Microfluidics for studying cellular signaling and performing microscale tissue analysis

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MICROFLUIDICS FOR STUDYING CELLULAR SIGNALING AND PERFORMING MICROSCALE TISSUE ANALYSIS
MICROFLUIDICS FOR STUDYING CELLULAR SIGNALING AND PERFORMING MICROSCALE TISSUE ANALYSIS

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ETH ZURICH

For the degree of

Doctor of Sciences

presented by

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ABSTRACT

Microfluidics, the manipulation of liquids using small structures in the tens of micrometers, is an emerging field of research and development that serves as a continuously growing toolbox for researchers in life sciences and medicine. The broad range of applications, where microfluidics have been applied, spans from nanoliter-volume chemical synthesis to microscale capillary-driven diagnostics, single cell analysis, high-throughput drug screening, electrophoretical separation of DNA, protein crystallization and many more. These applications take advantage of some useful capabilities of microfluidics, such as the small size of the devices, the ability to perform separations and detection with high resolution and selectivity, fast response, low costs, high precision and the possibility of performing experiments/analyses which can hardly been done using conventional methods. Such applications include, for example, single cell handling, electro-osmotic flow manipulation or confining liquids hydrodynamically over surfaces.

In the work presented here, microfluidic systems have been developed as tools to address specific biomedical challenges in the context of brain cell signaling and for local analysis of tissue samples in pathology. Understanding the pathways of inflammatory signaling cascades for unraveling the complex mechanisms of diseases such as Parkinson's or Alzheimer's is highly challenging. The major mediators are still unknown and the sequential flux of molecular information between different cell types of the brain is undefined. Microfluidic networks (MFNs) were used to plate precisely populations of primary brain cells and study their response in the context of neuroinflammation. The developed MFNs comprised chambers to accommodate either microglia, astrocytes or neurons, microchannels for bringing the cells in fluidic connection and user-chip interfaces for manipulating the fluid flow using external pumps and valves. For use with primary brain cells, the MFNs were equipped with features that allowed the incubation of the cells in an “open” MFN using standard cell culture techniques and closing of the MFN for subsequent microfluidic experiments. In addition to testing the device performance in terms of handling, microfluidic functionality and reliability, biological experiments were performed to examine the neuroprotective capability of astrocytes derived from different regions of the brain. For example, hippocampal neurons were cultured in one chamber of a MFN, while either hippocampal or cortical astrocytes were grown in an adjacent chamber. An oxygen-glucose deprivation (OGD) protocol was applied to the cells being either in fluidic communication or isolated. After a recovery period of 24 h, the cells were subject to viability assays. The results of this study showed a significant neuroprotective role played by cortical astrocytes (2.9 % propidium iodide (PI) positive neurons), whereas neurons in fluidic communication with hippocampal astrocytes did not show significant differences in viability compared to isolated neurons (both ~19 % PI positive neurons). Similar results were found when the cells were exposed to another neuroinflammatory stress, namely the exposure to amyloid β fibrils. The developed MFNs and the method shown proved to be a valuable, versatile and flexible analytical solution to unravel how different cell types of the brain contribute to the crosstalk events leading to neurodegeneration.
The amount of information received from pathological staining procedures (immunohistochemistry, IHC) is most often crucial for the best possible treatment of cancer patients. There is a critical need for a flexible method to extract more high-quality information from tissue sections for both drug discovery and clinical pathology. We developed a microfluidic technology called the microfluidic probe (MFP), which allows local (bio)chemical processing of samples in a non-contact scanning mode and hypothesize that this tool may be of great value for pathologist to analyze tissue samples, allowing multiplexed, local and fast staining for markers, while being conservative with reagents and tissue. The main component of the MFP is a microfabricated head comprising at least two microchannels, one to inject and the other to aspirate liquid at the apex of the head. The MFP head is brought close to the sample to be processed and simultaneous injection and aspiration of a processing liquid within immersion liquid, results in hydrodynamic confinement (~100 μm diameter) of the processing liquid on the sample. This work demonstrates the capability of the MFP to perform microscale immunohistochemistry (μIHC) on cancerous tissue thyroid and breast tissue sections in a multiplexed and adaptive manner. Within the standard workflow of IHC, the MFP was used to deliver locally antibodies against specific cancer markers to tissue sections. Subsequent conventional processing of the tissue sections with secondary antibodies and chromogen solutions then revealed the staining results. By varying the residence time of the MFP on the tissue, different staining intensities were achieved within a single experiment. This allows for easy adaption of staining conditions using only one tissue section, even for multiple antibodies, because the processing liquid can be exchanged during an experiment. Good staining contrasts were found at 5 to 20 s residence time, which is considerably shorter than the 30 min recommended by the supplier of the antibodies. The capability of the MFP to stain for multiple markers on a single tissue section was exemplified by delivering 3 different antibodies to invasive ductal carcinoma breast tissue and in addition multiplexed staining was performed on individual cores of tissue microarrays including the formation of 100 μm wide hematoxylin lines for counterstaining. Performing μIHC with the MFP proved to be useful in several aspects. (1) It allows for staining specific regions of a tissue, interpret the results and reprocess another region with adapted conditions, (2) the non-processed areas of a tissue remain unchanged, which improves the contrast for histological analysis, (3) very low volumes of agents are needed (e.g. only a few nanoliters of antibody solution per staining spot), (4) multiple processing solutions can be used, including counterstain agents, and (5) due to the possibility of scanning the hydrodynamic flow confinement, even gradients can be performed, which can be particularly useful for fundamental cancer/tissue research.
ZUSAMMENFASSUNG


Neuroinflarnations-Stress, der Behandlung mit Amyloid β Fibrillen, ausgesetzt wurden, ergaben sich vergleichbare Resultate. Die entwickelten MFNs und die gezeigte Methode erwiesen sich als wertvolle, vielseitige und flexible analytische Lösung um Klarheit zu verschaffen, wie verschiedene Typen von Hirnzellen zu Ereignissen, welche zur Neurodegeneration führen, beitragen.

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1 Introduction

1.1. Scope and outline of the thesis

Microfluidics has proven to be a highly valuable, ever growing family of related technologies enabling researchers in life sciences novel experiments/analyses which conventionally have not been possible.\textsuperscript{1,2,3} The most prominent features of microfluidics over larger fluid handling and analyzing systems originate from the unique behavior that liquids exhibit at the micrometer length scale.\textsuperscript{4,5} Flow of liquids in microfluidics is typically laminar, thereby making rinsing efficient and mixing challenging. Surfaces in microfluidic devices play an important role not only by defining flow paths but also by interacting with species transported in the confined liquid. Volumes well below 1 nL can easily be handled by fabricating structures with lateral dimensions in the micrometer range. Reactions that at large scales are limited by the diffusion of reactants can be accelerated.\textsuperscript{6} Finally, parallel streams of liquids can be made in microchannels with a high degree of control and reproducibility, which allows chemical reactions and gradients to be made at liquid/liquid and liquid/solid interfaces.\textsuperscript{7} Thus, it is not surprising to find that microfluidics are increasingly being used for applications in life sciences, where samples often are limited in volume, reagents are expensive or rapid diagnostics is needed. Many ongoing developments and studies are pushing the limits of microfluidics, for example, in diagnostics, single cell studies and handling, localized chemistry or even microscopic observation of living cells.\textsuperscript{8,9,10} The aim of this thesis is to explore two new microfluidic concepts/technologies for studying cellular interactions and tissue analysis, with the motivation that these microfluidics may help collecting new findings in biology and medicine, specifically within the scope of the two challenging topics: neuroinflammation and pathology.

Neuroinflammation, which is hypothesized to contribute to neurodegenerative diseases such as Parkinson’s or Alzheimer’s, involves complex interactions between various cells, e.g. neurons, microglia and astrocytes, which are still poorly understood.\textsuperscript{11,12,13} Although significant efforts are ongoing to study the complex underlying mechanisms of these diseases, the understanding of the role of neuroinflammation is still a major challenge.\textsuperscript{14} For more insight in the complexity of neurodegenerative diseases, the mechanisms of intercellular communication between different types of brain cells need to be unraveled.\textsuperscript{15} Microfluidic networks (MFNs) that allow individual stimulation and liquid transfer between cell populations or single cells can support ongoing studies on how neuroinflammatory processes take place.\textsuperscript{16,17} One example for this is to study the effect of amyloid beta oligomers on brain cells and observing their response with modern readout systems. One part of this thesis is assigned to such studies. The work described in chapter 2 aims to provide a new concept of a polydimethylsiloxane (PDMS)-based MFN that allows for studying populations of primary brain cells with state of the art read-out possibilities. A background section explains the motivation for the work and points out the relevance of the work. The development of PDMS microfluidic networks for seeding, culturing and studying mammalian cells is described and
key results shown. A separate sub-chapter on overflow microfluidic networks (oMFNs),
allowing culturing and studies of primary brain cells, is provided, which is the basis of the
recent “Brain Chip” technology. The chapter links to chapter 6.2 in the appendix, where the
results of the project are presented. There, neuroinflammatory insults (metabolic stress and
exposure to amyloid β fibrils) were mimicked using oMFNs and primary brain cells. With
this approach, regional differences in glial control of neuronal physiopathology could be
demonstrated. During the work with MFNs, a method for fabricating such devices with
enhanced optical properties was developed. MFNs fabricated using the new method allowed
the acquisition of “clean” brightfield microscopy images. The method and results are
presented in chapter 6.1 of the appendix. A recent review on pharmacology using
microfluidics is provided in chapter 2.2. It discusses the role of microfluidics for cell studies
based on recently published literature.

One challenge in pathology is the acquisition of high quality data through immunohistochemical staining.\textsuperscript{18,19} Staining for specific markers on thin tissue sections provides invaluable information to the pathologist.\textsuperscript{20} The current protocols for immunohistochemistry are usually performed on an entire tissue cut and only a single marker is stained for. These procedures have a number of limitations: (1) There could be not enough tissue available to generate the number of sections required if the sample needs to be analyzed for multiple markers. (2) Optimizing staining procedures to avoid over- or understaining consumes a significant amount of sample. (3) The time for staining procedures may be an issue and limits, for example, the analysis of tissue during surgery.\textsuperscript{21} These limitations of traditional immunohistochemistry suggest that there is room for improvements, and microfluidics can be part of it. The microfluidic probe (MFP), a microfluidic device for “contact free” chemical processing of samples in the micrometer range, can offer ways of (bio)chemically processing tissue sections locally and serve as a tool for multiplexed and interactive staining. The work described in chapter 3 was performed based on the hypothesis that the MFP can address the limitations of traditional immunohistochemistry. The technology and strategically driven technical improvements are described in chapter 3.1. There, the working principle of the MFP as well as its benefits, uniqueness and potential applications are explained in detail. The next two chapters are based on published articles. The topic of the first publication (chapter 3.2) is micro-immunohistochemistry. Here, the MFP was used to stain tissue sections at the micrometer scale for improved pathology. The results confirm that the MFP can be a powerful technology for local processing of biological substrates, such as tissue sections, and may become a tool of choice for future pathological studies/diagnostics. The MFP in the context of microfluidic technologies operating in the “open space” is then reviewed in the second article (chapter 3.3). The review discusses three classes of such microfluidic systems based on microelectrochemistry, multiphase transport, and hydrodynamic flow confinement of liquids.

A retrospective view on the two microfluidic technologies is provided in chapter 4. I look at
the work from the outside and comment on the potential of the two technologies used and
provide suggestions for further improvements and applications.
1.2. Background on how the Projects developed

1.2.1 Microfluidics for studying cellular signaling (the “Brain Chip”)

In 2008, the “Brain Chip” project started on the basis of a collaboration between the ‘Experimental Biosciences’ group at IBM Research – Zurich, NeuroZone srl., a startup company in Milano, and Professor Michela Matteoli from the department of Medical Pharmacology at the University of Milano. The goal of the project was to jointly develop a microfluidic platform for studying intercellular pathways in the context of neuroinflammation. Closed and open microfluidic devices were developed to seed, cultivate, stimulate and study cells from the brain. First results on seeding precisely different brain cell line populations and demonstrating cellular pathways were achieved, followed by experiments with primary astrocytes and neurons that allowed the observation of diverging levels of neuroprotection caused by astrocytes, depending on the origin of the cell in the brain (hippocampus or cortex).

1.2.2 Microfluidics for tissue analysis (the “Microfluidic Probe”)

The vast majority of microfluidic devices have closed flow paths. Closed flow paths facilitate the integration of functional elements (such as heaters, mixers, pumps, UV detectors, and valves) into one device while minimizing problems related to leaks and evaporation. However, many samples cannot pass inside closed microfluidics. This is in particular the case for adherent cells and tissue sections. Similarly, processing or patterning surfaces under microfluidic conditions is difficult. Inkjets can deliver ink in a non-contact mode, but not in the presence of an immersion liquid. Other techniques than inkjet technology can also pattern surfaces and at even higher resolution, but are limited in their ability to operate in a liquid environment. Liquid environments minimize drying artifacts and denaturation of biomolecules, and thus enable working with living microorganisms. For patterning surfaces and analyzing samples on a surface in the presence of a liquid environment, several strategies were developed to overcome the limitations of closed microfluidics. Some strategies relied on confining liquids near a surface or on delivering a precise amount of biomolecules in a well-defined region of a liquid. Scanning nanopipettes and hollow AFM probes were also developed for patterning biomolecules on surfaces with micrometer accuracy.

In the Microfluidic Probe (MFP) project, a microfluidic technology for local (bio)chemical processing of surfaces within a liquid environment is developed and its potential for biological and medical applications explored. The device (MFP) was first presented in 2005. The basic principle of the technology is to bring a microfabricated probe head close to the surface of interest in an aqueous environment. Two adjacent openings in the probe head, facing the sample surface, are used to inject and aspirate a processing liquid to perform a local chemical reaction on the sample. Aspiration takes place at a higher flow rate than the liquid is injected, which results in a hydrodynamic confinement of the processing liquid within the surrounding liquid (immersion fluid). As the MFP is well suited for local
deposition of proteins and staining of cells was demonstrated\textsuperscript{10,31}, one logical step was to explore the capability of the technology for performing immunohistochemistry on tissue cuts.

1.2.3 References

20. www.ihcworld.com
2 THE “BRAIN CHIP” FOR STUDYING CELLULAR SIGNALLING
2.1. Introduction to the “Brain Chip” project

Some parts of this chapter have been extracted from the following publications:

**Lovchik, R.D., Bianco, F., Matteoli, M. and Delamarche, E.**  
Controlled deposition of cells in sealed microfluidics using flow velocity boundaries  

**Lovchik, R.D., Tonna, N., Bianco, F., Matteoli, M. and Delamarche, E.**  
A microfluidic device for depositing and addressing two cell populations with intercellular population communication capability  

**Lovchik, R.D., Bianco, F., Tonna, N., Ruiz, A., Matteoli, M. and Delamarche, E.**  
Overflow Microfluidic Networks for Open and Closed Cell Cultures on Chip  

**Contributions** – I lead the development of the PDMS microfluidic networks used for the project. In particular, I came up with the concept of molding the microstructures in PDMS and sealing them with a hard lid comprising vias for fast and easy connection to pumps or valves. The concept of cell deposition using flow velocity boundaries originated from the experiments I performed using PDMS MNFs. I optimized the deposition procedures for cell lines in MFNs and developed the method of filling and operating the MFNs without having or generating air bubbles. I designed the MFNs and fabricated the replicas from Si molds supplied by our technicians. The various holders for easy assembly and alignment of the chips were designed by me and fabricated by our workshop staff. Some of the custom made equipment I directly fabricated myself in the mechanical workshop.

I developed the concept of overflow MFNs (oMFNs), designed the oMFNs and optimized closing procedures for leak free assembly of the devices. I performed experiments using cell lines (e.g. HeLa, neuroblastoma, fibroblasts) to test the performance of the devices. The biological pathway studies were performed together with our project partners. They gave suggestions on the biological systems to be used, as well as on the cultivation procedures and experimental setups. Their core expertise lies in studying neuronal development and intercellular communication among different types of brain cells (e.g. neurons, microglia, astrocytes) under physiological and pathological conditions. I was involved in all experiments presented in this chapter. Most of the biological results shown in the appendix of this thesis were acquired in Milano at NeuroZone. I lead the writing of the above listed publications and prepared the figures, some of them with content provided by our project partners, who did the confocal microscopy.
2.1.1 Background

Microfluidics are emerging as invaluable tools for research on cells for a number of reasons. First, they enable experiments on cells to be performed with well-defined chemical and topographical environments. Second, they can reduce the number of cells needed for experiments. Third, they can be used for experiments in which a precise number of cells can be stimulated and observed accurately. Last, they permit experiments to be performed in parallel and/or with high throughput. Despite these possibilities, microfluidics are not well established for daily research work on cells, as they lack user friendliness and require specific and often cumbersome methods for depositing and culturing cells. This is further complicated when taking into consideration more relevant cell systems such as primary cells which may require longer culturing times before being ready for specific assays (i.e. primary neurons need to be cultured several days in vitro (DIV) before establishing mature synaptic contacts). Although progress on sealing a Petri dish with a hybrid microfluidic-vacuum platform was recently demonstrated by Chung et al., interfacing cell cultures grown in “open” dishes with microfluidics that are only a few microliters in volume remains a great challenge. Similarly, depositing cells on a treated glass slide and gluing a lid having ports onto the slide provides a method for enclosing living cells inside a microfluidic flow path. Such a method would be hard to implement with multiple types of cells and can be simplified if the gluing step could be omitted. Finally, Shuler et al. developed powerful micro cell culture analogs to assess the cytotoxicity of drugs on cells. In this work, cells were mixed with a gel at a temperature of 4 °C, then added to the micro cell culture analogs, and entrapped in the gel by raising its temperature to 37 °C. Using a low temperature (especially if primary cells are used) as well as challenges with air bubbles and leaks when screwing a cover on the micro cell culture analogs also entail technical challenges.

In addition to the above-mentioned advantages and drawbacks of microfluidics when working with cells, entirely new types of experiments become possible when distinct cell populations are placed within a microfluidic device, which allows to simultaneously carry out a functional analysis on stimulated versus control cells in a miniaturized, highly-controlled microenvironment. Besides enabling the analysis of cellular behaviors, such as cell differentiation, motility, or response to external trophic or toxic stimuli, microfluidics open the possibility of dissecting cell-to-cell communication in complex intercellular scenarios, such as those represented by the nervous or the immune systems, where the deep comprehension of molecular interchanges among different cell types (i.e. in the central nervous system, Figure 2.1.1) is crucial for the correct understanding of the mechanisms involved in the onset of diseases. In the brain, this is particularly true for the neuroinflammatory events which contribute to neuronal death in neurodegenerative diseases. The latter are among the most devastating diseases, which account for 30% of healthcare costs in industrialized countries and an estimated 35% of the disease burden within the seven major pharma markets. Notably, today there are no drugs available that can arrest or reverse neurodegeneration.
In the last years it has become clear that dysfunctions of the synapse (the functional contact between neurons) are central to the aetiology and progression of a wide range of neurological and psychiatric disorders, including neurodegenerative diseases, schizophrenia, autism, depression and many others, which can therefore be collectively regarded as synaptopathies. Besides specific defects in neuronal proteins, activation of immune mechanisms and inflammation play a crucial role in synaptopathies. Bidirectional functional interactions among neurons, astrocytes and microglia, through the release of soluble chemical mediators, govern both the sequence of inflammatory events (cascades of inflammatory mediators) and the pathological outcome (damage or absence of damage to the neurons). However, the pathways of these inflammatory signaling cascades remain cryptic, the major mediators unknown, and the sequential flux of molecular information among the different cell types still undefined. The scientific community is currently facing the challenge of dissecting the neuroinflammatory cascades among microglia, astrocytes, and neurons by breaking down the cellular networks and controlling the flow of inflammatory mediators.

Figure 2.1.1 – Different types of cells in the brain. (a) Section of a rat brain tissue, stained with fluorescence dyes to visualize key cells and structures, courtesy of Bjornsson, Roysam et al., *J. Neurosci. Methods* 2008, 170, 165–178. (b) Simplified illustration of the complex interactions between some of the cell populations involved in neurodegenerative diseases. (N: neurons, A: astrocytes, M: microglia, O: oligodendrocytes, C: blood capillary). Figure taken from [21], drawing made by Edith Lovchik.
among these cells. This is crucial for understanding the molecular mechanisms that are at the basis of neuronal damage, in order to define smarter strategies for tackling neurodegenerative processes.

Together with our partners from the University of Milano and Neuro-Zone, we started a project on the development of microfluidic platforms to study cellular signaling in the context of neurodegenerative diseases. We focused on the development of novel microfluidic devices inside which two or more types of cell populations can easily be deposited and cultured prior to exposing them to stimulating factors independently or sequentially. The device should be reversibly sealed with a lid, which can be removed for additional staining or imaging of the cells. The microfluidic devices should have small dead and total volumes, a fast response time for exchanging liquids, and should allow usage with a high degree of interactivity without the need for technical expertise. Our primary goal in devising such devices was to investigate the reciprocal chemical communication among pure cultures of different brain cell types. Because accumulating evidence suggests that development of brain diseases is associated with changes in synergistic interactions among neurons, astrocytes and microglia, the possibility of dissecting altered flows of information occurring in neurodegenerative disorders, and synaptopathies in general, would provide novel diagnostic and therapeutic strategies.

The next chapter shows the evolution and experimental results of the different generations of microfluidic devices, which I developed.
2.1.2 Development of microfluidic networks for studying cellular signaling

Despite progress in designing microfluidics and refining the approaches taken to study cells in microfluidics, a substantial challenge remains: microfluidics usually are closed systems, and cells must pass inside micro-conduits to be deposited in areas permitting their stimulation and observation. Strategies for immobilizing cells in a closed microfluidic device employ mechanical trapping, balancing hydrodynamic forces exerted on cells with optical tweezers or dielectrophoresis, hydrodynamic vortices generated by ultrasounds, or entrapment inside gel matrices. Adherent cells critically rely on cell-cell or cell-surface interactions for proper functioning. In principle, adherent cells can be immobilized in selected areas of a microfluidic device by patterning CAMs in the device. In practice, however, patterning of proteins and CAMs in nonplanar microfluidic structures is a challenging issue. Even for microfluidic devices composed of a planar element and a microstructured element, it may be cumbersome to functionalize the planar element with CAMs and to assemble the micro-fluidic device. Furthermore, adherent cells can be deposited/lost in peripheral microfluidic structures or user-chip interfaces, and deposition must be well balanced with rinsing strategies to ensure that cells locate where desired.

Within the “Brain Chip” project, I developed a simple and reliable method for the controlled deposition of adherent cells onto planar regions of a sealed MFN. The method takes advantage of antagonist forces, which are (1) the adhesive forces between adherent cells and CAMs, and (2) the shear stress exerted by a moving liquid on attaching/adherent cells. Even though CAMs are indifferently deposited in the various regions forming the microfluidic device, cells are not able to adhere in regions where the flow velocity is kept sufficiently high. In contrast, cells adhere in regions having low flow velocity. The juxtaposition of regions of low and high flow velocity defines boundaries in the microfluidic device where the chances for a cell to adhere vary greatly.

