Multilayer Microfluidic Devices for Cell Studies

A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

presented by

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2012
“Believe you can and you’re halfway there”

(Theodore Roosevelt)
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Abstract

It is important to understand cells to understand life, and hence, obtain insights into Mother Nature’s secrets. In this thesis studies of (single) cells are addressed by the use of multilayer microfluidic devices (MMDs). These devices consist of three layers, whereby an intermediate permeable membrane physically separates two fluid channel networks. Thus, exchange of molecules and interaction between both fluid layers is only possible via the membrane. The introduction of another fluidic layer adds a level of spatial and temporal control compared to planar devices. In this thesis two devices are developed and optimized, one for single cell studies and the other one for cell co-culture studies. In the first application the additional level of fluid control is used to precisely supply an inducer molecule and in the second device it is used to generate different microenvironments.

The first MMD facilitates long-term measurements of single cell responses to external stimuli. The top layer is designed for the trapping and the culturing of cells. During the development of the traps for hydrodynamic single cell trapping several steps of designing, re-designing, and optimization are performed. The bottom layer is employed for supplying chemical compounds that can be transported towards the cells in defined concentrations and temporal sequences. The performance and the potential of the device are demonstrated using human cells transfected with an inducible gene expression system. In the top channel the expression of a fluorescent protein is observed while varying the concentration and exposure time of the inducer tetracycline via the bottom channel. Different aspects of induced gene expression, i.e. inducer concentration dependency, transient induction and parallel supply of different inducer concentrations, are explored. This is achievable by using different flow profiles for the supply of tetracycline. The study reveals the averaged response over tens of cells that are analyzed in parallel. More importantly, the heterogeneous responses of individual cells are revealed as well. Thus, it is possible to obtain a final average result and many individual results within the same experiment.

The second device is a microfluidic test system for cell co-cultures on opposite sides of the intermediate membrane. Here, for fast and precise cell positioning dielectrophoretic trapping is used. Therefore, microelectrodes are designed on the membrane. The physical characterization reveals no differences before and after processing of the membrane. Furthermore, dielectrophoretic cell capture and viability of cells trapped in a microfluidic device are demonstrated. The combination of these membranes with MMDs forms a powerful tool to study cell-
cell interactions in co-cultures, whereby spatial separation of different cell types and/or microenvironments is required. Initial efforts to design such a multilayer cell co-culture device are presented. A cell co-culture with cells on opposite sides of the intermediate membrane is generated by combining dielectrophoretic and electrostatic forces. Both cell lines remain attached to the membrane after switching off the dielectrophoretic forces, even though one cell type is positioned against gravity.

The presented microfluidic platform enables systematic studies under defined conditions and is a valuable tool for single cell and cell co-culture studies to obtain insights into mechanisms and kinetics that are not accessible by conventional macroscopic methods.
Zusammenfassung


Diese mikrofluidische Plattform ermöglicht systematische Studien unter definierten Bedingungen und ist ein geeignetes Werkzeug für allgemeine Einzelzellstudien und für Zell Ko-Kulturen um neue Einsichten in Mechanismen und Kinetiken zu erhalten, die mit herkömmlichen makroskopischen Methoden nicht umsetzbar sind.
Abbreviations

List of prefixes

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<th>Symbol</th>
<th>Prefix</th>
<th>Exponent</th>
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<tbody>
<tr>
<td>d</td>
<td>deci</td>
<td>$10^{-1}$</td>
</tr>
<tr>
<td>c</td>
<td>centi</td>
<td>$10^{-2}$</td>
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<tr>
<td>m</td>
<td>milli</td>
<td>$10^{-3}$</td>
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<td>µ</td>
<td>micro</td>
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<tr>
<td>n</td>
<td>nano</td>
<td>$10^{-9}$</td>
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<tr>
<td>p</td>
<td>pico</td>
<td>$10^{-12}$</td>
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<tr>
<td>f</td>
<td>fempto</td>
<td>$10^{-15}$</td>
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List of volumes in liter

<table>
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<th>Unit</th>
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<tr>
<td>L</td>
<td>liter</td>
<td>$1 \text{ dm}^3$</td>
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<tr>
<td>mL</td>
<td>milliliter</td>
<td>$1 \text{ cm}^3$ $10^{-3} \text{ L}$</td>
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<tr>
<td>µL</td>
<td>microliter</td>
<td>$1 \text{ mm}^3$ $10^{-6} \text{ L}$</td>
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<tr>
<td>nL</td>
<td>nanoliter</td>
<td>$(100 \mu \text{m})^3$ $10^{-9} \text{ L}$</td>
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<tr>
<td>pL</td>
<td>picoliter</td>
<td>$(10 \mu \text{m})^3$ $10^{-12} \text{ L}$</td>
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<tr>
<td>fL</td>
<td>femtoliter</td>
<td>$1 \mu \text{m}^3$ $10^{-15} \text{ L}$</td>
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List of Greek symbols

<table>
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<td>ς</td>
<td>zeta</td>
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List of Organisms

- **E. coli**  *Escherichia coli*
- **HEK293**  Human embryonic kidney cell line
- **HepG2**  Liver hepatocellular carcinoma cell line
- **NIH-3T3**  Mouse embryo fibroblast cell line

General abbreviations

- **AFM**  Atomic force microscopy
- **CAD**  Computer-aided design
- **CM**  Clausius-Mossotti
- **DMEM**  Dulbecco’s Modified Eagle’s Media
- **DEP**  Dielectrophoresis
- **EDTA**  Ethylenediaminetetraacetic acid
- **eGFP**  Enhanced green fluorescent protein
- **EOF**  Electro-osmotic flow
- **EROD**  7-ethoxyresorufin-O-deethylase
- **FACS**  Fluorescence activated cell sorting
- **FEP**  Fluorinated ethylene propylene
- **GFP**  Green fluorescent protein
- **hCAM**  Hybrid cell adhesion multilayer
- **HEPES**  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **ID**  Inner diameter
- **ITO**  Indium-tin-oxide
- **MCS**  Multiple cloning site
- **MEM**  Minimum Essential Media
- **MEMS**  Microelectromechanical systems
- **MMD**  Multilayer microfluidic device
- **MODC**  Mouse ornithine decarboxylase
- **OD**  Outer diameter
- **PAH**  Poly(allylamine hydrochloride)
- **PBS**  Phosphate buffered saline
- **PC**  Polycarbonate
- **PCR**  Polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PEEK</td>
<td>Polyether ether ketone</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethyleneimine)</td>
</tr>
<tr>
<td>PEM</td>
<td>Polyelectrolyte multilayer</td>
</tr>
<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate), polyester</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PTFE</td>
<td>Poly(tetrafluoroethylene)</td>
</tr>
<tr>
<td>PSS</td>
<td>Poly(styrene sulfonate)</td>
</tr>
<tr>
<td>RMS</td>
<td>Root-mean-square</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds-per-minute</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>tetR</td>
<td>Tetracycline repressor protein</td>
</tr>
<tr>
<td>tetO²</td>
<td>Tetracycline operator</td>
</tr>
<tr>
<td>T-REx™</td>
<td>Tetracycline-regulated expression system</td>
</tr>
<tr>
<td>μTAS</td>
<td>Micro total analysis systems</td>
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1 General Introduction

1.1 Cells: the Building Blocks of Life

In 1665 R. Hooke discovered the cell while examining a thin slice of cork with a newly developed instrument, the microscope. In the 19th century the observations of several scientists led to the development of the cell theory. The cell theory states that all living organisms are made up of cells, the basic units of life, and that new cells arise from old ones. The cell theory holds true for all kinds of living organisms, whether unicellular or multicellular; simple or more complex. This theory explains the importance of studying cells, because the study of cells is fundamental to the study of life. Thus, cell research leads to new discoveries, which help to improve the quality of life. Without cell studies cells would have remained as black boxes of the unknown (Figure 1.1). The studies on cells, which were performed over the last 350 years, resulted in the knowledge of today. In former days the research focused on fundamental aspects such as cell structure, composition, and organization. With time the focus shifted to more complex aspects like cell behavior, interaction, and communication. Later on, the knowledge was sufficient to be used to improve life, e.g. applications in medicine. Nowadays, simple questions might be answered; however, there still remain enormous uncertainties to be addressed. Prominent examples of cell studies of the presence focus on curing cancer or elimination of inherited diseases.

Figure 1.1 Without cell studies cells still would be black boxes of the unknown.

Many different techniques such as western blotting and mass spectrometry have been developed and optimized to improve knowledge of cells; this includes their signaling pathways, their communication, and their responses. Nevertheless, fluorescence spectroscopy is the most important technique in cell biology. For
example, it is used for fluorescence microscopy, fluorescence activated cell sorting (FACS), and in microplate readers. The mentioned techniques require large quantities of cell material. Moreover, the analysis of a large cell population by standard techniques results in an average analysis, which obscures the heterogeneous characteristics of individual cells.\textsuperscript{3,4} For example, averaged results can prevent the discovery of more complex mechanisms, as shown in Figure 1.2. On the basis of the overall cell responses it is not possible to distinguish between a graded and an ‘all-or-nothing’ cell response. Only single cell measurements can help determine the underlying cellular mechanisms.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1_2.png}
\caption{Why is there a need for single cell studies? The cell responses can be (A) an all-or-nothing signal or (B) graded. (C) The overall cell response of both cases is the same. Only studies on the single cell level can shed light into the underlying mechanisms.}
\end{figure}
Already back in 1957 differences in gene expression between cells could be experimentally observed; dramatic variations of protein levels were found between cells within a single population deriving from the same parental cell.\textsuperscript{5} However, only in 1990 reliable single cell assays for gene expression studies enabled the examination of the underlying stochastic phenomena.\textsuperscript{6} Since then, several studies were performed to shed light onto the understanding of cell heterogeneity.\textsuperscript{7-13} Many cellular components are present at very low copy numbers.\textsuperscript{14} Therefore, stochastic fluctuations in gene expression can become significant and led to cell-cell variations (even in clonal populations).\textsuperscript{15-18} Nowadays variations in the protein levels, also referred to as non-genetic heterogeneities, are attributed to two main determining factors:\textsuperscript{19-21} (i) the intrinsic noise, caused by the inherent stochastic noise of the biochemical events during gene expression and (ii) the extrinsic noise, caused by additional variations of other cellular species that are involved in gene expression, e.g. polymerases. These molecular components are gene products themselves. Hence, they will vary over time and from cell to cell. On the other hand there is the genetic heterogeneity, i.e. mutations in the DNA sequence are causing cell-to-cell variations.\textsuperscript{21} The publication of the complete DNA sequence of the human genome\textsuperscript{22, 23} in 2001 started a new era: the molecular/genetic basis of all human diseases, first and foremost cancer, should be elucidated. New methods such as DNA microarrays that allow the simultaneous gene-expression profiling of thousands of genes accelerate these studies. Their use to sub-classify cancer based on the innate level of gene expression of the tumor has already been demonstrated.\textsuperscript{24-28}

Measurements of cell-to-cell differences are challenging because they require methods to manipulate and address single cells and analytical instrumentation that is sufficiently sensitive to measure cell processes or target compounds in low concentrations. Moreover, to reach reliable statistical confidence, the analytical platform should preferentially allow parallel analysis of large cell numbers. These challenges can be tackled using miniaturized platforms as they offer remarkable promises for single cell handling and open the way to novel methods that are unfeasible to realize with macroscopic instrumentation.\textsuperscript{29-33} For example, it is possible to monitor single cells over defined time periods, which is not achievable with standard single cell techniques like FACS.
1.2 Introduction to Miniaturization

In the research field of lab-on-a-chip many efforts towards miniaturization in chemical and biological processes are described (Figure 1.3).29 The dimensions of structures fabricated by means of microfabrication technology are designed to match biologically-relevant length scales34-36; hence, valuable platforms for cell analysis and cultivation can be designed, and completely new applications without macroscopic equivalent can be developed. Examples are miniaturized sensors, micro-electromechanical systems (MEMS) and biochips for DNA analysis.

Figure 1.3 Microfluidic technology is used to improve conventional chemical, biological, and physical methods in the laboratory. (Adapted from Ref.37)
1.3 Microfluidic Research

The term microfluidics refers to scenarios where liquids or gases are confined in micrometer-sized channels and reservoirs. The history of microfluidics as an enabling engineering science dates back not longer than 25 years. The first microfluidic devices were used for acid-base titrations, and measurements of pH in blood. The introduction of microfluidic systems for separation processes, such as chromatography and electrophoresis, improved separation time and quality. In 1990 Manz et al. proposed the idea of a miniaturized total analysis system (µTAS), a system that integrates all steps regarding sample handling and measurement. The evaluation of different chip materials, chip designs, and fluid transport followed. Various chip designs and injection methods were presented and parallelized; even multi-step automated systems were demonstrated.

Efforts to miniaturize (bio)chemical reaction systems were made as well. Improvements on reduced reaction times, lower energy consumption, high product yields, and improved selectivity were observed for fast and highly exothermic or endothermic reactions. Furthermore, the safe handling of toxic and radioactive reagents was achieved. There was a further milestone in 1998 when the biological reaction for DNA amplification (polymerase chain reaction, PCR) was realized on a microchip. This demonstrated the practicability of precise heating and cooling in microfluidic systems. In the following years further advances in DNA amplification, sequencing, analysis, and mapping were presented. Microfluidic devices to determine DNA-protein interactions were developed as well.

Nowadays, microfluidic research is widely performed. It covers fabrication issues, theoretical aspects such as simulation and modeling of fluid streams, distributions of compound concentration and temperature, experimental work on fundamental micro- and nanofluidic phenomena, and applications in chemistry, biology and medicine.
1.4 Basic Principles in Microfluidic Research

Microfluidic devices provide a number of advantages compared to large-scale and non-microfluidic systems (Figure 1.4). These include the low sample/analyte consumption, the fast mass and heat transfer, the small residence time distributions, the integration of several components, the possibility of parallelization, the ability to control/handle small volumes, the high portability, and the possibility to handle and manipulate microscopic objects due to similar dimensions.

Figure 1.4 Simple scaling considerations demonstrate the benefits in micro-sized reaction systems. The short distances enable fast heat and mass transport. High surface-to-volume ratios support effective heat exchange to the environment. In a flowing system, serial processing is possible. Due to small scales, massive parallelization is feasible. (d: length of edge, n and m: numbers of reaction systems serial and parallel, respectively).
Although, the nature of a chemical reaction or a biochemical process will not be changed in a miniaturized system the reduction in size of a reaction system gives rise to phenomena that are usually not observed on the macroscopic scale. For example, the surface-to-volume ratio increases at small dimensions. The larger interface will have an effect on any interaction between molecules in the liquid and the surface. Therefore, phenomena such as adsorption, wetting, and capillary forces have to be considered for many applications.

1.4.1 Flow Generation

Various methods such as hydrodynamic flow, electro-osmotic flow (EOF), flow induced by centrifugation, and shear-driven flow between two plates being moved against each other have been explored to generate flow in microfluidic systems. Sometimes, capillary forces are sufficient to drive fluids into small microchannels. This is often used to (pre-)fill a microfluidic network. However, hydrodynamic flow is mostly used for flow generation. The pressure-driven flow generates a parabolic flow profile with a maximal flow velocity in the center and a flow velocity of zero at the walls of the channel (Figure 1.5). As a consequence, molecules are transported at different velocities depending on their position within the channel. The necessary pressure difference between the channel inlet and its outlet can be achieved by connecting the microchannels to syringe pumps that provide a precise volume flow rate. Typically, flow rates from nL/min to mL/min can be achieved with these pumps. However, the stepwise movement of the pump motor can cause a pulsed flow at low flow rates. Hence, low flow rates are best realized with pressure-driven pumping systems. In these systems a gas pressure on the fluid reservoir is used to control the flow rate. Other alternatives to create pressure differences between inlet and outlet include bubble formation or acoustically driven pressure.57
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Figure 1.5 Generation of flow in a microchannel. A parabolic flow velocity profile emerges from a hydrodynamic flow. (A) Illustration of the flow generation resulting in a hydrodynamic flow. (B) The flow is visualized by activating a caged fluorescent dye at a certain position and taking fluorescent images at selected time delays.

Flow can also be generated by the use of an electric field. EOF relies on the movement of charged particles relative to a stationary, charged surface upon application of an electric potential across the microchannel. The presence of an electrical double layer at the interface between channel wall and solution is required. In EOF the fluid moves as a whole, i.e. there is a constant flow velocity across the microchannel (Figure 1.6). However, the intensity of the applied voltage, the surface charge, the pH, and the ion concentration of the buffer influence the EOF. As a consequence, these conditions have to be controlled carefully.
Figure 1.6 Generation of flow in a microchannel. An EOF results in a constant flow velocity across the channel. (A) Illustration of the flow generation resulting in an EOF. (B) The flow is visualized by activation of a caged fluorescent dye at a certain position, and taking fluorescent images at selected time delays.

Another fluid transport method uses centrifugal forces, where the fluid moves to the outside of the microchip, depending on the rotation speed. The centrifugal forces must be higher than the capillary forces in order to move the liquid. This requirement can be used to create valves simply by changing the channel diameter or the surface properties. By changing the rotation speed the valve can easily be opened and closed. This method enables easily-controlled parallelization of process steps.59

1.4.2 Laminar Flow

One key characteristic of flow movement in microchannels is laminar flow, i.e. the absence of turbulence (Figure 1.7). The flow regime can be characterized by the dimensionless Reynolds number (Re). This represents the ratio of inertial to viscous force. The Re number can be calculated by:

\[ Re = \frac{\rho \cdot v \cdot l}{\mu} = \frac{\text{inertial force}}{\text{viscous force}} \]

(with \( \rho \): fluid density, \( v \): flow velocity, \( l \): typical length scale (e.g. radius of the channel), and \( \mu \): dynamic viscosity)

The transition from laminar to turbulent flow occurs in a straight circular pipe for \( 2000 < Re < 3000 \). For \( Re < 2000 \) a laminar flow and for \( Re > 3000 \) a turbulent flow can be expected. In a microfluidic system with channel sizes between 1 µm
and 100 µm and flow velocities in the range of µm/s to mm/s, the Re numbers range between 0.001 and 1. Thus, the inertial force is low compared to the viscous one. Dependent on the designed application, the absence of unpredictable turbulences has either positive or negative implications.

Figure 1.7 When viscous forces become important over inertial forces, laminar flow can be observed. (A) Co-flowing solutions in a microfluidic chip (microchip from Micronics, Inc.). (B) Contrary, the turbulent mixing of the same solutions in a glass flask.

The principle of laminar flow has been used in several applications for chemical and biological reaction and analysis systems. The fluid movements within microchannels can be calculated and furthermore this predictability allows for a precise control, e.g. over distribution and transport times of compounds.

i) Simulation and modeling: Fluid movement (e.g. distribution of compounds and transport times of analytes in microchannels) is predictable and can be calculated by the Stokes equations.

ii) Diffusion and mixing: Two merging fluid streams are not actively mixed, but passive mixing on the basis of molecular diffusion will occur. This molecular diffusion over a time increment $\tau_D$ can be estimated according to the Einstein-Smoluchowski relation:

$$\tau_D = \frac{l^2}{2 \cdot D}$$

(with $l$: distance, the molecule has traversed, and $D$: diffusion coefficient)

For example, for solute ions with a diffusion coefficient of $10^{-9} \text{ m}^2/\text{s}$ it will take approximately 10 s to cross a distance of 100 µm. For proteins with a diffusion coefficient of $10^{-11} \text{ m}^2/\text{s}$ it will take approximately 16 min to cross the same
distance. It is possible to calculate the length of the channel required for complete mixing for a given flow velocity. Whether mixing based on diffusion is sufficient in a given channel at a given velocity is indicated by the Péclet number (Pe).

\[
\text{Pe} = \frac{1 \cdot v}{D} = \frac{\text{convection}}{\text{diffusion}}
\]

(with l: typical length scale, v: flow velocity, and D: diffusion coefficient)

A closer look at the actual concentration distribution within a microchannel reveals that in hydrodynamic flows, the parabolic flow profile has to be considered.\(^6^2\) Narrow microchannels are able to enhance diffusive mixing, but they are not always practical because of their high fluidic resistance. Therefore, different strategies have been introduced to achieve mixing. In laminating mixers fluid streams are separated and united in turn to decrease the diffusion distance.\(^6^3\) In other mixers grooved surfaces are implemented\(^6^4\) or electrokinetically induced instabilities\(^6^5\) are used.

iii) Reactions at the interface of two laminar streams: It is possible to carry out fast reactions at stable interfaces between two co-flowing solutions (Figure 1.8 A). For example, Kenis et al. presented the formation of a silver wire at the interface of two streams. One stream contained a silver salt and the other stream a reductant.\(^6^6\)

iv) Separation and filtration: The laminar flow regime can be exploited to separate molecules with different diffusion coefficients. Small molecules diffuse across the channel, while larger ones remain at the original position in planar devices where several channels merge into one main channel. So-called T-sensors (Figure 1.8 B) have been used for measurements on concentrations and diffusion coefficients\(^6^7\), and for the characterization of antigen-antibody binding at the inter-diffusion zone\(^6^8\). A channel network with an H-design (Figure 1.8 C) can be used for the filtration of molecules.\(^6^9\)

v) Focusing of an analyte stream: The hydrodynamic focusing of a stream by two side streams (Figure 1.8 D) was introduced\(^7^0\) and further modified\(^7^1,7^2\). The focusing of the analyte stream can highly increase the detection efficiency. It is frequently used in (miniaturized) cytometers to enable the excitation of individual cells by laser light.\(^7^3\) The width of the focused stream depends on the applied flow rates, the higher the flow rates of the side stream the more focused is the analyte stream. The position of the focused stream can be controlled as well, by varying the flow rates of the side streams. Hence, the precise directing of the focused stream into a specific outlet channel is achievable.
vi) **Multi-laminar streams:** Different co-flowing laminar streams can be employed to generate stepwise changes of analytes or continuous concentration gradients (Figure 1.8 E). Multi-laminar streams can be used for the analysis of single cells or cell populations that are localized within the microchannel. Depending on their position, the cells are exposed to different streams, and hence, treated with different conditions. Examples for applications of this method include the monitoring of cellular responses on different stimulating reagents, the local activation of gene expression or the probing of subcellular changes upon local treatment.

