Abstract

This thesis presents methods to combine 3D cell culture, microfluidics and gradients on a controlled cellular scale. 3D cultures in biological extracellular matrix gels or synthetic gels bridge the gap between organ-tissue cultures and traditional 2D cultures. A device for embedding, anchoring and culturing cells in a controlled 3D flow through micro-environment was designed and evaluated. The device was realized using an etched silicon pillar flow chamber filled with gel mixed with cells. The pillars anchor and stabilize the gel as well as increase the surface to volume ratio, permitting higher surface flow rates and improving diffusion properties. Within the structure cells were still viable and proliferating after six days of cultivation, showing that it is possible to perform medium- to-long term cultivation of cells in a controlled 3D environment.

This concept was further developed to include controllable and time stable 3D microgradient environments. In this system stable diffusion gradients can be generated by the application of two parallel fluid flows with different composition against opposite sides of a gel plug with embedded cells. Culture for up to two weeks was performed showing cells still viable and proliferating. The cell tracer dye calcein was used to verify gradient formation as the fluorescent intensity in exposed cells was proportional to the position in the chamber. Cellular response to an applied stimulus was demonstrated by use of an adenosine triphosphate gradient where the onset of an intracellular calcium release also depends on cell position.
Papers


Contributions by the author

The contribution of Susanna Rydholm to the publications listed above is:

I. Major part of experiments, analysis and writing.
II. Major part of experiments, analysis and writing. Contributed to design of devices
III. Major part of experiment design. Contributed to experiments, analysis and writing.
IV. Major part of experiment design, device design, experiments, analysis and writing.
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>PAPERS</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td>CONTENTS</td>
<td></td>
<td>V</td>
</tr>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>MATERIALS</td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>CELLS</td>
<td>3</td>
</tr>
<tr>
<td>2.1.1</td>
<td>COS-7 and MDCK</td>
<td>3</td>
</tr>
<tr>
<td>2.1.2</td>
<td>DI TNC1</td>
<td>4</td>
</tr>
<tr>
<td>2.2</td>
<td>GELS</td>
<td>5</td>
</tr>
<tr>
<td>2.3</td>
<td>FLUORESCENT PROBES</td>
<td>6</td>
</tr>
<tr>
<td>2.4</td>
<td>CONFOCAL MICROSCOPE</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>MICROMACHINED DEVICES</td>
<td>8</td>
</tr>
<tr>
<td>3.1</td>
<td>MICROFABRICATION METHODS</td>
<td>8</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Deep reactive ion etching (DRIE)</td>
<td>8</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Anodic Bonding</td>
<td>9</td>
</tr>
<tr>
<td>3.2</td>
<td>MICROMACHINED SILICON CHAMBER FOR 3D CELL CULTURE</td>
<td>9</td>
</tr>
<tr>
<td>3.3</td>
<td>MINIATURIZED DEVICE FOR GRADIENT STUDIES</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>DEVICE EVALUATION</td>
<td>13</td>
</tr>
<tr>
<td>4.1</td>
<td>3D CULTURE CHAMBER</td>
<td>13</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Evaluation of gel and fluidic properties</td>
<td>13</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Cells in 3D culture chamber</td>
<td>15</td>
</tr>
<tr>
<td>4.1.3</td>
<td>Discussion regarding 3D culture chamber evaluation</td>
<td>16</td>
</tr>
<tr>
<td>4.2</td>
<td>GRADIENT DEVICE</td>
<td>17</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Diffusion theory and calculations</td>
<td>17</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Experimental evaluation of gel filling and diffusion gradient</td>
<td>19</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Cells in gradient device</td>
<td>22</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Discussion regarding gradient device evaluation</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>DISCUSSION AND CONCLUSIONS</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>ACKNOWLEDGEMENTS</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>REFERENCES</td>
<td>30</td>
</tr>
</tbody>
</table>
1 Introduction

Most cell biological studies are carried out in traditional petri dishes or on microscope slides. These techniques are easy to use, with well established culturing methods and allow a multitude of cells to be observed. Two trends are however emerging, culturing cells in three dimensions and miniaturization of experimental assays. In vivo, cells are organized in three-dimensional patterns surrounded by other cells as well as extracellular matrix, ECM, all components collectively forming tissues. Culturing cells in an artificial ECM makes it possible to perform single cell studies of cells adapted to a three dimensional (3D), and thus more in vivo like, environment. Such culture conditions bridge the gap between traditional organ-tissue 3D cultures and conventional 2D cultures. Experiments made with cell cultures in 3D ECM gels in petri dishes show that cells exhibit different behaviour than 2D cultures due to changes in morphology and cell adhesion properties [1-2]. For example, cancer cells do not respond to drugs the same way in gels as on plastic surfaces [3-7]. As a consequence, much effort is invested in creating good gels for cell growth and support [8-13].

Culturing cells in gels is a step towards the development of more versatile and in vivo like cell examination assays. Miniaturization offers additional possibilities, such as increased flow control due to laminar flow profiles, reduced material consumption and facilitation of high throughput screening. Further, the diffusion lengths are shorter, leading to a better control of the cell micro environment and the delivery of treatments to the cells. The experiments can thus be made more complex, with controlled flows reaching the cells from different directions creating diffusion gradients. Also, the small dimensions used result in steep gradients even on the cell scale.

Gradients are of interest for several reasons. During developmental growth, it is known that morphogen gradients help to control differentiation processes and thus organization of the organs in the living body [14-16]. Processes such as axon growth and guidance in nervous tissue as well as cell migration and chemotaxis are driven by chemical gradients [17-19]. Stable gradients also enable screening studies in a wide concentration range of drugs and treatments.