Figure 2.1.2 shows the design of a microfluidic device having two cell chambers. It is made of a transparent elastomeric PDMS MFN that holds the cell chambers and connecting channels. The MFN is closed with a Si lid having vias for the entrance and exits of liquids in and out of the MFN. Contact between the MFN and the lid is conformal and therefore results in a leak-free device when assembled. PDMS is transparent and also allows direct optical observation of liquids and cells being moved throughout the device. In principle, a harder transparent material, such as plastics or glass, can be used instead of PDMS but this would require the MFN and lid to be bonded together. In practice, PDMS has the additional advantages of (1) being hydrophobic so that cell adhesion molecules can directly adsorb to it from solution, (2) providing reversible contact making the lid reusable after each experiment, (3) being cheap and easy to mold, (4) being slightly permeable to gases (e.g. N\textsubscript{2}, O\textsubscript{2}, CO\textsubscript{2}), which can be important to help remove trapped air, (5) being easy to clean and sterilize with heat or using ethanol, (6) being compatible with cells, and (7) having a low background autofluorescence. The MFN has two adjacent cell chambers, which can be serviced independently with microfluidic channels. These channels are connected to the cell
Figure 2.1.2 – Design and assembly of a MFN having two chambers for depositing and studying cell populations. (a) A molded PDMS MFN having two cell chambers and connecting microchannels is aligned to a Si lid having vias. The chambers are serviced using one or several of the vias for depositing, culturing and stimulating living cells. A central microchannel brings the chambers into fluidic communication, if needed. (b) The ports are connected to pumps or valves using capillary tubes and fittings or can be kept open. Figure taken from [37].

chambers via flow-distribution structures. The geometries of the microchannels and cell chambers create flow velocity boundaries: for a given flow rate of cell suspension passed in a MFN homogeneously covered with cell adhesion molecules, high shear stress on cells near the walls will prevent cells from adhering to the walls, whereas low shear stress in the cell chamber permit cell adhesion there, see Figure 2.1.3.11

Figure 2.1.2(b and c) are photographs of the assembled microfluidic device. The six microchannels numbered in Figure 2.1.2(b) are in regard to vias in the lid that bear corresponding ports for standard fittings and tubings. Three of the ports are connected to high-precision motorized pumps and the other three to valves and reservoirs. A typical workflow for experiments is to fill the device with buffer, perform cell deposition in the chambers, perfuse the chambers with fresh medium, stimulate cells in one chamber, and transfer some liquid from one chamber to the other. All these steps can be observed in the MFN in real time using an inverted microscope. The compact design of the chambers permits viewing both chambers in a small field of view, and the fanning of the vias and bonding of the ports on the lid keeps the footprint of the device small (~3.2×2.6 cm²).37

The assembly of the devices for experiments was the following: the Si lid and PDMS MFN were aligned and assembled by hand with the help of alignment marks and under cleanroom conditions. The assembled device was filled with liquid (e.g. deionized water or buffer containing cell adhesion molecules). Typically, air bubbles would be trapped in some areas of
Figure 2.1.3 – Principle of depositing cells in a microfluidic chamber using flow velocity boundaries. (a) Despite the presence of CAMs on the bottom of successive microfluidic areas, cells do not adhere in narrow areas when their flow velocity is kept sufficiently high. (b) Following this principle, a microfluidic device comprises a narrow inlet and outlet, narrow flow distributing structures, and a wide capture area wherein cells can be deposited, cultured, and studied. (c) Qualitative illustration of the flow velocity profile along the main axis of the chip depicted in (b). Flow in the chip can be adjusted interactively to vary the fraction of cells attaching in the different areas of the chip and to identify a critical velocity. Figure taken from [11].

the MFN and in the cell chambers in particular. This air was removed by centrifugation of the device for 30 to 40 min. For this, the ports were filled and the device was placed with the PDMS side on a glass slide using a cleanroom paper as spacer between the slide and the PDMS. Centrifugation took place at ~250 RCF using a rotor for standard multiwell plates. During the centrifugation step, trapped air was displaced by water and moved out of PDMS. The spacing paper was found to be important to ensure that air only had to diffuse out across the thickness of the PDMS MFN. The device was then hooked up to prefilled tubings that were connected to pumps or valves. Unused ports were close using threaded plugs (Upchurch). The microfluidic device was positioned on the stage of an inverted microscope (Nikon Eclipse TE300, Egg, Switzerland, equipped with a Camcorder, Sony CDR-SR100E, Schlieren, Switzerland), with the PDMS MFN directly in contact with the microscope stage.
Device performance

Prior to biological experiments, the microfluidic device was tested using colored water. A prefilled microfluidic device was connected to three pumps (ports 4, 5 and 6) and three shut-off valves (ports 1, 2 and 3). The pumps were equipped with valves to quickly start or stop the flow. Liquids were drawn into the MFN by pulling the liquid rather than pushing because applying a positive pressure for pushing a liquid into the MFN at flow rates above ~7 µL min\(^{-1}\) resulted in the delamination of the PDMS from the Si chip or in the formation of liquid cushions between these layers, especially around the inlet vias. Pulling liquids at flow rates in the range of 0.1 to 10 µL min\(^{-1}\) did not pose problems. Flow rates above ~20 µL min\(^{-1}\), however resulted in the formation of air bubbles, presumably owing to the permeability of PDMS to air. Another benefit of pulling the liquids is that exchanging liquids was possible without having to exchange syringes on the pumps: the liquids were supplied from reservoirs positioned directly at the shut-off valves and controlled by manually switching the valves.

Figure 2.1.4 shows some of the capabilities of the microfluidic device. Here, flow control is demonstrated using four different liquids: water, water colored red and black using food colorants, and a suspension of 10-µm polystyrene beads in water. Plugs of liquids as shown in Figure 2.1.4(a) can be used for rapid cell stimulation, staining cells, fast rinsing, or transfer of sub-microliter volumes of liquids between the chambers. Here, a volume of ~0.5 µL of red-colored water was supplied through port 5 and inserted into a water stream moving from port 6 to port 1 at a flow rate of 2 µL min\(^{-1}\). Plugs of liquid can be repeated by continuously pumping a liquid through the MFN and rapidly switching the incoming port to a second liquid and back. A ~0.5 µL plug of liquid was able to pass a chamber in <5 s or on the course of a few minutes using low flow rates (e.g. 0.1 µL min\(^{-1}\)). Keeping a plug of liquid immobile for a long time is not recommended because it was observed that air bubbles tended to appear in the MFN when pumping was stopped for more than 30 min. It was unclear whether this air outgassed from the liquid or diffused through the PDMS MFN. Figure 2.1.4(b) illustrates a configuration in which ports 2, 3, 4 and 5 are simultaneously opened and two independent flow streams between ports 5 and 3 (red) and between 4 and 2 (black) are established through each chamber. This setting can be used for depositing different cell populations in the chambers or perfusing the chambers with different liquids. In this example, the flow rate was 2 µL min\(^{-1}\) in both fluidic paths. The central S-shaped microchannel linking both chambers is 15.6 mm long and 250 µm wide. Diffusion of chemicals coming from either side of this microchannel is negligible. Convection of liquid through it can occur if different flow rates in the right and the left chamber are used: viscous coupling between the liquids at the junctions between microchannels will pull the liquid in the central microchannel toward the side with the highest flow rate. If needed, a port to purge the connecting channel can be added to avoid this situation.
Figure 2.1.4 – The MFN can be used for a broad range of experiments as exemplified here using colored liquids and 10 µm polystyrene beads. (a) A MFN initially filled with water has one of its chambers flushed with a plug of liquid before moving the plug to the second chamber by switching on/off the appropriate valves and high precision syringe pumps. (b) Optical micrograph showing both chambers flushed with liquids in an independent manner. The micrographs in (c) and (d) show the supply of two laminar streams of colored liquid to each chamber. (e) Complex gradients can be achieved using simultaneously four ports (as 3 inlets and 1 outlet). Here, polystyrene beads, 10 µm in diameter, were deposited in the chambers by sedimentation. Then a first gradient formed by a red and a clear stream of liquid flowing through the left chamber received an additional stream of darkly colored solution before entering the right chamber. Figure taken from [37].

Adjacent laminar flows can be created in one chamber by opening two inlet ports located on the same side of a chamber and pulling liquid from any outlet port. In Figure 2.1.4(c and d), two colored liquids are injected in the microfluidic device via ports 1 and 2 and pumped through port 4 with a rate of 2 µL min⁻¹. Figure 2.1.4(c) illustrates the high degree of control over the flows of liquid in the microfluidic device because the laminar flow is centered in the chamber. Gradients were created having a width ranging from ~20 to > 500 µm (visual determination) and with the main axis centered at any lateral position of a chamber. Complex gradients were performed using ports 1 (red liquid), 2 (transparent liquid) and 3 (black liquid) for injecting liquids and port 5 for pumping liquid at a rate of 2 µL min⁻¹, Figure 2.1.4(e). In this example, polystyrene beads having a size similar to that of cells (10 µm) were deposited by sedimentation in the chamber to verify that these particles can be homogenously distributed inside the chamber and to provide some optical contrast and a size reference for experimenting with gradients. A homogeneous distribution of the beads, suitable for cell seeding, was observed at flow rates between 0.5 and 5 µL min⁻¹. Valuable experiments can be done using such gradients. A subpopulation of cells in the chamber can be exposed to selected chemicals, another subpopulation to a chemical gradient, and a third subpopulation to a reference liquid. Using this methodology, toxicity assays on cells, cell adhesion assays, or cell stimulation and differentiation studies may be done.⁴¹²
Improving the optical properties of the PDMS MFNs for brightfield microscopy

The PDMS material Sylgard® 184 (Dow Corning, Midland, MI, USA), although from the mechanical properties very well suited for microfluidic applications, has an obvious drawback when used in liquid contact and brightfield microscopy. Filler particles swell due to liquids, e.g. buffers or culture medium, and appear in brightfield microscopy images as objects with sizes in the several micrometer range. This can be disturbing, especially when PDMS microstructures are used for cell studies, where the cells of interest and bulk PDMS from the MFN are on the same optical plane. Our observation during experiments with the first generations of “Brain Chips” was confirmed by many brightfield images of PDMS chips found in the literature. Most micrographs of PDMS structures show swollen filler particles of dust particles which (1) give the image a “dirty” appearance, and (2) could cause problems during image recognition for automated data acquisition, e.g. cell counting.

To overcome this issue of the commercially available PDMS Sylgard® 184, we developed a method for the fabrication of PDMS MFNs based on a two layer system with a custom made optically clear PDMS bonded to a layer of Sylgard® 184. The optically clear PDMS, we call it “bioclear” comprises the microfluidic structures, which normally within the focal plane during observation, and the Sylgard® 184 PDMS layer gives the system the required mechanical stability. Dust or filler particles in the Sylgard® 184 are out of focus and do not disturb the optical imaging during experiments, Figure 2.1.5

Figure 2.1.5 – Cross-section of a two layered PDMS MFN with enhanced optical properties. Microfluidic structures are molded in a thin layer of clear PDMS (yellow, ~700 μm thick) and are supported by a thick layer of Sylgard® 184 PDSM (purple). The dust and swollen filler particles visible in the Sylgard® 184 layer are typically out of focus during brightfield microscopy. The materials were artificially colored for this illustration.

A detailed description of the development and characterization of the two layered optically clear PDMS is given in the appendix of this thesis in chapter 6.1. MFNs used for the “Brain Chip” project were fabricated using this method, when the experimental plan proposed to acquire micrographs in the brightfield.
Deposition of two different cell lines

Depositing cells and establishing communication between cell populations were exemplified using two mammalian cell lines, which are widely used for research activities in the field of neuroscience: immortalized murine N9 microglia and human SH-SY5Y neuroblastoma. The first cell line is used to assess the functional behaviour of microglia, the immunocompetent resident cells within the central nervous system; the second cell line is a third-generation neuroblastoma-forming clusters of undifferentiated cells which can differentiate neurites, and therefore provide a mimic of neuronal response for in vitro studies.

Microfluidic devices were coated with fibronectin (Sigma) by adding a 50 μg mL⁻¹ solution of fibronectin in PBS (Sigma) into the ports of the device before the centrifugation step. The device was then hooked to three pumps (ports 4, 5 and 6) and three shut-off valves with reservoirs. The reservoir connected to port 2 was filled with PBS, and a flow was started by pulling through port 4 at a flow rate of 4 μL min⁻¹. After rinsing for ~2 min, the PBS in the reservoir was replaced by a N9 microglia suspension having 4×10⁵ cells mL⁻¹. The cells entering the chamber were monitored using an inverted microscope, and the flow rate was slowly reduced until the cells started to deposit in the chamber, Figure 2.1.6(a). After ~20

Figure 2.1.6 – Micrographs showing N9 microglia and SY5Y neuroblastoma deposited in the cell chambers of a microfluidic device using “flow velocity boundaries”. The PDMS MFN was homogeneously coated with FN. (a) A suspension of freshly trypsinated N9 microglia in growth medium was introduced into the right chamber at a flow rate of 2 μL min⁻¹ and cells locally deposited in the chamber. After ~15 min, the chamber was rinsed with fresh medium at the same flow rate. (b) Subsequent deposition of SY5Y neuroblastoma in the left chamber was performed at a flow rate of 0.5 μL min⁻¹ for ~20 min and was followed by rinsing with medium without changing the flow rate. Arrows indicate the flow direction in the chamber during deposition and rinsing and crossed arrows denote that the inlet for the chamber had its valve closed. Figure taken from [37].
minutes of deposition at a flow rate of 2 μL min−1, the cell suspension in the reservoir was replaced with PBS and the chamber rinsed until all the cells in the channels before and after the chamber had been flushed. The same procedure was applied for depositing SH-SY5Y neuroblastoma in the second chamber, but with a flow between ports 3 and 5 and a flow rate reduced to 0.5 μL min−1. Deposition of SH-SY5Y cells took place for ~20 min, and the chamber was then flushed with PBS. Figure 2.1.6(b) shows the cell populations in each chamber.

**Biomolecular cascade between two cell populations**

N9 microglia (chamber 1) and SH-SY5Y neuroblastoma (chamber 2) were deposited as described above. The cells were then stained or stimulated by selective exposition to specific agonists using different flow paths as depicted in Figure 2.1.7(a-b). SH-SY5Y neuroblastoma in chamber 2 were stained (data not shown) using the lipophilic dye FM143 by flushing the chamber with a staining solution at a flow rate of 2 μL min−1 for ~2 min using the same valve/pump configuration as for cell deposition. The purpose of this staining was to assess the viability of the cells and to help visualize the distribution of the cells in the chamber. Microglia were stimulated by flushing a 1 mM solution of ATP for 20 min through chamber 1. This exposure to ATP is known to induce shedding of microvesicles from microglia, a process which represents a way of intercellular communication within the central nervous system during neuro-inflammatory events.  Microvesicles, which contain cytokines such as IL1 beta, are then released into the extracellular environment.  Vesicles shed from N9 microglia were transferred to chamber 2 containing SH-SY5Y neuroblastoma (Figure 2.1.7 (c)). Vesicle transfer was carried out at a flow rate of 1 μL min−1 for ~300 s. To verify that transfer of microvesicles occurred from chamber 1 to chamber 2, ~5 μL of the supernatant in the exit port of cavity 2 was collected using a micropipette and loaded into an Eppendorf tube for specific staining with a TRITC-labeled IB4 antibody (Sigma-Aldrich, Italy; used at a 1:100 dilution in a Krebs Ringer solution: 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2 mM CaCl2, 6 mM D-glucose, and 25 mM HEPES/NaOH, pH 7.4). After 30 min, the solution containing the stained vesicles was placed on a glass slide and covered with a glass coverslip. Figure 2.1.7 (d) shows confocal fluorescence microscope images of IB4-positive microvesicles, which were shed from ATP-challenged microglial cells. A second verification that stained vesicles were indeed derived from microglia contained in chamber 1 was performed by fixing and staining the cells in both chambers. This was done by flushing a solution of 4% paraformaldehyde through the chambers at a flow rate of ~0.5 μL min−1. The PDMS MFN was carefully removed from the Si lid and both types of cells were stained with the TRITC-labelled IB4 antibodies and DAPI (Invitrogen, Italy). DAPI passes the cell membrane of intact living or fixed cells and interacts with DNA in the nucleus of cells. For this reason, both N9 microglia and SH-SY5Y neuroblastoma are visible in the fluorescence microscope images in Figure 2.1.7(e and f). In contrast, N9 microglia, but not SH-SY5Y neuroblastoma, are IB4 positive (Figure 2.1.7(g and h)), thus univocally indicating that the IB4-positive microvesicles retrieved in chamber 2 were derived from cells plated in chamber 1.
Figure 2.1.7 – Experimental workflow for establishing a biomolecular cascade between two cell chambers. A layout of the MFN device is shown with the flow path for (a) depositing N9 microglia, (b) depositing SH-SY5Y neuroblastoma, and (c) directing vesicles shed from stimulated N9 microglia to SH-SY5Y neuroblastoma. (d) Confocal fluorescence images showing vesicles that were retrieved from the outlet port of chamber 2, stained with a TRITC-labelled IB4 antibody, and deposited on a glass slide. (e) – (h) Fluorescence images of fixed N9 microglia and SH-SY5Y neuroblastoma after dual staining with the selective nuclei stain DAPI and with a TRITC-labelled IB4 antibody. (i) Closeup of image (g) that shows a vesicle being shed from a N9 microglia. Figure taken from [37]. Experiment conceived by Fabio Bianco and Michela Matteoli, experiments performed at IBM.

With this setup of MFNs, we could show that supplying culture media, buffers, chemicals and staining reagents through the cell chambers is not limited to moving chemicals from one chamber to the other but can also involve organelles such as microvesicles. The stimulation of cells can vary in duration and can even be done using a sequence of chemicals. The viability of cells can be verified and cells counted prior to investigating a pathway because cells in the chambers are observable (directly or with the help of a staining dye) using optical microscopy. Creating gradients and using laminar streams of liquid passing through a chamber can in principle be done to address a fraction of the cells in a chamber and to have a subset of the cells used as a control population. Two types of cells were used here but it should be straightforward to scale the microfluidic device shown here to create “cascades” with three or more types of cells. While pathways of living organisms can be extraordinarily complex, pathways in many diseases tend to be dominated by anomalous concentration levels of one or a few (bio)chemicals. Synapthopathies belong to this category of diseases. In synapthopathies, the flux of soluble inflammatory mediators or even the transfer of cytokine-containing microvesicles may contribute to the onset of neuroinflammation, an event which leads to neurodegeneration. This suggests that this concept of microfluidic networks can be used for dissecting the flux of information occurring among different brain cells.
Toward cell studies using primary cells from the brain

Primary cultures from the central nervous system, in contrast to cell lines, represent a physiologically more relevant cell model, given that they largely maintain the features of their correlates in situ and therefore represent a more reliable system for investigating neuronal development and molecular processes of brain diseases. For this reason, leading neurobiology labs worldwide use primary cultures to investigate neuronal development, cell-to-cell signalling, and molecular mechanisms of brain diseases. Also, primary neuronal cultures are increasingly used for preclinical studies on potential drug candidates.42

The step of going towards cell studies using primary cells, e.g. astrocytes or neurons, was accompanied by the challenge of long term culturing of cells within the MFN before microfluidic experiments can be performed. Culturing of primary brain cells within a closed PDMS microfluidic device is cumbersome. The microfluidic platform has to supply a high degree of control of the pH, gas exchange, nutrient supply, flow conditions (e.g. long term stop flow without air bubble formation) and temperature to maintain the best possible environment for the cells. Also, when working with more than one cell type, it is likely that the cell populations have different culturing periods and would have to be introduced at different times. We developed a next generation of MFNs that represent a bridge between the typical workflow in cell biology and the field of microfluidics. They offer the possibility of plating cells in a sterile environment, keeping them in standard culturing conditions within incubators, and retrieving them at the right developmental state to use them in a microfluidic device. The working principle and results achieved with primary cells is described in the next chapter.
2.1.3 Overflow microfluidic networks for culturing and studying primary cell populations

This chapter describes a concept for microfluidics in which cells are plated and cultured in an open part of the chip and studied under “microfluidic conditions” after sealing the open chip with a lid. Plating and culturing the cells with this chip is extremely simple: the surface of the chip is treated with cell-adhesion molecules such as fibronectin or poly-L-lysine, then a few microliters of cell suspension and medium are added to the open chip, and the open chip is placed in a cell incubator to provide cells with the appropriate temperature and level of CO₂ to enable development of their phenotype. Whenever needed, the culture medium is changed by pipetting. In this way, cells can be maintained for any duration of time akin to cells cultured in T-flasks.

The open “Brain Chip” was named an overflow microfluidic network (oMFN), the design of which is shown in Figure 2.1.8(a). The oMFN is molded in PDMS, 150 µm deep, and possesses a central cell chamber having a volume of ~0.5 µL. The chamber is connected to inlet and outlet microchannels via flow-distributing structures, similar to the design described in chapter 2.1.2. The central features of the oMFN are the “overflow” zones containing capillary microstructures, which are adjacent to the cell chamber.

After cells had sufficient time to grow and develop their phenotype in the cell chamber, a Si lid having vias (800 µm in diameter) and fittings is placed by hand over the oMFN using alignment marks as visual aids. The lid (4 to 15 g in weight, depending on the type of ports and fittings used) is approached slowly until it touches the droplet of cell suspension located in the cell chamber. It is then let come into contact with the oMFN under its own weight.

Figure 2.1.8 – Design (a)-(b) and photographs of a PDMS oMFN before (c) and after (d) assembly with the Si lid bearing ports, fittings and tubings. Figure taken from [21].
Sometimes, gently pressing the middle of the lid using tweezers is necessary to trigger contact between the oMFN and the lid. During this step, excess liquid in the cell chamber flows over the chamber sidewall and connects with the overflow areas, Figure 2.1.8(b). The liquid is pulled away from the chamber owing to the capillary pressure generated by the microstructures arrayed in the overflow areas, and the lid conforms to the elastomeric oMFN. Not having the overflow areas results in having a thin film of liquid squeezed between the MFN and the lid, which makes the surfaces slide against each other unpredictably during assembly and the interstitial liquid will fill remote microfluidic structures, thereby entrapping air bubbles. The oblong microstructures visible in Figure 2.1.8 (b) have a length of 100 µm, a width of 60 µm, an intra-row spacing of 60 µm and inter-row spacing of 30 µm. These structures and their lattice were selected from earlier work on capillary pumps for microfluidic diagnostic chips. Excess liquid quickly fills the overflow zone with a straight filling front, thereby preventing air entrapment in the overflow area and pushing air toward the periphery of the oMFN. One overflow area can accommodate 4.35 µL of liquid; by connecting these areas to the alignment marks using microchannels, venting and draining of the overflow areas are achieved. Figure 2.1.8(c and d) respectively show a photograph of a PDMS oMFN and of an oMFN sealed with a Si lid having ports. Si was selected as a material for the lid because of its mechanical stability, flatness (the face of the Si wafer in contact with PDMS is polished), chemical resistance, and compatibility with adhesives such as those used for bonding ports. The footprint of the ports and tubings, visible in Figure 2.1.8 (d), can be reduced by soldering metallic wires directly to the lid if needed.