![Figure 1.8](image)

**Figure 1.8** Schematic drawings to illustrate the consequences of the laminar flow present in microfluidic devices: (A) Chemical reactions can take place at the interface between the laminar flows of reagents. (B) Analyte concentration and diffusion coefficients can be measured in this geometry, called T-sensor. (C) A so-called H-design network can be used to filter molecules. (D) The analyte stream is focused by two side streams. (E) Multi-laminar streams can be used to generate stepwise gradients of analytes.
1.5 Cells on Chip

In the last years the use of microfluidics has gained a big impact in academic research regarding cell biology, systems biology, and pharmaceutical and medical applications. For example, studies on the selection, lysis and analysis of (single) cells have been presented. Microfluidic devices are suitable for the generation of controllable and reproducible microenvironments as well. The development of miniaturized cell counters and cell sorting devices as well as the caging of suspended cells by the use of dielectrophoretic forces were the first cell analytical devices introduced for the analysis of suspended cells. Microfluidic devices for studies on adherent cells and cell cultures to provide new insights into cellular processes followed. The introduction of pneumatic/hydraulic valves in poly(dimethylsiloxane) (PDMS) microdevices allowed the formation of small liquid volumes employed for fluid metering in protein crystallization studies or single-cell assays. In other studies, cells were exploited to act as a pump or to deliver other kinds of energy, or organ-like systems were created to mimic natural conditions.

1.5.1 Cell Trapping Techniques

For cell or even single cell studies in microfluidic devices the ability to control and to define cell positioning in precise locations is crucial. Various principles for cell entrapment have been developed (exemplarily shown in Figure 1.9). It can be executed via physical or chemical trapping methods. Among the physical methods mechanical, electrical, magnetic, fluidic, optical, and acoustic trapping principles and among chemical methods surface modification and gel-based systems shall be mentioned in more detail.
1.5 Cells on Chip

**Figure 1.9 Various cell trapping techniques.**\(^{94}\) Several examples for physical and chemical cell trapping approaches are shown.

**Hydrodynamic Trapping**

Hydrodynamic cell trapping (Figure 1.9 a-c) is the most common principle of cell entrapment in microfluidics. Cells are positioned in defined regions of the microdevice after being separated from the flow by variations of surface topographies.\(^{95}\) Therefore, mostly mechanical obstacles or barriers that match the size of the cells (to be captured) are integrated into the device. The mechanical hurdles can be compared to a sieve: they sieve the cells by providing a passage for the fluid only. Different topographies have been developed that include vertical walls either the height of the channel\(^{96}\) or slightly smaller\(^{97}\), pores either in the channel side walls\(^{98}\) or in the bottom\(^{99}\), or niches of little flow and low shear stress in the walls of a microchannel\(^{100}\). These strategies are easy to implement into a microfluidic design, rapid, scalable, and possibly automatable. On the other hand, cell retrieval and dynamic manipulation might be challenging. Therefore, designs for non-contact cell trapping by use of microeddies\(^{101}\), and valves\(^{102}\) were presented as well. Hydrodynamic cell trapping is not limited to one cell type as shown by Skelly *et al.* in their microdevice for cell fusion.\(^{103}\)
Electrical Trapping

Cell manipulation in electrical trapping principles rely on electrical forces. Dielectric cell properties as well as the negative charged cell surfaces are exploited for dielectrophoretic respectively electrophoretic methods. To be able to use electrical forces in the microdevices a series of electrodes has to be implemented into the chip design. Therefore, the microfabrication becomes more challenging. Additionally, the potential damage of an electric field on cells might limit these techniques. Nevertheless, electrical cell trapping is widely used due to its ability to retrieve cells, to use the electrodes for different reasons, e.g. cell trapping and lysis, and to vary the electric field by changing the design.

Dielectrophoresis

Dielectrophoresis (DEP, Figure 1.9 e) describes the phenomenon of the manipulation of neutral particles (such as cells) in a non-uniform electric field. It was first described 1951 by Pohl et al. The use of electrodes with different geometries generates the required non-uniform electric field due to:

\[ F_{\text{DEP}} = p \cdot \nabla E \]

(with \( F_{\text{DEP}} \): DEP force, \( p \): cell’s dipole moment, \( \nabla E \): gradient of electric field)

There is no net force for a neutral particle, if the dipole is located in the same field due to \( F=F^+ \) (Figure 1.10 A). However, if the dipole is located in a field of different magnitude, then the net force will be non-zero and the cell will be attracted towards the electric field maximum due to \( F>F^+ \) (Figure 1.10 B). The DEP forces do not depend on the polarity of the electrical field.
Figure 1.10 Neutral particle movement in two different electric fields. (A) In a uniform electrical field the induced polarization of a neutral particle cannot be used for its manipulation due to the lack of a net force. (B) In contrast, in a non-uniform field a neutral particle will be attracted to the field maximum due to its induced polarization and the different magnitude of the field.

More precisely, the DEP force acting on a spherical particle is given by:

\[ F_{\text{DEP}} = 2\pi r^3 \varepsilon_m f_{\text{CM}} \nabla E^2 \]

(with \( r \): particle’s radius, \( \varepsilon_m \): absolute permittivity of the suspending medium, 
\( f_{\text{CM}} \): Clausius-Mossotti (CM) factor)

The CM factor is given by:

\[ f_{\text{CM}} = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \]

(with \( \varepsilon_p^* \) and \( \varepsilon_m^* \): complex permittivities of the cell and the medium, respectively)

The complex permittivities for a dielectric material can be described by:

\[ \varepsilon^* = \varepsilon - \frac{i\sigma}{\omega} \]

(with \( \varepsilon \): permittivity, \( \sigma \): conductivity, \( \omega \): angular frequency of applied electrical field, \( i \): imaginary unit)
If the relative polarizability of the cell is greater than the relative polarizability of the suspending medium, the CM factor will be positive and the cell will be attracted toward regions with high electric field strength. This phenomenon is known as positive DEP (Figure 1.11 A). On the other hand, if the CM factor will be negative, i.e. the cell is less polarizable than the suspending medium, the cell will be directed down the field gradient. This is known as negative DEP (Figure 1.11 B). Thus, the DEP force is depending on the electrical properties of the cell and the suspending medium, and the frequency of the electric field. To alter DEP forces variations in conductivity is most common due to its easy adjustment, although it is also possible to vary permittivity by the use of buffers that consist of molecules with large dipole moments.\textsuperscript{94,106,107}

\textbf{Figure 1.11} Positive and negative DEP. It can be controlled if a neutral particle is attracted to (positive DEP) or repelled from (negative DEP) the field maximum. (A) For positive DEP the polarizability of the suspending media has to be lower than the one of the particle. (B) For negative DEP it is vice versa, the polarizability of the suspending media has to be higher.
Electrophoretic Trapping

By use of electrophoresis charged particles can be manipulated in an electric field. The used Coulomb force is given by:

\[ F = q \cdot E \]

(with \( q \): net charge on the particle, \( E \): applied electric field)

The relation between electric field intensity and velocity of the dispersed particle is given by the electrophoretic mobility \( \mu \). This can be describes as:

\[ \mu = \frac{\varepsilon_m \cdot \zeta}{\eta} \]

(with \( \varepsilon_m \): permittivity of the liquid, \( \zeta \): zeta potential, \( \eta \): liquid viscosity)

The zeta potential is primarily related to the ionic strength of the liquid and the particle’s charge density. The difference in the zeta potentials of different cells enables their electrophoretic separation.\(^{108}\)

Alternative Cell Trapping Techniques

Magnetic Trapping

Magnetic cell manipulation (Figure 1.9 f) is becoming very popular in the resent years.\(^{109}\) There are three main types of magnetic trapping, depending on the properties of the cell: (i) using paramagnetic properties of cells\(^{110,111}\), (ii) using diamagnetic properties of cells\(^{112}\), and (iii) using surface chemistry to label cells with magnetic particles\(^{113}\). The third type is independent on the cell type; any cell could be labeled with magnetic particles. Two main advantages of this technique are (i) the very selective binding of magnetic particle to cells and (ii) the selective isolation from sample mixtures by magnetic forces.\(^{114}\) The surface of magnetic particles can be modified with functional groups or biomolecules using conventional surface principles. The available sizes range from a few nanometers to tens of micrometers. For cell manipulation an external magnet can be used and possible influences like pH, ionic strength, and surface charges can be neglected. In general magnetic trapping is used for cell separation, enrichment, and isolation.
Droplet-based Trapping

When two immiscible fluids are brought together in a microchip droplets are formed. Monodisperse microdroplets can be formed on a microchip by introducing an aqueous flow into a hydrophobic oil phase. In these processes, capillary forces play a crucial role as they are competing (and exceeding) the viscous forces. Since the first demonstration in 2002 by Quake and co-workers, droplet-based microfluidics has attracted great interest. Droplet-based microfluidics facilitates the fast generation of fL-nL volumes, in which cells can be enclosed. The droplets can be filled at the very moment of formation, and the tiny, finite volume enables rapid mixing of reagents and cells. The droplets are transported along channel networks, without dispersion of their content or contamination between individual droplets. Hence, microdroplets offer extremely valuable conditions for many screening applications. Droplet-based microfluidics for biological applications include PCR, protein crystallization, cell-free protein expression, and single cell analysis.

Optical Trapping

For optical trapping (Figure 1.9 g) a highly focused laser beam is used to generate forces in the piconewton range that are exploited to precisely manipulate particles. The momentum of the laser beam is transferred onto the particle and the Gaussian profile of the laser beam forces the cell to the center of the beam, where it is trapped. The technique is used due to its non-invasive character, the availability of user-friendly platforms, and the increasing resolution. However, optical tweezers can photo-damage the cells, and cell manipulation above single cell level is challenging. Nevertheless, optical tweezers were already combined with microfluidics for single cell trapping and manipulation, cell sorting, and biological analysis.

Acoustic Trapping

Another technique for cell manipulation is based on ultrasonic standing waves (Figure 1.9 h). These are generated by ultrasonic transducers, which subject the cells to a mechanical force. The mechanical force is dependent on the cell volume and frequency, and is the result of the primary and secondary radiation forces. The primary force originates from the standing wave and the secondary from the waves scattered by the cells. Acoustic standing waves have been used for cell separation, cell enrichment, cell proliferation, and biological analysis.
Chemical Trapping

Cell entrapment by chemical trapping techniques mainly refers to chemically modified surfaces (Figure 1.9 i). Chemical modifications of microfluidic surfaces provide an attractive approach for cell immobilization along given patterns. Microcontact printing is most commonly used to pattern polymers and proteins to facilitate cell attachment and adhesion prevention in a controlled and precise manner.\textsuperscript{132-137} Besides cell entrapment by surface modification, the use of hydrogels is common (Figure 1.9 j). The huge variability of precise controllable physical and chemical properties of hydrogels allow applications in many biological research areas such as biological sensing, cell encapsulation, (co-) culturing of mammalian cells, drug delivery, construction of an extracellular matrix or tissue engineering.\textsuperscript{138-141}

1.5.2 Multilayer Microfluidic Devices (MMDs)

The majority of microfluidic devices have a planar design, i.e. a channel system is embedded in a substrate and closed by a cover plate so that the processes are performed in a downstream mode. Contrary, by the use of MMDs the introduction of a second fluidic layer enables a further level of control. The use of multilayer microchips generated out of three layers of PDMS has already been shown.\textsuperscript{88, 89} However, packaging of multiple layers to form a single microchannel network is an approach for other applications as well. Multilayer microchips, in which the intermediate PDMS membrane has been replaced by a permeable membrane made of polycarbonate or polyester to physically separate top and bottom microchannel networks (see Figure 1.12), have been introduced for the use in cell culture studies.

**Figure 1.12** Scheme of a multilayer chip with an integrated porous membrane.
Takayama and co-workers\textsuperscript{142} cultured pulmonary epithelial cells on the permeable membrane and mimicked the flow conditions present in many pulmonary diseases. The researchers reproduced the typical sound, which is created in the lungs of patients suffering from lung injuries, e.g. asthma (Figure 1.13 A). In the follow-up study a device that reconstitute a functional alveolar-capillary interface was presented.\textsuperscript{143} In nanotoxicology studies the accentuation of toxic and inflammatory responses of the lung were mimicked (Figure 1.13 B).

\textbf{Figure 1.13} MMDs used to mimic organs on a chip. (A)\textsuperscript{142} (B)\textsuperscript{143}

In our research group MMDs were first designed for induced gene expression in yeast cells\textsuperscript{77} and the characterization of human urothelial cells as a potential model system for toxicological and carcinogenetic studies\textsuperscript{144}. Latter application focused on the applicability of the MMD to the assessment of cytochrome P450 activity, and the expression of the enzymes involved, to individual urothelial cells. The cells were pre-treated with benzo[a]pyrene, a pro-carcinogen, which induced the synthesis of the cytochrome P450 enzyme CYP1A1. Thereby the CYP1A1-dependent 7-ethoxyresorufin-O-deethylase (EROD) was activated. EROD catalysed the oxidative dealkylation of 7-ethoxyresorufin, which resulted in the formation of the fluorescent resorufin. Therefore, enzyme activity was specified by fluorescence microscopy (Figure 1.14). In the MMD the top layer was used for cell culture and the bottom layer for cell manipulation.
Figure 1.14 Kinetics of CYP1A1-dependent resorufin formation. (A-D) After cell exposure to 7-ethoxyresorufin an increase of enzyme activity was detected in benzo[a]pyrene pre-treated cells. (E-G) After applying a washing step (between D and E) enzyme activity decreased again. (H) Bright-field image of the adherent cells before the experiment was started. To induce gene expression of the enhanced green fluorescent protein (eGFP) yeast cells were provided with the inducer galactose. As counterpart glucose was used to repress gene expression. The different stimuli were supplied by use of multi-laminar streams in the bottom channel of the MMD, whereby both streams were separated by a stream of buffer. The spatial resolved initiation of the expression of eGFP was demonstrated (Figure 1.15). Again, cells were cultured in the top layer and manipulated via the bottom layer of the MMD.

Figure 1.15 MMD used to induce gene expression in yeast cells. (A) Yeast cells were positioned in the top layer of a MMD, while the supply of galactose, buffer and glucose was achieved via the bottom channel. (B) Time course of eGFP fluorescence under inducing and non-inducing conditions.
1.6 Scope and Structure of the Thesis

Studies on the single cell level are crucial to obtain new insights, while taking cell heterogeneity into account. In bulk cell studies responses of single cells can be obscured by the overall cell response. Hence, cell behaviors that differ from the main behavior, e.g., by following recessive pathways, might not be detected. However, cell heterogeneity has to be considered, if all secrets of Mother Nature should be discovered. In this thesis perturbations on mammalian cells induced by small molecules will be addressed. A powerful tool to address this problem on the single cell level offers the research field of microfluidics. To overcome disturbing issues like influences on cells due to flow switching, a MMD is developed further. The introduction of a second fluidic layer enables another level of spatial and temporal control. One channel network will be used for cell culture and the other one for cell manipulation. The intermediate permeable membrane allows for an exchange of small molecules between both layers. For proof-of-concept studies the induced gene expression of a fluorescent protein will be monitored. To show the broad range of different possibilities to manipulate cells in the developed device the inducer will be supplied in various flow patterns as well.

Additionally, the MMD will be used to generate cell co-cultures. Cell co-cultures are used to mimic in vivo conditions to study cell-cell communications and interactions. The aim is to adapt the multilayer microchip for fast and reliable cell positioning. Two different cell types will be positioned on both sides of the intermediate membrane to mimic organ-like conditions. The separated channel networks also enable the generation of different microenvironments not achievable in planar devices.

The thesis is structured according to the following. In chapter 2 the microfabrication techniques used are introduced and described. Detailed fabrication protocols, however, are not depicted to avoid misunderstandings in the respective chapters. The microchip material used, how the microdevice is connected to the macroscopic world, and the surface coating used is explicated in this chapter as well.

In chapter 3 different pumping systems will be evaluated due to the need of a pumping system that allows for precise and stable flows over a long period of time and at the same time the supply of high volumetric rates in chapter 4.
In chapter 4 a MMD is designed and developed for single cell studies. The performance of the device is proven by monitoring the induced gene expression by fluorescence microscopy. Therefore, the cells are modified with the T-REx™ system and a GFP-like protein as gene-of-interest prior to use. The modified cells are trapped in the top channel of the device and the inducer tetracycline is supplied via the bottom channel. Different flow patterns in the bottom channel enable the induction of the gene expression in a continuous, and a transient manner, and as gradient.

In chapter 5 another interesting application of the MMD is presented; its use for cell-cell communication and interaction studies. Therefore, a cell co-culture is generated with cells on opposite sides of the intermediate membrane.

In chapter 6 the thesis will be summarized and an outlook will be given.
2 Microfabrication

Several microfabrication processes were used in this thesis. This chapter focuses on their introduction. The material PDMS is evaluated for its use in microfabrication, the fabrication techniques photolithography, metallization and soft lithography are introduced, and the interfacing between the microdevice and the macroscopic surrounding and the used surface coating are described. The detailed microfabrication protocols are not depicted in this chapter but in the corresponding chapters to obtain a clearer structure.

2.1 Polymers as Microchip Material

The most common materials used for microchip fabrication are various polymers. For the fabrication of polymer microchips casting, injection molding and hot embossing techniques are widely used. These techniques all require the fabrication of a master mold (Figure 2.1).

![Photography of a mold used for soft lithography. The negative photoresist SU-8 on the silicon wafer contains the reliefs of different microchannel designs.](image)
By far the most commonly used polymer is PDMS. The material has some favorable properties, but also some weaknesses. The fabrication of PDMS microdevices by molding is particularly simple, easy to learn and suitable for rapid prototyping. Additionally, only for the fabrication of the master for molding cleanroom facilities are required. PDMS microdevices are mostly of single-use. The sizes of the features within a microchip typically range between ~0.5 µm and a few hundred micrometers. However, high aspect ratios of the features are critical. The polymer is transparent for visible light down to ~300 nm. Therefore, the adaptation to optical microscopy is straightforward. The elasticity of PDMS still allows tight bonding if tubes and other components are incorporated into the device. Its flexibility enables the fabrication of multilayer chips and valves, and thin membranes as well. If required, PDMS can also be used as an insulator. The thermal stability of the material up to ~200 °C is e.g. used to decrease the curing time during microchip molding. In general, PDMS is regarded as a non-toxic material, i.e. it can be used as cell culture device. Cell culturing is possible as long as only up to three side walls of the channel are made out of PDMS and the other one(s) is out of a material that enhances cell adhesion. Depending on the cell type the direct growth of cells on the polymer is weak or not possible. PDMS is a very appreciated material in research applications, in which the fluid is water or an aqueous buffer due to its good stability. However, PDMS dissolves or swells in most organic solvents. Its hydrophobic surface can be transferred to a hydrophilic surface by plasma exposure. This facilitates bonding or surface coatings. However, the hydrophilicity of the surface is only transient. The surface converts back into a hydrophobic one over time. As shown in Figure 2.2 the converting time is dependent on the storage conditions; the surface hydrophilicity remains longer when the device is stored in water. Nevertheless, it is difficult to precisely predict the surface hydrophilicity because any contact to air has an affect on it. The polymer is gas permeable. This property has a positive impact for many applications. For example, it allows for a gas exchange when culturing cells. Thus, the cells can be cultured at their individual culturing atmosphere. On the other hand, the gas permeability of PDMS is a drawback due to e.g. the disturbing formation of air bubbles.
Figure 2.2 Hydrophobicity of the PDMS surface, illustrated by placing a water droplet on top of the surface. Freshly prepared PDMS is hydrophobic (A), but can be transferred into a hydrophilic surface by plasma treatment (B). However, the surface converts back to be hydrophobic over time (C). Within a few hours up to few days, the surface properties of plasma treated PDMS alter significantly. After a few days storage in ambient air (B1), the hydrophobicity is even stronger than for freshly prepared PDMS. In contrast, the plasma-activated, hydrophilic surface can be partly retained when the microchip is stored in water (B2). (Courtesy by A. Cavegn)
PDMS is commercially available as a two-component system: the oligomer rubber (vinyl-terminated PDMS) and the curing agent. The curing agent is a mixture of a platinum catalyst and a copolymer of methylhydrosiloxane and dimethylsiloxane (Figure 2.3). Variations in the mixing ratio of both solutions determine different rigidities of the hard polymer. Typically a mass ratio of 10:1 of oligomer and curing agent is used.

![Formation of reticulated PDMS (right) from oligomer and copolymer (left).](image)

**Figure 2.3** Formation of reticulated PDMS (right) from oligomer and copolymer (left).

### 2.2 Fabrication Techniques

#### 2.2.1 Photolithography

Photolithography is a widely used technique in microfabrication. It refers to the process of transferring a pattern into a photosensitive polymer (photoresist) by its exposure to UV-light. Therefore, the photoresist is deposited onto a substrate such as silicon or glass. The pattern is defined in the used photolithographic mask. Typically, these masks are chromium-coated glass wafers. A computer-aided design (CAD) drawing of the pattern is first transferred onto the mask by e-beam lithography or direct laser writing, then developed and finally etched into the chromium. Low-cost alternative high-quality printouts of film masks can be used as well. However, due to their lower resolution these film masks are only suitable for feature sizes bigger than 6-10 µm.

The protocols for photolithography are equal in their step-after-step procedures. First, the substrate has to be coated with a layer of photoresist. Second, this substrate is placed on a hotplate to remove the volatile solvent, in which the photoresist is dissolved, and to partially harden the resist. Third, the mask and the pretreated substrate are placed into a mask aligner, phasing each other, whereby the mask is placed between the light source and the coated substrate. If necessary, the pattern and the substrate can be aligned before illumination. They are exposed
to UV-light for typically a few seconds to transfer the pattern into the photoresist layer. During illumination, the photoresist undergoes chemical and physical rearrangements, which change its solubility in a particular developer solvent. The exposure time is dependent on the energy needed to transfer the pattern, and can be calculated by taking the lamp intensity into account. Fourth, the pattern is developed, and if necessary, there is a final post-bake to completely harden the photoresist. By repeating these individual steps it is possible to fabricate a multilayered patterning of the substrate.

