. ISSN 2041-1723. doi: 10.1038/ncomms5250.

[13] Mimetas. OrganoPlate® 3-lane 40. URL `https://www.mimetas.com/en/organoplate-3-lane-40/`.

[14] Marc A Unger, Hou-Pu Chou, Todd Thorsen, Axel Scherer, and Stephen R Quake. Monolithic Microfabricated Valves and Pumps by Multilayer Soft Lithography. Technical report.

[15] A. R. Vollertsen, D. de Boer, S. Dekker, B. A. M. Wesselink, R. Haverkate, H. S. Rho, R. J. Boom, M. Skolimowski, M. Blom, R. Passier, A. van den Berg, A. D. van der Meer, and M. Odijk. Modular operation of microfluidic chips for highly parallelized cell culture and liquid dosing via a fluidic circuit board. *Microsystems and Nanoengineering*, 6(1):107, dec 2020. ISSN 2055-7434. doi: 10.1038/s41378-020-00216-z.

[16] Shantanu Bhattacharya, Arindom Datta, J.M. Berg, and Shubhra Gangopadhyay. Studies on surface wettability of poly(dimethyl) siloxane (PDMS) and glass under oxygen-plasma treatment and correlation with bond strength. *Journal of Microelectromechanical Systems*, 14(3):590–597, jun 2005. ISSN 1057-7157. doi: 10.1109/JMEMS.2005.844746.

[17] Lin Wang, Bing Sun, Katherine S Ziemer, Gilda A Barabino, and Rebecca L Carrier. Chemical and physical modifications to poly(dimethylsiloxane) surfaces affect adhesion of Caco-2 cells. *Journal of Biomedical Materials Research Part A*, 9999A:NA–NA, 2009. ISSN 15493296. doi: 10.1002/jbm.a.32621.

[18] George Sitterley. Fibronectin (FN) — Sigma-Aldrich, 2008. URL `https://www.sigmaaldrich.com/technical-documents/articles/biofiles/fibronectin.html`.

[19] Jyrki Heino. The collagen family members as cell adhesion proteins. *BioEssays*, 29(10):1001–1010, oct 2007. ISSN 02659247. doi: 10.1002/bies.20636.

[20] Sigma-Aldrich. Collagen Coating Protocol — Sigma-Aldrich. URL `https://www.sigmaaldrich.com/technical-documents/articles/biofiles/collagen-product-protocols.html`.

[21] Dhavan Sharma, Wenkai Jia, Fei Long, Shweta Pati, Qinghui Chen, Yibing Qyang, Bruce Lee, Chang Kyong Choi, and Feng Zhao. Polydopamine and collagen coated micro-grated polydimethylsiloxane for human mesenchymal stem cell culture. *Bioactive Materials*, 4:142–150, dec 2019. ISSN 2452199X. doi: 10.1016/j.bioactmat.2019.02.002.

[22] SuSos Surface Technology. PLL-g-PEG Polymere. URL `https://susos.com/beschichtungstechnologien/pll-g-peg-polymere/`.

[23] Ammar Azioune, Marko Storch, Michel Bornens, Manuel Théry, and Matthieu Piel. Simple and rapid process for single cell micro-patterning. *Lab on a Chip*, 9(11):1640–1642, jun 2009. ISSN 14730189. doi: 10.1039/b821581m.

[24] Marc A. Unger. Monolithic Microfabricated Valves and Pumps by Multilayer Soft Lithography. *Science*, 288(5463):113–116, apr 2000. ISSN 00368075. doi: 10.1126/science.288.5463.113.

[25] Seunghwan Lee and Janos Vörös. An Aqueous-Based Surface Modification of Poly(dimethylsiloxane) with Poly(ethylene glycol) to Prevent Biofouling. *Langmuir*, 21(25):11957–11962, dec 2005. ISSN 0743-7463. doi: 10.1021/la051932p.