Figure 2.1.9 shows the sealing of an oMFN the cell chamber of which was covered with ~5 µL of water colored in red. The ports on the lids were connected via tubings to a reservoir (inlet port) and a high-precision computer-controlled pump (outlet port, Cetoni GmbH, Korbussen, Germany). The ports and tubings were filled with water prior to assembling the lid and oMFN to prevent the trapping of air in the microfluidic flow paths. Positioning the lid and having all excess liquid moved to the overflow areas took 5 – 10 s. After assembly, the liquid (colored in green) was pumped into the assembled microfluidic chip at a flow rate of 2 µL min⁻¹. Liquid was pulled through the chamber to create a negative pressure in the oMFN, and flow rates between 1 and 3 µL min⁻¹ were typically used. Prior to depositing cells, PDMS oMFNs were treated with a solution of polylysine (neurons) or fibronectin (other cells). PDMS oMFNs were exposed for 30 s to an O₂-based plasma to oxidize their surface and create the negative charges needed to immobilize polylysine via electrostatic interactions.
Cell lines (HeLa and SY5Y neuroblastoma) and primary cells from central nervous system (microglia, astrocytes, and neurons) were deposited in oMFNs. First, 5 µL of culture medium was placed on the cell chamber and then 2 µL of cell suspension was pipetted into the medium. Cells reached the surface of the chamber by sedimentation and were allowed to attach. Incubation of the cells proceeded for 1 (HeLa, SY5Y) to 6 DIV (primary cells). The oMFN was then closed as described above, and the morphology of cells and functionality of the sealed oMFNs were tested by staining the cells using vital dyes and using an inverted microscope. Figure 2.1.10(a) is a brightfield image of HeLa cells in the closed chamber. Images in Figure 2.1.10(b-c) were taken after cell membrane live staining in the closed microfluidic system. The images in Figure 2.1.10(a-c) show that cells grow normally in PDMS chambers. Figure 2.1.10(d-f) are fluorescence microscope images of primary cells fixed and retrospectively labeled with cell-specific marker to show that primary brain cells attach and grow normally on oMFNs. If needed, the lid can also be made from glass so as to visualize cells using an upright microscope or differential interference contrast microscopy.
We were not able to close and remove the lid repeatedly without compromising the viability of the cells. We think that the shear stress exerted on the cells by the liquid pulled with the cover and/or the small volume of liquid (<1 µL) left in the chamber account for this. Separating the lid from the oMFN while pumping medium into one port and having the second port closed, might solve this problem.

The concept of the oMFN was extended to a chip having two cell chambers. Figure 2.1.11(a) shows the photograph of a two-chamber oMFN assembled with a Si lid having 6 ports for fluidic connection. The oMFN has a footprint of 32 × 26 mm² and its two cell chambers are only 3 mm apart to enable visualization of both chambers simultaneously using a 4× microscope objective. The layout of the channels and corresponding ports allows liquids to be drawn sequentially, or independently if needed, through the chambers. The entire overflow zone can accommodate up to 48 µL of excess liquid. Typically, ~7 µL of cell suspension is used to plate and culture the cells in each chamber. Then, the chip is closed with the lid and cell culture medium is pumped through the chambers using ports 1 (inlet) and 6 (outlet).

A known biochemical pathway of intercellular communication between primary astrocytes and microglia was performed to validate the system: primary cortical astrocytes in the first chamber were exposed to 50 µM glutamate, a condition which is known to induce the regulated release of ATP. Upon transfer into the second chamber, the gliotransmitter activated purinergic receptors on microglia cells. For these experiments, astrocytes were cultivated in the left cell chamber for 3 DIV, while microglia were plated in the right chamber for 1 DIV. The oMFN was then closed using the lid, and regular morphology of the
plated cells was assessed by inverted microscope observation. A growth medium was perfused for ~3 min at a flow rate of 1 µL min\(^{-1}\) from port 1 to 6 before introducing a buffered solution containing 50 µM of glutamate and 20 µg mL\(^{-1}\) of propidium iodide (PI) dye for 20 min at a flow rate of 1 µL min\(^{-1}\). It is well known that glutamate-induced release of ATP results in activation of purinergic receptors on microglia cells, and in particular activation of ionotropic P2X7 receptor.\(^{48}\) Prolonged activation of P2X7 is known to induce the formation of an aspecific pore, measured by dye uptake, which is known to induce sustained intracellular calcium levels in microglia leading to the release of neuroinflammatory mediators.\(^{40}\) In our experiment, we showed that ATP conveying in the second chamber as a consequence of astrocyte stimulation with glutamate leads to an uptake of PI by microglia. The micrographs in Figure 2.1.11(b-g) show images (10× objective) of microglia after three independent experiments. Microglial dye uptake in Figure 2.1.11(c) indicates cell exposure to ATP, which was released by astrocytes, and convected to the second chamber. Direct PI uptake by the microglia following glutamate exposure is excluded because omitting the astrocytes in the first chamber did not lead to staining of the microglia by PI, Figure 2.1.11(e). As additional control experiment, the astrocytes were stimulated with glutamate, this time having an addition of oxidized ATP (oATP, 100 µg mL\(^{-1}\)) to the glutamate/PI solution. Oxidized ATP inhibits P2X7 receptor of microglia, and, as expected, a strong reduction of dye uptake by microglia was observed (compare Figure 2.1.11(g) with Figure 2.1.11(c)). Figure 2.1.11(h) shows the percentage of PI positive cells relative to the number of cells observed in brightfield for the two experiments (with and without receptor inhibition). Moreover, the oMFN is not permanently bonded to the Si lid, so cells can be fixed after the experiments by perfusing a 4% paraformaldehyde solution for 15 min followed with PBS, and then further analyzed after removal of the lid. The astrocytes and microglia used in the last experiment (glutamate + oATP, Figure 2.1.11(g)) were stained specifically for either GFAP or IBA (see supplementary information) after removal of the lid, and imaged using a fluorescence microscope, Figure 2.1.11(i) and (j).

These experiments are significant because they demonstrate that a pathway between two primary cells can be studied using an oMFN in the presence of, for example, inhibitors of cellular receptors. An obvious application of this method is to carefully screen for neuroprotective action of selected compounds in the context of synapthopathies. For more complex pathways or experiments requiring very precise stimulation of cell populations, flow tracers, such as polystyrene beads, can be added.

**Biochemical analysis of brain cell interactions in neuroinflammatory scenarios**

With the oMFNs, a microfluidic platform is available for researchers from the biological community to study cell interactions in more complex scenarios, like neuroinflammation. The microfluidic setup (oMFNs, Si lids with ports, syringe pumps and chip holders) were transferred into a biological laboratory in Milan to be used in a “non-microtechnological” environment. The simplicity of the oMFN concept together with the uniqueness of allowing cell culturing as with conventional cell culture dishes, made it easy to use by neurobiologists,
who are not experts in exploratory microfluidics. Our partners were able to perform experiments and observe brain cell interactions within two types of neuroinflammatory insults. Brain cells (astrocytes and neurons) were independently cultured in two separate chambers of oMFNs. The cell populations were individually primed with different stimuli and subsequent microfluidic connection allowed the observation of the specific contribution of each cell type by analyzing the cells using standard methods, such as electrophysiology, calcium dynamics, morphological characterization and vitality tests. With these experiments, they demonstrated the regional differences in glial control of physiopathology. No neuroprotective role was observed from hippocampal astrocytes, whereas a significant neuroprotective behavior could be accredited to astrocytes from the cortex. These observations were also made during intensified inflammation scenarios by exposing the cells to Aβ + IL-1β. The results of the study and a detailed description of the experiments are provided in the appendix in chapter 6.2.
2.1.4 References


L. Gervais and E. Delamarche, Lab Chip 2009, 9, 3330–3337.


2.2. The “Brain Chip” in the context of microfluidics for studying cell-cell interactions

The content of this chapter has been published:

Delamarche, E., Tonna, N., Lovchik, R.D., Bianco, F. and Matteoli, M.
Pharmacology on microfluidics: Multimodal analysis for studying cell-cell interaction
Current Opinion in Pharmacology, 2013, 13, 1−8.

Contributions – I contributed to the figures and helped writing one third of the review (The role of microfluidics in pharmacology).

2.2.1 Introduction

Cell-cell interaction studies represent a key topic in modern pharmacology. The ability of cells to exchange information with the surrounding microenvironment is fundamental in normal tissue homeostasis and defects in this crosstalk are at the basis of many pathologies, including cancer, neurodegenerative and cardiovascular diseases. Cell-cell interaction processes represent therefore primary targets for defining correct strategies to modulate disease progression. Although allowing identification of cell-cell interactions and cell signaling within native microenvironments, in vivo studies are limited by expensive experimental manipulations, lack of control over local conditions and need of complex imaging setups. Studies of cell-cell interactions in vitro have several advantages, due to more tightly controlled experimental conditions, higher experimental throughput, and lower costs. Microfluidic techniques are advancing the cell-cell interactions studies, by increasing our understanding of the effects of homotypic and heterotypic intercellular cross talks in fundamental processes, such as tissue morphogenesis, and by providing cell-based and organ-based platforms for preclinical drug and toxicity testing.¹,² These ‘cells on a chip’ and ‘organs on a chip’ will presumably have an important role in expediting early stages of drug discovery and help reduce reliance on animal testing. Finally, microfluidics helps clinical diagnosis of individual patient samples, allowing accurate biomarker expression analysis as an early step toward personalized medicine (see as an example Ciftlik et al.³).

2.2.2 The role of microfluidics in pharmacology

Microfluidic offers a broad range of potential benefits when working with cells. Microfluidic chips necessitate much less cells, biological samples and reagents than Petri dishes. Furthermore, cellular microenvironments and culture conditions can be tuned precisely and parallelization is readily achieved by splitting cell chambers and servicing them using microchannels. Using microfluidics for studying cells harbors specific challenges. First, the surface-to-volume ratio of microstructures increases rapidly with diminishing dimensions and can lead to depletion of chemicals in culture media. For this reason, cell chambers in
microfluidics are usually connected to inlet channels for continuous delivery of culture medium using external reservoirs and pumps. Second, materials used to fabricate microfluidic devices should promote cellular adhesion and not release cytotoxic chemicals. Third, cells should be monitored using transparent materials such as glass or PDMS or by integrating electrodes to measure cellular functions and integrity. Finally, the deposition of cells in microfluidic chambers should be efficient and simple. This is particularly challenging when distinct cell populations should be deposited in several chambers. The next part of the review specifically addresses this challenge for both arraying single cells as well as depositing two types of cell populations in microfluidics. We illustrate how cells can be deposited in "closed" microfluidic devices or in an "open" microfluidic device that can be sealed after cell deposition. The fabrication of microfluidics for long term cultures of cells has been described in detail elsewhere.4,5,6,7

**From single cells to multiple cell populations in closed microfluidics** – Early attempts for immobilizing single cells in closed flow paths used microfabricated cups that retained cells flowing in a buffer.8 Typically, such cups have a middle opening or limited height to let a stream of liquid pass and can be arrayed on a hexagonal lattice to increase trap-cell interactions. This method was recently revisited by Dittrich et al. who designed arrays of "microhurdles". The microhurdles are covered with a deformable, patterned PDMS membrane. Pressurization pushes down the membrane and creates a row of cell chambers, Figure 2.2.1(a). Typically ~60 chambers having a volume of 625 pL are used and have an occupancy of 0, 1 or 2 cells per chamber.9 Moreover, the surface of the chamber can be functionalized with a specific antibody for capturing intracellular proteins, secondary messengers or metabolites using ELISA.9 Lee et al. recently showed that single cells can be directed toward cavities adjacent to a main channel.10 There, the flow paths and cavity geometry limit cell occupancy: when one cell enters the cavity it increases its flow resistance, which diverts incoming flow. Migration of the trapped cell toward the center of the cavity restores flow and a second cell can enter. This ingenious strategy enables the formation of heterotypic cell pairs for studying intercellular communication.

An alternative method is to deposit cells in a chamber by balancing cellular adhesion with shear stress of a liquid on cells, Figure 2.2.1(b). In this example, Delamarche et al. used a PDMS microfluidic network (MFN) having a 0.5 µL cell chamber.11 The chamber is serviced by narrow inlets, all surfaces of the MFN are covered with fibronectin and the MFN is placed on an inverted microscope. Passing a suspension of cells at gradually lower flow rates increases the probability of depositing cells in the chamber. The optical image in Figure 2.2.1(b) shows deposited microglia (N9 type from mouse). This approach permits near single cell accuracy deposition.11

**Cells in open microfluidics** – The deposition of cells inside closed microfluidics can be challenging when working with neurons due to a slower adhesion process of neurons to surfaces. A variant of MFNs solves this problem by culturing neurons in open chambers that can be closed in a leak-free manner in the presence of liquid, Figure 2.2.1(c).12 These microfluidic chips, called "overflow" microfluidic networks (oMFNs), are made from PDMS; a cell suspension is placed in the cell chamber of the chip where cells sediment and adhere.
Figure 2.2.1 – From single cells to multiple cell populations in microfluidics. (a) A microhurdle can retain a single cell moving through a chamber and the cell chamber can then be sealed upon pressurization of an elastomeric sealing layer (1). Such chambers can be arrayed as exemplified in (2, 3) where cell chambers and distribution channels are filled with a blue or red solution, respectively. (4) Example of histiocytic lymphoma cells immobilized in a cell chamber array (cells are not visible at this magnification). (b) Deposition of cells in a closed microfluidic chamber by gradual reduction of the flow rate of a cell suspension passing in the chamber (1) and optical microscope image showing microglia deposited in the bottom of the chamber after 900 s (2). (c) Loading cells in the chamber of a oMFN proceeds with seeding and culturing cells in the chamber (1−3) before closing the oMFN using a lid having macroscopic ports for connection to pumps (4). (5) Fluorescence microscope image showing primary neurons in a chamber after 7 DIV. Images reproduced with permission and their source: (a) ref. [7], copyright 2012 RSC, (b) ref. [10], (c) ref. [11].

The chip can be kept in a conventional cell incubator. A lid having vias, fittings and tubings is then placed on the PDMS chip for sealing. During this step, excess liquid wicks from the chamber into adjacent microstructured areas. oMFNs are scalable and support multimodal analysis of cell-cell interactions.13,14

"Digital" microfluidics for cellular studies – Digital microfluidics (DMFs) refers to the precise positioning of droplets using electrowetting phenomena.15 Wheeler et al. recently developed a DMF chip for cell co-cultures.16 They defined openings in a Teflon® layer to create hydrophilic sites that can trap a fraction of a moving droplet of cell suspension, Figure 2.2.2(a). The device is flipped upside-down depending on how it is used: either the array of addressable electrodes (droplet manipulation) or the plate with cells and hydrophilic areas (cell culture) is at the bottom. Figure 2.2.2(b) shows 3 types of porcine aortic cells after a week of culture. This approach can be used to fix, permeabilize, stain, and stimulate cells with cytokines for endothelial cell/monocyte adhesion experiments.16 The authors also
monitored cell surface density and proliferation using impedance spectroscopy.\textsuperscript{17} Combining impedance spectroscopy with microfluidics has gained momentum in the last two years for studying, for example, drug resistance of cells,\textsuperscript{18} stem cell differentiation,\textsuperscript{19} and tauopathy in hippocampal slice cultures.\textsuperscript{20}

**Figure 2.2.2** – Cell co-cultures on a DMF chip. (a) The chip is composed of a top plate having hydrophilic sites and a bottom plate having addressable areas for directional wetting control (1, 2). Microliters of a cell suspension are moved across a hydrophilic site for trapping some of the suspension (3). Flipping the chip upside down enables cell sedimentation and culture (4, right) or moving solutions by means of electrowetting (4, left). (b) Phase contrast images showing 3 types of cell cultures in distinct hydrophilic areas. Images reproduced with permission from the Royal Society of Chemistry, ref. [15].
Microfluidic chips with "forbidden" zones – In contrast to the examples above, "forbidden" zones for cells can be implemented in chips. Jeon et al. demonstrated this by creating two parallel compartments, which are connected by an array of microgrooves through which only neurites can extend.21 This allows localizing chemical insults to the neuron bodies. Placing two sets of neurons in each chamber, Schuman et al. studied synapse-to-cell signaling.22 In a variant approach, Peyrin et al. connected chambers using microchannels of diminishing lateral dimensions. They called this array of microchannels "axonal diodes" because the channels imposed unidirectional axon connectivity with a high selectivity of 97%.23 In such devices, co-culture of neurons over 2 weeks was demonstrated. Microfluidic chips, which had neurons co-cultured with pituitary cells releasing growth hormone, were also coupled to a mass spectrometer for the analysis of cell secretion and the authors demonstrated inhibition of growth hormone secretion upon dopamine release from the neurons.24 Finally, an ingenious concept was implemented on similar microfluidic devices by Chang et al. who pre-diced the glass bottom layer of microfluidic chips around the regions where axons develop.25 Cleaving the chip yielded glass stripes from which axons were harvested for analysis by means of gel analysis and RT-PCR.

2.2.3 Integrating microfluidics for studying cell-cell interaction

Microfluidics to reproduce the vascular microenvironment – Vascular endothelial cells are highly responsive to shear stress caused by the flow of fluid over their surface.26 Since cell–fluid and cell–cell interactions are critical components of many physiological and pathological conditions in the microvasculature, it is no wonder that one of the biological fields that has greatly improved from the implementation of microfluidics is the study of vascular physiology. By taking advantage of the small dimensions and laminar flow which typically characterize microfluidic systems, novel in vitro models have been created which reproduce many characteristics of the in vivo vascular microenvironment.26,27 Using microfluidics, it has been shown that a flow rate of less than 200 µL per hour is enough to make the sheared endothelial cells elongate and orient in the direction of the flow, which is a prominent feature of the endothelial mechanoresponse that is also found in vivo.26 Fluid shear stress has been shown to attenuate endothelial cell sprouting in a nitric oxide-dependent manner, while positive VEGF gradients initiate sprouting, thus indicating that endothelial cells are able to integrate signals from fluid forces and local VEGF gradients to achieve varied goals as vessel dilation and sprouting.28 In vivo-mimetic microfluidic devices have been recently developed which accurately reproduce microvascular networks digitized from in vivo images of rodent vasculature onto a microfluidic chip and which have been used for cell culturing and to study adhesion profiles of functionalized particles.29 Microfluidic systems integrating control over paracrine and direct physical cell-cell interactions under controlled microenvironmental conditions have been also utilized for improving scaffold vascularization, which is of critical importance in tissue engineering applications.30 Notably, a microfluidic device has been developed for the fixation and long-term culture of an isolated blood vessel on a microfluidic chip. This platform enabled long-term culture of the explants, on-chip imaging, immobilization, and spatiotemporal controlled delivery of drugs.31
could foresee that, in the future, this type of devices may enable the use of automated and standardized screens, providing important information about the bioavailability of new drugs through blood–tissue interfaces.

**Microfluidics in cancer research** – In cancer, the communication between tumor and surrounding cells is key in driving the process of tumor progression, with the microenvironment providing a fertile ground of cellular and humoral factors supporting tumor growth, angiogenesis, invasion and metastasis. The recent application of microfluidics to cancer research offers enormous potential to develop improved therapeutic strategies, by supporting the investigation of tumor angiogenesis and metastasis under physiologically relevant flow conditions. Moreover, working on the microscale enables to carry out functional studies, otherwise hampered, also on selected populations of cancer cells which typically represent a very rare subtype of a larger population. The use of microfluidics in cancer research has led to major findings, allowing to demonstrate that brain tumor cells are stiffer than benign cells and that membrane capacitance of ovarian cancer cells increases as the stage of malignancy advances from very benign to the most aggressive stage and allowing isolation of circulating tumor cells from the blood samples. In relation to cell-cell interaction in cancer, the Kamm’s group has developed microfluidic-based chips to monitor tumor cell-endothelial cell interactions in real-time, while enabling accurate control of biochemical and biophysical factors within a 3D matrix. In this system, not only paracrine interactions through soluble factor communication between both cell types were recreated, but also physical contact interactions once the tumor cells adhere to the endothelial monolayer. Use of this device allowed to show that signaling with macrophages via secretion of TNF-α results in endothelial barrier impairment and that blocking macrophage-secreted TNF-α reduced the intravasation rate and normalized endothelial barrier integrity. Microfluidic devices which allow cell seeding in multiple adjacent 3D gel channels to more accurately resemble the tumor microenvironment have also been designed, and some of them allowed to obtain 3D tumor models avoiding the frequent non-uniform distribution of cells and allowing more accurate studies of drug uptake. At the level of cancer treatment, microfluidic devices offering high-throughput operational capability and massive parallelization have been designed, including a robust automated microchannel platform for 3D cell cultures and a microfluidic-based multiplex platform for empirical chemosensitivity assays.

**Microfluidics to investigate neuroinflammation** – Microfluidics represent a valuable tool also for analyzing intercellular communication mediated by transmission of soluble signals, which is typical of inflammatory scenarios. A great example of this process is neuroinflammation, which is thought to contribute to the processes of neurodegeneration. Neuroinflammation is mainly sustained by the brain resident cells, microglia, i.e. the brain immune cells, and reactive astrocytes. In spite of the evidence indicating that chronic inflammation might influence the pathogenesis of neurodegenerative diseases, such as Alzheimer’s and Parkinson’s Diseases, there is considerable debate concerning which
molecules are synthesized and released, how astrocytes and microglia interact reciprocally and with neuronal cells, and how the kinetic responses and the precise connectivity of the inflammatory cascades are perturbed in chronic disease states. Microfluidics has been recently applied to investigate the role played by astrocytes derived from different brain regions in controlling neuronal viability under exposure to amyloid beta oligomers (Aβ), Figure 2.2.3. Primary astrocytes from different brain regions were plated and cultured in separate chambers of an open microfluidic network oMFN, either in isolation or in microfluidic biochemical communication with hippocampal neurons. A significant detrimental effect was observed in neuronal viability and functionality when cultured in the presence of hippocampal but not cortical astrocytes. These data, besides providing support to the view that astrocytes from different brain regions differently affect neuronal viability upon exposure to inflammatory stimuli, confirm microfluidics as a powerful method for investigating the specific dynamics of molecular mechanisms involved in the crosstalk among different cell populations during neuroinflammatory events.