The photolithography protocols differ in the details of the individual steps. For example, the viscosity of the photoresist and the spin speed define the final height of the pattern. Higher features can be achieved by the use of more viscous resists and lower spin speeds, and lower features can be achieved by the use of less viscous resists and higher spin speeds. The baking temperatures and times and the needed exposure energy vary as well. The correlation between photoresist height and spin speed, the bake temperatures as well as the exposure energy are specified for each photoresist in the manufacturer’s protocols. Another big difference is also the type of photoresist; there are so-called positive and negative photoresists. Positive photoresists become soluble, and negative ones become insoluble after light exposure. Thus, positive resists transfer the transparent areas of the pattern on the photolithographic mask into holes, grooves and chambers, whereas negative resists produce columns, ridges and plateaus (Figure 2.4).

Figure 2.4 Photolithography. A substrate (e.g. silicium) is coated with a photoresist. Afterwards a photolithographic mask is placed on top of the photoresist
layer, and an exposure to UV-light follows. During development non-illuminated areas are eliminated using a negative photoresist (left) or illuminated areas are eliminated using a positive resist (right).

Positive photoresists are often used when the substrate will be processed further. On the other hand, negative photoresists are chosen when a master form is needed, e.g. for PDMS molding. The most important negative photoresist is SU-8. It can be used to generate resist layers up to several hundred micrometers on the substrate and features with high aspect ratios. After development and post-baking the substrate can directly be used as mold for soft lithography. The microfabrication of an SU-8 master is depicted in Figure 2.5.

![Microfabrication of an SU-8 master](image)

**Figure 2.5** Individual steps of the microfabrication of an SU-8 master.
2.2.2 Metallization

For the deposition of metals various physical vapor deposition techniques are used, among them e-beam evaporation is (next to sputtering) very common. For feature sized >25 µm a shadow mask can be used to define the pattern. For smaller feature sizes the substrates have to be photolithographically prepared before metal deposition. The samples are placed into sample holders in a vacuum chamber phasing the metal crucibles at the bottom. For e-beam evaporation an intense e-beam is focused into the crucible containing the metal to be deposited. Thus, part of the surface of the metal is melted and evaporated. The evaporated atoms travel through the vacuum and condense on the surface of the substrate resulting in a metal layer. By means of e-beam evaporation it is also possible to deposit multiple layers of different materials in one run. The number of different materials is only limited by the amount of crucibles in the vacuum chamber. The number of samples within one run is dependent on their sizes. With decreasing diameters, the number of possible substrates in the sample holders increases within one run. The benefits of e-beam evaporation are the high deposition rate, the low costs, the low damage due to the low energy of the deposited atoms, and the low contamination because only the e-beam is in contact with the material source.\textsuperscript{149, 150}

After the metal is deposited excess metal around the pattern has to be removed. For this so-called lift-off process the samples are placed into a remover solution and incubated until the remaining photoresist is dissolved and hence, redundant metal is removed (Figure 2.6, left). The addition of heat and agitation can accelerate that process. Sometimes, however, the one-layer lift-off process is not evident, because the features are too close together or the adhesion of the metal to the substrate is too weak. In these cases a bilayer lift-off process is an alternative (Figure 2.6, right).\textsuperscript{151} For a bilayer lift-off the substrate is first coated with an additional lift-off resist during photolithography. The subsequent steps are the same as used for a one-layer lift-off. Due to a faster dissolving rate of the lift-off photoresist compared to the positive photoresist, the lift-off resist undercuts the positive resist during development. This results in stepped edges at the features. Therefore, there is a non-continuous metal layer after film deposition and hence, it is easier to reach and finally dissolve the photoresist during lift-off.
Figure 2.6 Scheme of the metallization process shown for a one-layer respectively bilayer lift-off process. The substrate is photolithographically processed, the metal deposited and the photoresist/metal stack lifted-off resulting in the desired metal film. Left: For the one-layer lift-off only the positive imaging photoresist is deposited during photolithography resulting in a continuous metal film to be fractured and lifted-off. Right: For the bilayer lift-off the substrate is coated first with a lift-off resist. Due to different dissolution rates the lift-off resist undercuts the positive imaging photoresist during development. This results in a non-continuous metal film and eases the lift-off of the photoresists/metal stack.
2.2.3 Soft Lithography

Soft lithography refers to molding and patterning techniques used to fabricate microdevices out of soft elastomers, such as PDMS, with patterned relief structures on its surface.\textsuperscript{152-155} For PDMS molding (Figure 2.7) the oligomer and curing agent are mixed thoroughly and degassed. This mixture is poured on top of a master form and hardened in an oven or on a hot plate. Temperatures between 70-150 °C and times between a few minutes and several hours are chosen to harden the polymer. PDMS spreads out on the master. Thus, a frame is required for microchips with a thickness of a few millimeters. After curing, the PDMS block is peeled of the master, cut into size (if required), and access holes are punched, e.g. for the connection of tubing. There are several techniques to seal the microchip with a cover, e.g. glass slide or foil. Freshly prepared PDMS microchips are sticky to glass and other materials and hence, can directly be attached onto a cover. For high flow rates a stronger bonding is required. This is mostly accomplished by activation of the PDMS microchip and the cover by means of air plasma.\textsuperscript{156,157} For a leakage-free bonding in MMDs, which contain an intermediate permeable membrane, the use of chemical crosslinking agents is sufficient as well.\textsuperscript{158,159}

![Figure 2.7 Sequence for fabrication of a polymer (PDMS) microchip by soft lithography.](image)
2.3 From Fabrication to Application

2.3.1 Interfacing

After establishing protocols for the fabrication of microfluidic devices, their replication is straightforward. However, the connection to peripheral devices, i.e. bridging the microscopic and the macroscopic world often requires additional care.\textsuperscript{160} Methods for liquid supply, interfaces for flow control and imaging/detection systems are required. Figure 2.8 shows the connection of a microchip to tubing by means of bent metal connectors.

![Connection of a microchip to tubing](image)

**Figure 2.8** Interfaces between micro- and macroscopic world. Leakage-free connection to tubing is mandatory for reliable flow rates. A PDMS microdevice with metal plugs and PTFE tubing is shown.
2.3.2 Surface Modification

Physical and chemical surface characteristics have to be considered, especially in biological applications. Hence, it is often required to modify the surface, either during microchip fabrication or by pre-treatment of the microdevice before use. Flushing the microchannel with the dissolved coating material and allowing some time for adsorption to and reaction on the channel walls enable a covalent or transient coating. In this thesis the surface chemistry was altered by the use of polyelectrolyte multilayers (PEMs). PEMs are thin alternating films of cationic and anionic polyelectrolytes from aqueous solutions deposited on a surface. Among the available surface treatments the use of PEMs has gained a lot of attention due to the simplicity of the deposition. Decher et al. introduced their formation by alternating adsorption of polyelectrolytes in the 1990s.\textsuperscript{161-163} The layer-by-layer deposition of the electrostatic self-assembly process is depicted in Figure 2.9. A negatively charged surface adsorbs alternating layers of polycations and polyanions.

![Layer-by-layer deposition of polyions to form PEMs on a substrate.](image)

Figure 2.9 Layer-by-layer deposition of polyions to form PEMs on a substrate.

There are no restrictions regarding the size or topology of the substrate and the possible automation of the process, because the deposition only consists of adsorption from solution.\textsuperscript{164} The only requirement for a successful PEMs formation is the reverse of the surface charges after each polyelectrolyte. This so-called surface charge overcompensation results in the freedom to incorporate more than two different polyelectrolytes and in the self-assembly of monolayers, due to repulsion of equally charged polyelectrolytes.\textsuperscript{165, 166} Furthermore the charge overcompensation is rather a property of the polyelectrolyte than of the surface, because the polyelectrolyte can bridge over small defects of the underlying surface. Hence, the conformation at
the newly created film surface is dependent on the polyelectrolyte itself and the adsorption conditions. Different macroscopic properties of the PEMs, e.g. function and thickness, can be obtained by varying the microscopic structure. The incorporation of various materials with a function like proteins can change the utility of PEMs. The thickness of the PEMs can be regulated by varying the concentration of salt, which is present in the polyelectrolyte solution, due to coiling of the polyelectrolyte. Dubas et al. showed an almost linear correlation between PEMs thickness and sodium chloride concentration. PEMs have also been combined with microfluidics. One of its first uses was the coating of capillary inner walls for capillary electrophoresis. Nowadays, there is a tremendous variety of applications, where PEMs and microfluidics are combined.
3 Evaluation of Pumping Systems
In this chapter the performances of several pumping systems are evaluated. For the subsequent study on the monitoring of induced gene expression in single cells stable flows at low flow rates are required i) to minimize shear stress on the cells that might influence the experimental data and ii) to avoid cell loss during the experiment due to instabilities in the provided flow. Additionally, a total volume of about 1 mL is needed to carry out experiments over a time period of at least 15 h. Hence, it is crucial to test different pumping systems and choose the one that can fulfill both requirements best. Four syringe pumps and two pressure-driven pumps are evaluated.

3.1 Introduction

The use of microfluidics has become prominent in a wide and diverse range of research applications. The vast prospects offered by microfluidic systems have attracted newcomers to the field from different scientific backgrounds. Regardless of the application the microfluidic system will be used for, a number of important experimental conditions must be considered before starting any experiments, e.g. operating temperature, surface characteristics, or used solvent. Although there is a wide variety of materials to choose from, the requirements and restrictions of the application lead mostly straight to one distinct material. In addition, the right pumping system has an important influence on the experiments. Specific requirements have led to the development and characterization of various pumping systems, which are widely used. The performance of some of these commercial pumps has been evaluated. For example, Garstecki and co-workers characterized in detail the influence of oscillations in pumping systems on the dynamics of droplet formation. Biological applications, specifically cell studies, require low and stable flow rates to minimize negative effects on the cells caused by shear stress. Additionally, extremely high flow rates can result in the loss of cells before, during or after the experiments are carried out. For example, for the studies on monitoring induced gene expression the incorporation of hurdle structures into the microfluidic device for mechanical trapping of single cells is required. Three posts, two smaller at the top and the bottom and one longer at the side, form a U-shaped hurdle structure (Figure 3.1). The flow through empty traps is used to lead single cells into the hurdle structures and to trap them there. However, high flow rates or an unstable flow could cause the loss of the trapped cells due to cell movement. To carry out long-term live cell imaging experiments a total volume of media of approximately 1 mL is needed. Therefore, it is critical to choose a microfluidic pumping system that can generate stable flows at low flow rates, while at the same time delivering a high volumetric flow rate.
Figure 3.1 Design of the hurdle structures used to trap single cells. Left: Scheme of a cell trap including its dimensions. Middle: Micrograph of an actual trap. The arrows indicate the flow through it. Right: Micrograph of a trapped cell (green). Too high or too instable flows cause cell loss, e.g. through the spaces between the posts of the trap (indicated by arrows).

3.2 Experimental Section

In order to obtain stable flow rate conditions for single cell studies the performance of four syringe pumps (mechanically driven) and two pressure-driven pumping systems was assessed. The syringe pumps neMESYS (cetoni GmbH, Germany), NanoJet (Chemyx Inc., USA), PHD ultra (Harvard Apparatus, USA), 55-2222 (Harvard Apparatus, USA), and the pressure-driven systems MAESFLO (Fluigent, France) and a home built system were chosen for the evaluation. The following two setups were used to measure the flow stability and precision: (i) syringe pumps were connected to a flow sensor (Flowell, Fluigent, France) via PTFE (poly(tetrafluoroethylene)) tubing (OD: 1/16 inch, ID: 800 µm, 10 cm, Peter Klaus Müller SA, Belgium) and PEEK (polyether ether ketone) capillaries (OD: 1/32 inch, ID: 250 µm, 19 cm, BGB Analytik, Switzerland). (ii) Pressure-driven pumps were connected to a 15 mL flask via silicon tubing (OD: 3 mm, ID: 1 mm, Unico Haberkorn AG, Switzerland) and subsequent to the flow sensor via PEEK capillaries (OD: 1/32 inch, ID: 50 µm, 50 cm, BGB Analytik, Switzerland). The smaller ID of these capillaries resulted in a higher resistance in the system. This enabled the measurements at low flow rates. In both setups another PEEK capillary (OD: 1/32 inch, ID: 250 µm, 19 cm) connected the flow sensor with a water reservoir. For flow rate measurements filtered, deionized water was pumped through the setups described above and depicted in Figure 3.2.
Figure 3.2 Setups to measure the performance of different pumping systems. In image (A) the setup for syringe pumps and in (B) the setup for pressure-driven pumps is shown. 1: syringe pump with a 1 mL syringe (here: neMESYS), 2: flow sensor, 3: PTFE tubing, 4: PEEK capillary (ID: 250 µm), 5: PEEK capillary (ID: 250 µm) and water reservoir, 6: pressure-driven pump (here MAESFLO) and silicon tubing, 7: 15 mL flask, 8: PEEK capillary (ID: 50 µm)

Before any measurement was taken the pumps were allowed to run for 10-15 min in order to stabilize the flow. The measurements of the syringe pumps were performed with 1 mL glass syringes (Hamilton Bonaduz AG, Switzerland), 1 mL plastic syringes with an o-ring (Codan Medical ApS, Denmark) and 1 mL plastic syringes without an o-ring (B. Braun Melsungen AG, Germany). For the
3.2 Experimental Section

Pressure-driven pumping systems PEEK capillaries with smaller ID, i.e. high resistance, were used. The flow rates were measured for one hour at 500 nL/min and 5000 nL/min, each. The flow rate was set directly for syringe pumps, however, for pressure-driven pumps this was not applicable, because only the applied pressure could be regulated. Therefore, the flow sensor had to be used to set the pressure that resulted in the aimed flow rate. Although, these pumps were passive regulated there was no feedback control.

The flow measurements were carried out at least three times. For each measurement a histogram of the distribution of flow over time was Gaussian fitted to reveal maxima values, $x_c$, and the width, $w$. The $x_c$ value gave information about the precision of the pump, i.e. the closer the value of $x_c$ was to the set value (500 nL/min or 5000 nL/min) the more precise the system. For the pressure-driven pumps higher variations could occur due to the relatively poor control of these systems; only the applied pressure could be varied and therefore, it was more difficult to achieve the preset flow rate. The width, $w$, of the Gaussian fit is defined as the area in between $\pm \sigma$, i.e. this area contains 68% of all measured points. Thus, the width gave information about the stability of the system, i.e. the lower the value of $w$ the lower the spread of measurements obtained, and therefore, the more stable the pump. For clarification the difference between $x_c$ and $w$ is depicted in Figure 3.3. Very precise and stable pumps will have $x_c$ values close to $x_{set}$ and low $w$ values. For the comparison of the different pumping systems the respective means of the $x_c$ and $w$ values of the triplicates were calculated.

![Figure 3.3 Depiction of information obtained from Gaussian fit. (A) The Gaussian fit gives information about the position of the maximum, $x_c$, and the width, $w$. The position of the maximum is correlated to the precision and the width to the stability of the pump. (B) Precision information. In B1 the deviation of $x_c$ from the set value, $x_{set}$, is higher than in B2, i.e. B2 is more precise. (C) Stability information. The width in C1 is wider than in C2, i.e. C2 is more stable.](image)
3.3 Results and Discussion

In Figure 3.4 representative raw data traces of the measurements for the NanoJet and neMESYS pumps using glass syringes. The variations in flow of the neMESYS system were smaller for both flow rates (500 nL/min and 5000 nL/min) compared to the NanoJet system. This suggested a more consistent fluid delivery by the use of neMESYS pumps. As expected, the stability increased for both pumps at a flow rate of 5000 nL/min when compared to 500 nL/min. The higher flow rate traces show a more linear behavior when compared to the lower flow rates, which exhibit a more variable pattern.

Figure 3.4 Representative flow rate traces over one hour period for the pumping systems neMESYS and NanoJet. Raw data of triplicate measurements for 500 nL/min (A and B) and 5000 nL/min (C and D) using two pumping systems with glass syringes.
For each raw data trace a separate histogram of the flow distribution was generated. In Figure 3.5 the histograms of one measurement per pump for each flow rate are shown. They each correspond to one raw data trace in Figure 3.4. At both flow rates the histograms were narrower for the neMESYS pumping system when compared to the NanoJet pump. Hence, the measurement spread was less and the stability of the neMESYS pump was better. Further analysis was required to obtain more information about the performances of the pumps. In general, these histograms were Gaussian fitted to obtain the values $x_c$ and $w$, and therefore, information about the precision and stability of the pumping systems.

**Figure 3.5** Representative histograms of measurements for two pumping systems, taken at flow rates of 500 and 5000 nL/min. The distributions of the flow over time raw data (Figure 3.4) were visualized as individual histograms. Each histogram was Gaussian fitted to obtain values for $x_c$ and $w$. The flow rate distribution of the NanoJet pump was broadened compared to the flow rate distribution of the neMESYS pump, resulting in higher $w$-values.
Figures 3.6 and 3.7 and Table 3.1 summarize all results of the performance measurements: Here, the comparison of the pumping systems containing all the different configurations concerned, e.g. the pumping system and syringe type, are graphically shown. The table gives an overview of the percentage deviation from the set flow rate. For the syringe pumps the most stable and precise flows at 500 nL/min were achieved using the neMESYS and the PHD ultra pumps with glass syringes, whereby the precision of the system was even better for the neMESYS pump (Figure 3.6). On the other hand, the two other syringe pumps (55-2222 and NanoJet) used with plastic syringes without o-rings created the most unstable and imprecise flows at 500 nL/min. In general, the performance of each syringe pump was better by combining it with glass syringes. The pressure-driven pumping system MAESFLO achieved very precise and stable flows, even with the lack of direct control over the flow settings. The results of the measurements for the higher flow rate were similar (Figure 3.7). However, for all pumping systems, under all conditions, more stable flows were observed at 5000 nL/min.

**Figure 3.6** Measured flow rate at 500 nL/min of all six pumps studied. The histograms of the raw data were Gaussian fitted, and $x_c$ (symbols) and $w$ (columns) plotted. The syringe pumps (neMESYS, NanoJet, PHD ultra, 55-2222) were measured.

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3 For notification: all fabricants recommend the use of glass syringes.
using three different syringes (glass, plastic with o-ring and plastic without o-ring). The flow rate of the two pressure driven pumps (MAESFLO and the home built system) was measured with one set of capillaries.

![Flow rate comparison for a flow of 5000 nL/min.](image)

**Figure 3.7** Flow rate comparison for a flow of 5000 nL/min.

The percentage deviation of w around the set flow rate is shown in Table 3.1. The better performance of all pumping systems at higher flow rates was confirmed, even where plastic syringes without o-rings were used.
### Table 3.1 Percentage deviation of \( w \) around the set flow rate.

<table>
<thead>
<tr>
<th>500 ± x%</th>
<th>neMESYS</th>
<th>NanoJet</th>
<th>PHD ultra</th>
<th>55-2222</th>
<th>MAESFLO</th>
<th>Home built</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>4.5</td>
<td>10.5</td>
<td>4.2</td>
<td>9.3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Plastic (with o-ring)</td>
<td>8.5</td>
<td>12.9</td>
<td>10.3</td>
<td>19.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Plastic (without o-ring)</td>
<td>10.9</td>
<td>23.3</td>
<td>10.1</td>
<td>19.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Capillary</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1.4</td>
<td>7.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5000 ± x%</th>
<th>neMESYS</th>
<th>NanoJet</th>
<th>PHD ultra</th>
<th>55-2222</th>
<th>MAESFLO</th>
<th>Home built</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>2.7</td>
<td>6.2</td>
<td>1.9</td>
<td>3.8</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Plastic (with o-ring)</td>
<td>2.9</td>
<td>9.4</td>
<td>1.6</td>
<td>4.2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Plastic (without o-ring)</td>
<td>4.1</td>
<td>10.5</td>
<td>2.5</td>
<td>5.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Capillary</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

The variations in the performances of the syringe pumps using the different syringes can be explained by comparing the different surface tensions inside the syringes. Glass has the smoothest surface of the three tested syringes, and hence, the lowest friction and probability of tilting during its operation. The o-ring inside the plastic syringe lowers its friction and the probability of tilting and allows a better performance compared to the plastic syringe without an o-ring. The performance of the pressure driven pumping systems is better than the mechanical driven one due to the lack of mechanical parts that can cause tilting, stumbling and friction.
3.4 Conclusions

The use of low and stable flows is of paramount importance when performing cell studies in vitro. In particular, low flow rates are necessary to achieve reliable and physiologically relevant results. However, long-term cell measurements will consume more media and therefore higher volumes are needed to avoid disruption of the flow while an experiment is being carried out. For these reasons it is critical to choose the right pumping system to obtain stable and precise flows for higher volumes. By using the appropriate pumping system with the right components (e.g. glass syringes vs. plastic ones) these requirements can be reached. On the other hand, higher flow rates are less restrictive when choosing the pumping system. This is due to the increase in stability that all pumps and components will show at higher flow rates. Each researcher is reliant on very careful planning of experiments to achieve meaningful results. As shown here, even the choice of a pumping system can be especially crucial. Thus, the time spent doing pumping and flow evaluation is spent well.

In this thesis the evaluation of several pumping systems was important for the studies on the induced gene expression. It was possible not only to see the differences in the performances, but also to choose the pumping system that fulfilled the requirements (stable flows at low flow rates and supply of adequate volumes) best: the pressure-driven MEASFLO system.
4 Induced Gene Expression in a Multilayer Microfluidic Device
In this chapter the monitoring of the induced gene expression in single cells is addressed. Therefore, a MMD is introduced, where the cell handling is separated from cell manipulation. The individual steps during development of the optimal design of the cell traps, as well as the performed molecular cloning and cell modification steps are pointed out. After proving the performance of the designed device different aspects of induced gene expression, i.e. inducer concentration dependency, transient induction, and parallel supply of different inducer concentrations, are monitored.