Miniaturized gradients have previously been made by using laminar flows in channels [20-28], diffusion through membranes or microvalves [29, 30] or by use of microfluidic injectors in a reservoir [31]. These gradients have the advantage of being formed and switched rapidly. However, such systems have two main disadvantages. First, they can only be used on 2D cultures, and second, such gradients are difficult to keep temporally
and spatially stable. Maintaining laminar flow gradients over cells often requires a constant flow. Flow will exert shear stress forces which the cells can be sensitive to. Gradients over gels have also been made by microfluidic control and hydrodynamic focusing during polymerization [32, 33]. The main disadvantage with such systems is the relative complexity of the gel loading, as simultaneous control over four or five independent flows are necessary. Further, the gel is not accessible once formed. This makes supply of nutrients during cultivation difficult and offers less versatility as an analysis system. Also, different gel widths, and thus different gradient slopes, cannot be achieved and tested simultaneously. High throughput assays with such systems are not readily feasible.

Another method to immobilize cells in 3D is through dynamic seeding (mechanical fence trapping) [34] of cells. This method causes somewhat high and non-variable cell densities in the device.

This thesis focuses on methods to combine cell culture in gels with microstructures. The first paper shows a method to fill a silicon pillar structure with gel and cells and to maintain culture environment. The second paper further develops this concept into more complex fluidic guidance for cell-gel filling, facilitated culturing and generation of stable gradients. It also enables a variety of gel assay widths as well as simultaneous reference assays not exposed to the gradient. This concept enables parallel screening and can be further developed into a high throughput screening system. The third paper utilizes the previously developed microstructures and focus on the chemistry and mechano stability of hydrogels. New formulations for hydrogels were tested and discussed in this study. The fourth paper deals with flow induced cellular signals. Here cells are not embedded in gels but are cultured in microstructures and later subjected to asymmetric flow conditions. The third and fourth papers build on the first two studies but with a different focus, why they are not discussed in detail in this thesis but referred to the original publications.
2 Materials

2.1 Cells

In this thesis three cell types were used COS-7, MDCK and DI TNC1. COS-7 and MDCK are renal (kidney) epithelial cells whereas DI TNC1 are astrocytes which appear as support tissue for neurons in brain. These cell types are further described below.

2.1.1 COS-7 and MDCK

The COS-7 and the MDCK cells are renal epithelial cells derived from African Green Monkey embryos and adult Cocker Spaniel respectively. Epithelial cells make up the epithelial tissue that covers or lines all body parts, both internal and external. They form a tight single layer that is boundary to the external environment. Epithelial tissue is found not only as linings of the respiratory, urinary and digestive tracts, but also in more complex invaginations such as sweat glands, lungs, liver and kidney. The main functions of the epithelium are protection, containment of body fluids and transport in and out of the body. Figure 2-1 shows cultured MDCK cells.

In the proximal tubule of the renal nephron the main function of the epithelium is to regulate reabsorption of water and solutes from the primary urine of the lumen. This transport is partly driven by asymmetric salt solutions on each side of the epithelium. The epithelial cells are thus exposed to an asymmetric environment, and in response the cells themselves are asymmetric, i.e. polarised. Polarisation means that there are different membrane proteins at the apical (facing the lumen) and the basolateral sides (blood sides).

COS-7 cells are robust and comparably easy to culture. They are cultured in petri dishes in medium with the addition of serum, penicillin and L-glutamin, and are kept at 37 °C in an atmosphere containing 5% CO₂. When they have reached confluence, usually after 3-4 days, they are harvested by exposure to trypsin, after which they can easily be removed from the substrate by gentle flushing, and replated in fresh culture dishes. MDCK cells are slightly more difficult to culture as they are more strongly attached to the growth substrate. As a result of this a cell scraper is used after trypsination to detach the cells into suspension.

Culture medium has the disadvantage of being auto fluorescent, and for experiments with fluorescent microscopy phosphate-buffered saline (PBS) is commonly used.
Figure 2-1: MDCK cells in 2D culture stained for the cytoskeletal component tubulin (the two cells in the middle are undergoing cell division).

instead. The PBS used in this thesis contains (in mM) 100 NaCl, 4.0 KCl, 1.0 CaCl₂, 1.2 MgCl₂, 20.0 HEPES, 1.0 NaH₂PO₄, 10 D-glucose, and the pH was adjusted to 7.4.

2.1.2 DI TNC1
The DI TNC1 cells are astrocytes which are one of three different types of glial cells, or neuroglia, that are present in nervous tissue. Astrocytes have three major functions. Firstly they provide mechanical support for neurons and mediate the exchange of metabolites between neurons and blood vessels. Secondly they help in maintaining extracellular ionic homeostasis by buffering the extracellular concentration of ions. Thirdly, they clean up and remove dead neurons. The other types of glial cells are oligodendrocytes, which produce myelin sheets that insulate axons, and microglia, which clean up neuronal debris and play a role of the immune system in brain. Figure 2-2 shows cultured astrocytes.

The astrocyte cell line used in this thesis (DI TNC1) is derived from diencephalon tissue of a one day old sprague-dawley rat. The cells were cultured in DMEM GlutaMAX-1 medium with the addition of serum at 37 °C and 5 % CO₂. Before confluence they were harvested by exposure to TrypleExpress enzyme for 5 minutes at 37 °C. Experiments with astrocytes using fluorescent microscopy were also performed with PBS as described above.
2.2 Gels

Tissues are not made up exclusively by cells, but a substantial amount is extracellular space filled with a network of macromolecules called the extracellular matrix (ECM). The ECM is produced locally by the cells in the matrix, and the composition thus differs between different tissues. It is the ECM that determines much of the physical properties of tissues, but apart from being a mechanical support structure the ECM is also important in cell signaling, wound repair, cell adhesion and tissue function.