[26] Dhananjay Bodas and Chantal Khan-Malek. Hydrophilization and hydrophobic recovery of PDMS by oxygen plasma and chemical treatment—An SEM investigation. *Sensors and Actuators B: Chemical*, 123(1):368–373, apr 2007. ISSN 09254005. doi: 10.1016/j.snb.2006.08.037.

[27] Marinke W. van der Helm, Mathieu Odijk, Jean-Philippe Frimat, Andries D. van der Meer, Jan C.T. Eijkel, Albert van den Berg, and Loes I. Segerink. Fabrication and Validation of an Organ-on-chip System with Integrated Electrodes to Directly Quantify Transendothelial Electrical Resistance. *Journal of Visualized Experiments*, 2017(127):56334, sep 2017. ISSN 1940-087X. doi: 10.3791/56334.

Appendices

A Protocols

A.1 Chip production

The fabrication process for the PDMS device was provided and is based on Unger et al. [24].

1. Mix PDMS (RTV 615) (1:7 (w/w)) for the flow layer and (1:20 (w/w)) for the control layer. Control layer: 2 gram + 0.1 gram. Flow layer: 3.5 gram + 0.5 gram.
2. Degas the PDMS for 2 hours
3. Cast the PDMS on the flow layer mould. For the control layer, spincoat the PDMS on the micro milled PMMA mould at 750 rpm for 60 seconds, resulting in an approximately 70 µm thick membrane at the valve site.
4. Place the control layer on a flat surface after spinning for 20 minutes at room temperature.
5. Pre-cure both layers for 45 min at 60°C.
6. Punch the inlets for the flow channel.
7. Align the two layers and press (gently) together to bond.
8. Cure overnight (ON) at 60 °C.
9. Punch the inlets for the control channels.
10. Cast PDMS (Sylgard) 10:1 on a large glass slide.
11. Cure for 20 min at 60 °C.
12. Oxygen plasma treat the control layer and the PDMS on the glass slide and bond.
13. To promote the bond, and fully cure the 10:1 PDMS, place in an oven ON at 60 °C.
14. The final device now consists of four layers, three PDMS layers (flow and control, 10:1 PDMS) and one glass layer.

A.2 Protocol coating

Simplified chip:

1. Oxygen plasma treat the chip.
2. Flush all channels with 70% ethanol.
3. Flush all channels with PBS and place pipette tips with 2.5 µL PBS in the inlets and 0.5 µL in the outlets.
4. Flush desired channel with a 100 µg/mL PLL-g-PEG in PBS solution and place pipette tips with 2.5 µL PLL-g-PEG in the inlets and with 0.5 µL in the outlets.
5. Incubate at RT for 30 minutes.
6. Flush some of the coated channels with PBS and place pipette tips with 2,5 µL PBS in the inlets and with 0.5 µL in the outlets.
7. Flush the other coated channels and some uncoated channels with a 0.1 mg/mL collagen type I in PBS solution put pipette tips with 2,5 µL collagen type I in the inlets and with 0.5 µL in the outlets.
8. Incubate for 60 minutes at 37 °C.
9. Flush coated channels with PBS.
10. Directly use the chip for cell culture.

Multiplexed chip:

1. Oxygen plasma treat the chip.
2. Fill the chip with 70 % ethanol.
3. Flush the chip with PBS and place pipette tips with 100 µL PBS in all inlets and outlets.
4. Pressurize control channels 1, 2, 4, 5 and 6.
5. Flush the culture chambers with a 0.1 mg/mL collagen type I in PBS solution via the flush inlet and outlet and place pipette tips with 100 µL collagen type I in this inlet and outlet.
6. Incubate for 60 minutes at RT.
7. Flush the culture chambers with PBS via the flush inlet and outlet and place pipette tips with 100 µL PBS in this inlet and outlet.
8. Depressurize all control channels.
9. Directly use the chip for cell culture.