4.1 Introduction

The systematic analysis of cellular responses in single cells requires precise control of the surrounding environment to mimic in vivo conditions and to precisely regulate the supply of effectors. Often only a small number of molecules are required to induce cell responses. Therefore it is essential that small liquid volumes are handled without loss of the compounds. Standard tissue culture techniques lack the ability to create controllable and reproducible microenvironments.

A MMD is designed, which enables a significant improvement of fluid handling by separating the channel system for cell handling and cell maintenance from the channels used for cell stimulation. The two required microfluidic layers are positioned on top of each other with an intermediate porous membrane at the interface to facilitate the transfer of dissolved molecules between the layers (Figure 4.1). In this design, the top layer is used exclusively for cell introduction, single cell trapping and supply of media, and guarantees optimized cell viability by use of weak flow rates for media supply and removal. The bottom layer contains three input channels to supply stimulating agents to the cells. Dissolved compounds for cell stimulation can be supplied either continuously, periodically or pulsed. Additionally, the delivery of stimulants can be spatially resolved using multi-laminar flows allowing the formation of chemical gradients. Hence, the multilayer microfluidic platform allows a further level of temporally and spatially control concerning cell handling and analyte supply, previously unavailable to cell biologists, and not achievable on a planar microfluidic device.
4.1 Introduction

Figure 4.1 Schemes of the microdevice. (A) Exploded view of the MMD with the top and bottom channel and the intermediate membrane. (B) Side view of the assembled device. Cells are trapped at the microstructured columns in the top channel, while the inducer solution is introduced from below and reaches the cells via the micropores in the membrane.

Based on previous designs\textsuperscript{77, 144, 186} the cell handling was improved by implementation of a cell culture chamber with a trap array in the top channel. Precise single cell positioning simplifies the currently semi-automated analysis of the individual mammalian cells, and will pave the way for fully automated data analysis in future. The performance of the microdevice is demonstrated by studying the expression levels of a fluorescent protein in human cells. In this system the gene expression is inducible and hence, serves as a convenient model system to demonstrate the potential of the device for spatial and temporal controlled gene expression.

The gene-of-interest encodes the ZsGreen1-DR protein, a destabilized variant of the green fluorescent protein ZsGreen1. Therefore, gene expression can be monitored online by time-lapse fluorescence microscopy. This protein is used to establish the parameters for titration and determine the time and spatial resolution on induced gene expression.
4.1.1 Tetracycline-Regulated Expression (T-REx™) System

The inducible gene expression in the tetracycline-regulated expression (T-REx™) system (Invitrogen, Germany) for mammalian cells can be controlled by tetracycline as an external effector. In the absence of tetracycline the expression of the gene-of-interest is inhibited and in the presence of tetracycline the expression is induced. The regulatory unit in the T-REx™ system is adapted from the E.coli Tn10-encoded tetracycline resistance operon. The major components of the T-REx™ system include tetracycline as external effector and two plasmids: the regulatory pcDNA6/TR vector and the inducible expression vector. The detailed mechanism of repression and induction of the gene expression in the T-REx™ system is depicted in Figure 4.2 and described in the following:

1) The regulatory pcDNA6/TR plasmid continuously expresses tetracycline repressor proteins (tetR). These tetR proteins form homodimers.

2) Two tetR homodimers bind with a binding constant of $K_B=2\times10^{11}$ M$^{-1}$ to two tetracycline operator (TetO$_2$) sequences on the inducible expression plasmid. This results in the repression of the transcription of its gene-of-interest.

3) Upon addition, tetracycline is taken up by the cells and binds with an association constant of $K_A=3\times10^9$ M$^{-1}$ to the tetR protein in a 1:1 stoichiometry.

4) The binding of tetracycline to the tetR homodimers causes a conformational change. Thus, the homodimers cannot bind to the TetO$_2$ sequences anymore and the tetR:tetracycline complex dissociates from the TetO$_2$. This finally results in the derepression of the transcription of the gene-of-interest.
Figure 4.2 Overview of the mechanism of gene expression repression and induction. (Only DNA components of T-REX™-system are shown). A conformational change of the tetR protein, caused by the inducer tetracycline, results in dissociation of the repressor and hence, protein expression is initiated.\textsuperscript{190}
An overview of the experimental outline is given in the following and in Figure 4.3:

1) The gene-of-interest is inserted into the inducible expression vector.
2) The inducible expression vector and the regulatory pcDNA6/TR vector are co-transfected into mammalian cells.
3) The expression of the gene-of-interest is inhibited. Addition of tetracycline induces its transcription.
4) The desired gene can be assayed.

Figure 4.3 Overview of the T-REx™-system. The gene-of-interest is inserted into the inducible expression vector and mammalian cells are co-transfected with both plasmids (expression and repressor plasmid). The addition of the inducer tetracycline enables the expression of the gene-of-interest.190

4.1.2 Gene-of-Interest: ZsGreen1-DR

For the understanding of biological systems the ability to visualize, track and quantify molecules and events in living organisms at high temporal and spatial resolutions is a key requirement.191 The discovery and further development of fluorescent proteins such as the green fluorescent protein (GFP)192, 193, its derivatives194 and a whole family of GFP-like proteins195-197 has boosted biological research. Nowadays, the spectral profiles of fluorescent proteins span almost the entire visible light spectrum.198 The various colors originate from the formation of a chromophore in the interior of the protein. This formation is caused by the covalent modification of three amino-acid residues that are consecutive in the protein’s sequence.199 The generation of fluorescence does not require any
substrate or cofactor\textsuperscript{200}, and hence, gene expression can be quantified easily. The protein family shares the native three-dimensional structure, termed $\beta$-can, which consists of an 11-stranded $\beta$-barrel with the chromophore embedded in the middle of an internal $\alpha$-helix directed along the $\beta$-barrel axis.\textsuperscript{201, 202}

One of the original Anthozoa coral reef proteins from \textit{Zoanthus} sp. (Figure 4.5) has become a commercial product (Clontech) under the trade name ZsGreen. Matz \textit{et al.} introduced this GFP-like protein in 1999.\textsuperscript{195} The protein consists of 231 amino acids and forms tetramers in the natural state. The homology to the original GFP from \textit{Aequoera victoria} is 20\%. The excitation maximum at 496 nm and the emission maximum at 506 nm allow its imaging with standard light and filter combinations in confocal and widefield microscopy.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{ZsGreen.png}
\caption{ZsGreen – a GFP-like protein. (A) The GFP-like protein was isolated from the coral \textit{Zoanthus} sp.\textsuperscript{203} (B) In the natural state ZsGreen exists as a tetramer.\textsuperscript{204}}
\end{figure}

The ZsGreen protein has been re-engineered due to a non-optimal expression in mammalian cells. For an optimized expression the coding sequence was adapted with a series of silent base-pair changes based on the findings for GFP.\textsuperscript{205} The new variant was termed ZsGreen1. Like all GFP variants the ZsGreen1 protein is very stable. This allows its accumulation and easy detection, however, prevents its use for rapid turnover studies. For this purpose a destabilized variant, termed ZsGreen1-DR, was constructed by the fusion of the amino acid residues 422-461 of the mouse ornithine decarboxylase (MODC) to the C-terminus of ZsGreen1. With a half-life of approximately 30 min MODC is one of the most short-lived proteins in mammalian cells. The fused sequence, amino acids 422-461, causes the fast degradation of MODC.\textsuperscript{206, 207} The fused sequence also contains a PEST region. PEST regions are rich in the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T) and are often flanked by clusters containing positively charged amino acids.\textsuperscript{208} A search among short-lived proteins confirmed the hypothesis
about the correlation between rapid protein degradation and the presence of PEST sequences. 

4.2 Experimental Section

4.2.1 Molecular Cloning

The model gene-of-interest encoded the ZsGreen1-DR protein, a destabilized variant of the GFP-like protein ZsGreen. Therefore, the ZsGreen1-DR gene had to be inserted into the inducible gene expression vector of the T-REx™ system. The ZsGreen1-DR gene was isolated from the pZsGreen1-DR plasmid (Clontech, France, Figure 4.6) using the restriction enzymes BamHI and NotI (both New England Biolabs GmbH, Germany). Both restriction sites were unique in the plasmid’s DNA sequence, whereby the restriction site of BamHI was located in the multiple cloning site (MCS) and the restriction site of NotI directly after the ZsGreen1-DR stop codon. The cleavage of the plasmid-DNA resulted in sticky ends for both restriction enzymes. Hence, the right orientation of ZsGreen1-DR was given for the ligation of the ZsGreen1-DR into the inducible expression vector pcDNA4/TO/mycHisB (Figure 4.7).

Figure 4.6 Vector map and MCS sequence of pZsGreen1-DR. The restriction site of BamHI is located within the MCS and the restriction site of NotI directly after the stop codon of ZsGreen1-DR.
4.2 Experimental Section

Figure 4.7 Vector map of the inducible expression vector pcDNA4/TO/mycHisA, B, C. There are three different plasmids to ensure that the cloning of the gene if interest is in frame with the C-terminal c-myc epitope and the polyhistidine tag.\textsuperscript{211}
Table 4.1 depicts the composition of the digestion mixture that was used to isolate the ZsGreen1-DR gene from the pZsGreen-1DR vector. The digestion was carried out at 37 °C over night.

**Table 4.1 Composition of the digestion mixture to isolate the ZsGreen1-DR gene.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pZsGreen1-DR (10 µg)</td>
<td>16.16</td>
</tr>
<tr>
<td>BamHI</td>
<td>0.5</td>
</tr>
<tr>
<td>NotI</td>
<td>0.5</td>
</tr>
<tr>
<td>10x BamHI buffer</td>
<td>3</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>9.84</td>
</tr>
</tbody>
</table>

Table 4.2 depicts the composition of the digestion mixture that was used to cleave the pcDNA4/TO/mycHisB vector to allow a ligation with the ZsGreen1-DR gene. The digestion was carried out at 37 °C over night.

**Table 4.2 Composition of the digestion mixture to cleave the pcDNA4/TO/mycHisB vector.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA4/TO/mycHisB (10 µg)</td>
<td>13.5</td>
</tr>
<tr>
<td>BamHI</td>
<td>0.5</td>
</tr>
<tr>
<td>NotI</td>
<td>0.5</td>
</tr>
<tr>
<td>10x BamHI buffer</td>
<td>3</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>12.5</td>
</tr>
</tbody>
</table>

The isolated ZsGreen1-DR gene was ligated into the pcDNA4/TO/mycHisB plasmid. The composition of the ligation mixture is depicted in Table 4.3. The ligation mixture was incubated at room temperature for 1 h, followed by 10 min at 65 °C.

**Table 4.3 Composition of the ligation mixture.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZsGreen1-DR (170 ng/µL)</td>
<td>2</td>
</tr>
<tr>
<td>pcDNA4/TO/mycHisB (150 ng/µL)</td>
<td>1</td>
</tr>
<tr>
<td>10x T4-DNA ligase buffer</td>
<td>2</td>
</tr>
<tr>
<td>T4 ligase</td>
<td>0.5</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>14.5</td>
</tr>
</tbody>
</table>
The new construct, inducible ZsGreen1-DR expression vector, was transformed into E-coli to be amplified. Positive clones were selected on LB-media containing 50 µg/mL ampicillin. Ampicillin is a β-lactam antibiotic that inhibits cell wall biosynthesis in bacteria. The resistance gene of the inducible ZsGreen1-DR expression vector encoded a β-lactamase, which hydrolyzed ampicillin. After selection the plasmid-DNA was isolated by alkaline lysis, purified by a phenol/chloroform extraction, and digested with the restriction enzyme HindIII (Fermentas, Germany) that cleaved once in the plasmid backbone and once in the ZsGreen1-DR gene. Table 4.4 depicts the composition of the digestion mixture. After identification of positive transformants their plasmid-DNA was prepared, i.e. amplified, isolated and purified, for the co-transfection of mammalian cells.

Table 4.4 Composition of the digestion mixture to cleave the inducible ZsGreen1-DR expression vector.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inducible ZsGreen1-DR expression vector (1 µg)</td>
<td></td>
</tr>
<tr>
<td>HindIII</td>
<td>0.25</td>
</tr>
<tr>
<td>10x FastDigest™ buffer</td>
<td>2</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>Up to 20</td>
</tr>
</tbody>
</table>

4.2.2 Cell Modification and Cell Culture

Evaluation of Transfection

Several transfection systems (TransIT®-LT1 (Mirus Bio LLC, Maddison, WI), TransIT®-293 (Mirus Bio LLC, Maddison, WI), Lipofectamine™ 2000 (Invitrogen, Switzerland), TurboFect™ (Fermentas, Switzerland), and jetPrime™ (Polyplus-transfection SA, France)) were compared to evaluate the transfection system, which yielded the most efficient cell transfection and the lowest cell toxicity. Therefore, human embryonic kidney cells (HEK293, DSMZ, Germany) were transfected with GFP according to the individual manufactures’ protocols (see Table 4.5 and Table 4.6). The use of the GFP allowed for an observation of the transfection efficiency by fluorescence microscopy. The cytotoxic effect of the transfection agents was estimated visually. Additionally, the transfection efficiencies of different batches of prepared inducible ZsGreen1-DR expression vector DNA were compared to choose the plasmid-DNA that was most efficient for further generation of a stably transfected HEK293 cell line.
Table 4.5 Cell seeding protocols for individual transfection systems (the day before transfection).

<table>
<thead>
<tr>
<th>Transfection system</th>
<th>Number of cells to seed (6-well plate)</th>
<th>Media per well (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransIT®-LT1</td>
<td>200000</td>
<td>2.5</td>
</tr>
<tr>
<td>TransIT®-293</td>
<td>200000</td>
<td>2</td>
</tr>
<tr>
<td>Lipofectamine™ 2000</td>
<td>200000</td>
<td>2</td>
</tr>
<tr>
<td>TurboFect™</td>
<td>200000</td>
<td>4</td>
</tr>
<tr>
<td>jetPrime™</td>
<td>200000</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.6 Individual transfection protocols.

<table>
<thead>
<tr>
<th>Transfection system</th>
<th>DNA (µg)</th>
<th>Transfection reagent (µL)</th>
<th>Serum free media (µL)</th>
<th>Buffer (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransIT®-LT1</td>
<td>2.5</td>
<td>7.5</td>
<td>250</td>
<td>---</td>
</tr>
<tr>
<td>TransIT®-293</td>
<td>2</td>
<td>4</td>
<td>200</td>
<td>---</td>
</tr>
<tr>
<td>Lipofectamine™ 2000</td>
<td>4</td>
<td>10</td>
<td>500</td>
<td>---</td>
</tr>
<tr>
<td>TurboFect™</td>
<td>4</td>
<td>6</td>
<td>400</td>
<td>---</td>
</tr>
<tr>
<td>jetPrime™</td>
<td>2</td>
<td>4</td>
<td>---</td>
<td>200</td>
</tr>
</tbody>
</table>

Cell Modification and Cell Culture

HEK293 cells were stably co-transfected with the modified T-REx™ system, i.e. the inducible ZsGreen1-DR expression vector and the regulatory vector pcDNA6/TR. HEK293 cells were co-transfected with the regulatory and the inducible ZsGreen1-DR expression vector in a ratio of 6:1 to ensure complete repression of ZsGreen1-DR gene expression without tetracycline. For the selection of positive clones, i.e. cells that were transfected with both plasmids, 5-10 µg/mL Blasticidin S HCl and 100-800 µg/mL Zeocin™ (both Invitrogen, Switzerland) were added to the cell culture media. Blasticidin S HCl was dissolved in Millipore water to a 5 mg/mL stock solution before use. Zeocin™ was purchased as stock solution of 100 mg/mL. Blasticidin S HCl is a nucleoside antibiotic that inhibits protein synthesis in both prokaryotic and eukaryotic cells.\textsuperscript{216, 217} The resistance gene of pcDNA6/TR encodes a deaminase, which converts Blasticidin S to a nontoxic deaminohydroxy derivate.\textsuperscript{218, 219} Zeocin™ is a member of structurally related bleomycin/phleomycin-type antibiotics, which binds to DNA and cleaves it.\textsuperscript{220} The
resistance gene of the inducible gene expression vector encoded a protein that bound to Zeocin™ and hence inhibited it.

The T-REx™-modified HEK293 cells were cultured in DMEM low glucose media containing 10% fetal bovine serum (tetracycline negative), 1% MEM and 1% penicillin-streptomycin at 37 °C and 7% CO₂ atmosphere. For experiments, the cells were harvested in the same media that additionally contained 25 mM HEPES buffer, 5 mM EDTA and 1 µg/mL propidium iodide. All cell culture media and substitutes were received from PAA Laboratories, Germany. Propidium iodide (Sigma-Aldrich, Switzerland, 1 mg/mL) is a common agent for dead cell staining. It is a membrane impermeable dye that intercalates into nucleic acids. After its binding to nucleic acids the fluorescence is enhanced 20-30 fold and red fluorescence can be detected. HEK293 cells tend to aggregate in suspension; therefore, ethylenediaminetetraacetic acid (EDTA, Titriplex® II, VWR, Switzerland) was added to minimize cell aggregation during transport to the light microscopy laboratory. Titriplex® II was dissolved in Millipore water to a 0.5 M stock solution before use.

**Cell Aggregation Study**

Cell aggregation complicated single cell trapping in the multilayer microchip. To minimize cell aggregation the effects of different additives on cell dissociation were compared. Due to the need for viable cells the effect on toxicity was taken into account as well. EDTA, trypsin, PEG 6000, and Accumax™ were chosen as additives. EDTA is a chelating agent for different metal ions like calcium, hence, preventing cell junctions formed by cadherins. Trypsin, a serine protease, cleaves extracellular proteins and is conventionally used in cell culture as an agent to detach cells from cell culture flasks. PEG 6000 is a biocompatible polymer that prevents cells from adhesion to surfaces. Accumax™ (Innovative Cell Technologies, Inc., USA) combines different proteases, collagenolytic and DNase activities to dissociate cell aggregates. For the experiments the cells were washed three times in PBS buffer and harvested in cell culture media containing 25 mM HEPES buffer and 1 µg/mL propidium iodide and an additive. Three different concentrations of EDTA (0.75 mM, 5 mM and 10 mM), 0.1x trypsin, 5% PEG 6000, or Accumax™ (in a 1:1 ratio) were added to the cells. Then, the cells were incubated at room temperature for approximately 30 min to mimic the transport to the light microscopy laboratory.
Cell Growth and Viability on Chip

The surface of the polyester membrane was hydrophilic and promoted cell adhesion. To confirm practicability of the membrane for cell handling T-REx™-modified HEK293 cells were seeded into the microchip and cell growth was observed over several days. Tips with media containing 25 mM HEPES buffer, 0.1 µg/mL tetracycline (Sigma-Aldrich, Switzerland) and 1 µg/mL propidium iodide were placed in the inlets of both channel systems. Tetracycline was dissolved in ethanol p.a. (Scharlab SL, Spain) to a 1 mg/mL stock solution before use. A low flow was achieved until the pressure difference between the empty tips placed in the outlets and the filled tips in the inlets was too low to cause a flow. The tips were exchanged every day to ensure the supply of fresh media and the removal of the used one. Tetracycline was added for visualization of healthy cells and propidium iodide to stain dead cells.

4.2.3 Multilayer Microfluidic Device

Microchip Design

The microfluidic device for single cell trapping in the top layer and their manipulation via a channel network in the bottom layer was designed. The use of the bottom layer for the supply of the inducer in (i) a continuous pattern, (ii) a pulsed pattern, or (iii) at different concentrations led straight to the final design. However, the designing of a cell chamber in the top layer for studies on single cells was more challenging. During the development of the final design to monitor induced gene expression, different strategies were used to capture cells. These included the use of channels without any features and the implementation and optimization of cell traps.

Microchip Fabrication and Interfacing

The MMD was prepared out of PDMS (Sylgard 184, Dow Corning, Germany) by casting both layers from a SU-8 master. This SU-8 master was fabricated by photolithographic processing of a silicon wafer following standard protocols. First, the silicon wafer was dehydrated for 1 h at 200 °C before it was coated with the negative photoresist SU-8 2015 to a height of 20 µm. After a soft bake (240 s at 95 °C) the coated wafer was exposed to the mercury i-line (365 nm) until the transfer energy of 150 mJ/cm² was reached. The used photolithography mask (film
mask, JD Photo Tools, UK) contained features for both layers of the microchip. The microchannel in the bottom layer had a width of 370 µm and the microchamber in the top layer a width of almost 5 mm. After illumination, the wafer was placed on a hotplate for the post exposure bake (300 s at 95 °C). Next, the features were developed in SU-8 developer for 240 s, and the master was rinsed with isopropanol. For the final hard bake the master was placed over night at 160 °C. The whole process resulted in an SU-8 master with channel structures of the designed width and a height of 20 µm. Finally, the master was silanized overnight under vacuum using 1H,1H,2H,2H-perfluorodecyl-dimethylchloro-silane (ABCR, Germany) to ease the detachment of cured PDMS.