The ECM consists of three major types of macromolecules – fibers, proteoglycans and glucoproteins. The main fibrous components are collagen, elastin and laminin, and their primary function is to give the tissue strength and elasticity. Proteoglycans stabilize the collagen networks, give the tissue ability to withstand compressive forces and function as cell surface receptors. The third group, the glycoproteins, provides a linkage between cells and matrix components.

In this thesis one artificial ECM gel and two types of hydrogels were tested. The ECM gel is the commercially available Matrigel (BD Biosciences, Bedford, MA, USA). It mainly consists of proteins such as laminin, collagen IV, heparan sulphate proteoglycans, entactin and nidogen. It also contains growth factors such as TGF-B, fibroblast growth factor and tissue plasminogen activator. In addition to the Matrigel, two types of hydrogels were used. Hydrogels are networks of water-insoluble polymer chains. The first hydrogel consists of an equimolar mixture of Phenylalanine (Phe) dipeptide formed by solid-phase synthesis with a fluorenylmethoxycarbonyl (Fmoc) protector group on the N-terminus, and Fmoc-protected Lysine (Lys). This stable gel scaffold gives a good support for 3D culture compared to other peptide combinations [35]. The gel is shear sensitive, and returns to a liquid state with vigorous stirring. The second hydrogel consist solely of Phenylalanine. It was made by first creating a gelling agent [36] consisting of Fmoc-Phe-Phe dissolved in dimethyl sulfoxide. The agent is
added to the desired dispersion medium, here PBS, initializing an immediate gelling process.

## 2.3 Fluorescent probes

Fluorescent probes absorb light in one wavelength and emit it at another. This makes it possible to use them in the fluorescent microscope, and to achieve clear images of the stained structures. All fluorescent cell probes in this thesis are cell-permeant dyes with carboxylic acids that have been modified with acetoxymethyl (AM) ester groups, which results in uncharged molecules. Once inside the cell intracellular esterases hydrolyze the ester bonds reforming the carboxyl groups. The probe becomes polarized and is leaking out of the cell much more slowly than it entered. In some cases, amongst the probes used here, the dye is non-fluorescent until hydrolyzed. In this thesis two probes were used, calcein AM for cell viability tests and fluo-4 AM for detection of intracellular calcium signals.

In addition to the fluorescent cell probes two other fluorescent probes, fluorescein and rhodamine, were used. They were used to stain fluids and gels to enable microscope monitoring.

## 2.4 Confocal microscope

The confocal laser scanning microscope (CLSM) is an essential tool for many biomedical imaging applications. The CLSM has several applications, which include imaging of thin optical sections, multiple wavelength images, three-dimensional reconstructions and living cell and tissue sequences.
Before viewing in the confocal microscope the samples are stained with fluorescent probes. In the confocal microscope a single point of the sample is illuminated and detected at a time, and an image is made by scanning over the specimen. The laser light is reflected down to the sample by the dichroic mirror, i.e. a mirror that selectively reflects certain wavelengths while others are allowed to pass. Emitted light from the specimen has a longer wavelength than the laser light, and it can thus be separated from the reflected light on its way back towards the detector. Figure 2-3 shows the excitation and the emission beam paths of the confocal microscope.

Thin optical slices of thick specimens can be made in the confocal microscope by only allowing light from the focal plane to reach the detector. This is performed with use of a pinhole aperture, which is placed so that light from in focus regions of the specimen is also in focus at the pinhole. Mostly this light can pass through the small pinhole opening and reach the detector (whole line in figure 2-3 b), whereas light from other regions will mainly be blocked (dotted line in figure 2-3 b). In this way it is possible to make thin optical slices of thick specimens without physical damage. By adding together several slices from different focus positions a high resolution three-dimensional reconstruction of the specimen can be made. With an open pinhole the microscope functions as an ordinary fluorescent microscope.
3 Micromachined devices

3.1 Microfabrication methods

Two traditional microfabrication methods were used in this thesis, deep reactive ion etching (DRIE) and anodic bonding. They are both shortly described here.

3.1.1 Deep reactive ion etching (DRIE)

Microstructures are commonly made of silicon, much due to its mechanical and electrical characteristics and available microfabrication methods. The starting material is a thin (300-500 µm thick) monocrystalline silicon wafer. Onto this wafer the desired structure is patterned using a photosensitive polymer solution, photoresist, and a glass mask. The photoresist covers the wafer surface, and is exposed through the glass mask so that the mask pattern is transformed to the photoresist, see figure 3-1.

DRIE is a method for directed vertical etching of silicon. It is performed with alternating an isotropic etch step and a passivation step. The isotropic etch has the same etch rate in all directions, compared to anisotropic etching which has different rates in different crystal directions. The isotropic etch results in a shallow pit, see figure 3-2 b). During the passivation step a protecting fluorocarbon layer is deposited all over the structure, figure 3-2 c). The next etch step removes the fluorocarbon from all horizontal surfaces by directed acceleration of the etch gas, and another pit is formed. By continuing this process for several cycles the desired etch depth is achieved.

Figure 3-1: Photoresist patterning of silicon wafer. a) The photoresist covers the entire wafer and is b) exposed through the glass mask so that c) the mask pattern is transformed to the photoresist.
3.1.2 Anodic Bonding

Anodic bonding is a method to permanently bond glass to silicon. A Pyrex (borosilicate) glass wafer (170-300 µm thick) and a silicon wafer are heated to 350-500 °C, and a strong electric field (100-1000 V) is applied. After cooling electrical charges are located at the interface between the silicon and the glass, resulting in large electrostatic attraction forces holding the wafers together.