**Figure 2.2.3** – Investigation of cell-cell interactions in a two-chamber oMFN. (a) After depositing and culturing two cell populations in an oMFN (1), the device is closed with a lid carrying tubings and fittings for connection to pumps. (2) Table illustrating experimental combinations for stimulating cell populations in the chambers with a specific example of stimulating cells in chamber A and followed by flushing medium from chambers A to B. (b) Experiments and selected fluorescence images corresponding to the stimulation of hippocampal (HAs) and cortical (CAs) astrocytes with Aβ + IL-1β followed by co-culturing with hippocampal neurons (HNs). (3) HNs in microfluidic communication with HAs experience high death rates possibly due to glutamate excitotoxicity because the presence of APV significantly reduces cell death. Images reproduced with permission from ref. [14].
2.2.4 Conclusions

With comparison to the conventional drug research techniques, miniaturized cell chips possess significant inherent advantages from their microscopic size, thus providing versatile approaches to mimic in vivo conditions for more realistic cell-based drug research. Although cell chips hold immense promise as a universal platform and powerful tool to investigate cell–drug interactions, they are still at an infant stage of development and most of them are proof-of-concept demonstrations. There are many hurdles to overcome before cell chips can be used for practical applications. Non-specific protein adsorption and undesired liquid evaporation are just some of the issues which must be overcome. Cell chips need to be standardized in terms of materials, interfaces and channel geometries for experimental protocol assessment and data comparison. Ease of handling and system robustness have to be achieved for reliable and simple implementation. The device design should be user-friendly and avoid unnecessary operational barriers. In addition, data interpretation for a novel device is also challenging, thus, more research should be performed to reconcile differences with data collected using conventional methods. Nevertheless, once these aspects are taken care of, microfluidics will represent an irreplaceable tool for modern pharmacology that needs to study cell-cell interaction.

2.2.5 Acknowledgements

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2.2.6 References

3 MICROSCALE TISSUE ANALYSIS USING THE MICROFLUIDIC PROBE
3.1. Introduction to the Microfluidic Probe

Some parts of this chapter have been extracted from the following publications:

Lovchik, R.D., Drechsler, U. and Delamarche, E.
Multilayered microfluidic probe heads

Kaigala, G.V., Lovchik, R.D. and Delamarche, E.
A vertical Microfluidic Probe

Contributions – I developed the multilayer MFP heads and performed the experiments with this second generation MFP heads. I made all the illustrations and lead the writing of the first publication above. The new vertical MFP heads (third generation MFP) were my idea and I designed and assembled the first batches of chips. I developed the fabrication process and designed the holder for the vMFP heads. This approach was published in a proceeding, where I lead the writing. The subsequent experiments, published in Langmuir (second publication above) were performed with G.V. Kaigala. My contributions to this publication were: Designing and fabricating vMFP heads, setting up the vMFP platform, performing the experiments using cell cultures and developing the method for using vMFP heads with integrated immersion liquid dispensing. I co-wrote the publication and prepared the illustrations.

3.1.1 Background

Processing of surfaces using low volumes of liquids with spatial confinement is in many contexts a powerful capability. Developing such a technology to be used in conjunction with low volumes of liquids is one of the central goals of the microfluidic research community. It is common to make use of closed microchannels within which numerous applications have been demonstrated. However, high hydraulic resistance, the difficulty to introduce samples such as tissues, cells and mesoscopic objects into microchannels, and clogging by particulates and air bubbles strongly limit the practical use of closed microfluidic systems to perform localized bio(chemistry) on biological surfaces. Moreover, biological samples are sensitive to their physical environment and are prone to drying artifacts and denaturation.

Several strategies have been developed by the research community to overcome the limitations of closed microfluidics in their ability to pattern, process and analyze samples on surfaces. Inkjet is the most widely used technology to deposit liquids on surfaces; early development of this technology was done by the industry, in particular by IBM, HP and Cannon. Inkjet technology is presently limited by several artifacts, including drying effects, the inability to pattern surfaces in liquid environments and the physics of liquid ejection, which restricts the range of geometrically defined confinements of chemicals on surfaces.
Also, the thermal/pressure variations in the droplet ejection may result in denaturation of biomolecules employed as ink in inkjet technology.

One technique based on microfluidics for processing and interacting with surfaces involves the use of two capillaries placed atop a surface to form a closed fluidic system. With this technique chemical reactions are performed on surfaces and applied to: electronic wafer-probing, patterning fluid lipid membranes onto a glass substrate, a droplet-based system to enable localized electrical stimulation and recording of chemical species. While this technique is suitable for certain applications, it is desirable that the delivery/probing tools are not in physical contact with the surface. Another microfluidic approach is the use of micro/nano pipettes to process surfaces within liquid environments while not needing physical contact with a surface. This involves filling a pipette with electrolytes and submerging the pipettes within a liquid medium over a surface. The charged species are delivered on the surface by applying an electric potential. Some examples of the use of nanopipettes are in the controlled delivery of DNA and proteins, scanning of biological surfaces, delivery of individual molecules to cellular compartments and electrochemistry. A related implementation is scanning electrochemical microscopy to deposit gold on glass implemented in the form of a fountain pen. These techniques of applying an electric field need careful design of the electrolyte system, buffers and biological mediums to enable the electromigration of charged species – designing such a system can be particularly challenging for complex biological/clinical medium. What is critically needed is a non-contact approach that operates in a liquid environment and is minimally sensitive to the physiochemical properties such as charge and solubility of biological mediums and surface topographies.

Other demonstrations of localized bio(chemical) surface processing are dip pen nanolithography (DPN) and atomic force microscopy (AFM)-based approaches. A particularly interesting development of the DPN is the use of feed channels connected to the tip of the AFM to deliver liquids. Hollow AFM probes have also been developed for patterning biomolecules on surfaces to scan regions in the mm-scale for cellular applications and the deposition of oligonucleotides. In such demonstrations, the piezo stage is suitable to process hundreds of nanometers of surfaces in the horizontal dimension and only a few nm-range of variations in the vertical dimension. However, cellular and tissue assays often need processing in the mm-range (and beyond) in the horizontal, and in the µm-range in the vertical dimension. Additionally, operating such AFM-based techniques in conjunction with cells/tissues is challenging because the feedback needed to position the cantilever is confounded by its operation in liquid medium. Along these lines is a related demonstration of dispensing reagents to mammalian cells in liquid medium assisted by a polymeric aqueous two-phase system.

An approach where flow of liquids is created on the surface and confined spatially solely by liquid boundaries is highly desirable. It is for such implementations that the characteristics inherent to microfluidics such as, fluid dynamics under low-Reynold number conditions can be fully exploited. Further, performing chemical and biological assays in this regime of liquid flows can be applied to a mode of non-contact probing/processing of surfaces. Our
laboratory at IBM Research developed a microfluidic technology, the MFP, in which a microfabricated probe head is the key component and which interfaces with the surfaces during processing. The MFP head consists of two apertures, one to inject liquid and another to aspirate liquid. The injection/aspiration of the processing liquid is performed within an immersion liquid. The immersion liquid assists in the hydrodynamic focusing of the processing liquid at the MFP head. This approach overcomes the need to insert samples into closed microchannels. A few examples of what has already been demonstrated with the MFP are: the lysis of a single human breast cancer cell followed by collecting and analyzing the messenger RNA released from the lysate, microperfusion of brain tissue, delivery of multiple compounds for pharmacological screening of adhered cells. A variant to this approach is a dual pipette for controlled drug infusion.

I have been developing the MFP technology and demonstrated its capabilities to process surfaces and perform (bio)chemical events within liquid environments. The following chapters explain the working principle of the MFP and as well show the evolution of the technology during the past few years I was working on the project. Examples of applications for localized (bio)chemistry on surfaces, e.g. microscale tissue analysis in chapter 3.2, are provided and the potential of the MFP as a tool in life sciences discussed. A detailed review on the MFP within the context of other “open space” microfluidics is found in chapter 3.3.
3.1.2 Working principle of the MFP

The key component of the MFP is the microfabricated head. The head comprises at least two microfluidic channels that exit to the open space in close proximity. We call these openings of the channels apertures. During operation, the apex of the head comprising the apertures is brought into close proximity with the surface of the sample, surrounded by an immersion liquid. The injection of a processing liquid through one of the microchannels and simultaneous aspiration at a higher flow rate through the other channels form a hydrodynamic flow confinement of the processing liquid within the immersion liquid. At gap heights of 1 to 30 µm between the MFP and the sample surface, the confined processing liquid is in contact with the sample surface and allows for (bio)chemical reactions to take place. Figure 3.1.1 shows a MFP head and illustrates how the liquid flows hydrodynamically confine the processing liquid (red) on top of the substrate.

Figure 3.1.1 – MFP working principle. (A) Photograph of a microfluidic probe head consisting of glass and silicon sealed to create micron-sized channels. The MFP head consists of via/ports to couple liquid in and out and an apex that is polished to enable a stable flow-confinement of pL’s of processing liquid. (B) Schematic of a two aperture vertical microfluidic probe head showing two microchannels, the channel on the left (red) here is configured to inject the processing liquid, and the channel on the right is configured to aspirate both the processing liquid and the immersion liquid present on the surface. (C) Schematic of a close-up of the tip of the MFP head interacting with the surfaces shown here are typical dimensions and flow rates both for injection and aspiration to achieve a stable flow confinement. Figure taken from [1].
The MFP platform

The basic platform for an MFP typically consists of a microfabricated MFP head, peripherals for handling liquids, a motorized precision X-Y-Z stage and an inverted microscope, Figure 3.1.2. The head is a key component of the vMFP platform and is described in detail in the remainder of this introductory chapter. The equipment used to hold the head and to scan the substrate is mounted on the stage of a standard inverted microscope (Nikon Eclipse TE300, Egg, Switzerland). The head itself is housed within a custom-made holder connected to an angle table for tilt correction. The unit for leveling and scanning the substrate consists of an adjustable holder onto which the substrate is clamped and two motorized linear drive units for X and Y positioning. Processing of a substrate was performed by programming the movement of the substrate relative to the vMFP head that is maintained stationary during operation. For the injection and aspiration of liquids, Nemsesys pumps (Cetoni GmbH, Korbussen, Germany) with syringes of 500 µL volume (Hamilton 1705 TLLX, Banaduz, Switzerland) were used. The fluidic connection of the pumps to the head uses standard 1/32 inch transparent tubes with PEEK fittings. The surfaces were monitored using the inverted microscope in the brightfield or in fluorescence mode. The patterns on the surface were recorded using a digital camera (Sony CDR-SR100E, Schlieren, Switzerland). For high-resolution imaging of surface, an upright microscope (Nikon, Eclipse 90i, Egg, Switzerland) with a cooled, low-noise high sensitivity CCD camera (ST-8, SBIG, Santa Barbara, USA) was used.

Figure 3.1.2 – Key components of the vMFP platform. (a) A motorized, high precision X, Y, Z stage controls the movement of the head and the surface to be processed. The tubing leads to external pumps and visualization of the surface is performed using an inverted microscope (not shown). (b) Photograph of a custom-built holder that mounts the head and couples to the tubing that connects to the external pumps. (c) Patterns of fluorescently-labeled antibodies on a glass surface were formed using a vMFP and visualized with an inverted microscope. Figure taken from [1].
3.1.3 Technological roadmap of the MFP

First generation MFP (monolithic MFP heads)

The first generation MFPs were developed by Juncker et al. at IBM Research, Figure 3.1.3.\textsuperscript{21} The part of the head that confines the liquid is a Si chip that has two apertures and is brought into close proximity of a substrate of interest. Horizontal microchannels on the other face of the chip link the apertures with vias formed in a PDMS connection block, Figure 3.1.3(a). Capillaries inserted in the PDMS provide a connection between motorized pumps and the apertures. Accordingly, confinement of an injected liquid is realized by controlling the flow rate of the liquid injected into one aperture and by reaspirating it together with some surrounding immersion liquid, Figure 3.1.3(b). An assembled MFP head is shown in Figure 3.1.3(c).

Figure 3.1.3. – Assembly of a monolithic MFP head with a PDMS connection block. (a) A typical MFP head comprises a PDMS block having vias and a corresponding microfabricated Si chip. (b) Glass capillaries inserted into the vias of the PDMS ensure a fluidic connection to peripheral pumps. Injection of a processing solution through an aperture and simultaneous aspiration through a second aperture in the presence of an immersion liquid result in a directed flow of the processing solution along a nearby substrate. (c) Photograph of an assembled MFP head before insertion of the glass capillaries. Image taken from [22].
Although the MFP heads developed by Juncker et al. were versatile and applicable to a broad range of problems, some challenges remained to be solved. First, the assembly of the Si chip with the PDMS connection block followed by the insertion of the glass capillaries is labor-intensive. Second, these operations also have limited yield because the Si chip and PDMS are small and difficult to handle, operations also have limited yield because the Si chip and PDMS are small and difficult to handle, and stress in the PDMS during bonding to the Si chip and insertion of the capillaries can lead to the detachment of the PDMS. Third, PDMS is not compatible with many chemicals and solvents, which restricts the use of the such heads mostly to aqueous-based solutions and ethanol. Finally, microfabrication of small apertures in a thick Si wafer using DRIE can be challenging, time-consuming and expensive. Although these limitations may be considered as minor inconveniences in research, they can considerably hinder the large-scale deployment of the MFP technology in the fields of lithography and diagnostics.

**Second generation MFP (multilayered MFP heads)**

For improved yield in the fabrication of MFP heads and easier handling, we developed a second generation MFP heads, the *multilayered* MFP heads. These new heads were easier to fabricate and to package than heads made with unitary construction and could be interfaced with tubings using standard fittings. Figure 3.1.4 shows the concept of the multilayered MFP head with the ports used to connect the head to mechanical pumps (a) and a cross section of the assembled device (b). The most critical structures in the head are the apertures. Their

![Figure 3.1.4. – Concept of a multilayered MFP head. (a) The PDMS connection block is replaced by standard ports for tube fittings. (b) Side view of a multilayered MFP head, where a first layer having vias brings the ports into microfluidic communication with the microstructures of a second layer. The second layer faces a substrate to be processed and holds the structures responsible for hydrodynamic flow confinement. Figure taken from [22].](image-url)
shapes, dimensions and relative positions, which are defined on a design level, govern the HFC of a liquid over a surface to a large extent. Other parameters contributing to the HFC are the injection and aspiration flow rates, trajectory and speed of the head over the substrate, and the separation distance between head and substrate, all of which can be changed interactively. The need to interface the apertures to peripheral pumps is solved by linking the upper part of the apertures to microchannels that are spread out horizontally to leave sufficient space for bonding the macroscopic ports.

Multilayered MFP heads were microfabricated using Si wafers, although other materials can be used. We call the upper part of a multilayered MFP head the Si lid and the lower part the HFC chip. The Si lid has large (of approx. 1 mm in diameter) vias that connect structures between the ports and the HFC chip. The HFC chip comprises all microstructures. Microchannels on the upper side of the HFC chip provide a fluidic connection between the vias and the apertures located in the center of the HFC chip. These apertures, which have typical lateral dimensions of a few tens of micrometers, are etched completely through the HFC chip. Posts around the mesa are used as leveling aids when adjusting the MFP head for experiments. Setting the MFP head co-planarly with the substrate is necessary for preventing accidental contact between the mesa and the substrate.

A multilayered MFP head requires three photolithographic steps (e.g. coating, exposing and developing the resist followed by etching Si) to make the HFC chip and one step for the Si lid. In comparison, previous monolithic heads required only three steps. Those heads, however, also required molding a PDMS connection block that had to be plasma-treated and bonded to a Si chip. Using PDMS is more labor-intensive, and inserting the capillaries into the PDMS connection block without compromising the adhesion of the PDMS to the MFP chip or producing debris can be delicate. Employing PDMS also restricted the choice of liquids and solvents used for processing a substrate to those compatible with PDMS. These challenges were solved with the multilayered MFP heads. Moreover, in such heads apertures can be fabricated more accurately when a thin Si wafer is used for the HFC chip, whereas the lid wafer can remain thick to provide mechanical strength to the head. Finally, the Si lid can have a standard layout, and changing the geometry of the apertures only necessitates changing the design of the HFC chip.

Two different sizes of MFP heads were produced. In Figure 3.1.5(a), a small 3 × 7 mm² multilayered MFP head carrying the mesa and posts is shown from the side facing the substrate. The aspirating aperture of this particular head is hemispherical, and the injection aperture is round and centered in the mesa. This MFP head can be connected to the pumps by directly mounting capillaries into the vias. Figure 3.1.5(b) is a photograph of a 10 × 20 mm² MFP head with ports. A magnified view of a via over the beginning of a horizontal microchannel is shown in Figure 3.1.5(c). The oblong structures in the microchannels were designed to prevent the collapse of the materials tested during the optimization of the bonding step. Four materials were tested for bonding the two wafers: (1) a spin-coated 2-µm-thick Teflon AF adhesive (DuPont, Geneva, Switzerland), (2) a capillary underfill (Epotek U300-2, Polyscience AG, Cham, Switzerland), (3) a spin-coated 2-µm-thick polyimide, and (4) a ~1-mm-thick plasma-treated PDMS membrane. All materials were tested using the same MFP
head design. Best yields were achieved using polyimide as adhesive, and therefore this material was used throughout the work. With polyimide as bonding adhesive, the oblong structures can be omitted. In Figure 3.1.5(d), a multilayered MFP injecting a red-colored liquid at a flow rate of 2 µL min⁻¹ in water as immersion liquid and aspirating at a flow rate of 15 µL min⁻¹ is shown. The substrate was a glass slide, and the gap between the mesa and the substrate in this image is ~10 µm.

**Figure 3.1.5.** – Multilayered MFP heads. (a) Photograph of the bottom side of a MFP head with the apertures, the mesa and the four supporting posts. (b) Photograph of a MFP head with ports mounted on the Si lid of the device. (c) Micrograph showing the via of a Si lid before mounting the port. The oblong structures belong to the beginning of the horizontal microchannels of the HFC wafer. (d) Micrograph of a MFP in operation. A red solution flows from one aperture to the other with an injection flow rate of 2 µL min⁻¹ and water as immersion liquid. The aspiration flow rate is 15 µL min⁻¹. The gap between the mesa and the substrate (glass slide) is ~10 µm. Figure taken from [22].

The development of multilayered MFP heads proved to be particularly useful for surface-processing applications, which, unlike biological applications, deal with potentially smaller patterns and a broader range of liquids and chemicals. Employing a thin Si wafer (e.g., 100 µm in thickness) to fabricate the HFC chip, it should be possible to fabricate well-defined apertures with lateral dimensions of less than 10 µm using conventional DRIE or focused ion beam. In this case, the mechanical strength of the head would be provided by the Si lid. The use of standard ports and fittings and replacing the molded block of PDMS reduces the work needed for assembling a head. Multilayered heads are also more amenable to using many processing liquids because apertures can be small and close to each other with the horizontal microchannels fanning out sufficiently, thus leaving sufficient space for adding many ports on the Si lid.

Illustrations of the potential of multilayered MFP heads for patterning continuous and discontinuous patterns of biomolecules on surfaces as well as for direct processing of resist materials in a non-contact mode are shown in chapter 3.1.4.
Third generation MFP (vertical MFP heads)

The concept of multilayered MFP improved the ease of use and yield in the fabrication of classical MFP devices. From the microfluidic point of view, the multilayered MFP heads worked just fine. Using microfabrication it is simple to fabricate many channels and apertures in parallel, in principle allowing multi-aperture MFP heads to be designed. This would allow multiplexing of different chemical reactions and generation of complex flow profiles, e.g. parallel laminar flows of processing liquids within one flow confinement. The limiting factor for this is typically the chip-to-world interface. The MFP heads become larger, the more tubings for liquid supply or removal are needed. This was the main reason to reconsider the concept of using microfabricated through-silicon vias facing the substrate to be processed. A, retrospective obvious, solution for keeping the footprint of MFP heads small and still being able to multiply the number of liquid connections, was to develop microfluidic chips as MFP heads, which instead of being operated co-planar to the surface, are vertically oriented. The result was a vertical MFP head (vMFP) with all features in-plane and with the flow confinement at the apex of the vMFP head. The base configuration of the vMFP head, has a single channel for injection of the liquid and another channel for aspiration. The vMFP head is a hybrid Si/glass microfluidic device consisting of several elements such as vias, microchannels (flow-paths), filter structures and an apex that altogether play a critical role in defining the key characteristics of the flow confinement and system response time. Vias interface with the tubing and the external pumping peripherals. The preferred dimension (diameter) of the vias designed were between 100 \( \mu \text{m} \) and 500 \( \mu \text{m} \): vias having these dimensions are reasonably large to enable simple interfacing with the external fluidic peripherals, have sufficiently low dead volume resulting in a system response time in the order of seconds, and are easy to fabricate without requiring to etch high aspect ratio structures. The apex physically supports the flow confinement, comprises apertures and had an area of approximately 1 mm\(^2\). We chose this area as our earlier work suggests that the apex (termed ‘mesa’ in the context of planar MFPs\(^1\)) should be more than 10-fold larger than the spacing between the apertures to ensure that the flow confinement remains unperturbed to conditions such as convection in the immersion liquid. In addition, an area of 1 mm\(^2\) is still sufficiently small to easily level the apex and manipulate the head relative to the surface.

The microchannels connect the vias to the apex providing the flow-paths to the apertures. For optimal flow confinement and switching times, we designed appropriate width, depth, spacing and number of channels. We typically used microchannels that are 20 \( \mu \text{m} \) deep, 200 \( \mu \text{m} \) in width near the vias and which taper to 20 \( \mu \text{m} \) at the apertures. These structures have sharp walls, and if needed can be isotropically etched to form curved walls.\(^{28}\) Structures such as posts/pillars are included along the flow-paths to form filter elements to capture particulates. We designed the dimensions and spacing of the posts to ensure that they do not contribute more than 10% of the total resistance of the flow path. Such posts were grouped in areas for filtering particles consecutively in the ranges of 10 \( \mu \text{m} \), 5 \( \mu \text{m} \) and 1 \( \mu \text{m} \). The overall dimension and shape of the head is determined by the functional elements that need to be integrated, the fabrication process and the application. The heads were typically a rhombus with a surface area of 72 mm\(^2\), apex with 1 mm\(^2\) and thickness of ~0.9 mm, see Figures in chapter 3.1.2.
Fabrication of vMFP heads

Vertical MFP heads were composed of a Si layer (400 μm thick) holding the microfabricated structures that was bonded with a glass layer (500 μm thick), Figure 3.1.6(a). The microchannels and vias were made in a 4 inch Si wafer (Siltronix, Geneva, Switzerland) using standard photolithography with a photoplotted mask (64,000 dpi, Zitzmann GmbH, Germany) and deep reactive ion etching (DRIE) as shown in Figure 3.1.6(b). The microchannels were typically 20 μm deep. A glass wafer (Borofloat® 33, SCHOTT AG) was anodically bonded to the processed Si wafer at 450 °C and 1250 V. Channels/apertures of the vMFP heads were loaded with (molten) wax (OCON 199, Logitech GmbH, Germany) heated to 80 °C. After dicing, four vMFP heads were simultaneously mounted on a custom-made holder for lapping and polishing of the apex. The vMFP heads were immersed in heptane for two hours at room temperature to remove the wax.