For PDMS molding, the PDMS oligomer and curing agent were thoroughly mixed at a mass ratio of 10:1, and the mixture was degassed in a desiccator. For the cell-trapping channel in the top layer, a frame was placed onto the master form, filled with the pre-polymer and cured at 150 °C for 20 min. After curing, excess PDMS was removed and access holes for the fluids were punched using a biopsy puncher (1.5 mm diameter, Miltex, Switzerland). For the bottom layer the PDMS pre-polymer was poured onto the master form and fixed with a 24×40 mm glass slide (No.1, Menzel, Germany) at a height of approximately 100 µm and cured at 150 °C for 3 min. Thus, the bottom layer was directly prepared on the glass slide. After both layers were cleaned and the surfaces were exposed to an oxygen plasma (Plasma Cleaner PDC-32G, Harrick Plasma, Ithaca, NY), a piece of a track-etched polyester (polyethylene terephthalate, PET) membrane (11 µm thickness, 1.2 µm pore size, 1.6×10^6 pores per cm², it4ip, Belgium) was placed on top of the cell trapping structures in the top layer. To ensure complete sealing between the two layers and the PET membrane, tiny drops of uncured PDMS were placed onto the edges of the membrane. Placing the bottom layer on top, whereby the main channels of both layers were perpendicular to each other, assembled the microchip. The device was placed another 2-5 min onto a hotplate at 100 °C to ensure the curing of the PDMS drops for sealing and complete bonding of the assembled chip (Figure 4.8).
Figure 4.8 Assembled MMD. The PDMS drops at the edges of the membrane sealed the chip, i.e. an exchange between the channel in the top and the bottom layer was only possible through the intermediate membrane. The microdevice was filled with a fluorescent dye for a better visualization. (Scale bar: 1 mm)

Custom-made metal connectors, FEP (fluorinated ethylene propylene) tubing (OD: 1/16 inch, ID: 250 µm, BGB Analytik AG, Switzerland) and PEEK capillaries (OD: 1/32 inch, ID: 50 µm and 250 µm, respectively) were used to connect the MAESFLO pressure flow control system with the microfluidic device. The 15 mL tubes containing the reagents were connected to the flow control system by PEEK capillaries (ID: 50 µm). The outgoing capillaries (ID: 250 µm) were connected to custom-made bubble traps and finally to the chip via FEP tubing. FEP tubing was also used to connect the outlets of the microdevice with the waste reservoirs. A closed system was achieved by connecting the waste reservoirs with the MAESFLO.
4.2.4 Monitoring of Induced Gene Expression and Data Evaluation

Fluorescent signals were monitored with an inverted microscope (IX81, Olympus, Switzerland) equipped with a 20x 0.45NA Ph LUCPLFLN (long-distance) objective using the Olympus CellR 3.0.x software. The filter FITCQ (ex: 485/20; beam splitter: Quadband 410/504/582/669, em: 531/22) was used for ZsGreen1-DR protein expression and TRITCQ (ex: 560/25; beam splitter: Quadband 410/504/582/669, em: 624/40) for dead cell staining. Images were recorded every 10 min with a digital camera (Orca ER, Hamamatsu, Switzerland) at an exposure time of 40 ms. To maintain relative fluorescence intensities the data were processed using ImageJ 1.43g software (NIH, USA). Only single cells that showed a positive response (detectable increase of fluorescence intensity) after 15 h were considered. Circular regions of interest of the size of the particular cell, i.e. diameters of about 15 µm, were manually defined in the bright field images to determine the mean value of the fluorescence intensity of individual cells. Afterwards, these mean values were normalized to the starting value.

4.3 Results and Discussion

4.3.1 Molecular Cloning

For the monitoring of the induced gene expression the ZsGreen1-DR gene had to be inserted into the inducible gene expression vector of the T-REx™ system. First, the pZsGreen1-DR plasmid was transferred into E.coli to be amplified, followed by its isolation and purification. Next, the pZsGreen1-DR plasmid-DNA the ZsGreen1-DR gene was isolated by a digestion with the restrictions enzymes BamHI and NotI. The results of the digestion and the DNA isolation are shown in Figure 4.9. The left panel shows the agarose gel after digestion of the pZsGreen1-DR plasmid. In pocket 1 and 2 the linearized plasmid is shown after cleavage with BamHI and NotI, respectively. In pocket 3 two bands are visible, which refer to the two plasmid-DNA fragments of the double digest with BamHI and NotI. The bands were located approximately at the expected sizes of 839 bp and 3412 bp for the ZsGreen1-DR gene and the backbone of the pZsGreen1-DR plasmid, respectively. Pocket 4 was used for the DNA ladder to give reference for the size of the fragments. The right panel of Figure 4.10 shows the agarose gel after the isolation and purification of ZsGreen1-DR. In pocket 5 another marker, and in pockets 6 and 7 the linearized plasmid for reference purposes were loaded. Pocket 8 shows one band, which referred with a size of approximately 850 bp to the isolated and
purified ZsGreen1-DR. It is obvious that the second band at approximately 4000 bp is missing, i.e. the gene was successfully isolated from the backbone of the plasmid.

**Figure 4.9** Agarose gels of the digestion of the pZsGreen1-DR plasmid (left panel) and after the isolation of the ZsGreen1-DR gene (right panel). In pockets 1, 2, 6 and 7 the linearized plasmid, in pocket 3 the plasmid’s double digest, in pockets 4 and 5 DNA ladders, and in pocket 8 the isolated ZsGreen1-DR gene were loaded. All bands were located at the expected sizes. Note: For a better visualization the original images were recolored, and the contrast and brightness were adjusted.

Before the ZsGreen1-DR could be ligated with the inducible gene expression vector, the vector had to be prepared. Therefore, it was cleaved with the same restriction enzymes as used for ZsGreen1-DR to achieve complementary DNA sequences for ligation. A digestion of the newly constructed inducible ZsGreen1-DR expression vector with the restriction enzyme HindIII was performed to confirm the success of the ligation. The restriction enzyme HindIII had two restriction sites within the plasmid-DNA, one in the backbone of the plasmid and the other one in the ZsGreen1-DR gene. This resulted in two fragments of 5223 bp and 723 bp in size, respectively, but only, if the insertion of the gene into the vector was successful. Figure 4.10 shows the agarose gel of the digestion for three different
plasmid-DNA batches (pockets 2, 3, and 4). In all three cases two bands were visible located at the expected sizes. Thus, the plasmid-DNAs contained the gene-of-interest and could be used to generate a stably transfected HEK293 cell line to monitor the induced gene expression. The difference in the intensities of the bands referred to different concentrations of plasmid-DNA.

![Agarose gel of the digestion of the newly created inducible ZsGreen1-DR expression vector.](image)

**Figure 4.10** Agarose gel of the digestion of the newly created inducible ZsGreen1-DR expression vector. The two bands in the pockets 2, 3, and 4 had the expected sizes, and hence, the successful insertion of ZsGreen1-DR into the inducible gene expression vector was confirmed. In pocket 1 a DNA ladder was loaded as size reference.
4.3.2 Evaluation of Transfection

Evaluation of Transfection Systems

There are many different commercial transfection systems available that differ in their transfection efficiency and cytotoxicity, depending mainly on cell type and DNA to be transfected. Therefore, five transfection systems were tested to obtain the best transfection efficiency at the lowest cytotoxicity in HEK293 cells. HEK293 cells were transient transfected with GFP according to each manufacturer’s protocol. Transfection efficiency and cell morphology were assessed 24 h after transfection (Figure 4.1). A transient transfection efficiency of 40-50% was observed for Lipofectamine™ 2000, TurboFect™, and jetPrime™, whereby the efficiency of the latter seemed to be a bit lower compared to the other two. The transfection systems TransIT®-LT1 and TransIT®-293 resulted in an efficiency of only 20%. Cell viability, i.e. growth and morphology, were observed as well. The most viable and healthy cells were detected for cells treated with the transfection systems TransIT®-LT1, TransIT®-293, and jetPrime™. Many cell clusters were seen for TurboFect™, and the highest cytotoxicity showed cells treated with Lipofectamine™ 2000. To summarize, Lipofectamine™ 2000 was the system with the highest transfection efficiency, but unfortunately also with the highest cytotoxicity. The transfection systems TurboFect™, TransIT®-LT1, and TransIT®-293 resulted in either good transfection efficiency or low cytotoxicity. The only system with good transfection efficiency and a feasible cytotoxicity was seen for jetPrime™. Hence, jetPrime™ was chosen as transfection agent.
Figure 4.11 Comparison of different transfection systems: (A) TransIT®-LT1, (B) TransIT®-293, (C) Lipofectamine™ 2000, (D) TurboFect™, and (E) jetPrime™. Bright cells indicate fluorescence, the brighter the cell the more GFP was expressed. (Scale bars: 60 µm)
Evaluation of Inducible ZsGreen1-DR Expression Vector DNA

For an efficient transfection the purity of the plasmid-DNA to be transfected is important as well. The plasmid-DNA of five positive clones (E.coli that contained the inducible ZsGreen1-DR expression vector) was amplified, isolated, and purified as describe in the molecular cloning section above. HEK293 cells were transient transfected using jetPrime™, and the transfection was assessed after 24 h. Fluorescence microscopy revealed green fluorescence for four out of five plasmid-DNAs (Figure 4.12), though at different amounts and intensities. The transient transfection of HEK293 cells with plasmid-DNA (B) and (D) showed the highest number of transfected cells. Hence, these two plasmid-DNA batches were used for further experiments. The differences in the plasmid-DNA batches were a result of variations in the previous performed molecular cloning. The plasmid-DNA sequence is the same, however, slightly variations in an individual step during the molecular cloning procedure have an effect in the outcome. For example, differences in efficiencies during the digestion of plasmid-DNA with restriction enzymes, the ligation or the transformation of E.coli with the plasmid-DNA can be listed as possible sources. However, slightly alterations in the procedures of the isolation of the amplified plasmid-DNA from E.coli and its subsequent purification might be the main causes for differences in the inducible ZsGreen1-DR expression vector DNA.

![Figure 4.12 Transfection efficiency of four different inducible ZsGreen1-DR expression plasmid-DNA batches. The fifth DNA did not result in any detectable fluorescence. Bright cells indicate fluorescence, the brighter the cell the more GFP was expressed. (Scale bar: 60 µm)](image)
Evaluation of Co-Transfection

HEK293 cells were co-transfected with the two plasmids of the T-REx™ system in a ratio of 6:1 (pcDNA6/TR: inducible ZsGreen1-DR expression vector). 22 h after transfection no green fluorescence was observed, i.e. the expression of ZsGreen1-DR was inhibited. However, the gene expression could be initiated by addition of tetracycline. In Figure 4.13 fluorescent images after 22 h of gene induction are shown for three different tetracycline concentrations (0.1/0.5/1 µg/mL). With increasing tetracycline concentrations the number of fluorescent cells increased as well, suggesting a concentration dependent induction. In general, the number of induced HEK293 cell was increased by the generation of a stable transfected cell line.

**Figure 4.13** Induction of gene expression. 22 h after addition of tetracycline green fluorescence was detectable. The number of fluorescent cells was increasing with increasing tetracycline concentration (A) 0.1 µg/mL, (B) 0.5 µg/mL, and (C) 1 µg/mL. Bright cells indicate fluorescence, the brighter the cell the more GFP was expressed. (Scale bar: 75 µm)

### 4.3.3 Cell Aggregation Study

The influence of different additives on minimizing cell aggregation was compared. For this study the experimental conditions were simulated. This included cell preparation, and cell incubation to mimic the transport to the light microscopy laboratory. Figure 4.14 shows examples of the influence of the different additives on cell aggregation. Table 4.7 summarizes the influence on cell viability and cell aggregation. In the negative control there were many medium-to-large cell aggregates detectable, and this confirmed the need for an additive. Only a slight decrease in cell aggregation size and amount was observed for 5% PEG 6000. A positive influence, i.e. a decrease in the size and amount of cell aggregates, was observed for 0.75 mM EDTA and Accumax™. However, the lowest aggregate amounts and sizes were observed at 5 mM and 10 mM EDTA and 0.1x trypsin.
Unfortunately the toxic influence of trypsin and Accumax™ on cell viability was evident as well. Therefore, EDTA was the additive of choice; its addition resulted in a single cell suspension and the cells remained viable. The influence of 5 mM and 10 mM EDTA were comparable and hence, a concentration of 5 mM EDTA was chosen for further experiments.

**Figure 4.14** Influence of different additives on cell aggregation. (A) In the negative control there were medium and big cell aggregates formed. The addition of 5 mM EDTA (B) or 0.1x trypsin (C) resulted in single cells with only a few small cell aggregates. However, the toxic effect of trypsin could be observed as well. After the addition of 5% PEG 6000 only a slight decrease in cell aggregates was detected. (Scale bar: 60 µm)

**Table 4.7** Influence of different additives on cell viability and cell aggregation.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Cell viability</th>
<th>Cell aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>95%</td>
<td>Medium – high</td>
</tr>
<tr>
<td>EDTA [0.75 mM]</td>
<td>95%</td>
<td>Low – medium</td>
</tr>
<tr>
<td>EDTA [5 mM]</td>
<td>95%</td>
<td>Very low</td>
</tr>
<tr>
<td>EDTA [10 mM]</td>
<td>95%</td>
<td>Very low</td>
</tr>
<tr>
<td>Trypsin [0.1x]</td>
<td>80%</td>
<td>Very low</td>
</tr>
<tr>
<td>PEG 6000 [5%]</td>
<td>95%</td>
<td>Medium – high</td>
</tr>
<tr>
<td>Accumax™</td>
<td>85%</td>
<td>Low</td>
</tr>
</tbody>
</table>
4.3.4 Cell Growth and Viability on Chip

Comparing cell growth on the membrane with cell growth on the cell culture flask assessed the biocompatibility of the PET membrane. No visual differences could be observed by comparing HEK293 cell growth and morphology over several days (Figure 4.15). The possibility of observing cell growth underneath the membrane instead of on the membrane was excluded by adding a washing step of the PET membrane.

Figure 4.15 Biocompatibility of PET membrane. HEK293 cell growth and morphology on the membrane were similar to cell culture plates. (Scale bar: 60 µm)
To further verify the compatibility of the MMD for living cell studies, the cell growth of the modified HEK293 cells was observed over 6 days in the microfluidic device (Figure 4.16). The cells adhered visibly on the membrane after about 1 day and showed morphologies and growth rates similar to cells in a culture plate, proving that the microdevice did not significantly compromise cell viability and could be employed for long-term studies.

**Figure 4.16** Bright field image (7 h) and fluorescence images (23-146 h) showing T-REx™-modified HEK293 cells trapped in the chip and cultivated over a period of 6 days. Cell growth and morphology were similar to culture plates. After 146 h only a few dead cells were detectable (propidium iodide staining). Tetracycline was added continuously to induce production of fluorescent ZsGreen1-DR for better visualization. Note that only weak fluorescence was observed at 7 h. (Scale bar: 100 µm)
In the studies monitoring the induced gene expression the cells were typically measured over 15 h, i.e. the cells were not yet adhered and still exhibited a round-shaped morphology. The cell viability during gene expression studies was always determined before and after finishing the experiments by dead cell staining (propidium iodide). Bright field images as well as fluorescence images were taken directly before and after the experiments to ensure that dead cells were not included in the data analysis (Figure 4.17). During the experiments only few cells died, and the average cell survival rate was high (approximately 96%).

**Figure 4.17** Overlay of bright field images with the fluorescence images showing the green fluorescent from ZsGreen1-DR and the red fluorescence originating from dead cells (propidium iodide) before (left) and 15 h after (right) tetracycline supply. (Scale bar: 30 µm)
4.3.5 Microchip Design

The microfluidic device was designed to position single cells in the top layer and manipulate them via a channel in the bottom layer. During the development of the final design used to monitor induced gene expression, different strategies were used to position cells at the crossing section between both channels. The design #1 did not include any trapping features in the top channel. The cells were positioned on the crossing section just by applying higher flow rates in the bottom channel than in the top channel to create suction towards the membrane. Figure 4.18 shows an actual image of a device with three trapped cells (indicated by arrows) on a 250 x 250 µm area as crossing section. This reveals a very low cell trapping efficiency of only 0.3% in these microdevices.

Figure 4.18 Cell capture design #1. Two empty channels were designed in the device. The suction created by maintaining a higher flow rate in the bottom than that in the top channel did not result in any evident cell capturing. (Scale bar: 50 µm)
In the design #2 hurdle structures were implemented to disturb the flow and mechanically trap the cells in the top channel. The SU-8 master for these microchips had a one-layer design. Thus, the traps were as high as the channel, i.e. 20 µm. This in turn resulted in a flow only around the traps. The lack of a driving force for the cells to enter the cell traps explained the low trapping efficiency of only about 5%. In Figure 4.19 sketches of the side (A) and top view (B) of the trap are shown. The flow profile in (B) indicates the flow around the traps. In (C) the low trapping efficiency is visible, i.e. only one trap is occupied. Additionally, not one single cell but two or more cells were captured in one trap.

**Figure 4.19** Cell capture design #2. Features for mechanical cell trapping were designed in the top channel. The hurdles spanned the whole channel height (A) and hence, the flow failed to force the cells into the traps (B). This resulted in a trapping efficiency of only 5%, as visible in (C). (Scale bar: 50 µm)
To overcome the lack of a driving force for cell capture two possibilities for improvement of the design evolved: i) the use of open trapping structures, facilitating a flow through the trap or ii) the use of a two-layer design, promoting a flow underneath the trap. The latter was tested in the design #3. A two-layer SU-8 master was used to cast the PDMS, i.e. the hurdles did not span the whole channel height (40 µm hurdle vs. 47 µm channel height). The 7 µm space between the trap and the channel bottom allowed a flow underneath the hurdles. This forced the cells to enter the traps where they were captured. The trapping efficiency in this design was at approximately 60-65%. However, the size of the hurdles was too big, so that often more than one cell was trapped, and the channel became clogged. Additionally, cell loss over time was problematic. The cells were not trapped within, but lay on the surface of the trap. Hence, small irritations of the flow or cell movement caused the cell loss during long-term experiments. In Figure 4.20 sketches of the side (A) and top view of the trap with the flow indicated (B) and an actual image showing the trapping efficiency (C) are depicted.

**Figure 4.20** Cell capture design #3. The design of the features for mechanical cell trapping was improved. There was an additional flow underneath the traps (A). This forced the cells to enter them (B). The trapping efficiency, as shown in an actual image (C), was at approximately 60-65%. However, multiple cell trapping per feature, channel clogging, and cell loss during experiments became an issue. (Scale bar: 50 µm)
A channel height of almost 50 µm could be critical concerning multiple cell trapping and channel clogging. The probability that small cell aggregates enter the channel is bigger for higher channels than for lower ones. Once these aggregates entered the channel they would be transported to the cell traps where they got trapped. This would result in multiple cell trapping and eventually in channel clogging. Therefore, the idea of using a two-layer SU-8 master was abandoned, and open traps were designed in design #4. A gap of approximately 5 µm was inserted in the middle of the traps. Hence, the fluid could not only flow around, but also through the empty traps. This flow forced the cells to enter the traps. The trapping efficiency was with 65% promising. The clogging of the channel was minimized due to additional space between individual traps. Cell loss during long-term experiments remained challenging. This and multiple cell capture needed further optimization. In Figure 4.21 sketches of the side (A) and top view of the trap design with the flow indicated (B) and an actual image (C) are shown.

**Figure 4.21** Cell capture design #4. The hurdles had the same height as the whole channel (A). However, the insertion of a gap allowed a flow around and through the traps. A captured cell blocked this gap and was held in place by the flow (B). The trapping efficiency was with about 65% promising (C). (Scale bar: 50 µm)
In the design #5 the traps received their final improvements (Figure 4.22). Three post features formed an open cage. Two smaller posts (10×10 µm in size) were separated by a 6 µm gap from the longer third post (10×20 µm in size) to form the open cage. With a depth of 16 µm of the cell trap it was possible to capture one mammalian cell (approximately 15 µm in diameter). Cell loss was minimized due to the fact that the size of the inner trap matched the cell size; the cell was surrounded directly by three features that decreased the cell’s ability to move. This even allowed the use of relatively high flow rates (up to 7.7 µL/min) to wash out non-trapped cells. Single cell trapping was possible at an efficiency of 70-75%, and long-term experiments could be carried out.

**Figure 4.22** Cell capture design #5. The hurdles spanned the whole channel height (A). The insertion of gaps allowed a flow around and through the traps. A captured cell blocked the gaps and was held in place by the smaller posts (B). Single cell trapping could be performed efficiently (C). (Scale bar: 35 µm)
In the final MMD the cell culture chamber in the top layer contained the traps of design #5. Additionally, the microfluidic chamber was enlarged to a width of almost 5 mm. Here, the traps were not only used to capture cells, but also to increase the stability of the chamber. Otherwise the chamber would have collapsed due to its high aspect ratio and the flexibility of PDMS. The channel in the bottom layer was designed as a straight channel with three inlets and one outlet with a width of 370 µm and a height of 20 µm. Its width was limited to these 370 µm. For wider channels the aspect ratio would have been too high, which would result in the collapse of the channel, unless some supporting structures would be implemented. These supports in turn would influence the flow profile in the bottom channel. Another reason for the limitation in the channel width was the microscopic setup used. It did not allow the documentation of a wider area. Figure 4.23 shows a sketch of the final device and images (A-C) of the final cell trap design.

**Figure 4.23** Final MMD. The sketch shows the final channel networks in the top and bottom layer. Additionally, SEM and actual images of the cell traps are depicted in (A-C). (A) SEM image of the PDMS top layer showing the trap array. (B) SEM image of an individual trap. (C) Actual image of the assembled PDMS device with entrapped cells (the pores of the porous membrane are visible as well).
4.3.6 Chip Operation

Directly after assembly, the chip was quickly prefilled with deionized water to maintain the hydrophilicity of the plasma-activated PDMS surface. Moreover, the filling process by centrifugation prevented the formation of air bubbles, which would be otherwise induced during priming of the chip. The later introduction of air bubbles was avoided by implementing custom-made bubble traps in the tubing that delivered media and reagents, respectively. For filling, the inlet was connected to the pumping system, the outlet was initially blocked, and the air was allowed to leak from the chamber via the opened ventilation access. Afterwards, the ventilation access was closed and the bubble trap was connected to the chip via the outlet. The chips were used directly after preparation, without any further surface treatment. In Figure 4.24 the experimental setup is depicted.

Figure 4.24 Photographs of the experimental setup. (A) The pressure pump system was placed on top of the incubation chamber that surrounded the microscope. The microchip, the bubble traps, the waste reservoirs and the pump formed a closed circuit for stable flow rates. (B) Actual image of the assembled microfluidic device. In
the image the channel in the top layer, containing the cell chamber, run from left to right and the channel in the bottom layer from top to bottom. Only one out of three inlets were used in the bottom channel. (C) Photograph of the motorized x-y-stage of the microscope onto which the microchip was placed. The device and the custom-made bubble traps were integrated in the tubing for media, buffer and tetracycline supply.