A.3 Cell culture

Off-chip:
Medium renuwal in T25 flask, every two to three days:

1. Aspirate the culturing medium from the flask.
2. Wash the cells with 2 mL PBS and aspirate the PBS.
3. Add 3 mL new DMEM+ (DMEM with 10% FBS and 100 units/mL pen/strep).
4. Incubate at 37 °C.

Passaging in T25 flask, every four to five days:

1. Aspirate the culturing medium from the flask.
2. Wash the cells with 2 mL PBS and aspirate the PBS.
3. Add 750 µL 10% trypsin
4. Incubate for 3-5 minutes at 37 °C until cells are detached.
5. Add 5 mL of DMEM+ and resuspend cells, flushing cells off of the culturing surface.
6. Transfer cells in DMEM+ and trypsin to a 10 mL tube and centrifuge for 6 min at 130 g.
7. Aspirate medium from the cells
8. Add 600 µL DMEM+ and resuspend.
9. Mix 15 µL of the cell suspension with 15 µL of trypan blue.
10. Add mixture to a counting slide and count cells using an automated cell counter.
11. Add 3 mL DMEM+ to a new T25 culture flask.
12. Add enough cells to create a seeding density of 10.000 cells/cm^2.
13. Incubate at 37 °C.

Simplified chip

1. Place DMEM+ in an half open tube in an incubator at 37 °C and incubate for 1 hour.
2. Flush all channels with DMEM+ and place pipette tips with 2.5 µL DMEM+ in the inlets and with 0.5 µL in the outlets.
3. Incubate for 2 hours at 37 °C.
4. Flush all channels with a cell suspension to create a 100.000 cells/cm^2.

5. Place pipette tips with 2.5 µL cell suspension in the inlets and with 0.5 µL in the outlets.
6. Incubate for 1 hour at 37 °C.
7. Carefully flush the channels with DMEM+ and place pipette tips with 10 µL DMEM+ in the inlets and with 0.5 µL in the outlets.
8. Repeat step 7 twice a day for three days.

Multiplexed chip

1. Place DMEM+ in an half open tube in an incubator at 37 °C and incubate for 1 hour.
2. Flush the chip with DMEM+ and add pipette place pipette tips with 200 µL DMEM+ in each inlet and outlet.
3. Incubate for 1 hour.
4. Pressurize control channels 1, 2, 4, 5 and 6.
5. Flush a cell suspension through the culture chambers via the flush inlet and outlet to create a seeding density of 200.000 cells/cm^2.
6. Incubate for 1 hour at 37 °C.
7. Carefully flush the chambers with DMEM+ via the flush inlet and outlet and place pipette tips with 200 µL DMEM+ in this inlet and outlet.
8. Depressurized the control channels.
9. Incubate for 17 hours at 37 °C.
10. Pressurize control channel 7.
11. Flush flow channels with PBS by flowing from the normal inlet to the flush inlet and from the normal outlet to the flush outlet.
12. Place pipette tips with 200 µL DMEM+ in the flush inlet and flush outlet.
13. Address all chambers one by one by pressurizing the right control channels and flush them with PBS via the normal inlet and outlet.
14. Address chambers 2, 4, 6 and 8 one by one by pressurizing the right control channels and flush them with 15 % trypsin in PBS via the normal inlet and outlet. Pressurize control channel 7 directly afterwards to close off the culture chambers.
15. Incubate for 10 minutes at 37 °C.
16. If cells are not detached, repeat step 14 and 15.
17. Address all chambers one by one by pressurizing the right control channels and flush them thoroughly with PBS via the normal inlet and outlet.

A.4 Cell staining

12-well plate

1. Aspirate culturing medium from the cells.
2. Wash cells 3x with PBS.
3. Add 1 mL of 4% formaldehyde in PBS solution to each well.
4. Incubate for 20 minutes at RT.
5. Was cells 3x with PBS.
6. Add 1 mL of 0.3% TritonX in PBS solution to each well.
7. Incubate for 30 minutes at RT.