3.2 Micromachined silicon chamber for 3D cell culture

A flow-through microchamber for 3D cell culture was designed and manufactured using the microfabrication methods described above. It has one inlet and one outlet for fluid connections and a chamber cavity in the middle. Inside the chamber there are pillars which help to stabilize the gel-cell mixture. The chamber is 400 µm wide, 90 µm deep and the diameter of the pillars is 40 µm. Figure 3-3 a-b show a conceptual sketch of the whole device and a SEM picture of the chamber cavity with pillars. Three distances between the pillars were used, 20, 35 and 70 µm. The chamber structure was etched in silicon and is sealed with an anodically bonded 300 µm thick glass lid to permit optical detection.

The chip filling and usage procedure is schematically pictured in figure 3-3 c. In this system only one gel type, the Matrigel, was used. Matrigel is liquid at low temperatures, enabling pipetting of cell-gel mixture down into the chip. During polymerization (transition from liquid into gel) Matrigel shrinks creating flow paths for further perfusion, as shown in figure 3-3 c-ii. Further perfusion is necessary for supply of culture medium and test substances. Fluid transport inside the chip is controlled by pipetting or hydraulic pressure (syringe pump). The device was evaluated in terms of fluidic properties, mechanical stability of the gel and cell viability and proliferation.

Figure 3-2: DRIE of silicon. a) Photoresist patterned silicon. b) Etch step that results in a shallow pit. c) Passivation step placing a protective fluorocarbon layer over the structure. d) The next etch step results in another pit.
Figure 3-3: a) A conceptual image of the device and b) a SEM picture of the chamber with pillars. c) Schematic image of chip filling and usage procedure. i) Axial cross section of microchamber filled with gel and cells at 5 °C. ii) Gel shrinkage during polymerization at 37 °C creates channels that enable cell treatment through perfusion. iii) Schematic illustration of perfusion.

3.3 Miniaturized device for gradient studies

The microchip for 3D cell culture was further developed to enable more controllable and specific environments. The new design allowed cells to be exposed to controlled and stable chemical concentration gradients in three dimensions. This was achieved by maintaining separate flows on each side of a gel plug, and thus initiating a diffusion process through the gel as shown in figure 3-4. The gradient is maintained by constant flow through the channels, ensuring that the concentrations are preserved at the gel boundaries. In theory this should result in a linear gradient of solutes, determined by the concentration difference in the channels, the permeability of the gel for the solute and the size of the gel plug. The theory describing diffusion properties in such a system is further described in section 4.2.1.
The gradient chip consists of two inlet and two outlet channels connected by three gel-cell culture assay chambers, see figure 3-5 a. The assay chambers are marked with squares. Each channel is also divided into separate side channels with reference cell assay chambers marked with circles. These reference chambers were designed to allow simultaneous study of cells not exposed to a gradient. This feature has however not been used or characterized in the present work. Figure 3-5 b shows a SEM image of a separate cell assay chamber. The assay chambers contain pillars with diameter 20 μm and spacing 50 μm, as they help to reinforce the gel. The width of the assay chambers range from 100 to 500 μm, and the height is 50 μm.

Figure 3-6 a shows a sketch of the filling and usage process of the chip. The gel was filled into the assay chambers through a hole etched through the back side of the silicon above each assay chamber. These holes are not present in figure 3-5. As seen from figure 3-6 a-iii the cell assay chamber is in direct contact with the external environment through the fill hole during culture, meaning that no perfusion is necessary to supply the cells with fresh medium. Instead, the chip can simply be placed in a traditional petri dish with culture medium, where supply of nutrients and oxygen to the cells relies on

**Figure 3-4: Schematic image of gradient chamber function.**

**Figure 3-5: a) Gradient chip with 2+2 reference cavities (marked by circles) and 3 gradient cavities in middle (marked with squares) with different lengths, here 300 μm, 400 μm and 500 μm. The varying lengths enable three different gradient slopes within the same chip. Perfusion channel widths are 500 μm. b) SEM image of single 300 μm x 200 μm cell assay chamber with pillars. Pillar diameters are 20 μm and spacing is 50 μm.**
diffusion. Figure 3-6 b schematically shows the fabrication process, with patterning of photoresist onto a silicon wafer, etching of channels and assay chambers, glass bonding and finally etching of holes (inlets, outlets and assay chamber fill holes) through the back side of the wafer. Two separate masks were used to achieve the different patterns of photoresist on each side of the silicon wafer. The glass was bonded to the front side to seal the channels. After etching of the back side holes the assay chamber pillars were attached only through bonding to the glass. The thickness of the glass was 170 μm (equivalent to cover slip thickness no. 1.5) to enable high resolution microscopy imaging of the cells. The chamber was evaluated in terms of sharpness and stability of the gradient and cellular responses.

**Figure 3-6:** a) Schematic image of chip filling and usage procedure and b) schematic side view of chip manufacturing process: i. patterning of photoresist (green) on silicon wafer, ii. etching of channels, iii. bonding of glass and iv. etching of holes through back side of silicon wafer.
4 Device evaluation

4.1 3D culture chamber

The 3D culture device was evaluated in terms of fluidic properties and gel stability which are both important factors when maintaining a controlled microenvironment for living cells. Further, cell viability and proliferation was examined. In this device only one gel type, the Matrigel, was used. The methods used and the results achieved are presented here, followed by a discussion.