After setting up the experiment, the T-REx™-modified HEK293 cells were harvested in their cell culture media containing 25 mM HEPES buffer, 5 mM EDTA and 1 µg/mL propidium iodide at a concentration of 1.5×10^6 cells/mL, and transported to the light microscopy laboratory. Directly before cell injection into the microdevice, the cells were filtered using a 20 µm nylon mesh (Celltrics®, LabForce AG, Switzerland) to minimize cell aggregates in the chip that would clog the channel. Between 10 µL and 20 µL of the filtered cell suspension were injected into the top layer of the device at flow rates between 0.5-1 µL/min. After 2-5 min about 40% of the 450 traps in the crossing section of the top and the bottom channel, i.e. area of measurement, were occupied with one cell, 27% with several cells and 33% remained empty. Remaining cells were washed out using flow rates up to 7.7 µL/min. A longer supply time of the cell suspension resulted in higher trapping numbers, but double or triple occupancy of single traps was increased as well. Furthermore, an increased settling of cells occurred at positions where no cell trap was implemented. During the experiments, low flow rates between 0.2-0.35 µL/min in the top layer and 0.35-0.5 µL/min in the bottom layer were applied. Higher flow rates over long periods of time resulted in loss of the trapped cells.
4.3.7 Monitoring of Induced Gene Expression

The microdevice facilitates systematic studies of the dependence of gene expression on (i) inducer concentration and (ii) exposure time. Furthermore, it has the potential to study concentration gradients of inducer in parallel (iii). By use of this MMD the flow pattern of the inducer can be individually controlled, i.e. completely independently of the supply of media. The different modes of inducer supply are presented in the following sections. They were realized by the supply of buffer and tetracycline solutions through the different input channels in the bottom layer and by changing the flow rates of each channel separately (Figure 4.25).

![Figure 4.25](image)

**Figure 4.25** Different flow profiles in the bottom channel were used to supply the inducer continuously (A), in temporal pulses (B) or spatially resolved by using multi-laminar streams (C) to monitor the dependence of gene expression on (i) inducer concentration, (ii) exposure time, and concentration gradients (iii), respectively.
4.3 Results and Discussion

In all cases, tetracycline was transported to the crossing section between bottom and top layer. It reached the top channel across the permeable membrane and hence, was taken up by the trapped cells. A higher flow rate in the bottom channel compared to the top one caused an active transport of the tetracycline solution from the bottom to the top channel, where the solution was removed to the outlet. Figure 4.26 illustrates the transport and diffusion of molecules from the bottom to the top channel to introduce or remove the inducer.

![Figure 4.26](image)

**Figure 4.26** Transport of molecules from the bottom to the top channel and vice versa, illustrated with food dye solutions. (A-B) Different flow rates (top channel: 0.2 µL/min; bottom channel: 2 µL/min) were used to allow transport and diffusion of solution from the bottom channel (vertical, blue channel) into the chamber of the top channel (yellow dye). (C-D) After 90 s the flow rates were switched (top channel: 2 µL/min; bottom channel: 0.2 µL/min) and hence, the blue dye solution was washed out of the chamber, and the yellow dye was transported to the bottom channel. The images show the transport at different time points: 0 s, 80 s, 111 s and 154 s (left to right). (Scale bar: 1.2 mm)

The use of the ZsGreen1-DR protein allowed an online monitoring by the use of fluorescent microscopy. The increase in fluorescence over time could be conveniently attributed to the level of ZsGreen1-DR expression. The emerging fluorescence was clearly visible after a few hours in about 40% of the cells. The further increase in the fluorescence intensity was monitored for up to 15 h. Only cells that remained in the cell traps over the full period of time and expressed the ZsGreen1-DR protein were analyzed. Cells that moved and escaped from the trap during the experimental time were neglected.
**Gene Expression: (i) Inducer Concentration Dependency**

First, the expression of ZsGreen1-DR was observed over time and for various tetracycline concentrations ranging between 0.05 µg/mL to 20 µg/mL. Cells were introduced into the microdevice and trapped at the designated positions. Afterwards, the inducer tetracycline was supplied (Figure 4.25 A) at a constant flow rate of 0.35 µL/min via the bottom channel and the increase in fluorescence intensity was measured for each cell. Data were obtained from at least two different microdevices. The chip-to-chip variability was low, i.e. the efficiencies of single cell trapping and of gene expression induction did not significantly differ (p>0.1 in one-way ANOVA test).

Figure 4.27 A shows representative curves of the normalized fluorescence intensity of individual cells over time after induction with 1 µg/mL tetracycline. The fluctuations of the curves can be attributed to instabilities in flow, instabilities of the auto-focusing module as well as fluctuations of the excitation lamp. Typically, the fluorescence increase started between 2.5-4 h after the initial supply of the inducer. This reflected the time required for tetracycline uptake until it was revealing its efficacy on the genetic level, and the subsequent protein expression and folding. While the uptake should be very fast\textsuperscript{225}, the expression of fluorescent protein typically occurs over hours\textsuperscript{226}. In general, a large heterogeneity of fluorescence intensity was observed, which further broadened over time, as illustrated in the histograms in Figure 4.27 B-D.
Figure 4.27  Increase of normalized fluorescence intensity over time. Representative data shown for a tetracycline concentration of 1 µg/mL. (A) Selection of raw data traces for individual, positively responding cells. An increase in fluorescence could be observed about 3 h after tetracycline supply. (B) Histograms presenting the distribution of the normalized fluorescence intensity that broadens and shifts over time. Underlying cell heterogeneity was already visible after 5 h, and increased further (10 h (C) and 15 h (D)).

After demonstrating that the device was properly functional, measurements of gene expression were performed in dependence of the tetracycline concentrations and the results are summarized in the box plots in Figure 4.28. About 140 single cells out of at least two different experiments were analyzed. Higher tetracycline concentrations resulted in an earlier and stronger increase in fluorescence with large cell-to-cell differences over time, while lower tetracycline concentrations caused a delayed and weaker expression of the fluorescent protein.
Figure 4.28 Gene expression after continuous induction. Distributions of normalized fluorescence intensities of single cells for several tetracycline concentrations at three different times. (A) 5 h, (B) 10 h and (C) 15 h. Higher inducer concentrations resulted in an earlier and stronger increase of fluorescence with large cell-to-cell differences over time.
Without addition of tetracycline, no increase in fluorescence could be observed. Based on these single cell measurements, the dose-response curve of tetracycline for the induced gene expression in modified HEK293 cells was derived (Figure 4.29). The curves for the time points 5 h and 10 h showed a linear trend on the inducer concentration. In contrast, the curve for 15 h resulted in a saturation of the induced gene expression, i.e. the amount of tetracycline within the cells was sufficiently high to prevent binding of the repressor homodimers to the promoter of the inducible gene expression vector. For this condition, the gene expression of ZsGreen1-DR was independent of the inducer.

Figure 4.29 Dose-response curve of tetracycline for the induced gene expression. Averaged values of fluorescence intensities are shown. For 5 h and 10 h the curves showed a linear trend. In contrast, the curve for 15 h resulted in a saturation of the induced gene expression. Here, the gene expression was independent of the inducer.
Tetracycline concentrations below 0.1 µg/mL did not result in a measurable expression, while for a high concentration of 20 µg/mL, the expression levels were reduced compared to the 10 µg/mL concentration. The reduced expression was most likely a consequence of the toxicity of the tetracycline solution at high concentrations. This assumption was confirmed by growth studies on a culture plate and FACS analysis for high tetracycline concentrations where the cells showed reduced growth rates and abnormal morphologies (Figure 4.30 and 4.31).

Figure 4.30 Toxicity of tetracycline solution. HEK293 cells were cultured in a 12-well plate. About 200,000 cells were seeded per well, and tetracycline was added at different concentrations. After 23 h at 37 °C and 7% CO₂ cell growth and cell morphologies were observed. The harmful effect of tetracycline solution is visible for concentrations of 20 µg/mL and higher. (The images were taken from the middle of each well; scale bars: 50 µm)
4.3 Results and Discussion

Figure 4.3.1 FACS analysis (FACSCanto II, BD Bioscience, Switzerland) of fluorescence intensities for different tetracycline concentrations. Gene expression was induced for 15 h. Only 40% of the cells were fluorescent (left). The toxic effect for higher concentrations of the tetracycline solution could be confirmed (right).

The results described above indicated that dose-response curves of the inducer could be determined reliably and conveniently on the single cell level in the MMD. The general dependencies of inducer concentration on the expression level of the fluorescent proteins were determined. Most importantly, heterogeneous behavior of the investigated cells was revealed. Interestingly, a small number of cells with fast emerging fluorescence and with high final fluorescence intensities, i.e. these cells expressed extremely large amounts of ZsGreen1-DR, could be observed. In contrast, other cells were producing no or low levels of ZsGreen1-DR, although it was confirmed that they were viable. This large heterogeneity of ZsGreen1-DR levels in the same cell culture could have different origins. Two major reasons are presumed: different transfection levels of the two plasmids of the gene expression system in individual cells, and/or the loss of plasmids during cell proliferation. Consequently, the very bright cells could be attributed to cells with high plasmid numbers. Furthermore, differences in the current state of cell cycles could be another reason for the observed heterogeneities. However, it was beyond this study to fully understand the details of the mechanism. Nevertheless, the experiments described here demonstrate the versatility of the device for such studies of single cell response.
**Gene Expression: (ii) Transient Induction**

Short-term exposure of cell-stimulating compounds could help to elucidate kinetic constants of the mechanisms as well as compound thresholds to provoke a cell response. On this microfluidic platform transient induction was easily realized by variation of the flow rates in the bottom input channels that were connected to tetracycline and buffer solution. To only deliver tetracycline solution (Figure 4.25 B), the buffer flow was stopped either manually or triggered by the software of the pressure driven pumps for periods between 1-10 min. Alternating flows of buffer and tetracycline were generated with flow rates of 0.35 µL/min in the bottom channel, while the flow of medium was constant in the top channel.

After switching the flows, it took a time of less than one second until tetracycline reached the cells. Approximately, the same time was required to wash out the tetracycline solution, when only buffer flow was supplied. Figure 4.32 shows the normalized fluorescence intensities over time for pulses of 10 min and 1 min (tetracycline concentration was 10 µg/mL) compared to a continuous induction. The cell response to the transient induction concerning the amount of expressed ZsGreen1-DR protein was clearly lower compared to continuous induction at the same concentration. The reduced expression of ZsGreen1-DR indicated that the repressor homodimers were expressed constantly (as expected). The uptaken tetracycline molecules bound to the repressor homodimers allowing transient ZsGreen1-DR expression. However, the blocking sites were reoccupied by the freshly produced repressor homodimers that prevented further ZsGreen1-DR expression. Additionally, it is assumed that tetracycline was removed out of the cells by multiple drug resistance proteins.227
4.3 Results and Discussion

**Figure 4.32** Fluorescence increase after transient exposure of the cells to tetracycline. Single tetracycline pulses of 1 minute (black open circles) or 10 minutes (red open squares) initiated the production of the fluorescent protein, but at a lower level compared to the continuous (blue open triangles) induction. The constantly expressed repressor protein re-inhibited the ZsGreen1-DR expression due to the lack of freshly supplied tetracycline.

**Gene Expression: (iii) Concentration Gradient of Inducer**

Finally, the application of the microdevice for systematic studies of inducer concentrations in parallel on a single device was shown (Figure 4.25 C). Here, the various solutions were supplied through the different input channels of the bottom channel at the same time. As a proof-of-concept, two tetracycline concentrations (0.1 µg/mL and 10 µg/mL) were simultaneously supplied to the cells, separated by a stream of buffer (all flow rates 0.35 µL/min). In this configuration, 1/3 of the cells were exposed to the low, 1/3 to the high tetracycline concentration and 1/3 to buffer only, respectively. As expected, the final protein levels differed, thereby reflecting the inducer concentration that initiated the fluorescence increase (Figure 4.33).
The concentration gradient of inducer resulted in a spatially resolved induction of gene expression. Here, multi-laminar streams in the bottom channel (1/3 each) delivered 0.1 µg/mL (black) and 10 µg/mL (red) tetracycline, separated by a buffer stream, to cells trapped in the microchamber of the top layer. Expression of the fluorescent protein was initiated at regions, where tetracycline reached the cells. The increase in fluorescence corresponded to the different tetracycline concentrations applied, i.e. cells exposed to 0.1 µg/mL expressed over time less ZsGreen1-DR protein than cells exposed to 10 µg/mL.

By use of concentration gradients for the supply of the inducer the influence of different stress factors, e.g. toxins, on the T-REx™ system could be observed in further studies.
4.4 Conclusions

A multilayer microdevice that enables independent regulation of flow patterns for cell handling and cell stimulation was developed. It allowed monitoring of induced gene expression on the single cell level with transient and spatially resolved induction, which is difficult to achieve on planar platforms. However, this is of particular interest for studies of single cell response, since it allows mechanistic studies, e.g. it facilitates the study of exposure times of compounds. Additionally, this microfluidic platform avoided adverse shear stress effects induced during necessary chip operations such as flow switching in other planar microdevices. The applicability of the multilayer microdevice for living cell studies was demonstrated by monitoring the tetracycline induced gene expression in individual cells. Different aspects of induced gene expression, i.e. i) inducer concentration dependency, ii) transient induction or iii) parallel supply of different inducer concentrations, could be achieved by using different flow profiles for analyte supply. This microfluidic platform will be of general interest for applications in systems biology, pharmacy or biotechnology, in which the effects of perturbations on cells, induced by small molecules, are investigated. In particular, the microdevice is useful for extended-time investigations, where slow processes are observed, as well as time-resolved stimulations, where the cells are exposed to only short pulses of a chemical inducer.
5 Dielectrophoretic Cell Capture on Polyester Membranes
In this chapter another interesting application of the MMD is described. The device is not only useful for single cell studies, but also for studies on cell-cell communication and interaction between different cell types. Therefore, a MMD with microelectrodes on the intermediate membrane was developed to allow the generation of cell co-cultures by positioning cells on both sides of the membrane by means of DEP. The use of DEP allows for a fast and efficient cell positioning. In the device both cell types are spatially separated, but communication is possible via the permeable membrane. Additionally, microenvironments can be created around the two cell types that differ from each other.

Due to the novelty of performing DEP on a permeable membrane the characterization of the fabrication of the microelectrodes on the membrane, their functionality for cell trapping, and the restoration of the membrane’s permeability was carried out first. Afterwards, a cell co-culture with cells on both sides of the membrane was generated in the MMD. Therefore, cells were positioned on the bottom side of the membrane by DEP and on the top side by gravity.

5.1 Introduction

The precise positioning of cells is a key requirement when dealing with microfluidic systems, specifically when cells are needed to be in defined areas for their stimulation and study. A number of approaches have been introduced to manipulate or capture cells within microchannels. They vary from mechanical traps\textsuperscript{96, 97} and flow control\textsuperscript{87, 119}, to optical\textsuperscript{228, 229} and electronic\textsuperscript{82, 230-232} techniques. Among the electrical techniques, DEP has gained a lot of attention in the microfluidics community. Although DEP is known since 1951\textsuperscript{105}, it was not until the last decade that the number of publications increased significantly for applications like biosensors\textsuperscript{233}, medical diagnostics\textsuperscript{234, 235}, particle filtration\textsuperscript{236}, nanoassembly\textsuperscript{237}, and DNA manipulation\textsuperscript{238}. DEP coupled with lab-on-a-chip devices have demonstrated suitability for DEP-based applications such as separation by size\textsuperscript{239, 240}, sorting\textsuperscript{241}, focusing\textsuperscript{242}, filtration\textsuperscript{243}, trapping\textsuperscript{238, 244}, and patterning\textsuperscript{245, 246}. The choice of platform will depend on the experiments to be carried out. The type of bio-particle (e.g., cells and viruses) to be manipulated defines guidelines such as the design of the electrodes or performing experiments with or without flow. For example, hematopoietic tumor cells were analyzed using a DEP system without applied flow. The electrodes generated cell trapping forces and at the same time created electro-thermal vortices that produced efficient drug mixing, allowing for the analysis of cancer drug-induced cytotoxicity.\textsuperscript{247} A similar
experiment was carried out where hepatitis A viruses were trapped in a microsystem using electro-hydrodynamic flow and DEP forces. These kinds of systems use non-adherent bio-particles and therefore provide platforms that can usually be reused several times. However, studies using adherent cells mostly require cell adhesive molecules on top of the electrodes to allow cell behavior and, hence, cell responses that would provide meaningful data. Since cells tend to rearrange the adhesive molecules they attach onto and leave behind residues from their own extracellular matrix after detaching, therefore limiting the number of times the devices can be reused. When cells are used in a microfluidic platform it is beneficial to have some form of trapping mechanism. However, the use of, for example, mechanical traps creates areas with different flow velocities, hence influencing the flow near the cells. This could likely affect the results of experiments in the cases where cells are sensitive to such shear forces. In contrast, DEP systems with planar electrodes render a channel without features that disturb the flow.

In general, DEP electrodes have been patterned on solid substrates such as glass slides and silicon wafers. However, there are a few publications on patterning electrodes onto permeable surfaces as well. For example, Duan and Meyerhoff showed that the metallization of permeable membranes was possible. They used patterned nylon membranes for sandwich enzyme immunoassays. Later, Švorčík et al. characterized the sputtering process to metallize polyethylene terephthalate (PET, also referred to as polyester). Nevertheless, the patterning of gold microelectrodes on a permeable membrane has yet to be demonstrated. Furthermore, no dielectrophoretic manipulation has ever been shown on a material such as a permeable PET membrane.

In conventional cell culture the use of permeable membranes (e.g. transwell/Boyden chamber) as cell attachment substrates has become prevalent for studies such as cell migration, cell-cell communication and drug transport. Cells cultured on permeable membranes can be exposed to different culture environments on their basal and apical sides. This allows for the exchange of molecules from both sides of the cell membrane, which promotes metabolic function similar to cells in vivo. Microfluidic multilayer permeable devices have the combined advantages of Boyden chambers and microfluidic systems. These devices represent a platform where cells could be interrogated independently of each other while maintaining control over their cell microenvironment. While others have demonstrated that cells can be assembled in distinct cell layers with or without a membrane to separate them, none of these approaches have taken advantage of the cell loading capacity of DEP. The combination of DEP with these multilayer systems offer the advantage of decreasing the total time of experiment
due to the increased cell loading capacity added by the DEP trapping forces. Cells can be trapped and concentrated on the membrane decreasing the wait period needed for cells, in some assays, to be confluent. The attachment of cells on opposite sides of a membrane in microfluidic devices using DEP on both sides of the membrane has yet to be demonstrated.

The combination of multilayer microsystems with dielectrophoretic cell capture onto the intermediate permeable membrane will enable in vitro co-culture systems that could shed light into the cell-cell interactions that occur in vivo.

5.2 Experimental Section

5.2.1 Fabrication of Microelectrodes

Membrane Preparation and Photolithography

Fixation of the PET membrane (11 μm thick, 1.2 μm pore size, 1.6 × 10⁶ pores per cm², cell culture treated, it4ip, Belgium) was necessary for photolithographic processing to prevent folding of the membrane, and hence to avoid problems with the gold patterning. Therefore, 495 PMMA A 11 (MicroChem, Newton, MA) was spin coated onto a 4 inch (10.16 cm) glass wafer (Valley Design Corp., Shirley, MA) to a thickness of 2.25 μm. A piece of PET membrane of about 2 × 2 cm was placed in the middle of the wafer, and then the wafer was baked at 110 °C for 5 min. For the bilayer lift-off process two different photoresists were spin coated onto the membrane. First, the membrane was coated with the lift-off resist LOR 3A (MicroChem, Newton, MA) to a thickness of 350 nm and baked at 155 °C for 10 min. Second, the positive photoresist S1813 (Rohm & Haas, Marlborough, MA) was spun to a thickness of 1.2 μm and baked at 110 °C for 5 min. Next, the wafer was placed into a mask aligner (MA/BA6, SUSS MicroTec AG, Garching, Germany) and exposed to UV-light (λ = 405 nm, 150 mJ/cm²) to transfer the pattern of the microelectrodes (1000 μm long and 10 μm wide with gaps between opposite electrodes of 10 μm) onto the photoresist. Finally, the pattern was developed in MF-319 (Rohm & Haas, Marlborough, MA) for 60 s. Afterwards, the wafer was placed overnight under vacuum to allow complete drying of the membrane. The individual steps are indicated in Figure 5.1 steps 1-3.
5.2 Experimental Section

**Metal Deposition and Microelectrode Patterning**

A 50 nm thick layer of gold was deposited onto the photolithographically processed wafer (E-bream evaporator Denton Infinity 22, Denton Vacuum LLC, Moorestown, NJ). Redundant gold was lifted-off in 1165 remover (MicroChem, Newton, MA). To support and accelerate the process, agitation and short pulses of sonication (3-5 s) were applied. The lift-off was completed within 5-10 min. After the lift-off process the sample was blow dried. The dimensions of the microelectrodes after all processing steps varied slightly from the design pattern (electrodes widths of approximately 11 µm and gaps of approximately 9 µm). The individual steps are indicated in Figure 5.1 steps 4-5.

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**Figure 5.1** Individual steps of the fabrication process of gold microelectrodes on a PET membrane.
5.2.2 Characterization of Microelectrodes

SEM Analyses

The distance to which gold was deposited into the pores was assessed by imaging a total of 10 pores, randomly selected, with field-emission SEM (Ultra-60 FESEM, Zeiss, Germany). To obtain the distance to which gold was deposited into the pores, the difference in the working distances of two SEM images in the same pore were measured: the first image was focused on the surface of the membrane (as outer value), and the second one was focused on the deepest point inside the pore where gold was still seen (as inner value). The difference between the inner and outer working distance corresponded to the distance to which the gold was deposited inside the pore.