Figure 3.1.6 – Fabrication of vMFP heads. (a) Schematic of a vMFP head and close-up view of the apex showing etched structures in Si. (b) Illustration of the process steps and photographs of key stages in the fabrication of vMFP heads. Figure taken from [1].
Design strategies for vMFP heads

Several designs and strategies for the fabrication of vMFP heads, which greatly helped fabricating the heads with high yield are given below. For example, an ubiquitous issue with fabricating microfluidic device is the risk of clogging microchannels with particulates, particularly during dicing and polishing. We used capillary forces to fill the apertures and microchannels of the heads with molten wax, Figure 3.1.7(a). This was done on wafer level using channels that connected rows of pairs of apertures and that were fed from a single channel. Dicing the vMFP heads disconnected the apertures from the channels servicing the wax, Figure 3.1.7(b). Figure 3.1.7(c) shows the apex of a non-polished vMFP head with wax filled in the apertures. It took only a few minutes of lapping and polishing to yield a vMFP head with a flat apex and, typically, a head was stored with wax in the apertures until it was used.

The microchannels linking the apertures to the vias used for liquid injection and aspiration provide ample room for implementing various microfluidic components. Figure 3.1.7(d) shows a series of posts, which have diminishing dimensions and spacing in the direction from the via to an aperture. These structures prevent clogging of the apertures by particulates that enter the head with the injection liquid. Other components than filters can be electrodes and heaters for electrochemical detection and temperature optimization of processing liquids, respectively.

The in-plane fabrication of microchannels provides interesting possibilities for vMFP heads that are not practical to implement in case of planar MFPs. For example, apertures can be inclined and serviced with curved microchannels as in Figure 3.1.7(e). This opens possibilities such as: (1) stretching the footprint of the processing liquid along a surface of interest, (2) giving more in-plane momentum to a processing liquid to move/align objects on a surface, or (3) defining specific shear stress conditions locally on a surface for studying cell adhesion, mechanotransduction and tissue engineering, as a few examples. Polishing small structures such as the apex of one vMFP head (with an area of about 1 mm²) can be challenging because small changes in the pressure applied to the head during polishing/lapping can dramatically affect the polishing/lapping rate. This is particularly a concern if the head has inclined injection and aspiration microchannels since the depth of polishing must be stopped exactly where a desired inclination of the channels is obtained. We therefore designed heads having repeated motives at the apex, Figure 3.1.7(f). Vertical trenches were also added on each side of a motif. As a result, polishing can be stopped and the head inspected using a microscope to verify that polishing/lapping has occurred to the desired depth. If excessive polishing has occurred, only one motif is lost but not the head, and polishing proceeds using the next motif.

Interesting footprint geometries of the processing liquid on a surface can be obtained by having more than two apertures and/or by implementing a symmetric flow aspiration. This may be achieved as shown in Figure 3.1.7(g) by splitting the aspiration flow path and implementing a hydraulic resistance on one of them to equalize flow resistances.
vMFP heads were used for many experiments on biological samples. Results on the performance of the device for local staining/treatment of cells or tissue cuts are shown in chapter 3.1.4 and 3.2.

**Figure 3.1.7** – vMFP head design and packaging strategies. (a) Photograph of a processed Si/glass substrate with 33 vMFP heads. (b) Schematic showing the filling of molten wax into the channels servicing several vMFP heads. (c) Unpolished vMFP head with wax filled in the apertures and microchannels. (d) SEM image showing posts with varying dimensions along the flow path to trap particles. (e) Illustration of a vMFP head consisting of an angular flow path to generate in-plane momentum to the processing liquid for manipulation of objects on surfaces. (f) Redundancy in structures and trenches designed as polishing aids to increase the yield in fabrication. (g) Schematic of a head showing three apertures/channels to support a symmetric flow confinement. Figure taken from [1].

**Integration of immersion liquid supply channels into vMFPs**

The immersion liquid is central to the flow confinement and local processing of surfaces. In previous work and preceding sections, the immersion liquid was supplied in hundreds of microliters to milliliter quantities onto the surface of interest prior to operating the vMFP using a pipette. Resupply of immersion liquid was sometimes needed due to its evaporation and fast consumption when high flow rates were used. Aspiration flow rates can reach up to 10 μL min⁻¹, approximately 80% of which is composed of immersion liquid. For these reasons, vMFP heads were developed integrating the immersion liquid dispensing. This
integration was obtained by adding a third via to the head, which serviced peripheral microchannels that open on the sidewalls of the vMFP, Figure 3.1.8a. Directing the channels for immersion liquid to the sidewalls of the vMFP helps keeping the apex small. These vMFP heads dispense the immersion liquid locally where it is needed while the surrounding area of the substrate can be kept dry. Further, the volume of the immersion liquid encapsulating the apex can be changed by iteratively adjusting the dispensing rate of immersion liquid flow. If the channels providing the immersion liquid are high on the vMFP head, grooves along the sidewalls of the head can be formed to guide the immersion liquid towards the apex. In order to maintain symmetry in the immersion liquid encapsulation at the apex and to reduce interfaces to the external fluidic peripherals, channels were designed with equal hydraulic resistances and merged the two dispensing channels into a single via, Figure 3.1.8a. The stability of the immersion liquid at the apex of the vMFP head was tested at a flow rate of 3 \( \mu \text{L min}^{-1} \), Figure 3.1.8b. During regular operation, dispensing of the immersion liquid was initiated followed by aspiration, ensuring encapsulation of the entire apex with liquid, Figure 3.1.8c. The injection of the processing liquid was then started, which resulted in a flow confinement, Figure 3.1.8(d and e). The stability of this flow confinement was tested for extended periods (~60 min) and it was found that evaporation, scanning in the X and Y directions, convection and surface characteristics (such as wetability and roughness) had no observable effect on the confinement of the processing liquid.

This integration is not only convenient but also opens interesting possibilities such as performing local electroless deposition of metals,\(^\text{32}\) eliminating corrosion of some metals due to the formation of a galvanic cell during the fabrication of conductive elements,\(^\text{33}\) treating fragile materials locally on a surface, and performing reactions at the interface of laminar flows.\(^\text{34}\) Arguably, a vMFP head having the immersion liquid integrated into it offers a shift in paradigm from processing surfaces using masks and bulk amount of chemicals towards a much more conservative use of chemicals.
Figure 3.1.8 – Integration of immersion liquid dispensing within a vMFP head. (a) Schematic of a vMFP head having immersion liquid dispensing channels (width 500 µm and depth 50 µm). (b) Photograph showing the encapsulation of immersion liquid at the apex of a vMFP head. (c) View of the apex with the outline of the immersion liquid marked with a dotted line. This image was captured in brightfield after initiating the dispensing of immersion liquid and aspiration. (d) vMFP head supporting a stable confinement of the processing liquid after significant in-plane movement. (e) Visualization of the flow confinement of fluorescent liquid formed between two apertures in the presence of a black dye (background) as the immersion liquid. Figure taken from [1].

3.1.4 Localizing (bio)chemical reactions on surfaces

Local deposition of antibodies using multilayered MFP heads

We chose to pattern antibodies on glass surfaces using multilayered MFP heads as shown in Figure 3.1.9, because antibodies deposited from solution onto a surface are widely used in diagnostics and research in life sciences. Patterning antibodies is needed, for example, for functionalizing the surface of biosensors for multiplexed detection of analytes. The substrate used was a functionalized glass slide having a proprietary chemical spacer, which had aldehyde terminal groups covalently bound to the glass (XENOBIND™, XENOPORE Corp., Hawthorne, USA). One mL of PBS was placed on the substrate to provide the immersion liquid. The injection flow path of the MFP (syringes, tubes, valves, and microchannels) was prefilled with the processing liquid containing fluorescently labeled antibodies (Anti-Guinea Pig IgG–TRITC antibody produced in rabbit, Sigma-Aldrich, Buchs, Switzerland) at a concentration of 100 µg mL⁻¹ and the aspiration path was prefilled with water. The MFP head was lowered into the immersion liquid and approached to a distance of 10 µm to the substrate.
Figure 3.1.9. – Continuous (a) and discontinuous (b) patterns of fluorescently-labeled antibodies deposited on aldehyde-functionalized glass. Both types of patterns were produced using an injection flow rate of 2 µL min\(^{-1}\) and an aspiration flow rate of 15 µL min\(^{-1}\) with a gap of ~10 µm and PBS as immersion liquid. (a) A horizontal movement of the substrate at velocities of 100 µm s\(^{-1}\) in the Y-direction and 500 µm s\(^{-1}\) in the X-direction was used to produce the antibody lines shown in the micrograph. (b) The discontinuous pattern was obtained through a movement of the MFP head at 5 mm s\(^{-1}\) between the different positions and pausing for 2 s at every position. The inset shows the typical shape of a deposition area produced using a MFP head having two 50-µm-large square apertures that are separated by 75 µm. Figure taken from [22].

Aspiration of immersion liquid started at a flow rate of 15 µL min\(^{-1}\), and thereafter the injection of the processing liquid at a flow rate of 2 µL min\(^{-1}\) was initiated. When the flow profile of the processing liquid appeared to be stable and had the desired size, computer controlled X- and Y-movements were done to generate the patterns programmed in WIN-Commander. Discontinuous patterns used a stop-and-go movement of the substrate with lateral velocities between 3 and 5 mm s\(^{-1}\) and pausing for 1 to 5 s at every deposition area. At these velocities, the injected processing liquid is shielded from the surface by a boundary layer of immersion liquid. This effect depends on the injection and aspiration flow rates, the gap and the geometry of the apertures.\(^{21}\) Optimal parameters to produce the patterns shown in Figure 3.1.9 were determined by changing the velocity of the substrate movement and the residence time at each position at the periphery of the sample.
**Microprocessing using multilayered MFP heads**

Maskless lithography and rapid prototyping have emerged as efficient techniques for patterning materials with a few micrometers of resolution. These techniques are used, for example, for prototyping microfluidics, substrates for cell culture technologies, and fabricating electronic circuit boards. Multilayered MFP heads can also write patterns on demand on a variety of surfaces. Figure 3.1.10 shows examples of patterns produced in a photoresist. First, a positive-tone photoresist (AZ 4562, Clariant, Muttenz, Switzerland) was spin-coated onto a glass slide to a thickness of 3 µm. After photo-exposure, the resist was patterned by locally processing it with a developer (AZ 400K, Clariant) using the multilayered MFP. Scanning using variable velocities and keeping the MFP head stationary can be done in one experiment, thereby making it possible to form structures of various depths and sidewall profiles in a single run. The fabrication of tapered structures is, for example, used in microelectronics for vias and electrical contacting of three-dimensional structures, MEMS and mold fabrication, and for the alignment of fiber optics. As shown in Figure 3.1.10(a-c), lines with sidewalls having a taper of 4° were produced using a scanning velocity of 75 µm s⁻¹ and injection and aspiration flow rates of 2 and 15 µL min⁻¹, respectively. Stationary development for ~5 s, using the same flow rates, resulted in the local removal of the resist down to the glass surface underneath and in the formation of sidewalls having a taper of 30°, see Figure 3.1.10(d and e). Similarly to the experiments with biofunctional molecules, discontinuous patterns were produced by performing a stop and go movement during scanning, Figure 3.1.10(f). The replacement of the PDMS connection block with adhesives and ports, as are conventionally used for liquid chromatography, and using a polyimide adhesive layer for bonding the Si lid and HFC chip permitted to use a processing liquid having a high pH (12.9). According to the specifications of the materials chosen for the fabrication of the multilayered heads used in this work, temperatures up to 180 °C (limited by the bonding adhesive and the gaskets of the ports) and a broad range of chemicals can be employed.
Localized (bio)chemistry on living cells using vMFP heads

Interacting and performing localized bio(chemical) reactions on cells is highly desirable in biomedical research for numerous applications such as engineering cellular architectures to create artificial tissues, modulation of stem cell microenvironments during differentiation for regenerative medicine and dispensing chemicals at varying concentrations on cells for screening and drug toxicology studies. For these reasons, we investigated whether the vMFP can be used to inactivate adherent cells and to dispense chemicals on living cells. To this end, heads were designed to match the spatial distributions appropriate to interact with a few cells

Figure 3.1.10. – Local processing of a 3-µm-thick positive photoresist film on a glass slide using a multilayered MFP. (a) Micrograph of lines directly developed in the resist by injecting a developer at 2 µL min⁻¹ and aspirating at 15 µL min⁻¹. Here, the MFP head velocity was 75 µm s⁻¹, the gap 10 µm and the immersion liquid water. (b) and (c) SEM image and corresponding profile of a line shown in (a). (d) and (e) SEM image of a single feature developed in photoresist and the corresponding profile of the sidewall between C and D determined using an AFM. (f) Assortment of discontinuous patterns made by locally developing a photoresist using conditions similar to those for the structures in (d). Figure taken from [22].
and sustain flow conditions that do not perturb their position. These head designs support flow confinements ranging from a few $\mu$m$^2$ (for single cells) to a few hundred $\mu$m$^2$ (for cell populations). In order to inactivate cells, a vMFP head supporting a flow confinement of sodium hypochlorite was positioned 30 $\mu$m above a confluent layer of NIH 3T3 mouse fibroblasts. The adherent cells in the path of the heads trajectory got in contact with sodium hypochlorite and were inactivated, Figure 3.1.11(a). The average residence time of the vMFP over a fibroblast was $\sim$1 s, corresponding to 50 nL of sodium hypochlorite dispensed. Inactivated cells were stained using trypan blue and rinsed with PBS; the living cells remained unstained. Alternate indicators of inactivated cells were morphological changes such as detachment and shrinkage, as seen in Figure 3.1.11(a). We further investigated local staining of living cells. Cellomics®, a whole cell fluorescent stain was used as the processing liquid. The head migrated at 0.1 mm s$^{-1}$ and stained adherent fibroblasts along its trajectory. An observation from Figure 3.1.11(b and c) is that the morphology of the stained fibroblasts remained unchanged and the cells continued to be adherent even after exposure to the flow confinement. Therefore, the flow conditions used (i.e. the sheer forces excreted on the cells) were adequate, and it did not inactivate or dislodge the fibroblasts.

The vMFP trajectories along the adherent cells did not result in sharp interfaces as with patterns formed by the immobilization of fluorescently-labeled antibodies on glass, Figure 3.1.9. This is likely due to the heterogeneity of the fibroblast population (in shape and distribution), internal diffusion of chemicals within cells, dislodging of cells when inactivated, and perturbations to the flow confinement due to topographical variations. In this work, we did not achieve single cell resolution but were able to interact with a few neighboring cells (2–4 cells).

Figure 3.1.11 – Interaction of vMFPs with fibroblasts. (a) Optical micrograph and corresponding inset of selective inactivation of fibroblasts using a vMFP head (apertures with 50 $\mu$m $\times$ 50 $\mu$m dimension and 50 $\mu$m spacing) using 2.5% sodium hypochlorite as processing liquid. The immersion liquid was PBS. (b) Image captured in fluorescent mode showing a pattern (‘MFP’) written on fibroblasts using a vMFP head with cellomics® as the processing liquid. (c) Detailed view of fibroblasts exposed to cellomics®. Figure taken from [1].
Besides using vMFP heads for local (bio)chemistry on cells, the technology has also been applied to tissues for performing localized immunohistochemistry. At description of this work is given in chapter 3.2.

3.1.5 References


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3.2. The MFP as a tool in pathology

The content of this chapter has been published:

Lovchik, R.D., Kaigala, G.V., Georgiadis, M. and Delamarche, E.
Micro-Immunohistochemistry Using a Microfluidic Probe
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Contributions – I conceived and performed the experiments together with the co-authors. In particular, I gathered information about classical immunohistochemistry and the state-of-the-art protocols and (bio)chemicals. I tested the suitability of the MFP for use on tissue sections and developed operating procedures for reliable processing of tissue section using the MFP. I developed ways to perform multiple control experiments on single tissue sections using PDMS stencils and selected suitable tissue/antibody combinations for high contrast stainings. Team work of the authors was required to obtain the key results for the paper, were GVK, MG and myself contributed equally. I analyzed the data together with GVK and MG. I made the figures for the paper and proposed a front cover for the journal, which was accepted.

3.2.1 Abstract
A flexible method to extract more high-quality information from tissue sections is critically needed for both drug discovery and clinical pathology. Here, we present micro-immunohistochemistry (µIHC), a method for staining tissue sections at the micrometer scale. Nanoliters of antibody solutions are confined over micrometer-sized areas of tissue sections using a vertical microfluidic probe (vMFP) for their incubation with primary antibodies, the key step in conventional IHC. The vMFP operates several micrometers above the tissue section, can be interactively positioned on it, and even enables the staining of individual cores of tissue microarrays with multiple antigens. µIHC using such a microfluidic probe is preservative of tissue samples and reagents, alleviates antibody cross-reactivity issues, and allows a wide range of staining conditions to be applied on a single tissue section. This method may therefore find broad use in tissue-based diagnostics and in research.

3.2.2 Introduction
We present a flexible method to perform IHC on tissue sections at the micrometer scale and using multiple antibodies; we call this method µIHC. IHC, a staining process based on specific antibody-antigen binding, is widely used in pathology to detect disease markers in
tissue sections. This staining method is used to visualize the level of expression of specific markers in tissues to identify the type and stage of diseases. It is also typical that a counterstaining (e.g. unspecific staining of nuclei or cell membranes) is applied for better contrast in the morphological characterization of the tissue (histology). Pathologists who routinely perform IHC need to screen biopsy samples for multiple disease markers to subtype and personalize treatment strategies. Some of the challenges they regularly encounter are the limited amounts of tissue sample, the qualitative nature of the staining results, and the need for human interpretation of complex IHC outcomes, which impact the life of patients. Therefore, methods that can extract more information from tissues and provide increased flexibility in IHC-staining procedures are needed. This need is even more pressing when considering tissue microarrays (TMAs). TMAs represent an interesting format in which only millimeter-sized tissue section cores are used to screen for new biomarkers while providing sufficient information for the reliable evaluation of specimens, including that of cancerous breast tissue. Recent attempts to increase the amount of information retrieved from tissues are the simultaneous incubation with multiple antibodies, which is limited by cross-reactivity, the use of double immuno-enzymatic reaction, which results in increased background and the use of multiple enzymatic substrates, which requires elaborate processing. Multiple antibodies labelled with quantum dots have also been used, but the antibody cross-reactivity persisted. Gannot et al. developed a technique, termed layered IHC, wherein several membranes were placed on a tissue section and incubated with antibodies. The immunoblotted antigens were detected on specific membranes. This approach, while suitable for multiplexing, was tedious, used the entire tissue section, and did not provide a path forward for interactive optimization of staining conditions. Sun et al. dislocated solid tumors and placed them in isolated chambers for immunocytochemistry. This approach, while suitable for systems biology analysis, does not provide histological information of the tissue. One clever demonstration to perform localized IHC in microchannels was reported by Kim et al., but flushing reagents within microchannels sealed on tissue sections limits the flexibility to stain specific regions of interest on the tissue section. Such microchannel approaches were also used for brain slice microenviroment modification, large area tissue section IHC using multiple streams of liquids, and in combination with lanthanide-based immunocomplexes.

Localizing liquids on surfaces appears to be critical for performing µIHC. Liquid localization has been pursued using AFM cantilevers, scanning electrochemical microscopy, nanopipettes, a chemistrole, and a polymeric two-phase system. While these techniques have been demonstrated in the context of specific applications, they are not suitable for non-contact processing of tissues and do not provide flexibility in scanning tissues at the length scales needed.

We developed a scanning non-contact microfluidic technology, the vertical microfluidic probe (vMFP), that confines picoliters of liquids on a surface of interest, and have shown that it can add and remove proteins from surfaces as well as inactivate and detach adherent cells. The main component of the vMFP is the head, a microfluidic device that has apertures at its apex to inject and aspirate liquids at a distance of 1-30 µm from the tissue section, Figure 3.2.1. This head is mounted on a stage that can scan areas with micrometer...
resolution up to the size of a microscope glass slide (1×3 inches), thereby enabling custom and precise patterning of biomolecules on biological interfaces. The patterning is reconfigurable in real-time, and the staining region can be as small as a few µm² (on the order of 5 to 10 cells). Apart from IHC, the MFP technology has been adapted by several research groups for diverse applications: Queval et al.\textsuperscript{18} performed microperfusion on brain slices, and Shiku et al.\textsuperscript{19} performed lysis on adherent cell layers for messenger RNA analysis.

### 3.2.3 Experimental and Results

The implementation of the vMFP for µIHC is in-line with the standard workflows used in conventional IHC, Figure 3.2.1. The vMFP head is compatible with (bio)chemical systems used for staining, is resistant to a broad range of chemicals and can be used indefinitely unless physically damaged. Its rhombic shape and its small apex (1 mm² or less in area) enable easy viewing of tissue sections both from above for positioning the vMFP and from underneath using an inverted microscope for quantitative fluorescence measurements.

For staining, we make use of a commonly used enzymatically amplified staining with the chromogen 3,3′-diaminobenzidine (DAB), which can be visualized in brightfield. The presence of an immersion liquid around the apex, in addition to enabling confinement of the processing liquid, prevents drying artifacts. The interaction of the confined processing liquid of the vMFP with the tissue section is critical and has therefore been assessed for an

**Figure 3.2.1** – Concept and workflow of µIHC using a vMFP. Dewaxing and rehydration of the tissue are performed according to conventional IHC (1). Using injection and aspiration apertures at the apex of a vMFP head, a solution of primary antibody is hydrodynamically confined (in the presence of an immersion liquid) to selected areas of a tissue section (2). Post-processing for visualization of the antigens on the tissue section continues as in standard IHC: the tissue section is incubated with secondary antibodies, and enzymatic precipitation of 3,3′-diaminobenzidine (DAB) chromogen leads to a visual signal, indicating the expression level of specific antigens in the tissue section semi-quantitatively. Typical parameters for the vMFP scans are indicated.
The footprint of the confined liquid on the tissue (see snapshots in Figure 3.2.2(a)) is comparable to the areas examined using laser capture microdissection\textsuperscript{20}, a technique widely used to isolate cells from tissue sections. Here, the difference is that vMFP-based μIHC preserves the tissue integrity for histological studies. Figure 3.2.2(a) shows the confinement of a fluorescein solution (i.e. processing liquid) over a 4-µm-thick human thyroid tissue section. The processed area is ~0.02 mm\textsuperscript{2} (i.e. confinement volume of ~400 pl for a gap of 20 µm) and varies by less than 5% during a period of 18 min. This area is a few times smaller than that of a biopsy sample obtained with a fine needle and many thousand times smaller than a section derived from a whole tumor. This reduction in size suggests that high-density multiplex antibody tissue processing with the vMFP can be realised. The vMFP allows adaptive scanning of multiple locations (or large areas) on a tissue section and with this, the implementation of tissue specific μIHC protocols for enhanced reliability. Modelling and analytical studies of the flow confinement gives guidance for designing and using different types of heads for a range of flow confinement conditions\textsuperscript{21}. Several factors together define the achievable level of staining. Some of these are intrinsic to the biological system.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3_2_2.png}
\caption{Interaction of a processing liquid with tissue sections. (a) Stability of the hydrodynamically confined liquid measured as the change in projected area of the flow confinement observed using an inverted microscope. The vMFP head was positioned 15 µm above either a glass slide or a 4-µm-thick human normal thyroid tissue section. 5 µM fluorescein in PBS-Tween 20 were used as the processing liquid (injection: 1 µl min\textsuperscript{-1}, aspiration: 5 µl min\textsuperscript{-1}), and the area of the flow confinement was monitored for 18 min. (b) Multiple regions of a normal human thyroid tissue section were incubated with a 700 µg ml\textsuperscript{-1} solution of α-TGB in PBS-Tween 20 for various residence times. The staining intensities for residence times between 1 and 100 s are shown in the graph. The marks in the micrographs indicate the boundary between stained (lower half) and unstained regions (upper half).}
\end{figure}
such as the receptor-ligand binding kinetics, the expression level of the antigen and the tissue morphology. Because the flow confinement is highly stable, it is straightforward to vary the residence time of the primary antibody solution on the tissue section to cover a large spectrum of antigen-antibody reaction times.