4.1.1 Evaluation of gel and fluidic properties

Filling the culture chamber is performed by injecting the exact chamber volume of liquid Matrigel with a pipette. The polymerization is then performed for 10 minutes in room temperature followed by 30 minutes of incubation at 37 ºC. The time in room temperature will allow for slow polymerization of the gel. When Matrigel is polymerized, i.e. turn from liquid into gel, it undergoes a volume decrease of about 30 %. This volume decrease is essential for the chamber function as it creates flow paths for further perfusion with cell culture medium during cultivation. The path formation is facilitated by the time in room temperature before incubation

Three dimensional imaging of the gel gives a clear view of its performance in the chamber. By mixing the gel with a fluorescent probe before injection such imaging is possible with the confocal microscope, as described in section 2.4. Such a 3D image of gel mixed with rhodamine is shown in figure 4-1. It reveals that the gel shrinkage is conical near the pillars. This non-uniform shrinkage leads to a more porous structure of the gel, increasing the surface area and thus the diffusion between the gel and the liquid.

![3D culture chamber](image)

*Figure 4-1: Matrigel stained with rhodamine 3D-imaged with confocal microscopy. Note the funnel shaped shrinkage of the gel around the pillars.*
Figure 4-2: Fluorescent to non-fluorescent flow at $t=75$, 120 s and 360 s in Matrigel-filled channels. The loss of signal (i.e. darker image) indicates the fast substitution of liquid. Imaged with time series in fluorescence microscope.

The rate of replacement of liquid in the chamber is evaluated by switching between fluorescent and nonfluorescent flow. This enables visualization and quantification of exchange rates and flow profiles, as seen in figures 4-2 and 4-3. This revealed a transition time of 120 s (flow rate 20 μl/min) for complete exchange of fluids.

Gel stability during perfusion was examined by mixing fluorescent beads (diameter 1 μm) into the gel. The movement of gel and possible gel fragments can now be visualized as displacement of the beads. During perfusion the gel was measured to remain undisturbed up to a flow rate of 10 mm/s and to be minimally disturbed at 12 mm/s. This is twice the rate as in a previously reported system [37], indicating that the pillars help to reinforce the gel.

Figure 4-3: Exchange rate between fluorescent and non-fluorescent flow in Matrigel-filled channel. Time constant $\tau=120$ s (rise time to 90% or fall time to 10% of final value) for the switch-over. The total exposure time during the switch-over is 0.06 s per pixel at a power of 35 μW, yielding a total energy of 2 μJ per pixel. The different intensity levels of the ROIs could be due to varying thicknesses of the gel layer and thus different amounts of fluorescein.
4.1.2 Cells in 3D culture chamber

When culturing cells inside gels the cells will connect to the protein scaffold of the gel instead of a surface as in 2D culture. To achieve this, the cells needed to be mixed into the gel. The Matrigel ECM was stored frozen and was thawed in refrigerator before use. At this point the Matrigel was liquid and would remain liquid when kept below 4 °C. Thus, mixing of liquid Matrigel and cell pellet were easily performed at low temperatures by gentle pipetting. The liquid cell-Matrigel mixture could then be filled into the desired structures. The devices were incubated, during which time the Matrigel proteins polymerized creating the protein network to which the cells attached.

During cultivation fresh culture medium was flowed through the chamber twice per day, and the chip was placed in culture medium to allow constant diffusion through the inlet and outlet ports into the chamber. No significant change in the viscosity of the Matrigel due to presence of cells was noticed during the filling procedure. It was, however, found that too narrow paths between the pillars, ~20 µm, resulted in cell clogging as seen in figure 4-4a. With enough space (> 35 µm) the cells nicely filled up the volume within the structure. Figure 4-4b shows a fluorescent image of calcein-stained cells and pillars within the structure. The distribution seems to be even, with similar cell densities over the whole chamber. Typical inter-cellular distances can also be seen in figure 4-4b.

Three cell concentrations were tested, 5 000, 10 000 and 20 000 cells/µl, in order to evaluate feasible densities with respect to growth space, clogging, distribution and visibility. The two higher concentrations resulted in densely packed cells with little space in between. This means that the possibilities for cells to proliferate will be limited. Therefore the lowest concentration was used for the majority of the experiments.

A common method to study cell viability is staining with the fluorescent cell tracer dye calcein AM. As described in section 2.3 the dye is cell permeable due to the AM ester group. Once inside the cell the AM group is cleaved by esterases, a process that will

![Figure 4-4](image1.png)

**Figure 4-4**: a) Too narrow paths (approx 20 µm) between the pillars results in cell clogging. b) Calcein-stained cells inside chamber, the cells distribute evenly throughout the structure.
Figure 4-5: Cells stained in chip after 3 days through secondary flow of calcein-AM (1 µM) in PBS. The large variance in cell sizes is likely due to cells being out of focus or clustered.

only occur if the cell is viable. The dye becomes fluorescent and also cell impermeable, i.e. it is trapped in the cell. Thus only viable cells will be positively stained. This method was used to evaluate cell viability and proliferation in the 3D cell culture chamber. During staining 1 µM calcein-AM was injected into the chamber and incubated at 37 ºC for 1 hour to allow for diffusion and homogenous distribution throughout the gel. Viable cells were then detected with fluorescent microscopy.

COS-7 cells were injected into the chamber at a concentration of about 5,000 cells per µl. After 3 days the cell density was ranging from 7,700 -10,400 cells per µl, with a mean value of 8,900 cells per µl, measured in 3 separate recordings. This corresponds to a mean cell density increase of 78 %, showing that the cells proliferate and function within our system and that medium to long term cultivation is possible. Figure 4-5 sows cells stained with calcein after 3 days within the chamber.

4.1.3 Discussion regarding 3D culture chamber evaluation

Gel filling is easily performed by pipetting of liqid gel. It is of great importance that the gel used decreases its volume during polymerization as it enables new flow paths through the chamber to be formed. These paths are necessary to maintain culture conditions. The 30 % shrinkage of Matrigel has proven to be sufficient. Overfilling should be avoided as it prohibits the path formation.