AFM Analyses

AFM images (Dimension 5000, Digital Instruments, Santa Barbara, CA) were acquired in tapping mode. Images were acquired at ambient conditions on dry samples. To obtain the possible differences in root-mean-square (RMS) roughness of the surfaces within the samples, seven independent areas of 10 x 10 µm were imaged and then analyzed using the Nanoscope 7.3 software. The analyzed surfaces were: (i) membrane before processing, (ii) membrane after processing, and (iii) the gold patterned.

Hydrophilicity Test

Contact angles were measured to characterize the hydrophilicity of the PET membrane during the processing. A drop of water was placed onto the sample, and a side-view picture was taken. The droplet curvature was fitted using the software FTA32 (First Ten Angstroms, Inc., Portsmouth, VA) to obtain the contact angle value. A contact angle between 0° and 90° is defined as a hydrophilic surface and a value between 90° and 180° as a hydrophobic surface. For each step during the electrode microfabrication the contact angle was averaged from four independent measurements.
Permeability Test

MMDs (Figure 5.2) were assembled to confirm that the permeability of the PET membrane was not affected during processing. The membrane was transferred onto a PDMS layer containing the top channel. Next, the PMMA was dissolved in acetone. The PDMS layer containing the membrane was plasma activated along with the PDMS layer containing the bottom channel. The membrane was sandwiched between both PDMS layers to complete the assembly of the device. Both layers contained a microfluidic channel of 30 µm in height and 1000 µm in width. These channels were perpendicularly aligned to each other, whereby the intermediate PET membrane allowed the exchange of reagents. Tubing was connected between the assembled microchip and syringe pumps (PHD 2000, Harvard Apparatus, PA). For the permeability test the flow rates varied between 0 µL/min, 0.5 µL/min and 10 µL/min.

Figure 5.2 Sketch of an assembled MMD. The processed PET membrane is aligned between the channels of the top and bottom PDMS layers. The top view sketch indicates the flow directions in both channels. The assembled device is comprised of a glass substrate, the bottom PDMS layer, the membrane, and the top PDMS layer. (For clarity purpose we are not showing the top PDMS part, but only its channel.) The dashed line in the top view denotes the position looked at in the cross section sketch. The cross section indicates the area where the exchange of reagents between the channels was possible, only through the pores of the PET membrane (see arrows).
5.2.3 Cell Culture

NIH-3T3 mouse embryonic fibroblast cells were cultured in DMEM modified with 10% bovine calf serum. For DEP cell capture experiments the cells were harvested in 0.147 M sucrose (Sigma-Aldrich Corp., St. Louis, MO). Sucrose, a non-electrolyte, was used as low-conductive media to perform positive DEP, i.e. the DEP forces attracted the cells towards the high field gradients (located on the membrane’s surface). HepG2 human hepatocellular carcinoma cells were cultured in EMEM modified with 10% bovine calf serum. For DEP trapping experiments the cells were harvested in 0.149 M sucrose to perform positive DEP.

5.2.4 Microfluidic Device: Fabrication and Operation

Fabrication of SU-8 Master

The SU-8 master for proof-of-concept experiments, i.e. testing the microelectrodes for DEP cell capture, was fabricated by photolithographic processing of a glass slide. Therefore, the glass slide was coated with the negative photoresist SU-8 2025 (MicroChem, Newton, MA) to a height of 36 µm. After a soft bake (150 s at 65 °C and 360 s at 95 °C) the coated glass was exposed to the mercury i-line (365 nm) until the transfer energy of 237 mJ/cm² was reached. The used photomask contained features for a microchannel with a width of 1000 µm. The glass slide was placed on a hotplate for the post exposure bake (60 s at 65 °C and 360 s at 95 °C). Next, the features were developed in SU-8 developer (MicroChem, Newton, MA) for 270 s, and the master was rinsed with isopropanol. For the final hard bake the master was placed over night at 160 °C. The whole process resulted in an SU-8 master with channel structures of 1000 µm width and 30 µm height. Finally, the master was silanized with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc., Bristol, PA) to ease the detachment of cured PDMS.

The SU-8 master for the MMD to generate cell co-cultures was fabricated by photolithographic processing of a silicon wafer. Therefore, the silicon wafer was dehydrated for 1 h at 200 °C before it was coated with SU-8 2025 to a height of 80 µm. After a soft bake (300 s at 65 °C and 960 s at 95 °C) the coated wafer was exposed to the mercury i-line (365 nm) until the transfer energy of 225 mJ/cm² was reached. The used photomask contained features for a microchannel with a width of 1000 µm. The wafer was placed on a hotplate for the post exposure bake (180 s at 65 °C and 540 s at 95 °C). Next, the features were developed in SU-8 developer for 510 s, and the master was rinsed with isopropanol. For the final hard
bake the master was placed over night at 160 °C. The whole process resulted in an SU-8 master with channel structures of 1000 µm width and 72 µm height. Finally, the master was silanized with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc., Bristol, PA) to ease the detachment of cured PDMS.

**PDMS Microfluidic Channel**

The SU-8 masters for PDMS layers with a thickness of a few millimeters were placed in a frame to stop PDMS (Sylgard 184, Dow Corning, Midland, MI) from spreading. PDMS was cured on the SU-8 master after mixing the PDMS elastomer and curing agent at a mass ratio of 10:1, respectively. Once thoroughly mixed and degassed, the mixture was poured onto the SU-8 master and cured for 4 h at 65 °C. Excessive PDMS was cut, and access holes of approximately 5 mm were punched. To reach the contact pad on the PET membrane in the multilayer device the covering PDMS was removed.

Thin PDMS layers with a thickness of approximately 100 µm were prepared directly on indium-tin-oxide (ITO) coated glass slides (Delta Technologies Ltd, Loveland, CO). The ITO-glass slides were rinsed with isopropanol before use. For molding the PDMS pre-polymer was poured onto the master, fixed with the ITO-glass slide, and cured for 3 min at 150 °C. The additional weight of approximately 1 kg on top of the ITO-glass removed the PDMS from the SU-8 structures and hence, open channels were achieved. There was no PDMS between the ITO and the microchannel, but only at its sides.

**Membrane Coating with PEMs or hCAM**

To enable cell anchorage and cell culture on chip after dielectrophoretic cell capture in proof-of-concept experiments, the area of the membrane containing the microelectrodes was coated with PEMs as described in Reyes et al.173 Briefly, 5 µL of a 1 mg/mL poly(ethyleneimine) solution (PEI, polycation, Molecular Weight (MW) = 70000, Polysciences, Inc., Warrington, PA) were placed on the microelectrodes. This first layer was incubated for 30 min, rinsed with water, and blow dried. Next, two bilayers of polyanion/polycation were deposited (polyanion = sodium poly(styrene sulfonate), PSS, MW = 70000, Polysciences, Inc., Warrington, PA; and polycation = poly(allylamine hydrochloride), PAH, MW = 70000, Sigma-Aldrich Corp., St. Louis, MO; 1 mg/mL each). Each layer was incubated for 10 min, rinsed with water, and blow dried. These procedures resulted in the deposition of
a total of five layers of polyelectrolytes on the microelectrodes. The chemical structure of the used cationic (PEI and PAH) and anionic (PSS) polyelectrolytes is shown in Figure 5.3.

![Figure 5.3 Chemical structure of used polyelectrolytes. Cationic polyelectrolyte: PEI (A) and PAH (B). Anionic polyelectrolyte: PSS (C).](image)

To anchor cells on the membrane in MMDs a hybrid cell adhesion multilayer (hCAM), a slightly adopted form of PEMs, was used. The hCAM improved cell viability by introducing a layer of fibronectin. The hCAM was prepared directly in the microchip after its assembly. The hCAM consisted of the PEMs as described above, but had an additional fibronectin layer embedded between the top PSS and PAH layers. For its preparation the channel in the top and the one in the bottom layer were filled with PEI solution and incubated for 40 min. Afterwards the channels were washed with water. Next, PSS, PAH, and PSS layers were deposited. Each layer was incubated for 10 min, and afterwards the channels were washed with water. For the subsequent layer the channels were filled with a fibronectin solution (50 µg/mL in PBS, Sigma-Aldrich Corp., St. Louis, MO), placed at 4 °C, and incubated for 90 min. For the washing step PBS was used. The final PAH layer was deposited during an incubation for 10 min at 4 °C. It was crucial to use water for the following washing step to avoid the precipitation of the PAH. Afterwards, the microchip was filled with PBS and stored at 4 °C.
Chip Assembly and Operation

For proof-of-concept experiments the membrane remained fixed on the glass wafer. Wires were connected to the contact pads using an electrically conductive adhesive (Epoxy Technology Inc., Billerica, MA) and cured for 1 h at 150 °C. Then, the area containing the microelectrodes on the PET membrane was coated with PEMs (see section above). Finally, the device was assembled by placing the cleaned PDMS microchannel on top of the gold microelectrodes, so that the channel was perpendicular to the microelectrodes. The assembled device was connected to a waveform generator (Agilent Technologies, Santa Clara, CA), and the channel was filled with 0.147 M sucrose using capillary forces. 150 µL of the NIH-3T3 cell suspension in sucrose were placed into the inlet of the microchannel. Suction was applied from the outlet to start the cell flow. NIH-3T3 cells were captured and anchored by applying a sine wave from 2-5 V p-p at a frequency of 10 MHz for less than 5 min. Subsequently, the cell/sucrose solution in the inlet was carefully exchanged with cell culture media, and the device was placed in the incubator at 37 °C and 5% CO₂ atmosphere. After 24 h a live/dead assay (Live/Dead® viability/cytotoxicity kit, Invitrogen, Eugene, OR) was performed according to the manufacturer's recommendation. Briefly, the cells were incubated in media containing 2 µM Calcein AM and 4 µM Ethidium homodimer-1 for 20 min before imaging. Green fluorescence indicated living cells and red fluorescence dead ones. For comparison, cell adhesion and viability of NIH-3T3 cells was tested directly on glass, on PEMs on glass and in a cell culture flask. Therefore, the cells were seeded onto these surfaces and incubated for 24 h at 37 °C and 5% CO₂. Afterwards a live/dead assay was performed as described above.

For the MMD the processed membrane had to be detached from the wafer. Therefore the membrane was cut to size (the smaller the lower the probability of leakage), and placed in acetone for 40 min to dissolve the PMMA. The acetone was rinsed out with water and the membrane was blow dried. Next, the channel of the cleaned bottom layer of the chip, i.e. the ITO-glass slide with the prepared PDMS layer, was aligned to the electrode array. By bringing the PDMS in contact with the PET membrane the piece of membrane containing the gold microelectrodes was transferred to the PDMS. The surfaces of both PDMS layers were plasma-activated and tiny PDMS pre-polymer drops were placed onto the edges of the membrane. The alignment of the top PDMS microchannel layer perpendicular to the microelectrode array, and bringing both PDMS layers together completed the device assembly. Wires were connected to the contact pad and the ITO-glass using an electrically conductive adhesive and cured for 1 h at 150 °C. Then, the channels in both layers were coated with hCAM (see section above). The microfluidic device was connected to a waveform generator, and the channels were filled with 0.149 M
sucrose using capillary forces. To determine between different cell types HepG2 cells were stained with calcein and NIH-3T3 with CellMask™ Plasma Membrane Stain (Invitrogen, Eugene, OR). Thus, HepG2 cells showed green fluorescence and NIH-3T3 cells red fluorescence. For the staining HepG2 cell culture media was replaced with PBS containing 2 µM Calcein AM and incubated for 20 min at 37 °C and 5% CO₂ atmosphere. NIH-3T3 cells were stained by adding 5 mg/mL of the dye to the cell culture and incubated for 5 min at 37 °C and 5% CO₂ atmosphere. HepG2 cells were harvested in 0.149 M sucrose. 150 µL of the HepG2 cell suspension in sucrose were placed into the inlet of the microchannel in the bottom layer. Suction was applied from the outlet to start the cell flow. HepG2 cells were captured and anchored at the gold microelectrodes on the PET membrane by applying a sine wave from 2-6 Vp-p at a frequency of 13 MHz between the electrode array on the membrane and the ITO at the bottom of the channel. Subsequently, the cell/sucrose solution in the inlet was carefully exchanged with cell culture media. Next, NIH-3T3 cells were harvested in cell culture media and 150 µL were placed into the inlet of the microchannel in the top layer. Suction was applied from the outlet to start the cell flow. To capture cells by electrostatic forces the cell flow was stopped and the cells were allowed to settle down and being anchored by the hCAM. This procedure was repeated several times to accumulate captured cells on the top side of the membrane.
5.3 Results and Discussion

5.3.1 Characterization of Microelectrodes

For the first time the fabrication of gold microelectrodes on a permeable PET membrane was described. The resulting DEP microelectrodes were characterized by several techniques.

SEM Analyses

In Figure 5.4 A a scheme of the gold pattern is shown. The interdigitated microelectrodes shown in the center are linked to contact pads. The micrograph in Figure 5.4 B shows an actual gold/PET surface; the continuous connection of the patterned gold is visible. The pores of the PET membrane appear as black spots in the coated as well as uncoated areas of the surface. The SEM images show the surface (Figure 5.4 C), and the insert (Figure 5.4 D) one single pore, used to characterize the gold deposition with respect to coverage or blockage of the micropores of the PET membrane. SEM imaging showed dark grey spots inside the pores, i.e. the gold did not completely block them. The 2-point z-stack scan was performed to obtain the distance to which gold was deposited inside the pores, and hence to characterize their partial blockage. The average distance to which the gold was deposited inside the pores was $2 \pm 1 \mu m \ (n = 10)$. These observations strongly suggested that the pores remained open and therefore permeable. However, even if the partial blockage by gold slightly affected the function of the membrane, half of the surface area, where cells would adhere, was not covered by it. Therefore, the permeability of the membrane remained effectively unaffected.
Figure 5.4 Scheme of the gold pattern and images of the gold patterned membrane. (A) The scheme shows the gold microelectrodes in the center, which are connected to contact pads. (B) Micrograph of the processed microelectrodes. (C) SEM image of the area used to measure the distance to which gold was deposited into the pores. The pores could be observed throughout the entire membrane. (D) Close up of one pore showing its partial blockage by the deposited gold. (Scale bars: B: 100 µm, C: 3 µm, D: 300 nm)
AFM Analyses

In Figure 5.5 AFM micrographs of the membrane before (A) and after processing (B) are shown. The pores could be observed throughout the membrane regardless of the patterned gold. The coated areas within the pattern were continuously connected to result in interdigitated microelectrodes. In addition, the surface roughness of the (i) unprocessed PET membrane, (ii) processed PET membrane, and (iii) gold pattern was measured to assess any possible changes during processing (n = 7, each). An RMS roughness of 28.7 ± 4.8 nm was observed for the membrane before processing, and an RMS roughness of 24.3 ± 10.6 nm was observed after treatment. When these results were analyzed they showed no statistical difference (ANOVA, analysis of variance, single factor, p = 0.33). Even the RMS roughness on the gold pattern (27.1 ± 9.6 nm) was not statistically different from the before and after processing values mentioned above (p = 0.70 and p = 0.61, respectively). The mechanical stability of the membrane was visually evaluated after processing, whereby no changes were detected.

Figure 5.5 AFM images of PET membranes before and after processing. Panel A shows the PET before processing, whereas panel B shows it with gold electrodes patterned. Both figures are 75 µm x 75 µm. The pores are randomly distributed and have an average diameter of 1.2 µm. The pores can be easily observed throughout the PET membrane including where the continuous layer of gold had been deposited.
Hydrophilicity Test

Contact angle measurements were used to monitor the hydrophilicity of the membrane during the processing steps. Water contact angles were measured at different points during the fabrication process to monitor changes in the membrane’s hydrophilicity (Table 5.1). With a water contact angle of 86° the membrane was slightly hydrophilic before any treatment. The sequential microfabrication steps decreased the contact angle to 69°, whereby the biggest change occurred after fixing the PET membrane onto the glass wafer via PMMA. Thus, the processing of the membrane changed its hydrophilicity. The decrease in contact angle means an increase in hydrophilicity of the membrane. This result should ultimately have a positive effect on the aimed use of the membrane as cell culture surface due to the fact that most cell types need a hydrophilic surface to grow and to proliferate.\textsuperscript{252, 253}

\begin{table}[h]
\centering
\caption{Water contact angles of the PET membrane (n = 4).}
\begin{tabular}{|l|c|}
\hline
Surface & Contact Angle [$^\circ$] \\
\hline
PET & 86 ± 1 \\
PET (on PMMA) & 74 ± 1 \\
PET (after development) & 73 ± 1 \\
PET (after lift-off) & 69 ± 2 \\
S1813 Photoresist & 83 ± 2 \\
PET (after TMAH) & 40 ± 3 \\
Gold (before lift-off) & 53 ± 4 \\
Gold (after lift-off) & 73 ± 2 \\
\hline
\end{tabular}
\end{table}
5.3.2 Permeability Test

A MMD was assembled to test the permeability of the PET membrane after processing. Two different colored food dyes were exchanged between the two layers by transporting them through the pores of the membrane (Figure 5.6). At equal flow rates the crossing revealed a green color as a result of a mixture of blue and yellow food dye. A higher flow rate of one dye resulted in its transport to the other channel via the membrane, and the crossing revealed the color of the transported dye. By varying the flow rates of either the one or the other dye its reversible transport to the channel on the other side of the membrane was possible. This ultimately confirmed that the permeability of the PET membrane was restored.

Figure 5.6 Permeability testing of the PET membrane after processing. Yellow and blue food dyes were exchanged between the channels in a MMD by a transport through the pores of the PET membrane. The white dashed lines indicate the position of the two channels; the yellow dye flowed from left to right and the blue dye from top to bottom. (A-D) show actual images, and in (E-H) the corresponding sketches are shown for better clarity. (A, E) At t = 0 both flow rates were 0.5 µL/min, resulting in a green color at the crossing. (B, F) After changing the flow rates (yellow: 10 µL/min, blue: 0 µL/min) the yellow dye was transported to the blue channel through the membrane and filled it (approximately t = 3 min). (C, G) After inverting the flow rates the blue dye was transported to the yellow channel and filled it (approximately t = 7 min). (D, H) The green color returned after setting both flow rates back to 0.5 µL/min (approximately t = 11 min). (Scale bars: 100 µm)
5.3.3 Dielectrophoretic Cell Capture: Proof-of-Concept

The microelectrodes were tested for proof-of-concept dielectrophoretic cell capture. A microfluidic device was assembled by placing a PDMS microfluidic channel perpendicular onto the microelectrodes and connected to a waveform generator (Figure 5.7).

Figure 5.7 Sketch of the assembled microfluidic device. A piece of PET membrane with deposited gold electrodes was fixed onto a glass wafer. The precise location of the microelectrodes is indicated by the red square. The zoom in shows an actual image of the gold microelectrodes. The PDMS microfluidic channel was assembled on top, perpendicular to the microelectrodes. Wires were glued to the contact pads and connected to a waveform generator.

Cell positioning by DEP is straightforward after the right parameters were determined. For example, for every cell type the frequency at which DEP can be performed has to be defined. Therefore, a capturing experiment has to be carried out; cells pass DEP electrodes at maximum applied voltage but varying frequencies until cell capture on the DEP electrodes is visible. Variations in the applied voltages direct cells either to electrodes that come first or to electrodes that are further down the array. A correct design of the electrode pattern is crucial to cell viability; the generated field should be large enough to significantly polarize the cell, but not too large to cause electrical or thermal damage.254, 255 Any potentially negative influence of the electrode material was minimized was well due to the presence of the different polyelectrolyte layers. Therefore, there was no direct contact between gold microelectrodes and cells. To avoid any possibly cell damage due to the
applied forces the dielectrophoretic cell capture was carried out within 5-10 min after harvesting the cells. This resulted in trapped cells in about half of the microelectrode surfaces by varying the applied voltage between 2 and 5 V_{p-p} at a frequency of 10 MHz. Variations in the applied voltage allowed for cell capture across the length of the microelectrode array. When cells experienced higher electric fields they were trapped on the first few electrodes of the array. On the other hand, when lower electric fields were applied cells tended to be trapped further down on the microelectrode array. The trapping efficiency could be increased further by either using a highly concentrated cell suspension or longer periods of DEP trapping. Additionally, the cell trapping efficiency can be influenced by the design of the microelectrodes as well.\textsuperscript{256} By modifying the configuration of the electrodes this could be further improved. Most of the trapped cells (approximately 90\%) still remained on the PEMs after switching off the DEP forces and exchanging the low-conductive media with cell culture media (Figure 5.8 A). A live/dead assay was carried out 24 h after cell attachment. The assay showed that about 99\% of the cells revealed green fluorescence, i.e. these cells were alive (Figure 5.8 B). The high number of living cells also confirms that the applied DEP forces and electrode material do not have a negative effect on cell viability.

**Figure 5.8** Efficient cell capture using DEP. (A) Micrograph after switching off DEP forces and exchanging low-conductive media with cell culture media (0 h). NIH-3T3 cell capture was evident, as soon as 5 min from the time the microelectrodes were energized. Cells flowed from bottom to top of the figure during DEP trapping. Red arrowheads point at some of the trapped cells. (B) Live/dead staining 24 h after
cell capture. The cells spread onto the membrane and green fluorescence could be observed in approximately 99% of the cells, demonstrating that cells were viable. White arrowheads point to some of the viable cells. Inserts show some of the trapped cells in more detail. The black lines in the images correspond to the microelectrodes. (Scale bars: 100 µm)

The images in Figure 5.9 compare the cell capture and cell viability on the membrane coated with and without PEMs. PEMs were deposited solely on the microelectrodes therefore the cell capture was expected to be low within the remaining channel. The left row in Figure 5.9 shows from top to bottom the inlet, the channel directly in front of the microelectrodes, the array itself, the channel directly after the array, and the outlet. The images were taken directly after cell trapping and exchanging the trapping media with cell culture media. As proposed, there was only sporadic cell adhesion detectable except on the surface containing the microelectrodes. Even the cell numbers on the membrane in the reservoirs of the inlet and the outlet were low. Thus, the dwell time of the cells was short enough to prevent high cell numbers that remained after media exchange. Cell viability assessed 24 h after cell capture revealed that the trapped cells spread over the surface and stayed viable independently of their location on the membrane (Figure 5.9 right row). The high cell numbers solely present on the microelectrodes confirmed the usability of the device for precise and fast cell positioning by DEP forces and the anchoring effect of the PEMs, which were only deposited there. Additionally, no difference regarding cell spreading and cell viability on the PEMs/PET surface compared to the PET surface itself was observed.