A human anti-thyroglobulin antibody (α-TGB) was delivered in approx. 100-µm-wide lines across a healthy human thyroid tissue and as expected, a correlation between the incubation time and the signal intensity was observed, Figure 3.2.2(b). This correlation reveals that specific interactions between antigen/antibodies from various tissues can be adjusted by varying the residence time. In the experiment, the incubation time of thyroid tissue with α-TGB was tested to achieve clear staining and was on the order of 20 s for the conditions used in the experiment. This duration is considerably shorter than the 30 min recommended by the supplier for standard IHC. We attribute this reduction in incubation time to convection, which resulted in increased transport of primary antibodies. While this reduction in processing time can be antibody-dependent, we do not know whether it will always exist because some markers may not be as accessible as others. The mechanical interaction between the injected liquid and the tissue may also improve the transport of molecules within the tissue.

Figure 3.2.3 – μIHC on cancerous breast tissue sections using different antibodies. (a) Monoplex staining of a 4-µm-thick well-differentiated invasive ductal carcinoma breast tissue section using a monoclonal mouse anti-human p53 protein antibody (54 µg ml⁻¹, residence time 200 s). A line of hematoxylin is patterned with 1 s residence time for counterstaining. (b) Multiplexed staining of a well-differentiated invasive ductal carcinoma breast tissue section for the presence of p53 (α-p53: 54 µg ml⁻¹, residence time of 300 s) and human progesterone receptor PR (α-PR: 125 µg ml⁻¹, residence time of 300 s) with an additional hematoxylin counterstain. (c) μIHC processing of selected cores of an infiltrating ductal carcinoma breast tissue microarray, each 2 mm in diameter. Each core of the tissue array is characterized a priori by the supplier for the expression of ER, PR and p53. Four cores of the tissue microarray (locations A5, B5, B9 and C5) were processed with the vMFP (α-ER: 370 µg ml⁻¹, α-PR: 125 µg ml⁻¹ and α-p53: 54 µg ml⁻¹).
µIHC was performed on invasive ductal carcinoma breast tissue using an antibody (α-p53) against the tumor suppressor protein p53. The two spots shown in Figure 3.2.3(a) were processed with a vMFP residence time of approx. 500 s. We also performed multiplexed µIHC with two antibodies, α-p53 and anti-progesterone receptor (α-PR) by exchanging manually the syringes containing different antibody solutions. As controls, conventional staining was performed following a similar IHC protocol. Counterstaining trajectories close to the µIHC spots were done with hematoxylin and a residence time of 1 s, Figure 3.2.3(b). Given the increasing use of TMAs, processing of small areas within individual cores of tissue microarrays was performed. Here, we screened four cores of an infiltrating breast cancer tissue microarray for the presence of three targets: estrogen receptor (ER), PR and p53. Hematoxylin was used as a counterstain, Figure 3.2.3(c).

3.2.4 Conclusion

µIHC is made possible by accurately localizing a solution of primary antibodies on a tissue section. In principle, the method shown here can be extended by stacking several vMFP heads for processing a tissue section with many primary antibodies in parallel or by optimizing staining conditions in real-time using fluorescently-labelled antibodies. Unique to µIHC and its implementation with the vMFP is its ability to increase the information gained from very small tissue samples, perform monoplex or multiplex staining of a tissue section, use extremely small volumes of antibodies on the order of a few nanoliters (i.e. 100× less than conventional IHC), and scan a tissue quickly and interactively. The vMFP operates at length scales that are common to the life sciences (µm to mm). Particularly useful is the ability to perform µIHC on a specific region of a tissue, interpret the result, and reprocess another region of the tissue using adapted conditions. This can even be done within individual cores of tissue microarrays. As local areas are biochemically processed and adjacent regions are left intact, the contrast for visualization is improved, facilitating histological statements. In summary, µIHC using a vMFP can not only support the work of pathologists but may also become a tool of choice for research and diagnostics involving biological substrates.

3.2.5 References

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3.3. The MFP within the context of “microfluidics in the open space”

The content of this chapter has been published:

Microfluidics in the "Open Space" for Performing Localized Chemistry on Biological Interfaces
Kaigala, G.V., Lovchik, R.D. and Delamarche, E.

Contributions – I co-wrote this review and prepared all the illustrations, including the journal cover. I critically contributed to the German version of the review for the German Chemical Society. The translation also involved adaption of all the illustrations.

3.3.1 Abstract

Local interactions between (bio)chemicals and biological interfaces play an important role in a wide range of fields such as surface patterning, single-cell analysis, local electrochemistry, electrophysiology, regenerative medicine, synaptic signalling, chemical locomotion, and cell toxicology. Here, we review microfluidic systems that operate in the “open space”, that is, without the need for sealed channels and chambers commonly used by the microfluidic community. This emerging class of microfluidics localizes chemical reactions on biological interfaces or biological specimens without imposing significant “constraints” on samples, such as encapsulation, pre-processing steps, or the need for scaffolds. They therefore provide new opportunities for organizing, patterning, manipulating, analyzing, and interacting with biological samples. This minireview first looks at the motivation for performing local chemistries, then outlines the requirements imposed on localization techniques, and finally describes three classes of microfluidics operating in the open space based on microelectrochemistry, multiphase transport, and hydrodynamic flow confinement of liquids.

3.3.2 Introduction

Microtechnology, microfluidics and soft lithography have provided the main steps of linking technology to biology and life sciences in general. These techniques are broadly explored by microtechnologists and chemists, and used by biochemists and biologists for protein patterning, assay miniaturization, diagnostics, advanced purification, and separation. The next critical step is to increase the compatibility of microfluidics with biological systems that are sensitive to the environment (such as fragile proteins on surfaces, living cells). In
addition, they should also be compatible with glass slides and Petri dishes. Moreover, as living matter “likes” surfaces, substrates that are functionalized for biological applications are increasingly used and commercially available. Microfluidics should be able to interact with such substrates in the “open space”, which will facilitate the study of biological samples. To succeed in these endeavors, microfluidics probably need to eliminate one of their major constraints: the walls.

Numerous applications require control of chemical and biochemical reactions on the micro- to nanoscale on biological interfaces. For example, interaction with adherent cells is highly desirable in biomedical research for engineering cellular architectures, modulating stem-cell microenvironments for regenerative medicine, presenting gradients of guidance molecules for understanding process in cell and developmental biology and dispensing chemicals at various concentrations for drug screening and toxicology studies.\textsuperscript{2,3,4,5} The volumes and length scales in microfluidics are generally compatible with those in the life sciences, but also the inherent technological components must be biocompatible.

There is now a greater need than ever before for developing technologies\textsuperscript{6} that could be used ubiquitously in life-science laboratories, akin to microscopes and micropipettes. In addition to precisely outlining the need and specification of a technology, driven by a biological/clinical investigation, careful considerations of the physical and chemical mechanisms underlying microfluidics are required. For example, widely used assays, such as DNA/protein microarrays and immunoassays, are based on analyte–receptor interactions, and understanding these allows their designs to be improved further. Therefore, the key to a successful implementation of tools for the localization of chemistries on biological interfaces is an in-depth knowledge of the interplay between diffusion, convection and reaction\textsuperscript{7} and of how a system will behave based on the fluxes, equilibration times and fundamental quantities of the chemical species involved.\textsuperscript{8}

### 3.3.2.1 Scope

This minireview first outlines the need and motivation for performing local chemistries on biological interfaces and the requirements imposed on localization techniques. A snapshot of the present microfluidic implementations for handling biological specimens within closed-systems is subsequently presented. The transition from closed to open microfluidic systems is outlined, starting from the removal of one wall (“the lid”), thereby making the system partially open. Finally it describes three classes of microfluidics operating in the open space based on microelectrochemistry, multiphase transport, and hydrodynamic flow confinement of liquids.

We bias this minireview towards biological systems and interfaces because we believe that this is where microfluidic technology is most needed to solve important problems in healthcare and life sciences and where it can have a broad impact.
3.3.2.2 Why Localyze Interactions with Biological Surfaces in the “Open Space”?

A fundamental characteristic of biology is compartmentalization. Biochemically speaking, reactions should occur at the right place and at the right time in cells and tissues. For this reason, most approaches for studying and transforming cells are based either on encapsulating liquids (e.g. nanodroplets, vesicles) or on engineering physical compartments for cells (e.g. scaffolds, microfabricated chambers). However, both approaches are restrictive. An excellent example illustrating the idea of spatial and temporal sequencing of biological events is described by Healy.9 During embryonic development, different cell populations contribute to the overall organization of the tissue, and chemical signaling within the developing tissue and organs controls the fate of cells. Biologists have long attempted to capture these processes outside of the body. Ex vivo methods for studying cell biology have contributed to the vast knowledge of mammalian cell function, but the spatial and temporal presentation of soluble and physical signals to cells continues to be difficult.9

Techniques offering the ability to study, work, stimulate, and locally interact with adherent cells and tissues in an “open space” would yield breakthroughs in understanding the biology and physics of biological interfaces, similar to the advancement of our knowledge enabled by other techniques. Patch-clamp, for example, is a well-established technique10 that relies on the pressure-based contacting of cells with a 1-2 µm diameter micropipette. It is widely used for measurements of the membrane potential and has provided significant insights into cell functions. Surface-based assays are inherently performed without total encapsulation (e.g. microtiter plates and glass slides), and there is a trend to use microfluidics to enhance their performance. We therefore believe that the power of microfluidics can be leveraged when implemented for use in the “open” space, without physical contact or constraints, allowing the interaction with surface assays and biological interfaces. Figure 3.3.1 aims to capture the set of emerging techniques for the local processing of biological interfaces and their applications in biology. Several of these examples are applicable in cell biology and tissue research. The scope of this minireview is not restricted to cellular analysis, and deals with localization on biological interfaces in general.

3.3.2.3 Microfluidic Technology for Localized Chemistry

Scientists developing microfluidics initially choose the path of using closed microfluidic channels and chambers. For specialized applications, such as in immunodiagnostics, closed-channel microfluidic devices have widely been used.11 However, the high hydraulic resistance, the difficulty to introduce samples such as tissues, cells and mesoscopic objects into microdevices, and the clogging by particulates and air bubbles limit the practical use of closed microfluidic devices. Moreover, biological samples are sensitive to their environment and are prone to drying artifacts and denaturation. Despite significant progress in microfluidic technologies, there is a strong motivation to develop new and improved approaches to control the chemical and physical environments around intact cells in tissue or cell cultures. Glass micropipettes have traditionally been used to alter the cellular environment and to deliver active compounds to biological interfaces, including to single cells. This is however not suitable for maintaining a localized environment because the active
substrate can diffuse outside the field of analysis. Sims and Allriton\textsuperscript{12} published a comprehensive review describing the motivation and microfluidic technologies for single cell analysis.

It is important to have technologies that can localize liquids on length scales that are commensurate with biology. The origin of liquid localization is inkjet technology, starting from printing ink to patterning biological molecules. In parallel, advances in microtechnology have enabled the fabrication of devices for measuring complex biophysical and biochemical characteristics of cells and sub-cellular components.\textsuperscript{13,14} A technology called microcontact printing (μCP),\textsuperscript{15,16} now widely used to pattern chemicals on a broad range of length scales on surfaces,\textsuperscript{17} is simple enough for use by non-experts. Microcontact printing has been applied in various fields, such as in the fabrication of protein biochips\textsuperscript{18} and in the preparation of surfaces to understand cell-surface interactions.\textsuperscript{19} It involves transferring molecules from a stamp onto a surface. For any modification of the pattern, the stamp has to be redesigned, which is tedious and time consuming.

Broadly speaking, the microfluidic approaches pursued in the context of localization are the following.

\textit{Microfluidics for handling cells:} A multitude of techniques such as electrophoresis, dielectrophoresis, electroporation, surface acoustics and Raman spectroscopy have been miniaturized and integrated into microfluidic devices, some of which exhibit great promise for cell analysis and interaction. However, the use of these closed channel microfluidics still poses many challenges, so that investigations of adherent cells and tissues within a closed device may not reflect the true biology of cells.
Microfluidics for compartmentalization: In the past few years, droplets encapsulating an aqueous phase have provided an even smaller and more powerful form of “compartmentalization” than microtiter plates and microarrays. The volumes of liquid encapsulated in these droplets can accommodate one or a few cells. They have been used for many applications, ranging from DNA sequencing, biochemistry, and drug screening to directed evolution. One of the techniques based on droplets is electrowetting on dielectrics, also called digital microfluidics. There, droplets are moved on a surface by means of an array of electrodes that modify the wetting characteristics of the surface. This technology shows great promise towards open-space microfluidics, but requires engineered surfaces.

Collectively, these microfluidic methods mainly rely on direct contact with a solid interface and do not allow the patterning of delicate surfaces of living cells as physical contact may damage them. Although existing microfluidic implementations of assays have made significant progress towards enabling capabilities otherwise infeasible, they still remain restrictive when dealing with surface-based assays. Some microfluidic implementations of biological assays are described below and shown in Figure 3.3.2.

Park et al. developed microfluidics for multiplexed immunohistochemistry (IHC) on tissue sections. They used microchannels made in Polydimethylsiloxane (PDMS) to draw solutions containing primary antibodies for detecting disease markers in tissue sections, see Figure 3.3.2(a). They showed that four biomarkers, namely, estrogen receptor, human epidermal growth factor receptor 2, progesterone receptor and Ki-67, can be detected in parallel on breast-cancer cells and human breast cancer tissues. Although this approach enables multiplexed IHC, it still is restrictive because the footprint of the microfluidic channels on the tissue section is determined arbitrarily by the design and fabrication of the microfluidic device. Small, damaged or heterogeneous tissue sections can render this approach challenging. Along the lines of local IHC, there is merit to develop techniques for multiplexed in situ hybridization assays on tissue sections. Dufva et al. demonstrated online monitoring of hybridization with fluorescein-labeled probes to 18S ribosomal RNA in mouse-brain tissue sections. They called their device HistoFlex. It provided uniform hybridization conditions across the reaction chamber, as determined by hybridization of spotted DNA microarrays. HistoFlex achieves a higher sensitivity than conventional techniques for the detection of miRNAs, but requires significant optimization a priori to account for varying tissue histology.

For visualizing, probing and manipulating the metabolic and structural machinery of mammalian cells, Whitesides and colleagues developed a microfluidic technique and called this “partial treatment of cells based on laminar flows” PARTCELL. They used it to study the subcellular processes of mitochondrial movement and changes in cytoskeletal structure in living cells. The microfluidic systems were prepared by placing PDMS having molded channels on top of a living cell. Parallel streams of different liquids were created in the microfluidic channel, Figure 3.3.2(b). The width of each stream and the position of the interface between adjacent streams were controlled by adjusting the flow rates. The cells were positioned between two adjacent streams, one with and the other without the molecule.
of interest. Figure 3.3.2(b) shows the disruption of actin filaments in selected cell regions after treatment with latrunculin A, a membrane-permeable molecule that binds to actin monomers. This approach increases the resolution in staining cells by using microfluidic elements with large features that are easy to fabricate. However, much like the device developed by Park et al., the footprint of the microfluidics needs to be altered for different cell distributions.

![Diagram](image1)

**Figure 3.3.2** – “Closed” channel microfluidics for processing local regions on tissue sections and cells. (a) PDMS with channels placed on tissue sections for the delivery of antibodies for local immunohistochemistry. (b) Partial treatment of cells using a microfluidic device. (c) “Microcanals” made in glass and PDMS with holes for pipetting nutrients/dyes to selected cells. Images reproduced with permission and their source: (a) from, copyright 2001 Macmillan Publishers; (c) from, copyright 2004 Royal Society of Chemistry.

Micropipette manipulation and probing of cells within a microfluidic environment can expand the functionality of several existing “closed” channel microfluidics. To this end, Folch et al. developed an open-air microfluidic device without roof. This technique, which they called “microcanals” constituted a cell-culture environment accessible with micropipettes, Figure 3.3.2(c). The microcanals were fabricated in PDMS and used glass as substrates. Despite being in open channels, the liquid was confined to the microcanals owing
to the wetting of the bottom corners and the hydrophobicity of the top surface of the microcanal walls. Cells were seeded in the microcanal floors by adding a cell suspension to the corresponding inlet and stopping the flow during cell attachment/spreading. Patch-clamp electrophysiological recordings from human kidney cells were performed. This approach provided a solution for interacting with the cells seeded on surfaces in semi-closed microchannels, but is only suitable for interacting with a limited number of cells.

The techniques described above are good examples of how cells and tissues can be investigated using microfluidics. The technique based on microcanals is a transition from closed to open microfluidics. By removing one wall, an open space over the biological specimen is created and the sample can be probed directly using patch-clamp technology, which improves the flexibility and interactive capability.

3.3.3 Requirements for Performing Localized Chemistry on Biological Interfaces in the “Open Space”

The previous section demonstrates how closed-channel microfluidics evolved to accommodate tissues and adherent cells for biological studies. More flexible and interactive tools are nevertheless needed to cope with the variability inherent in biological specimens. Only a limited number of techniques exist that are compatible with biological interfaces, particularly for processing in the “open” space. Techniques localizing chemical reactions on biological interfaces should ideally be:

- Non-invasive: they should preferably work in a “non-contact” mode to minimize the perturbation of the biological interface.
- Immersed: the presence of a buffer/liquid environment would prevent drying artifacts such as denaturation.
- Biocompatible: no toxic materials or chemicals should be needed, and the techniques should be compatible with typical ranges of pH, temperature, ionic strengths, shear forces, and pressures.
- Flexible: compatibility with different materials, topographies, length scales (µm to cm) and volumes inherent to biological systems would be beneficial.
- Interactive: the technique should provide feedback (e.g. current, voltage, force, optical signal) during interaction with the biological interface.

When using a partially or a fully open architecture, evaporation is not necessarily a problem, as it is easy and convenient to immerse the sample in larger volumes (mL range) of liquids or to provide an environment with high relative humidity. The key advantage with the open architecture, is that there is no need to flow cells and tissues through a closed microchannel for processing, instead, the (bio)chemicals are brought to the biological interface.
3.3.4 Strategies to Perform Localized Chemistry on Biological Interfaces

Shortly after its invention, scanning probe microscopy (SPM) was utilized to probe surfaces and interact with (bio)molecules on surfaces. With the addition of microfluidic functionalities, various techniques derived from SPM emerged for localizing (bio)chemical processes over biological interfaces. Interestingly, these techniques meet a fair number of the above requirements. We therefore review these techniques next, before we discuss microfluidic demonstrations based on microelectrochemistry, multiphase transport and hydrodynamic flow confinement of liquids, which meet even more of the above requirements.

3.3.4.1 From scanning probe microscopy to scanning techniques having microfluidic features

Scanning probe microscopy\(^{28}\) covers a set of techniques having high positioning accuracy that are used in material sciences, lithography, metrology and probing biomolecules on surfaces. In these techniques, a probe is moved line by line to record the probe–surface interaction as a function of the position. Among these techniques, the atomic force microscope (AFM) is the best known and most used. It uses a cantilever to probe surface properties, such as the topography, elasticity, electric potential, magnetism and mechanical resistance. Originally developed for investigating the physics of materials, the AFM soon started to be used to address some of the pressing needs in the life sciences such as the precise manipulation and interaction with biological samples. A key extension to AFM and related techniques was the recent inclusion of fluid-handling/dispensing capabilities, building a bridge between the SPM world and biological sciences.

The Mirkin group used AFM cantilevers to pattern a range of biomolecules on surfaces and called the technique dip pen nanolithography (DPN).\(^{29,30}\) Originally, DPN was used to transfer alkanethiolates from a cantilever to a gold surface in air, enabling self-assembled monolayers on gold to be patterned with sub-100 nm resolution. The DPN cantilever is coated with a chemical (“ink”) and transfers it locally to the surface by contact and diffusion, Figure 3.3.3(a). Cantilevers are made from silicon nitride and functionalized to increase the hydrophobicity of the tip. The liquid deposition properties are determined by the interactions between ink and tip, ink and surface, and the properties of the ink such as surface tension, viscosity and vapor pressure under the writing conditions. AFM-based approaches work in non-contact mode, but precise control of the distance between the cantilever and the surface is critical. This imposes limitations on the roughness and elasticity of the surfaces that can be probed as well as on the lateral displacement speed of the cantilever.

An attractive way to deliver chemicals in DPN is to use microfluidic elements to direct the liquid to the probe. The Espinosa group\(^{31,32}\) developed an approach for continuous feeding of inks to the tip of the probe. Kim et al.\(^{31}\) developed an AFM tip in which an aperture was implemented at the apex of a hollow pyramidal reservoir, Figure 3.3.3(b). They called this device nanofountain pen and it comprises a volcano tip, microchannels and an on-chip reservoir. In this design, the ink fed into the reservoir is driven by capillary action through the
microchannels to form a liquid–air interface around the tip. Patterns with lines as small as 40 nm were written.

Similar to the implementation of a “reservoir” for liquids in the vicinity of the cantilever, Zambelli and colleagues integrated microfluidic elements onto an AFM setup and called their technology FluidFM, Figure 3.3.3(c). This device comprised a hollow cantilever with microfluidic elements for use in air and liquid. The array of cantilevers was connected to a delivery system via a modified AFM probe holder, enabling force-controlled dispersion of a solution containing selected molecules into individual cells within a physiological environment. The cantilever geometry and the AFM force feedback allowed local delivery of molecules to cells either by gentle contact with their membrane or by perforation of the membrane. The FluidFM technology also made it possible to select microscopic objects such as living mammalian cells, yeast or bacteria.