The pillars help to anchor the gel in the chamber. Measurements with fluorescent beads reveals that double flow rate could be used compared to previously reported system [37]. Also, the non-uniform gel shrinkage near the pillars increases the surface to volume ratio, improving the diffusion between the gel and the culture medium. Better diffusion leads to better culture conditions.

Consecutive fluorescent and non-fluorescent perfusion revealed a time of 120 s (flow rate 20 µl/min) for complete fluid exchange in the chamber. This relatively slow exchange rate could be explained by accumulation of fluorescein in the gel and
subsequent slow fluorescein wash out. This is further supported by the steep curve slopes at the beginning of the fluid switch (see figure 4-3).

Cell viability was examined by staining with calcein. After 3 days culture the cell density was increased on average with 78 %, showing that the cells function and proliferate in the chamber and that medium to long term cultivation is possible. Cell proliferation is expected to be higher with more constant perfusion of culture medium.

4.2 Gradient device

4.2.1 Diffusion theory and calculations

The diffusion process is the flux of particles from a region of high concentration to a region of low concentration which arises from the collective random movement of particles, so called Brownian motion. According to Fick’s first law, the flux of particles $\Phi(x, t)$ in the positive x-direction at time t is proportional to the spatial gradient of particle concentration. This yields the expression

$$\Phi(x, t) = -D \frac{\partial c(x, t)}{\partial x},$$

where $D$ is the diffusion coefficient and $c$ is the concentration of particles. The diffusion coefficient states the diffusivity of a particle at a given concentration gradient. If the diffusion coefficient is zero the particle is not able to diffuse at all, and a high diffusion coefficient gives that the particle is highly diffusible. Fick’s first law thus states that there is a flux of diffusible particles from regions of high concentration to regions of low concentration, and that the flux is largest where the concentration gradient is largest [38].

At equilibrium the concentration is by definition independent of time and the flux, $\Phi$, is zero. According to Fick’s law, $\partial c/\partial x$ must be zero when $\Phi = 0$ and $D \neq 0$. At diffusive equilibrium the concentration is thus constant in space and time.

At steady state diffusion there is a constant influx and removal of particles in the system, but the observable macroscopic diffusion is constant. At steady state diffusion, both $\Phi$ and $c$ are independent of time, but the flux does not need to be zero. Instead a constant flow of particles is allowed. If $\Phi$ is constant Fick’s first law can be simplified to

$$\Phi = -D \frac{\partial c}{\partial x},$$

and after integration

$$c(x) = c(x_0) - \frac{\Phi}{D} (x - x_0),$$

at steady state.
where $x_0$ is a reference location. Fick’s first law thus implies that at steady state diffusion the concentration is a linear function of the distance $x$, as visualized in figure 4-6. As the steady state assumption says that the flux is constant there must be a source of particles outside this region where the concentration is changing. Therefore, the steady state assumption cannot be valid for all time in a physical system. It may, however, be a good assumption in cases where large reservoirs of particles interact diffusively with a small system.

![Figure 4-6: Under steady state conditions, the spatial distribution of concentration is linear](image)

Steady state diffusion could be a good approximation for the diffusion process in the gradient chamber, where a small gel plug is in connection with two large flow channels and the concentration in the channels are maintained by constant perfusion. But how does the gel-membrane affect the steady state gradient? Consider a membrane with thickness $d$ that separates two solutions containing solute $n$ at concentration $c_n^{(1)}$ on side one and $c_n^{(2)}$ on side two. The concentration of the solute in the membrane is $c_n(x)$ and the diffusion coefficient of the solute in the membrane is $D_n$. At steady state both the flux $\Phi$ and the concentration $c$ are independent of time. Insertion into equation 3 yields the expression

$$c_n(x) = c_n(0) - \frac{\Phi_n}{D_n} x.$$  \hspace{1cm} (4)

At $x = d$ equation 4 gives that

$$\Phi_n = \frac{D_n}{d} (c_n(0) - c_n(d)),$$  \hspace{1cm} (5)

and by combining equations 4 and 5 the following expression for the concentration $c$

$$c_n(x) = c_n(0) - \frac{x}{d} (c_n(0) - c_n(d))$$  \hspace{1cm} (6)

is achieved.

It is now necessary to formulate boundary conditions for the membrane-liquid interfaces. Assume that, at the interface, the solute is partitioned according to its solubility in the membrane and in the liquid. This means that if the solvent is more soluble in the membrane the concentration at the interface is higher in the membrane than in the liquid. This can be expressed as
4 Device evaluation

\[ k_n = \frac{c_n(0)}{c_n^{(1)}} = \frac{c_n(d)}{c_n^{(2)}}, \]  

(7)

where \( k_n \) is the membrane-liquid partition coefficient.

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4.2.2 Experimental evaluation of gel filling and diffusion gradient

Two different techniques were used to fill the gels into the gradient device, injection of final gel mixture or gel-forming within the microstructure. Matrigel and the shear-sensitive hydrogel are both mixed and ready before filling, and were injected into the device with precision pumping. This was performed with a piece of polyethylene (PE)
tube connected to a 10 μl syringe in a syringe pump with a minimum dispensing volume of 10 nl. The filling process of Matrigel is shown in figure 4-8. After Matrigel filling the chip is incubated at 37 °C for 30 min to ensure full polymerization before connection of perfusion tubing. The shear-sensitive hydrogel is stirred into liquid before injection and re-forms spontaneously after 5 minutes in room temperature.