8. Was cells 3x with PBS.
9. Add 1 mL ActinGreen 488 ReadyProbes Reagent 2 drops/mL PBS solution to each well.
10. Incubate for 30 minutes at RT, protected from light.
11. Wash cells 3x with PBS.
12. Add 1 mL NucBlue Live ReadyProbes Reagent 2 drops/mL PBS solution to each well.
13. Incubate for 20 minutes at RT, protected from light.
14. Wash cells 3x with PBS

Simplified chip

1. Flush channels thoroughly with PBS
2. Flush channels with 4% formaldehyde in PBS solution and place pipette tips with 2.5 µL formaldehyde solution in the inlets and with 0.5 µL in the outlets.
3. incubate for 20 minutes at RT.
4. Flush channels thoroughly with PBS.
5. Flush channels with 0.3% TritonX in PBS solution and place pipette tips with 2.5 µL TritonX solution in the inlets and with 0.5 µL in the outlets.
6. incubate for 30 minutes at RT.
7. Flush channels thoroughly with PBS.
8. Flush channels wit ActinGreen 488 ReadyProbes Reagent 2 drops/250 µ PBS solution and place pipette tips with 2.5 µL ActinGreen solution in the inlets and with 0.5 µL in the outlets.
9. incubate for 30 minutes at RT, protected from light.
10. Flush channels thoroughly with PBS.
11. Flush channels with NucBlue Live ReadyProbes Reagent 2 drops/250 µ PBS solution and place pipette tips with 2.5 µL Nucblue solution in the inlets and with 0.5 µL in the outlets.
12. incubate for 20 minutes at RT, protected from light.
13. Flush channels thoroughly with PBS.

Multiplexed chip

1. Address all chambers one by one by pressurizing the right control channels and flush them with 4% formaldehyde in PBS solution via the normal inlet and outlet.
2. Place pipette tips with 20 µL formaldehyde solution in all inlets and outlets and incubate for 20 minutes at RT.
3. Pressurize control channel 1, 2, 4, 5 and 6 and flush all chambers with PBS through the flush inlet and outlet.
4. Address all chambers one by one by pressurizing the right control channels and flush them with PBS via the normal inlet and outlet.
5. Address all chambers one by one by pressurizing the right control channels and flush them with 0.3% TritonX in PBS solution via the normal inlet and outlet.
6. Place pipette tips with 20 µL TritonX solution in all inlets and outlets and incubate for 30 minutes at RT.
7. Pressurize control channel 1, 2, 4, 5 and 6 and flush all chambers with PBS through the flush inlet and outlet.
8. Address all chambers one by one by pressurizing the right control channels and flush them with PBS via the normal inlet and outlet.

9. Address all chambers one by one by pressurizing the right control channels and flush them with ActinGreen 488 ReadyProbes Reagent 2 drops/250 µ PBS solution via the normal inlet and outlet.
10. Place pipette tips with 20 µL ActinGreen solution in all inlets and outlets and incubate for 30 minutes at RT, protected from light.
11. Pressurize control channel 1, 2, 4, 5 and 6 and flush all chambers with PBS through the flush inlet and outlet.
12. Address all chambers one by one by pressurizing the right control channels and flush them with PBS via the normal inlet and outlet.
13. Address all chambers one by one by pressurizing the right control channels and flush them with NucBlue Live ReadyProbes Reagent 2 drops/250 µ PBS solution via the normal inlet and outlet.
14. Place pipette tips with 20 µL NucBlue solution in all inlets and outlets and incubate for 20 minutes at RT, protected from light.
15. Pressurize control channel 1, 2, 4, 5 and 6 and flush all chambers with PBS through the flush inlet and outlet.
16. Address all chambers one by one by pressurizing the right control channels and flush them with PBS via the normal inlet and outlet.