![Diagram](image)

**Figure 3.3.3** – Scanning probe microscopy for local processing and delivery of chemicals to biological interfaces. (a) In DPN, ink migrates from the surface of an AFM tip to a surface in the area of contact. Using DPN, (1) nanoscale dot arrays, (2) letters written on a gold surface, (3) 25- and 13-nm gold nanoparticles hybridized to surface DNA templates, and (4) patterns of fluorescently labelled immunoglobulin G were written. (b) With a nanofountain probe, ink fed from a reservoir forms a liquid–air interface at the tip and molecules are transferred by diffusion from the interface to a substrate. (c) A cantilever fixed to a drilled AFM probe holder delivered a fluorescent stain to a single living neuron. Images reproduced with permission and their source: (a) left adapted from, copyright 2004 Wiley-VCH; (b) from, copyright 2005 Wiley-VCH; (c) from, copyright 2009 American Chemical Society.
3.3.4.2 Microelectrochemistry of (bio)chemicals

Miniaturization and precision positioning of needle-type electrodes are rapidly enabling new means of visualization and control of chemical and biochemical reactions on surfaces. There are several excellent reviews related to this topic, for example, the one by Schulte and Schuhmann\(^{34}\) written in the context of single-cell analysis. Combining microfluidics with microelectrodes operated in scanning electrochemical microscopy mode or with nanopipettes for scanning ion conductivity microscopy enables new applications, and these hybrid approaches are reviewed below.

**Scanning electrochemical microscopy (SECM):** The push-pull probe, developed by Momotenko *et al.*\(^{35}\) maps (electro)chemical information of surfaces with high spatial resolution. The device integrates a working electrode, a counter/reference electrode, and a microfluidic system, Figure 3.3.4(a). Microfluidic channels continuously replenished liquid between the probe tip and the interface hence electrochemical mapping of initially dry samples could be performed. In addition, because no immersion of the sample in electrolyte was necessary, the investigation of vertical or tilted substrates became possible. The push-pull probe was used in a “contact” mode, and therefore eliminated the time-consuming procedure of sample leveling prior to SECM imaging. This allowed the investigation of corrugated or curved samples. SECM images of various gold-on-glass samples provided the proof of concept for local surface activity characterization with high resolution. We believe that this probe can be coupled to other analytical techniques such as mass spectrometry, liquid or gas chromatography and capillary electrophoresis.

Cantilevers are suited for depositing biomolecules locally on surfaces. Bergaud and colleagues\(^{36}\) leveraged this concept and developed techniques to control the assembly of oligonucleotides on a surface. They adapted the polypyrrole technology to a cantilever-based deposition system for patterning surfaces for DNA immobilization. The array of cantilevers they designed consisted of ten cantilevers for deposition of molecules and two external force-sensing cantilevers for positioning the array, Figure 3.3.4(b). Each cantilever comprised a microfluidic channel and a reservoir. A gold electrode was patterned within the channel to enable electrochemical reactions to take place at the tip. Electrospotting was done by simultaneously filling the depositing cantilevers with electrolyte prior to positioning each of them at a precise location on the surface of a gold-coated substrate. Then an electrical pulse was applied between the anodically polarized gold substrate and the cantilever for 25 to 500 ms before releasing the cantilevers. The procedure to electropolymerize oligonucleotides is shown in Figure 3.3.4(b). The use of a cantilever array allowed electrochemical reactions in picoliter droplets to prepare biochips.
**Figure 3.3.4** – SECM techniques for local electrochemistry in liquid environments. (a) Push-pull probe with multiple channels for injecting and aspirating electrolytes. Shown is a photograph of two push-pull probes, a sketch of an exposed probe tip (below) and an image of a text pattern in gold (top right) together with the corresponding SECM image (bottom right). (b) Cantilever array and the process for local electrodeposition of oligonucleotides. Images reproduced with permission and their source: (a) from,\(^35\) copyright 2011 American Chemical Society; (b) from,\(^36\) copyright 2007 Wiley-VCH.

**Nanopipettes for scanning ion conductivity microscopy (SICM):** Nanopipettes were developed for the direct patterning of surfaces with biological material, and have since been used for numerous applications in biology. Ying\(^37\) published a review on the use of nanopipettes in bionanotechnology. An early demonstration of nanopipettes by Bruckbauer *et al.*\(^38\) was based on a variant of SPM called SICM, which was developed for scanning soft, nonconductive materials by using an electrolyte-filled nanopipette as probe. Key to this approach was the robust SICM distance control. The ionic current flowing between an electrode inside the nanopipette probe and an electrode in the bath was used to control the pipette–sample distance. Pipettes with inner diameters of 100-150 nm were positioned at a distance of about 100 nm above a glass surface. Biotinylated and fluorophore-labeled DNA or protein G were delivered to the surface and immobilized by biotin-streptavidin binding or electrostatic interaction. The electric field in the tip region is non-uniform along the axis of...
the pipette because of the conical shape of the pipette. The number of molecules exiting the tip depends on the combination of electro-osmotic flow, electrophoresis and dielectrophoresis.

Physical investigations of cells and the delivery of selected molecules on specific regions on the cell membranes require sensitive and specialized techniques. Bruckbauer et al. developed a tool for delivering fluorophore-labeled molecules in a controlled manner and, in addition, succeeded in performing single-molecule tracking. The sign and magnitude of the applied voltage controlled the number of molecules delivered. A nanopipette delivered individual fluorescent probes to preselected sites on the plasma membranes of living boar spermatozoa, Figure 3.3.5(a). The intent was to track the diffusion of membrane glycoproteins in different macrodomains. This technique enabled important investigations in membrane biology to study the heterogeneous distribution of component lipids and proteins. This high-resolution scanning and delivery technique also enabled the interrogation of the dynamics of submicron structures, such as lipid rafts or transient confinement zones. This was done by tracking and analyzing the diffusion of single molecules in real time, whereby the molecules could be located with a precision of 50 nm. In contrast to the use of fluorophores, which photobleached in short time, the nanopipette-based approach allowed multiple experiments to be performed on the same cell to probe different macro-domains and sub-regions in the plasma membrane, and the investigation of the existence of diffusion barriers without being limited by observation times.

Mechanobiology is a topic of increasing importance, but the techniques capable of probing the mechanical properties of membranes without damaging the latter are inadequate. Shevchuk et al. developed a method for imaging contracting cardiac cells using nanopipettes, Figure 3.3.5(b). A distance-modulated protocol for SICM provided a sophisticated distance control mechanism, enabling complicated physiological experiments to be made that required alteration of the ionic strength of the liquid media. When the cardiac myocyte contracted, the feedback control moved the sample stage to maintain a constant distance between the nanopipette and the cell surface. The technique measured rapid changes in cell height from 10 nm to several micrometers with millisecond time resolution. Nanopipettes were combined with laser confocal microscopy for the simultaneous measurement of the nanoscale motion of cardiac myocytes and the local calcium concentrations under the cell membrane. Interestingly, despite large cellular movements, a simultaneous tracking of the changes in cell height and measurements of the intracellular \( \text{Ca}^{2+} \) near the cell surface were possible with the nanopipette approach while retaining cell functionality.

Imaging living cells with nanoscale resolution provides great insight into the biomolecular mechanisms of cells. However, SICM is restricted to imaging relatively flat surfaces, whereas cells do not have a flat surface. When the nanopipette encountered a vertically protruding structure during scanning, it collided with the structure. Novak et al. overcame this problem by using SICM in a hopping mode. This allowed non-contact imaging of complex three-dimensional surfaces of living cells with resolutions better than 20 nm, Figure 3.3.5(c).
Hopping probe ion conductance microscopy (HPICM) was demonstrated by imaging nanoscale phenomena on the surface of living cells under physiological conditions. In conventional SICM, a nanopipette is mounted on a three-dimensional piezoelectric translation stage and an automatic feedback control moved the pipette up or down to keep the pipette current constant while the sample was scanned laterally. In contrast to conventional raster scanning, HPICM had the advantage that the order of imaging pixels did not have to be predetermined. The entire image was divided into equal-sized squares and, prior to imaging each square, the overall roughness of the sample was estimated by measuring the difference in height at the corners of the square. If the sample in a square was rough, the topography of that square was then measured at high resolution. If the sample was relatively flat, the squares were imaged at lower resolution. This adaptive technique was demonstrated by imaging of hippocampal pyramidal neurons in 15 min, Figure 3.3.5(c). It is very likely that this technique will increasingly be applied to biophysical studies of cells.
Biophysical studies of cell surfaces require techniques to map single active ion channels in intact cell membranes. This type of mapping is important because the spatial distribution of ion channels in the cell membrane plays a key role in providing precise and localized information on cellular functions. Using nanopipettes, Korchev et al. mapped the distribution of ATP-regulated K+ channels (KATP channels) in cardiac myocytes, Figure 3.3.5(d). The channels were organized in small groups and anchored in the Z-grooves of the sarcolemma. The distinct pattern of the channel distribution has important functional implications. This scanning method showed that the KATP channels hold their position on the cell surface for a relatively long time, and has the potential to be used for a range of precise biophysical investigations on cells.

3.3.4.3 Transport based on multiphase systems

Multiphase systems often are involved in compartmentalization in nature and essential for biochemical processes. In microfluidic systems, compartmentalization has been achieved using immiscible liquids. Closed-channel microfluidics exploit immiscible systems. Particularly appealing about this strategy is that it requires only inexpensive materials and off-the-shelf equipment, and that is easily accessible to researchers without microfabrication expertise. A review by Baret provides insight into multiphase systems using microfluidic systems. The comprehensive review on aqueous two-phase systems by Hardt and Hahn complements the former. In this section, we review the demonstrations in open microfluidic systems that leverage multiphase and immiscible systems.

Key aspects of using multiphase systems are the diffusive mass transfer and sample partitioning between two or more phases. Such systems can qualitatively be understood by considering the Gibbs free energy ($\Delta G_{\text{mix}}$) of mixing governed by $\Delta G_{\text{mix}} = \Delta H_{\text{mix}} - T \Delta S_{\text{mix}}$, where $\Delta H_{\text{mix}}$ is the enthalpy difference and $\Delta S_{\text{mix}}$ the entropy difference between the mixed and the phase-separated state. The timescale of the partitioning depends on the diffusion coefficient and may be on the order of several hours. Control of surface energy is important in partitioning within multiphase systems.

Polymeric aqueous two-phase system (ATPS): Takayama and colleagues contributed extensively to the implementation of aqueous two-phase systems in microfluidics. They explored polymers such as polyethylene glycol (PEG) and dextran dissolved in aqueous solutions because (i) these polymers form stable ATPSs over a wide range of temperatures, thereby increasing the convenience and stability of experiments; (ii) the high molecular weight of PEG and dextran form ATPSs at low polymer concentrations ensure that the bulks of both phases remain highly aqueous and non-toxic to cells, and (iii) this system works robustly in commonly used cell culture media. In Figure 3.3.6(a), the interfacial free energies between the two aqueous phases, the cell layer and the PEG phase, and the cell layer and dextran phase are represented by $\gamma_{12}$, $\gamma_{C1}$ and $\gamma_{C2}$, respectively, and the contact angle between the three phases is represented by $\theta$. 
Exposing selected cells in culture to reagents is important, for example, in single cell transfection, in toxicity studies and for creating living cell microarrays. Tavana *et al.*\(^4\) loaded a pipette tip with the dextran phase and lowered it into the PEG phase in proximity to a cell monolayer. Moving the pipette tip horizontally resulted in the formation of a continuous pattern of the dispensing dextran phase on the cells. This was demonstrated by patterning “UMICH” on a monolayer of HEK293H cells, Figure 3.3.6(b). The patterns were found to be stable over long incubation times. Critical for the stability of patterns is the low interfacial energy between the two immiscible phases and the roughness of the cell culture and the associated cell surface-dextran phase. The patterning technology was demonstrated with multiplexed cell-based studies of gene expression and gene silencing.

The generation of heterocellular niches by spatial patterning of one type of cells onto another cell type will likely be useful for a range of biological studies, including stem cell research and regenerative medicine. To this end, Tavana *et al.*\(^4\) used ATPS for non-contact printing cellular patterns on living cells in physiological environments. They engineered cellular niches to support neuronal differentiation of mouse embryonic stem cells (mESC) and showed that the density of printed mESC was an important factor for guiding mESC differentiation to neurons, Figure 3.3.6(c).

Platforms to study the role of cell-cell contact on various cellular phenotypes may strongly support numerous aspects of stem cell research. In the work by Tavana *et al.*,\(^4\) droplets of cell suspension in the denser aqueous phase were printed onto a substrate residing in an aqueous immersion phase. Because of their affinity for the denser phase, cells remained localized within the drops and adhered to regions of the substrate below the drops. Printing two different supporting cell types onto a gel surface created duplex heterocellular stem cell niches, which were overlaid with mESCs. To achieve multiple cell microarrays, the two cell types were mixed separately with the dextran phase, and the suspensions was transferred into a 1536-well plate, Figure 3.3.6(d). Slot pins mounted to a fixture were dipped into the wells to load the cell-containing dextran phase. The pins were then withdrawn from the source plate and dipped into a culture dish containing the PEG phase. The aqueous two-phase cell culture media nourished cells during incubation. After the cells attached, the ATPS were washed and replaced with regular culture media. This process resulted in uniformly sized islands of two different cell types with defined spacing. How the supporting cells were printed spatially determined the fate of the overlaid mESCs. The mESCs colonies placed on differentiation-inducing feeder cells exhibited enhanced neuronal differentiation and resulted in dense neuron networks.

Cell-to-cell communication in bacteria, called quorum sensing, allows bacteria to monitor their environment.\(^5\)\(^6\)\(^7\)\(^8\) Studying quorum sensing in the “open” space is representative of the true environment encountered by bacteria. Physically segregated, but chemically connected patterns of different bacterial populations are useful for studying the functions of bacterial and other cell populations.\(^9\) Interrogation of such quorum sensing is challenging because bacteria in suspension typically disperse due to diffusion, convection, and the motility of the bacteria themselves, which precludes spatial confinement. Yaguchi *et al.*\(^10\) developed a stable spatial patterning of sub-microliter droplets of bacterial suspensions using ATPS. Different
types of bacterial populations were positioned and maintained adjacent to each other without dispersion, Figure 3.3.6(e). ATPS was used to localize high concentrations of *E. coli*. When a chemical stimulus was applied, the droplet array produced a pattern of bacterial “illumination” that reflected the type of chemical to which the array was exposed. Microcolonies were created with different bacterial densities. Each microcolony essentially became a biosensor and, depending on the type of bacteria suspended, different chemicals could be sensed. As shown in Figure 3.3.6(e), the droplet were expected to (a) constitutively express bioluminescence, (b) constitutively express cyan fluorescence protein, (c) increase the expression of green fluorescent protein in response to acyl homoserine lactone, (d) decrease the expression of green fluorescent protein in response to acyl homoserine lactone, and (e) increase the bioluminescence in response to mutagens. Figure 3.3.6(e) shows the micrographs and plots of the response of the bacterial biosensor array.

**Figure 3.3.6** – Aqueous multiphase systems for patterning reagents or cells atop cells. (a) Dextran droplets containing reagents, either green or red dye in a PEG solution, were patterned on living cells. (b) Patterning of an aqueous dextran phase (blue) on a cell monolayer covered with the PEG phase (pink). Brightfield and fluorescent images of a patterned dextran phase on HEK293H cells spelling out “UMICH”. (c) Colony-size-dependent neuronal differentiation of mESC on PA6 supporting cells. Schematic and brightfield/fluorescent images of mESC printed on a PA6 monolayer at different densities. (d) Multiplexed aqueous two-phase cell printing. (e) Different bacteria (cyan or red fluorescent) suspended in dextran positioned inside a dish filled with a PEG-rich phase to pattern bacterial suspensions. The optical micrograph (1) of the bacterial suspensions and the chart (2) show the change in luminescence of the bacterial suspension without chemical stimulation. Images reproduced with permission and their source: (a) adapted from,²; (b) adapted from,⁴³ image supplied by the authors; (c) from,⁴⁸ copyright 2010 Wiley-VCH; (d) from,⁴⁹ copyright 2011 Wiley-VCH; (e) adapted from,⁵³ image supplied by the authors.
Other partitioning approaches: The demonstrations in the preceding section highlight the numerous advantages of multiphase systems for compartmentalization in the “open” space. Ismagilov and colleagues\textsuperscript{54} developed a device they called chemistrode and which combined multiphase systems with scanning probes. This microfluidic device used immiscible liquids to generate plugs to spatially separate liquids and enabled stimulation, recording, and analysis of molecular signals with high spatial and temporal resolution. The chemistrode therefore brought liquids transiently in contact with a chemical or biological substrate, and molecular signals were exchanged. Such an analytical tool could be useful to understand systems that rely on molecular signals, for example, chemical communication between cells. The chemistrode does this by chemical stimulation and recording of short pulses of 50 ms. Molecular signals are delivered by and captured in aqueous plugs surrounded by a fluorocarbon carrier fluid. The compartmentalization of these molecular signals eliminates dispersion and loss of sample due to surface adsorption. Recorded molecular signals were injected with additional reagents and analyzed off-line. When recombined, such analyses provided a “time stamp” on the chemical record of a system’s response to stimulation. As shown in Figure 3.3.7, insulin secretion from a single murine islet of Langerhans cells was measured using the chemistrode. The operation of the chemistrode consists of the following steps: (i) preparation of an array of aqueous plugs containing an arbitrary sequence of stimuli, (ii) delivery of the array of stimulus plugs to a hydrophilic substrate, (iii) coalescence of the stimulus plugs with the wetting layer above the hydrophilic substrate while the fluorocarbon carrier fluid remains in contact with the hydrophobic wall of the chemistrode, (iv) rapid exchange of diffusible signals between the plugs and the wetting layer, (v) delivery of response plugs to a splitting junction to form identical daughter arrays, (vi) injection of each daughter array with reagents required for further analysis, (vii) analysis of each daughter array by a different technique, (viii) and the recombination of data from the analysis of daughter arrays to provide a time-resolved record of molecular stimulation and response dynamics. This approach is therefore useful to measure molecular signals from multiple locations on a biological interface with high spatial resolution.
Figure 3.3.7 – Schematic of a chemistode in contact with a hydrophilic substrate. A fluorescent image of an islet showing an increase in fluorescence, corresponding to the rise in intracellular \([\text{Ca}^{2+}]_i\), in the islet upon simulation with the chemistode. Shown on the right are time-lapse brightfield images of an incoming stimulus plug merging with the wetting layer. The plots indicate the \([\text{Ca}^{2+}]_i\), response and insulin secretion of a stimulated cell islet. Traces measured by fluorescence microscopy during stimulation and recording showing fluorescence intensity of green dye as an indicator of \([\text{Ca}^{2+}]_i\), and the intensity of red dye as a marker of the stimulant solution (upper right). Traces with results of off-line analysis of plugs collected during recording (lower right). Images reproduced from,\(^54\) with permission from the authors.

3.3.4.4 Hydrodynamic focusing of liquids

Hydrodynamic focusing of liquids is well established in “closed” channel devices and routinely used in flow cytometers and Coulter counters for counting and determining the size of cells and particles. In this section, we review microfluidic approaches that leverage hydrodynamic focusing in the “open” space, Figure 3.3.8.

**Microfluidic probe (MFP):** The first to report on the microfluidic probe (MFP) was the IBM group in Zürich.\(^55\) The MFP combines hydrodynamic focusing with the concept of scanning probes for processing surfaces. The liquid boundaries formed by hydrodynamic forces underneath a MFP head confine a flow of “processing” solution. This is realized by setting the aspiration flow rate \((Q_a)\) of the processing solution to be greater than the injection flow rate \((Q_i)\), Figure 3.3.8(a). The base configuration of the head had two micrometer-sized
channels that injected/aspirated liquid. The surrounding liquid within which the MFP head is immersed, called immersion liquid, encapsulates the processing liquid. Therefore hydrodynamic focusing replaces the solid walls of closed microchannels, enabling the transition from closed microfluidics to microfluidics in the “open” space. In addition, because the MFP is mobile it can process large areas by scanning across them in an interactive manner. The liquid flows here are characterized by low Reynolds number (a measure of the ratio of inertial to viscous forces for a given flow condition) and high Péclet number (a measure of the convective versus diffusive transport, which helps to evaluate whether diffusion can broaden the hydrodynamically confined processing solution). Therefore, diffusion of the input liquid into the surrounding liquid is minimal, which enables local processing of biointerfaces in the “open space.” The size and shape of the confined liquid also depended on parameters such as, viscosity, density, interfacial tension between liquids, in addition to the aperture geometry and gap between the probe and the biological interface, Figure 3.3.8(b) (left). The MFP allows the aspirated solution to be used for further analysis, similar to what is done in perfusion or microdialysis. This tool was used to create chemical gradients on surfaces, protein microarrays, localized staining of cells, and the contact-free detachment of single cells, Figure 3.3.8(d). The MFP heads were fabricated in PDMS to be able to rapidly redesign the heads for different applications. To improve reliability and yield in fabrication, Lovchik et al. developed multilayered MFP heads that used standard microfabrication processes, ports and fittings. These multilayer heads were demonstrated by depositing antibodies on surfaces and by directly developing a layer of photoresist. Perrault et al. subsequently published a description of an MFP platform that largely focused on the instrumentation perspective.

With the goal of developing the MFP for broad application to biological interfaces, the IBM group developed a vertically oriented MFP, the vMFP. The vMFP overcomes challenges that limited the practical implementation of the original MFP technology. The device consisted of glass and silicon with the microfluidic features fabricated in-plane in the silicon layer. The vMFP head was oriented vertically, with the apex parallel to the surface and typical gaps of 1-30 μm. Several design and implementation strategies to achieve high yield in fabrication of the vMFP heads were reported. In addition, they also integrated functional elements such as particulate filters, redundant aperture architectures, inclined flow-paths, and multiple channels to enable symmetric flow confinements into the vMFP heads. A method to calibrate the distance between the apex of the vMFP and the biological interface was established. This approach involved visually monitoring the confinement of a solution containing fluorescently labeled antibodies on a glass surface while changing the gap distance between the probe head and the surface. The versatility of the vMFP was shown by patterning proteins on surfaces, inactivation of cells using sodium hypochlorite, and staining living NIH fibroblasts.
Figure 3.3.8 – Hydrodynamic focusing of liquids for performing local chemistries on biological interfaces. (a) Principle of the MFP. (b) Micrograph of a flow confinement and plot showing the dependence of the confined liquid on the injection/aspiration flow rates. MFPs with multiple apertures were used to generate complex shapes of flow confinements and “floating” chemical gradients (right). (c) Various MFP heads. (d) MFP-based local deposition of molecules (top left), complex gradients (top right), removal of proteins (bottom left) and structuring of photoresist (bottom right). (e) Staining confluent cells with dyes, local perfusion of brain cells, and local immunohistochemistry on cancerous tissue sections. Multiple regions of a normal human thyroid tissue section were incubated with α-TGB for various residence times. The staining intensities for various residence times are shown in the plot. The marks in the micrographs indicate the boundary between stained (lower half) and unstained regions (upper half). Images reproduced with permission and their source: (a), (b) top left, (d) top right, bottom left, and (e) upper center from, copyright 2005 Macmillan Publishers; (b) bottom left from, copyright 2011 Royal Society of Chemistry; (b) right top and bottom, and (c) bottom right adapted from, image supplied by the authors; (c) top, and (d) top left and bottom right adapted from, image supplied by the authors; (c) bottom left from, copyright 2011 American Chemical Society; (e) left adapted from, image supplied by the authors; (e) upper right and bottom adapted from, image supplied by the authors.