The second type of hydrogel was formed in the microstructure. 1 μl PBS was introduced to the fill hole of the assay chamber using a pipette. The chamber structure was instantaneously filled with the solution due to capillary forces. Phenylalanine dipeptide solution was then added to the fill hole and allowed to diffuse into the chamber, initializing spontaneous formation of peptide tubular structures and the generation of a stable gel plug after 3 minutes. Any protrusions of excess gel into the perfusion channels could then be removed by application of flow through the channels. A humid environment was maintained throughout the loading process to prevent excess evaporation of the filled suspension before the gel transition was initiated.

![Figure 4-8](image)

*Figure 4-8: Filling of the gradient chamber with Matrigel step by step, from a) before filling to d) filled. The gel is shown as darker color.*
Fluid flow was applied by placing the chip in a holder consisting of a PMMA (polymethyl-methacrylate, Plexiglas) lid and aluminium housing. Temperature control was incorporated in the aluminium housing. The tubing was inserted through the lid and sealed with a PDMS gasket, and the other end was connected to a syringe pump. Figure 4-9 shows a schematic image of the fluidic setup and a cross section of the chip in the holder.

Visualization of the diffusion process and the gradient was achieved by using the fluorescent dye fluorescein in channel 1 and PBS in channel 2. Figure 4-10 a) shows the achieved gradient in Matrigel after 80 minutes perfusion and b) intensity curves of corresponding gradient from between 5 to 80 minutes in arbitrary units. Figure 4-11 shows the development of gradient in a prototype chip up to 2.5 minutes.

Figure 4-10: a) Linear fluorescein gradient seen in microchip assay chamber containing Matrigel ECM. Gradient shown is achieved after 80 minutes of constant flow in both channels. b) Corresponding intensity curve in arbitrary units.
Figure 4-11: Gradient development during the first 2.5 minutes in a prototype device.

One advantage with the peptide hydrogels is that they can easily be removed from the microchamber. Removal of peptide hydrogel plugs was facilitated by perfusion of an enzyme wash solution consisting of SDS and proteinase K at pH 10 and 37°C. The flow rate was set to 50 µl/min and allowed to flush the perfusion channels for 30 minutes. To remove remaining gel residue, the flow rate was lowered to 20 µl/min and flow was prevented in one channel by blocking the outlet. This enabled crossover flow through the assay chamber. Pumping was continued until gel was entirely removed from the chamber. Additional washing with NaOH (1M) followed by repeated DI water rinsing was then performed before drying. After this cleaning process the chip was ready to be used in a new experiment.

4.2.3 Cells in gradient device

When filling cells into the gradient chip the cells are first suspended in the gels, and are thus filled simultaneously with the gel. Matrigel is liquid at temperatures below 4 °C, which enables gentle mixing of cells and Matrigel. This process must occur at low temperatures. The shear sensitive hydrogel is initially assembled into a stable gel followed by temporary liquification upon stirring. Effortless mixing with a cell pellet is thus possible while the gel remains liquid. For the gel formed in chip the cells are mixed into the PBS, and are thus injected into the chip before the gelling agent is added.

Culturing of astrocytes, MDCK cells and COS 7 cells were performed under static conditions, i.e. without perfusion, in the microchip. This is possible as the cell-gel plug is in direct contact with the surrounding culture medium through the gel fill hole when being placed in a petri dish. Over the first few days proliferating cells were identified. Long term culture was performed up to 14 days on astrocytes, demonstrating that the microstructure did not limit cell viability. Staining of cells with calcein allowed visualization of viable, metabolizing cells. Figure 4-12 a-b show astrocyte proliferation or migration during the first 48 hours of culture. The arrows mark the presence of new cells. Figure 4-12 c shows long term culture (14 days) of astrocytes. In 3D cultures cells
Figure 4-12: a-b) Migration or proliferation of phenylalanine hydrogel immobilized astrocytes in a microchip assay chamber after a) 24 hours and b) 48 hours of incubation at 37 °C. Arrows mark the presence of new cells. The cells were stained with calcein-AM. b) Long term growth of astrocytes in microstructure. Image show cells after 14 days of culturing in Fmoc-Phe hydrogel. d-e) Comparison between astrocyte morphology in d) 2D and e) 3D cultures.

Shift morphology from the flat, elongated cells seen on surface cultures to spherical cells. Figure 4-12 d-e compares astrocyte 2D and 3D cultures.

To confirm that the cells respond to the gradient they are exposed to, two tests were performed: calcein staining and cellular response to the energy-carrying molecule
Figure 4-13: Decreasing labeling efficiency of astrocytes embedded in hydrogel after 60 minutes with dual flow of calcein and culture medium. a) Micrograph of the cell culture and b) corresponding intensity curve where each peak represents a cell. Largest peak is closest to calcein flow channel.

Adenosine triphosphate (ATP). A calcein gradient over the cell-filled gel is established by calcein in one channel and cell culture medium in the other. The calcein concentration gradient enables visualization of cellular response according to the gradient. The concentration of calcein in the cell is directly proportional to the extracellular concentration. Thus cells positioned near the calcein flow will attain a higher intracellular concentration and thus fluoresce stronger, which can be detected with fluorescence microscopy. The results presented in figure 4-13 are achieved after 60 minutes with dual flows, which allows establishment of the gradient and completion of the cell staining. Each intensity peak in the curve represents a cell, and the maximum intensity decreases with cell position. The same result was achieved with Matrigel.