An influence of the material on cell adhesion and cell viability could be excluded due to similar cell behavior on various surfaces (Figure 5.10). To compare the NIH-3T3 cell behavior on the PEMs/PET membrane with standard cell culture, cell adhesion and cell viability was assessed on other surfaces, i.e. directly on glass, on PEMs on glass and on cell culture flask polystyrene. A live/dead assay revealed about 99% of living cells after 24 h. The cells showed similar behavior when seeded on the other surfaces to cells on the PET membrane, i.e. the different surfaces have no influence on cell adhesion and viability.
Figure 5.9  Cell capture using DEP. A sketch of the device is shown in the middle to visualize the locations of the images; the black arrows indicate the direction of the flow. Left row: Micrographs at various positions within the device after switching off DEP forces and exchanging low-conductive media with cell culture media (0 h). NIH-3T3 cell capture was evident within 5-10 min. Cell adhesion was significantly higher on the PEMs (only present on the microelectrodes). Right row: Live/dead staining 24 h after cell capture. The cells spread onto the membrane and green fluorescence could be observed in 99% of the cells, demonstrating that cells were viable. (Scale bars: 50 µm)
Figure 5.10 Cell adhesion and cell viability on standard cell culture surfaces. The behavior of NIH-3T3 cells did not significantly differ on the various surfaces. After 24 h approximately 99% of the cells were alive (green fluorescent cells). Note: the low number of cells in the images on a cell culture surface was not due to different cell behavior but to a lower number of seeded cells. (Scale bars: 50 μm)
5.3.4 Generation of a Cell Co-Culture in a Multilayer Microfluidic Device

In the second part of dielectrophoretic cell capture the first attempts for a co-culture MMD with cells on both sides of the intermediate membrane were carried out. Figure 5.11 illustrates the individual steps performed to generate a co-culture in a MMD. Gold microelectrodes were fabricated on a permeable PET membrane, and this membrane was assembled into the microdevice facing the ITO on the glass slide that closed the device. An interaction between the two channel systems was only possible through the permeable membrane. For the co-culture hepatocytes (HepG2 cells) were captured first in the bottom channel and then the positioning of fibroblasts (NIH-3T3 cells) in the top channel of the device followed. In the top channel gravity and in the bottom channel DEP forces were used for cell positioning. The electrostatic interactions between cell and hCAM deposited were used to anchor the cells.

Figure 5.11 Sketch of a cross-section of a MMD for the generation of cell co-cultures. (A) The device is comprised of two PDMS channels assembled perpendicular to each other. A PET membrane with patterned microelectrodes is sandwiched between the PDMS channels. (B) Cells are introduced into the channels in sequential fashion. The arrows indicate the direction of flow. (C) Cells are trapped and anchored with DEP and hCAM at the bottom and with hCAM at the top.

The attractive DEP forces pulled the HepG2 cells towards the PET membrane in the bottom channel and the hCAM held the cells in place even after the DEP forces were switched off and fluid flow continued. After exchanging the trapping media with HepG2 cell culture media in the bottom channel, the NIH-3T3 cells were positioned. Therefore, the cells were harvested in their cell culture media and 150 µL were injected into the top channel. Cell flow was started and after observing cells reached the crossing between the top and the bottom channel the flow was stopped to allow cell settlement. Settled NIH-3T3 cells were anchored on
the permeable membrane by the hCAM. The number of cells on the membrane was increased by repeating the cell flow, stopped flow, and cell settling steps several times. Cell trapping by means of gravity is not suitable for fast and efficient cell positioning. Here, however, it was acceptable due to the fact that the main focus was to show that HepG2 cells could be captured by DEP in the bottom channel and stayed hanging at the bottom side of the membrane. Figure 5.12 shows HepG2 (green fluorescence) and NIH-3T3 cells (red fluorescence) trapped on opposite sides of the membrane in the bottom and top channel, respectively. Both cell types spread throughout the membrane and were held in place by the hCAM.

![Micrograph of HepG2 (green) and NIH-3T3 (red) cells while being trapped in a multilayer device. (Scale bar: 100 µm)](image)

**Figure 5.12** Micrograph of HepG2 (green) and NIH-3T3 (red) cells while being trapped in a multilayer device. (Scale bar: 100 µm)

Figure 5.13 shows a higher magnification of the captured cells. To proof cell positioning on both sides of the intermediate membrane stack images were taken and with this information a 3D image was created. The insert in Figure 5.13 shows a 2D image in x-z direction taken from that 3D image. It is evident that one HepG2 cell (green) is spatial separated of one NIH-3T3 cell (red) by the PET membrane (black space between the two cells). This new approach demonstrated an easy and rapid way of cell enrichment and attachment by DEP and hCAM on the bottom side of the membrane and cell attachment via hCAM on the top side of the membrane in a multilayer microfluidic system.
5.3 Results and Discussion

Figure 5.13 Micrograph of NIH-3T3 (red) and HepG2 (green) cells after seeded. The cells were attached on both sides of the PET membrane. The black horizontal lines in the micrograph are the gold microelectrodes deposited on the PET membrane. The insert shows a 2D image from 3D image stacks (after deconvolution processing) of the spatial arrangement of one HepG2 and one NIH-3T3 cell. The black space between the cells indicates the position of the PET membrane. (Scale bar: 30 µm)

These results demonstrate the functionality of the MMD for the generation of cell co-cultures. However, the ultimate goal to present a MMD for cell co-cultures, where cells will be positioned on both sides of the intermediate membrane by use of DEP has yet to be shown. Therefore, the fabrication process of the microelectrodes has to be optimized. At the moment, the steps of fabricating electrodes on one side, turning over the membrane, and processing the PET membrane a second time result in functional microelectrodes, but only on one side. By overcoming these technical issues the MMD can facilitate a fast and reliable generation of cell co-cultures and specialized applications such as studies of drug transport, cell monolayer permeability and cell co-cultures can be carried out.
5.4 Conclusions

For the first time the use of gold microelectrodes on PET membranes as substrates to perform DEP cell entrapment in a microfluidic device is presented. The microelectrodes for DEP were fabricated using conventional photolithographic and metallization processes. The membrane was characterized with different techniques, and results showed that there was no difference in terms of hydrophilicity, roughness, and permeability of the membrane when comparing the before and after processing surfaces. The use of the patterned electrodes for DEP cell trapping experiments in a microfluidic channel was proven. The cell viability assessment showed that cells were viable 24 h after DEP trapping, demonstrating that long-term cell experiments can be carried out. Finally, the patterning of gold microelectrodes for DEP trapping on permeable PET membranes and the assembly of the membrane in a multilayer microfluidic system was accomplished. The trapping capabilities of DEP forces and the hCAM layer were demonstrated by the anchorage of HepG2 cells to the bottom of the membrane, despite gravity and fluid flow field. NIH-3T3 cells were anchored onto the top side of the membrane via the hCAM to generate a co-culture separated by the PET membrane. Cells were trapped and spread across the membrane on their respective sides.

The results demonstrate the functionality of the patterned microelectrodes on the permeable PET membrane for dielectrophoretic cell capture. This membrane along with DEP is suited for specialized applications such as studies of drug transport, cell monolayer permeability and cell co-cultures, among others. These applications gain the most when combined with MMDs. Here, the first attempts for multilayer co-culture devices were carried out. The added levels of control and the benefit of the localized cell enrichment by DEP trapping are at the heart of such devices. In addition, the combination of DEP and polyelectrolytes on a permeable membrane allows for a fast and reliable cell anchorage at a high efficiency, and hence subsequent long-term cell culture is achievable. The main advantages of using DEP to capture cells are the reliability of trapping and that it is a fast technique. Other co-culture studies in multilayer devices use only gravity for cell positioning. This results in a low efficiency of positioned cells compared to DEP. Additionally, the device cannot be moved to avoid a distortion because the cells need time to adhere and spread. An anchoring surface coating overcomes this drawback. The cells still need their time to adhere and spread properly, but the electrostatic interactions between the cells and the outermost layer of polyelectrolyte of both, PEMs and hCAM, hold them in place.
6 Summary and Outlook
In this thesis two different kinds of cell studies, single cell and cell co-culture studies, are addressed by means of microfluidics. Therefore, two different MMDs have been developed. A permeable membrane within these devices allowed an interaction between the two fluid channel networks and independent control over the operation of each channel network. This combination of physical separation but possible interaction at the same time enabled new techniques for studying cells.

In the first device studies on the single cell level were addressed to overcome the deficit of information due to averaged results of heterogeneous cell populations in bulk studies. Therefore, a MMD was developed, where single cells were positioned in the top channel network and their manipulation was achieved using the bottom channel. In the top layer cell traps were implemented into the cell culture chamber for hydrodynamic cell capture. The hydrodynamic trapping technique was chosen due to its ease of implementation and adaptation. Several steps of designing, re-designing, and optimization were performed during the development of the cell traps. The adopted trap design consisting of three mechanical posts that formed a U-shaped hurdle structure with an interior space of the size of one cell was found to be optimal. This enabled efficient single cell capture, which was evident within a few minutes. In the experiments monitoring induced gene expression 40% of the traps were occupied with one cell, 27% with several cells, and 33% remained empty. Additionally, by using the actual cell trap design cell loss during long-term experiments was minimized, resulting in sufficient data. A three-inlet design merging into one straight channel in the bottom channel enabled the performance of different cell manipulation strategies within this layer. Thus, it was not only possible to apply single- but also multi-laminar streams. This system facilitated measurements of the tetracycline-induced gene expression to terminate its dependence on inducer concentration, exposure time, and concentration gradients. In addition to the chip design the choice of the right pumping system was crucial as well. The requirements did not only involve a precise and stable performance of the pump, but also the supply of enough volume. Hence, it was necessary to test several pumping systems to be able to choose the one system that could generate precise and stable flow rates for relative large fluid flow volumes over long time periods. The evaluation of four syringe pumps, i.e. mechanically-driven systems, and two pressure-driven systems revealed significant differences in their performances. Generally, the performance was better for all systems operating at higher flow rates. However, for the monitoring of induced gene expression experiments pump performance at low flow rates was crucial. For the required conditions, the best performance was identified using the commercial pressure-driven pumping system MAESFLO, although the control of
this system was more complex due to its regulation of the pressure instead of the flow. With this pump it was possible to achieve required low flow rates of 0.2-0.5 µL/min over the required time period of the experiments, and the needed volumes of up to 1 mL were provided as well. After choosing the right pumping system and optimizing the design of the cell traps the monitoring of induced gene expression was performed. By the use of this device the gene expression in single cells was analyzed under different conditions (inducer concentration dependency, transient induction, and parallel supply of different inducer concentrations). In general, large cell heterogeneity was revealed. Most cells showed similar gene expression levels. However, a small number of cells fluoresced at an early stage of the experiments and reached high final fluorescence intensities. In contrast, in some other viable cells gene expression was either not induced or if induced, only at low levels. Based on the single cell measurements, dose-response curves of tetracycline for the continuous induced gene expression were derived for three time points (5 h, 10 h, and 15 h). For the 15 h time point the curve resulted in saturation. Thus, the time-limiting factor was now the gene expression and no longer the supply of tetracycline. Additionally, the transient gene expression induction resulted in lower final fluorescence intensities, and the parallel supply of different inducer concentrations resulted in final fluorescence intensities, which directly corresponded to the supplied inducer concentration. Overall, these results confirm the ability of the presented MMD to perform single cell studies.

The final design of the cell traps is optimized for the capture of single mammalian cells of about 15 µm in size, and their trapping over long periods of time. However, the trapping efficiency, i.e. how many traps are occupied with one cell can still be increased. There is potential to further adapt the design of the cell chamber and/or the channel network in the top layer to reach single cell trapping at almost 100% efficiency in the region of measurement. In the presented device one channel opens directly into a huge chamber, where single cells are captured. Most cells are transported in the middle of the stream due to the use of a hydrodynamic flow. As a result of both, the design and the hydrodynamic flow, the spatial distribution of cells within the cell chamber is bigger in the middle than at the walls. Thus, the trapping efficiency is highest in the middle. To capture more cells towards the walls of the chamber, the time period for the supply of the cell suspension has to be increased. On the other hand, a longer cell supply time results in multiple cell trapping in the middle. To overcome the quandary between efficient single cell trapping and excessive cell supply time branches can be inserted. Thus, the main stream does not directly enter the cell chamber, but is divided. Hence, the transported cells are more equally distributed, and single cell trapping is enhanced over the entire cell chamber.
The ease in adaptation of the design of the traps results in a wide range of possible use, i.e. the employment of the device is not limited to the use of mammalian cells of about 15 µm in size. Additionally, the defined pattern of cell traps allows for (semi-)automated data analysis due to the knowledge of the positions of trapped cells. Nevertheless, there might be limitations regarding the cell type used for to be studied. The presented MMD is designed to keep cells separated. Therefore, the device might be best used to study cells growing in suspension. Cells growing in suspension communicate with each other in a different way than adherent cells. For example, suspension cells lack the need to get in contact with each other. For adherent cells control experiments on cell behavior in suspension have to be taken into account. However, the importance of this factor is dependent on the type of experiment. For example, in a study that is focused on a fast event, such as the induced secretion the cell type can be neglected. In those experiments the event of the release of molecules, hormones or proteins is a sudden one. It is triggered by an on/off signal. Additionally, there is no need for the cell to produce the compound, which will be secreted, de novo, because it is already packed in vesicles. Hence, experimental data can be acquired immediately after cell treatment to induce secretion. It is also possible to focus the studies on the observation of fusion events (e.g. vesicle-cell fusion). A change of the conditions via the second fluid layer can induce fusion events. These types of experiments can be carried out in a short period of time, which results in short dwell periods of the cells inside the microchip. Hence, cell properties other than the ones directly involved in the event to be studied are not of interest and can be neglected.

Many different single cell studies can be performed using this device. For all applications the use of GFP or one of its derivatives (e.g. mutants, GFP-like proteins, and fusion proteins) as gene-of-interest is recommended since it facilitates the measurement of fluorescence levels by fluorescence spectroscopy. In promoter studies the T-REx™ system has to be replaced by a plasmid containing the promoter to be studied. The influence on gene expression of several transcription factors can be monitored in parallel by their supply in multi-laminar flows. Additionally, the minimal amount of transcription factors can be identified by their transient supply. After achieving basic information on the system it is possible to combine the study with e.g. toxicological effects. The presented MMD can be used for applications in drug discovery as well. Different effects of drugs can be observed by studying their flow supply patterns. Additionally, the use of the T-REx™ system allows a controlled gene expression, and even the possibility to have a negative control within the same experiment. Another interesting application is the study of toxicological effects on induced segregation. The induction of
segregation can be carried out in one channel and the supply of toxins in the other channel. In general, for all kinds of studies the use of the MMD is not strictly necessary, unless two different influences on the cells are to be observed in parallel.

In general, the device has potential for studies on perturbations of cells, caused by small molecules. Their influence can be monitored both on the single cell level, and also as an averaged cell population response. Additionally, cell responses to different inducer supply patterns can be observed. It is possible to carry out long-term investigations, where slow processes can be observed, time-resolved stimulations, where cells are exposed to short pulses of the molecules, as well as spatially-resolved stimulations, where cells are exposed to different types of molecules or varying molecule concentrations. These different applications are of interest not only for pharmaceutical studies and drug discovery, but also systems biology or biotechnology. The additional level of spatial and temporal control added due to the separation of cell culture and cell manipulation processes allows for precise single cell studies. Perturbations on cells can be studied in a manner not possible before, i.e. any perturbations caused by mechanical perturbations of fluid supply can be neglected. Additionally, isolated cell responses can be observed in studies on the single cell level. This might lead to new insights into biochemical pathways. Very often the average cell responses obscured individual cell responses. Different applications with cell traps allow for the simultaneous detection of both, averaged and individual cell behavior. The use of this device allows to address cell heterogeneity, the origin of individualism, which is not fully accounted for in bulk analysis techniques. The presented device is a new approach to perform individual cell studies, which are crucial to explore all of Mother Nature’s secrets.

Another interesting application of the MMD is the development of a device for the fast and evident generation of cell co-cultures that mimic in vivo conditions. Here, cells are located on both sides of the intermediate membrane. Thus, a more organ-like condition is created. In the human body different cell types are generally arranged in layer-by-layer structures, which are mimicked in the presented device. DEP was chosen as cell trapping technique mainly due to its flat properties, i.e. no obstacles were incorporated in the microfluidic channel that might disturb the flow and hence, result in perturbations on the cells. By its combination with PEMs a powerful tool for fast and evident cell capture was created. DEP was used to bring the cells to the membrane surface and PEMs to anchor them there. Thus, the DEP forces only needed to be switched on for short periods of time. With this any disturbing influences of the electric field were
The PEMs anchored the cells at the membrane surface allowing the cells to adhere and spread naturally. In this project only the first efforts towards a cell co-culture are shown. Preliminary development, optimization and most important characterization steps of the electrodes had initially to be performed, because of the novelty of fabricating microelectrodes on a permeable membrane. It was proven that the membrane did not physically differ before and after processing, and the use of the fabricated microelectrodes for DEP cell trapping was shown in a planar device. Next, these membranes were assembled into a multilayer device to begin with the generation of cell co-cultures. Unfortunately the DEP forces were not sufficient to trap cells directly on both sides of the membrane. Therefore, the fabrication of microelectrodes on both sides of the membrane was initiated. However, their function has still to be tested and evaluated in the near future. Additionally, the membrane was assembled in the microchip in another way, i.e. the microelectrode faced the bottom channel. Preliminary tests proved the ability to capture cells on the bottom side of the membrane by DEP/PEMs and in the top channel by gravity/PEMs.

This presented MMD is a powerful tool for the generation of cell co-cultures. Although further optimization is needed, the preliminary results are already very promising. The combination of DEP forces for cell positioning and PEMs for cell anchorage allow for fast and reliable cell capture. Great potential is seen in the area of mimicking organs on a chip. Two different cell types can be placed on top of each other and not only next to each other. Then, cell-cell interactions can be studied in different ways; e.g. stressing one cell type and monitoring the cell response in the other cell type. It is also possible to incubate the device for a defined period of time to allow the generation of tight cell monolayers. This can be used for studies on the transport of molecules through different cell types, and it is even possible to create different microenvironments. This system could potentially become a powerful tool to study cell-cell interactions in cell co-cultures (e.g. cell-cell communication and cell migration), where conditions can be tuned independently to accommodate the best growth and function conditions for each cell line. Cell exposure to different microenvironments would be possible, having two cell types physically separated.

These MMDs represent a unique platform to interrogate cells in ways that have yet to be realized. The main utility of the two devices are seen in drug discovery and organ-on-chip studies.
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References


Appendix
Chapter 4

AutoCAD chip designs.

A: Bottom channel, three inlets merge into one main channel.

B: Top channel, one channel opens into a microchamber containing cell traps. Alignment marks for the bottom channel are at the sides.

C: Microchamber for cell culture, zoom in of (B). The density of traps is higher in the middle of the chamber, where the crossing section between top and bottom channel will be in the assembled microdevice.

Recognition sites of the restriction enzymes *BamHI*, *NotI* and *HindIII*.
MCS of pcDNA4/T0/mycHisB.
Vector map of the newly designed inducible ZsGreen1-DR gene expression vector.
Chapter 5

AutoCAD designs.

A: Top channel, one main channel is used. The channel is bend due to the alignment with the bottom channel and the electrodes. Additional, alignment marks are visible.

B: Bottom channel, one straight main channel is used. The two smaller structures were not used.

C: Pattern of electrodes, the microelectrodes in the middle are connected to pads.

D: Microelectrodes, zoom in of (C).
Acknowledgments

I am very grateful to Prof. Dr. Petra Dittrich for supervising my Ph.D. and for offering me the opportunity to work in this inspiring, interdisciplinary research group.

I would like to thank Prof. Dr. Detlef Günther for the interest in my work and for accepting to co-examine my thesis without any hesitation.

Additionally, I would like to thank all people I met during my research stay at the National Institute of Standards and Technology, NIST, in Gaithersburg, MD, USA. Especially I would like to thank Dr. Darwin Reyes-Hernandez for giving me the opportunity to work with him. I want to thank everyone in the Semiconductor and Dimensional Metrology Division and in the NanoFab, who not only offered a lot of expertise and time helping me making progress with my project, but also for the nice chats during breaks.

The whole Dittrich group I would like to thank for the scientific support and discussions during my thesis. And of course also for the nice times we spent together apart from the labs, which was essential to keep a free mind.

Especially I like to thank my old and new office mates, Dr. Josep Puigmartí-Luis, Dr. Benjamin Cvetković, Andreas Cavegn, Felix Kurth, and Dr. Maik Hadorn. It was a pleasure to share an office with you.

I am very grateful to have met Christoph Bärtschi, Elisabeth Giger, and Carolin Blum here at ETH. Thank you for spending some time outside the lab with me. Additionally, I want to deeply thank Dr. Edward McCarthy, Dr. Yu-Chin Li, Lucia Gruber, and many more for making my time in Gaithersburg to what it was. Thank you very much!

I also want to thank everyone I met along my way to become a PhD graduate.

Of course I am grateful to everyone who gave me input on my thesis in form of proofs, discussions or any other kind of support. Thank you to finish it.

Mama, vielen vielen Dank! Ohne Deine bedingungslose Unterstützung durch die Hochs und Tiefs meiner Ausbildung wäre diese Arbeit nicht möglich gewesen.

Der allergrößte Dank gilt Harry&Bert! Ihr habt mir soo viel gegeben!
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Publications

Parts of this thesis have been published as scientific contributions or presented at conferences.

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Book Chapter


Patents

“Dielectrophoretic Cell Capture” (NIST Docket 11-024), pending

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