ATP was used to study cellular response to applied substances. ATP is known to trigger one rapid and distinct intracellular Ca\(^{2+}\)-peak initiated as soon as the ATP reaches the cell. Staining the cells with the calcium sensitive dye fluo-4 enables fluorescence detection of this Ca\(^{2+}\)-signal. Variations of the intracellular calcium level are seen as changes in fluorescence intensity. Staining is performed in the gradient chamber by covering the cell-gel mixture through the fill hole with 0.5 μM fluo-4. The chip is then kept dark in room temperature for 20 minutes as the dye enters the cells.

The ATP gradient is established by switching in 10 μM ATP in one channel and culture medium in the other. As the cells are positioned at different distances from the ATP-flow the Ca\(^{2+}\)-responses will arise at different times, depending on the position of the cell and when it is reached by the moving ATP front. The result from cells embedded in Matrigel is shown in figure 4-14. The first cell responds after 40 seconds followed by other cells according to their position in the assay chamber.
4 Device evaluation

Figure 4-14: Calcium response from COS 7 cells embedded in Matrigel with dual flow of ATP and culture medium. Cells respond in sequence as they are reached by the ATP front. Insert show cell position in assay chamber.

4.2.4 Discussion regarding gradient device evaluation

In this work three different gels were used, which all have advantages and disadvantages. Matrigel is good with regards to cellular microenvironment, as it contains more components present in the extracellular matrix produced in vivo. The hydrogels, on the other hand assemble much faster than Matrigel, which decreases the time needed for experimental preparations. They can also easily be manipulated in terms of stability by varying the peptide content. Long term cultures for up to two weeks showed that all gel types successfully support cell growth.

Gel filling by syringe pumping work well in new and unused chips, as the silicon surface is somewhat hydrophobic. The hydrophobicity arises from the protecting adhesive film that holds the silicon wafer together during the final dicing of the chips. After cleaning the native silicon and glass are hydrophilic. In cleaned and reused chips the gel tends to follow the walls of the side channels rather than remaining in the assay chamber. This problem is reduced, but not eliminated by the constraining effect of the pillars.

Filling by pipetting is the most straightforward method to fill the chamber as there is no need for precision pumping. Further the properties of the chip surfaces do not affect the filling result as the chamber structure is completely filled with PBS before the gelling agent is added. The pillars are beneficial also during this type of filling as they increase the confinement of assembling nanofibers, preventing unwanted displacement during gel formation.
Calcein staining of cells in the gradient device results in a clear fluorescence intensity increase from cells positioned near the flow of culture medium to cells near the calcein flow. The gel has then been exposed to constant perfusion for one hour. These results show that a stable calcium gradient can be achieved with this system, and that the cells exhibit labeling efficiency according to the gradient they are exposed to.

When the cells are reached by a diffusing ATP front they respond with an intracellular release of stored calcium. The initiation of the calcium response thus depends on the cell position, and range from between 40 to 100 seconds in figure 4-14.
5 Discussion and conclusions

As previously mentioned cells cultured in 3D gels behave different compared to cells cultured on flat substrates, due to changes in morphology and cell adhesion properties. It is thus often advantageous to study cells adapted to 3D growth. The majority of cells in tissues are embedded and surrounded by other cells and ECM, and can thus be expected to benefit from such culture conditions. This is the case for astrocytes, which \textit{in vivo} are embedded in brain tissue. Epithelial cells, such as COS 7 and MDCK, are however not embedded but instead lines the cavities and lumens of organs. These cell types were used anyway since they are both robust and easy to handle, and they make up a good test system for the devices.

In tissues the extracellular conditions are not stable as in traditional cell cultures. Diffusion of oxygen, carbon dioxide, ions and signaling molecules constantly change the properties of the extracellular space and create asymmetric environments. To be able to mimic such changes in cell microenvironment \textit{in vitro} increases the chance of cells maintaining a behavior more similar to \textit{in vivo}. Microfluidics offers an improved control over this microenvironment. To combine 3D culture with miniaturization and gradients we believe will add an extra dimension to traditional cell biological studies.

The 3D culture chamber was a first attempt to combine 3D culture and miniaturization to achieve a controlled 3D environment for cell assays. Cells were successfully embedded and cultured in a three-dimensional gel within the chamber. The microstructure design includes pillars in the cell assay cavity. The pillars help to reinforce and anchor the gel as well as increasing the surface to volume ratio after polymerization.

The culture chamber was further developed into a device focused on miniaturized stable gradients. The main advantages with our system compared to previously reported systems are the possibility to use two different filling methods and the facilitated culture. Both filling methods together allow for various types of gels to be used, depending on application and cell adhesion preferences. Culture conditions were improved by direct contact between the cells and the media through the gel fill hole. Perfusion was thus not necessary. Instead the chip was simply placed in a petri dish with culture medium. Experimental preparations are significantly facilitated when cultivation at static conditions is possible, as numerous chips can be loaded and cultured simultaneously. Also this device was designed with pillars in the assay chamber. The
pillars facilitate both filling methods as well as help to anchor and constrain the final gel plug.

In conclusion, this thesis focuses on methods to combine 3D cell culture, microfluidics and gradients. The 3D culture chamber opened up for relevant cell physiological studies in well controlled 3D chemical environments. The development into a more complex system for gradient studies further increased control over cell microenvironment and versatility of the test system. The widths of the gel plugs can be varied within the system, and it can be further developed into managing high throughput assays. In future studies, the gel fill hole can possibly be used not only to facilitate culture, but also to combine simultaneously gradients and systemic treatments. This would further improve control of the cellular microenvironment and provide a yet more versatile system. The reference chambers in separate side channels (figure 3-5 a) would enable simultaneous control experiments of cells not exposed to the gradient. Means of adjustment of culture conditions are always important as cell state and condition may vary from day to day due to a multitude of factors, such as culture passage, degree of confluence and state in the cell cycle.
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7 References


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5 Discussion and conclusions