More recently, the IBM group applied the vMFP for multiplexed and precise staining of tissue sections. They called their technique micro-immunohistochemistry (μIHC). This staining approach circumvents typical challenges encountered by pathologists, such as limited tissue samples and the need for qualitative staining results. The incubation of primary antibodies was performed using the vMFP, which is an important step in immunohistochemistry. In μIHC, nanoliters of primary antibody solutions were confined over micrometer-sized areas of tissue sections using the vMFP. This method was preservative of tissue and reagents, alleviated antibody cross-reactivity issues and allowed a wide range of
staining conditions to be applied on a single tissue section. The vMFP head was compatible with (bio)chemical systems used for staining and resistant to a range of chemicals and could be used indefinitely unless damaged physically. Its shape and its small apex enabled easy observations of tissue sections from both above and underneath using an inverted microscope. As expected, a correlation between the incubation time of the primary antibodies and the signal intensity was observed, Figure 3.3.8(e) (bottom right). This correlation revealed that specific interactions between antigen/antibodies from various tissues could be adjusted by varying the residence time of the MFP head on the tissue section. Incubation of anti-thyroglobulin (α-TGB) with thyroid tissue was tested to achieve visible staining and the necessary incubation time was found to be on the order of 20 s for the conditions used in the experiment. This incubation duration is much shorter than the 30 min recommended for conventional staining, a reduction that was attributed to the enhanced convective flows generated by the vMFP. Multiplexed μIHC was also performed on cancerous breast tissue sections and on selected cores of a tissue microarray.

Microfluidic approaches are well suited for culturing and studying single cells; however, the culturing of organized tissue, such as brain slices, continues to be difficult in microfluidic devices. Queval et al. used the MFP to perform local perfusion of brain tissue. This perfusion allows pieces of brain tissue to be maintained in vitro for extended durations without changes in the cellular composition of the brain tissue. This allowed many biological processes that occur in the course of days to weeks in the brain tissue to be studied, while also enabling high-resolution imaging using confocal microscopy. The setup for this comprised a perfusion chamber for the culture of organotypic slices and a transparent MFP for the microperfusion of the brain tissue. The MFP used was made from PDMS and had six apertures to perform perfusion of a small number of cells in a brain, Figure 3.3.8(e) (left).

Extending the single aperture pair in the MFP to multiple pairs not only parallelized the number of local chemical reactions, but also enabled the creation of stable soluble gradients in solutions. Qasaimeh et al. developed such a two-dimensional microfluidic quadrupole (MQ) consisting of 4 apertures, and generated local floating chemical gradients. The MQ was formed by simultaneously injecting and aspirating fluids from two pairs of opposing apertures in a narrow gap formed between the microfluidic probe and a substrate, Figure 3.3.8(c) (right). Because of the multiple flow confinements, a stagnation point formed at the center of the microfluidic quadrupole, as expected, and its position was adjusted hydrodynamically. The floating gradients enabled rapid spatiotemporal tuning of the gradient either hydrodynamically by adjusting the flow rates or physically by moving the MFP. MQs could easily be applied on different biological interfaces.

Although several applications of the MFP have been demonstrated, the design and operation of the MFPs were largely guided by empirical optimization. While Qasaimeh et al. reported a theoretical framework to generate microfluidic quadrupoles using the MFP, Christ and Turner provided insights into the underlying fluid mechanics. The latter undertook a comprehensive study to establish the relationships between the device geometry, inlet and
outlet flow rates, and the fluid physics. They investigated the effect of multiple flows and aperture design parameters on the size and shape of the flow envelope and the pressure drop between the apertures of several two-aperture devices. Trends were established based on 3D computational fluid dynamic predictions and experimental measurements for several geometries of devices with a range of gap heights, flow rates, and flow rate ratios. Their analytical and simulation results provided an elegant framework for understanding the fluid mechanics of MFPs and will likely aid in the design of the future MFPs.

**Dual/multi capillary probes:** Hydrodynamic focusing of liquids in the “open” space has also been performed using dual or multiple capillaries. Several interesting demonstrations used such capillaries to hydrodynamically confine liquids and are described in this section.

Collecting bioanalytes from selected cells in culture is generally challenging. Shiku *et al.* implemented a dual capillary probe for mRNA analysis of selected adherent cells. This probe comprised of theta (h)-shaped glass capillaries. A cell lysis buffer solution was introduced from an injection aperture without affecting cells in the vicinity, and the cell lysate was collected through the aspiration aperture for analysis, Figure 3.3.9(a). Human breast cancer cells NCF-7 and malignant human mammary epithelial cells HMT-3522 T4-2 were used, and the efficiency in collection of the mRNA was evaluated with real-time PCR.

Monitoring ion channel activity of single cells is critical in electrophysiological investigations to understand the functioning of a cell. Ainla *et al.* developed a microfluidic-pipette-based device made from PDMS having a tip in which circulating liquids generated a self-confining volume in front of the outlet channels, Figure 3.3.9(b). More recently, this group published a sophisticated version of this device, which handled multiple liquids streams and allowed a rapid switching of solutions. The device used flow recirculation of liquids to eliminate cross-contamination in the “open” space and enabled spatial control of the concentration of chemicals applied to selected objects on the surface. The tip dimensions were defined by the recirculating fluid and were less than 10 μm to address single cells. Using an uptake assay, *in situ* dose-response curves from adherent chinese hamster ovary cells expressing proton-activated hTRPV1 receptors were shown. With confined superfusion and cell stimulation, hTRPV1 receptors in single cells were activated, and the response measured by a patch-clamp pipette, Figure 3.3.9(b). Another implementation of a dual pipette-based system was that of Feinerman and Moses, which was developed for local and controlled drug infusion. Their system consisted of two concentric pipettes, in which each could be manipulated separately and pressurized independently. The inner pipette was loaded with the desirable solution and functioned as a source, whereas the outer one was a sink. This enabled a flow of the solution between the two pipettes that protruded only a small distance into the surrounding fluid. Time resolution of the infusion was found to be highly controllable, and oscillatory flows were generated, if required. This was useful for certain implementations in drug dosing. A similar PDMS-based MFP was recently used by Han and colleagues to measure cell kinase activity in adherent cells.
Another approach of local chemistries was shown by Smith et al., who used a 3D microfluidic system to create micropatterned lipid bilayer arrays to study protein-ligand and protein-membrane interactions in cell membranes. Micropatterned lipid bilayer arrays have been used for biophysical investigations and sensors. Their device maintained characteristics found in cellular membranes such as fluidity and biocompatibility. An array of fluid lipid membranes was patterned onto glass substrates using a continuous flow microspotter, Figure 3.3.9(c). Fluorescence microscopy was used to verify the formation of a bilayer on a glass substrate. Fluorescence recovery after photobleaching experiments demonstrated the fluidity the bilayers while being individually corralled on the substrate. The reproducibility of bilayer formation was demonstrated by the linear response of membrane fluorescence versus rhodamine-functionalized lipids incorporated into vesicles prior to fusion with the surface. The flexible nature of the micropatterned lipid bilayer arrays was demonstrated with several different fluorescently labeled lipids to generate a multi-component lipid array. Related techniques such as microcontact printing have been used to create lipid bilayers for studies of membrane biophysics, high throughput screening of compounds, and probing living cell with synthetic membrane interactions.

A variant of the MFP/dual pipette was developed by Routenberg and Reed and applied to process semiconductor wafers locally. They also called their device an MFP; however, there is a fundamental difference between this device and the MFP first developed. The MFP of Routenberg and Reed worked in a “contact mode:” it temporarily established a fluidic path when the probe was in contact with the surface. The probe is used in a fashion analogous to an electrical needle probe in which a micro-positioner serves to bring the probe into mechanical contact with the wafer to form a sealed channel. Fluids enter and exit through tubing attached to the top of the probe. Because there is no need to permanently bond the probe tip to the wafer or to rely on self-adhesion, the contact area has to be only slightly larger than the desired fluidic channel. This device was used for nanowire field-effect transistor sensor measurements.

There are several ways of fabricating MFPs and capillary probes, ranging from the use of polymers to established microtechnologies. Corgier and Juncker recently developed a simple but manufacturable approach to fabricate such a device, namely, a polymeric microfabricated electrochemical nanoprobe (MEN) with nanometer-scale electrodes. The probe and the electrodes are formed by embedding nanometer-thick metal lines between layers of UV-curable adhesive polymers, and then the MENs are diced and detached. With the MENs, hydrodynamically confined shapes of liquids could be generated to interact with biological interfaces.
3.3.5 Outlook

A surprisingly large variety of problems and applications require local processing of biological interfaces, many of them having only recently been reported. It is therefore timely to review the literature on microfluidics with a focus on what can be done with local (bio)chemistries on biological interfaces. This minireview captures the essence of this
emerging trend and lists a number of relevant tools and techniques. There are many variants in terms of how such devices that perform microfluidics in the open space are implemented. Several scientific breakthroughs have already been made using microfluidics that operate without closed channels. Such a shift from the technical implementation to applying the technology to ever more challenging problems indicates a quick maturation of the field.

We expect significant merit in combining a range of analytical techniques on biological interfaces while performing microfluidics in the open space. This will likely be used in pharmaceuticals, biotechnology and diagnostics. Some analytical methods already being applied to biological interfaces are local surface plasmon resonance to detect molecular binding events and changes in molecular conformation,\textsuperscript{73} surface-enhanced Raman spectroscopy to sense biomolecular interactions in real-time,\textsuperscript{74} and secondary ion mass spectrometry as well as laser induced breakdown spectroscopy for local material and tissue analysis.

In a world where liquid handling is inherent to life sciences and medical research, microfluidic technologies to control liquid dispensing and handling over several orders of magnitude (microliter to femtoliter) and with micrometer positioning accuracy will be developed and become ubiquitous. It is only a matter of time until this happens. We are convinced that techniques capable of interacting, manipulating and processing biological interfaces under physiological conditions will profoundly impact the fields of surface chemistry, biophysics, diagnostics, medicine, and biology.

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### 3.3.7 References

4 CONCLUSIONS AND OUTLOOK

Two projects, where microfluidic devices were used to study cellular signaling and performing micro-immunohistochemistry, yielded a number of promising results, thereby strengthening the widely discussed potential of microfluidics as valuable tools for research and in medical applications.

In the “Brain Chip” project, the designs and assembly of the microfluidic networks have significantly evolved through the last few years. The initial strategy of depositing living cells in sealed microfluidics using flow velocity boundaries was simple because the deposition could be monitored in real time and interactive control of the flow allowed precise numbers of cells to be placed in the chambers. Keeping the devices sealed during the deposition of cell adhesion molecules and cells helped minimizing the risk of contamination. From the beginning, it proved to be very convenient that the Si lid with the ports can be reused after cleaning and a new molded PDMS MFN can be used for each experiment. Supplying culture media, buffers, chemicals and staining reagents through the cell chambers is not limited to moving chemicals from one chamber to the other but can also involve organelles such as microvesicles. The stimulation of cells can vary in duration and can even be done using a sequence of chemicals. The viability of cells can be verified and cells counted prior to investigating a pathway because cells in the chambers are observable (directly or with the help of a staining dye) using optical microscopy. Creating gradients and using laminar streams of liquid passing through a chamber can in principle be done to address a fraction of the cells in a chamber and to have a subset of the cells used as a control population.

Long-term studies on living cells can eventually be done with sealed MFNs by perfusing culture medium, controlling the temperature, and regulating the level of CO\textsubscript{2} in the cell chambers. Although such experiments would be possible, sealed MFNs are cumbersome systems for longer cultivation of cells, especially when dealing with primary cells. Primary cultures from the central nervous system, in contrast to cell lines, represent a physiologically more relevant cell model, given that they largely maintain the features of their correlates \textit{in situ} and therefore represent a more reliable system for investigating neuronal development and molecular processes of brain diseases. For this reason, leading neurobiology labs worldwide use primary cultures to investigate neuronal development, cell-to-cell signaling, and molecular mechanisms of brain diseases. Also, primary neuronal cultures are increasingly used for preclinical studies on potential drug candidates. Overflow MFNs represent a bridge between the typical workflow in cell biology and the field of microfluidics, as they combine the convenience of working on primary cell cultures with the capabilities of microfluidics for high-throughput, low-volumes, automated, and accurate assays. The possibility of plating cells in a sterile environment, keeping them in standard culturing conditions within incubators, and retrieving them at the right developmental state to use them in a microfluidic device may serve a broad range of purposes, such as performing toxicity, motility, and adhesion assays, as well as cell differentiation studies. Scaling the concept of
oMFNs to having three or more interconnected cell chambers is possible, in view of depositing different cell populations in each chamber for dissecting the intercellular flows of information occurring among brain cells. This approach might help identify the mediators and the cellular sources of molecules involved in neuroinflammation, a process that plays a very active role in the pathophysiology of progressive neurodegenerative disorders, such as Alzheimer’s and Parkinson’s diseases, and synapthopathies in general. We exemplified this in our work, where we were able to distinguish the brain-region-specific glial contribution in two neuroinflammatory scenarios using oMFNs.

Similar to the evolution of the microfluidic networks for studying cell signaling, the microfluidic probe technology improved strongly over the last few years. This “open space” microfluidic technology has strong potential for patterning surfaces, processing materials, depositing and removing biomolecules and cells on surfaces, analyzing cells and biomolecules on surfaces, creating chemical gradients on surfaces, studying complex biological specimens, such as tissue sections, and creating structures with unusual profiles, such as tapered cavities. The development of the vMFP was a significant step forward in the area of technologies for surface processing and interaction with biological interfaces. The fabrication of vMFP heads is simple and efficient as long as good design rules are followed. The vMFP is highly versatile because it localizes chemical events in a simple and interactive manner on surfaces using hydrodynamic forces created by liquids instead of solid walls of microchannels. It exploits laminar flows and physics typical to the microfluidic regime and uses them to process, manipulate and study liquid-liquid and liquid-solid interfaces. The in-plane design of the vMFP head is well suited to integrate multiple functional elements such as heaters, electrodes, valves and sensors. Heating elements can be readily fabricated and used to regulate the temperature of the liquid prior to interacting with cells, to catalyze enzymatic reactions and for increasing the rapidity in hybridization of DNA/RNA. Electrodes could be used in interesting ways to perform electrochemical reactions, detection and analysis. Unique design of flow path geometries can be used to sort and deliver cells, retrieve beads after they capture analytes of interest and enable reactions at liquid-liquid interfaces. Valves along the flow paths can be integrated to perform rapid switching of multiple processing liquids and create low dead-volume environments to deliver and retrieve precious reagents. Further, the glass on the head allows optical access of the injected and aspirated liquids, therefore is well suited to integrate waveguides and sensors for optical or fluorescence detection.

Showing the proof of concept for highly controlled local immunohistochemistry on tissue sections using the MFP was essential for our decision in pushing the technology strongly towards biological/medical applications. Being able to analyze precious tissue samples in a multiplexed or more interactive way can help pathologists in their diagnosis and may, at the end, help patients. This is a strong argument for continuing the development of this tool. Along the MFP project, the technology became more mature over time and now reached a stage where operation of the tool is fairly easy, its performance is reliable and the peripheral equipment manageable also for non-microfluidic experts.
In terms of future applications for the “Brain Chip”, I have the following thoughts: The concept of PDMS MFNs still has more potential. Its simplicity is in my opinion the strength of the technology and allows for expansion as long as the complexity of the developed systems does not restrict its use for non-microfluidic experts. Adding more cell chambers or even building a modular system that allows combining more than one MFN if needed, is a straight forward approach and would allow for more complex experimental setups with cells. As in many microfluidics, the implementation of user-friendly interfaces for manipulating the liquid flow is crucial. At one point, one would need to think about smaller connectors and holders that allow for guided assembly of the devices. The technology is probably suited for a large range of biological experiments, as it can be adapted with little effort. Screening drugs, cell-signaling studies, tissue engineering, mechanobiological observations and many other applications may be feasible. Basically most of the biological experiments performed in closed microfluidics could be done using oMFNs, with the additional benefit of being cheap (disposable PDMS MFNs) and the option for “open” cultivation, which is comparable to conventional cell culture techniques.

For the MFP technology, I see a large number of possible applications in the future. The further development of the technology in terms of user-friendliness, reliability and costs, starts to make the tool attractive for all kinds of laboratories working in the life-sciences and medicine. Spreading the technology will most likely reveal many additional applications, where localized (bio)chemistry on the microscale is beneficial. Currently the application for pathological diagnostics appears to be one of the most promising. With technologies such as the MRI that already enable the detection of very small tumors, the amount of tissue material for pathological analysis may become critical. Here, the MFP has a very high potential. I believe that with the ongoing developmental effort spent, the perception of the MFP in this community will increase strongly and commercialization of the technology may become feasible.
ACKNOWLEDGMENTS

During my PhD, I never felt alone. There was always someone around to brainstorm, chat, support and listen. I am very grateful for this!

The quality of supervision, I experienced during my work, was outstanding. Whenever needed, Emmanuel Delamarche was there for discussions and great advice. Passing Emmanuel’s quality check before submitting a manuscript for publication was almost a warranty for getting the work published in a well-respected scientific journal. His great support in my personal and scientific career means a lot to me and I want to express my gratitude for all his efforts.

It was Prof. Viola Vogel who at the end made it possible for me to join the doctoral program at ETH. Having her as the supervising Professor was great. The scientific spirit she spreads in her group is catching and motivates to perform in the best way possible. I deeply want to thank Viola for believing in me, and giving me advice and the opportunity to be a member of her great team.

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Many special thanks are due to my great colleagues in Italy. I learned a lot from Fabio Bianco, without whom the “Brain Chip” project would not have been possible. It was also a great experience working with the team of NeuroZone and Michela Matteoli. The fruitful experimental sessions with Fabio, Noemi and Ana were a real pleasure and I want to thank all of them for being a great help!

It must have been luck that I had such a great environment to work within the nanofabrication group at IBM. Having so many great colleagues at work is on the one side very encouraging to focus on the scientific work, on the other side also a big portion of fun. I owe Martina Hitzbleck a great “thank you” for being such a big help during the last years and cheering me up continuously. In my early days at IBM, working with Dan Solis was a great experience for me, which I will not forget so quickly. His way of working and spreading a good spirit had a strong influence on my personal attitude, which for me is positive. I am thankful for that. The MFP project would not be at the level, which it is now, if Govind Kaigala wouldn’t have joined our group. Very soon he became my ‘pacemaker’ in this project and together we achieved quickly, what seemed to be very far away before. I want to thank him for his positive way of thinking and his input of any kind. I also had the pleasure of working with Marios Georgiadis, a “one of a kind” masters student, who helped us greatly in pursuing the work on micro-immunohistochemistry. Also, I want to thank Heiko Wolf for his on-demand support in many situations and all the other group members for fruitful discussions, support.
in fabrication and providing good ideas. Specially, my thanks go to Ute Drechsler, who was a great help and very supportive throughout the whole work.

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There is always a life next to the work in the lab, which has to function properly to ensure the right performance during work. I am very grateful to have a great family supporting my career and a girlfriend who is always there for me. Thank you mom, dad, Martin and Sarah for all you do for me.
6 APPENDIX

6.1. High-Grade Optical Polydimethylsiloxane for Microfluidic Applications

The content of this chapter has been published:

Lovchik, R.D., Wolf, H. and Delamarche, E.
High-Grade Optical Polydimethylsiloxane for Microfluidic Applications

Contributions – I initiated this work, based on observations I made during my experiments with PDMS microfluidic networks. The PDMS formulations were devised and provided by HW. I performed the literature research and designed the experiments, which were then discussed together with the co-authors. I performed all the experiments and analyzed the data. I lead the writing of the manuscript and made all the illustrations, except for the table with the PDMS formulations, which was provided by HW.

6.1.1 Abstract

Commercially available polydimethylsiloxane (PDMS) elastomers, such as Sylgard 184® are widely used in soft lithography and for microfluidic applications. These PDMS elastomers contain fillers to enhance their mechanical stability. The reinforcing fillers, often submicrometer small SiO$_2$ particles, tend to aggregate, swell with water, and thereby become cognoscible in a way that can strongly interfere with the visualization of micro-scale events taking place next to PDMS structures. As PDMS microfluidics are often used for studying cells and micro-/nanoparticles and for creating/handling nanodroplets, it has become highly desirable to employ a PDMS having high optical quality and that allows microscopy observation without artifacts. Here, we present a PDMS formulation that is free of fillers and has sufficiently low viscosity to perform a filtration step of the mixed prepolymer before curing. By molding a bi-layer microfluidic network (MFN), composed of a thin filler-free PDMS layer and a thicker Sylgard 184® backing layer, PDMS MFNs featuring both high optical quality and mechanical stability, can be fabricated.

6.1.2 Introduction

The vast majority of research involving microfluidics is exploratory in nature$^1$ and addresses a large number of problems ranging from analyzing minute amounts of samples, analyzing biological samples in a massively parallel manner, screening libraries using a highly miniaturized format, quickly detecting disease markers in body fluids, to scaling down
chemical synthesis that are hazardous or demand high pressures and temperatures\textsuperscript{2}. Among these examples, research on cell biology, toxicity, motility and differentiation are prominent\textsuperscript{3,4,5}. Studying cells in microfabricated chambers rather than in conventional, larger culture labware is useful for several reasons\textsuperscript{6}. First, less cells and reagents are needed. Second, accurate studies can be made by exposing cells to precise quantities of (bio)chemicals for well-defined periods of time. Third, many experiments can be done one after the other in a short time period or in parallel\textsuperscript{7}.

Microfluidics for these studies are often made from Sylgard\textsuperscript{®} 184 which is a thermally curable PDMS\textsuperscript{8}. PDMS can be microstructured by replicating a photolithographically-prepared mold, which allows chambers for cells, vias, mixers, and other structures to be made with high precision. PDMS, compared to other materials used in microtechnology, is relatively soft (Young’s modulus of Sylgard\textsuperscript{®} 184 is \(~2.5\) MPa)\textsuperscript{9} and is hydrophobic (advancing contact angle with water of \(\sim110^\circ\)). Its softness allows PDMS to seal spontaneously and reversibly microfluidic structures made in silicon, glass and other materials\textsuperscript{10}. Its hydrophobicity allows proteins such as cell adhesion molecules and antibodies to adsorb spontaneously from solution onto its surface. Other advantages of PDMS are, its relative transparency\textsuperscript{11}, which allows cells within microfluidics to be monitored under a microscope, its gas permeability\textsuperscript{12}, which helps regulate medium composition via the diffusion of \(\text{O}_2\) and \(\text{CO}_2\) through PDMS, its chemical resistance to aqueous solutions and biological buffers, and the possibility to sterilize it using ethanol or heat\textsuperscript{13}.

\textbf{Figure 6.1.1} – Optical artifacts generated by fillers used in commercial PDMS elastomers. (a, b) Typical brightfield optical microscope images of a Sylgard\textsuperscript{®} 184 MFN taken next to (a) and over (b) a cell chamber containing microglia. “L”, “P” and “W” respectively denote the lumen of the PDMS chamber, a post in the chamber, and a wall of the chamber. In contrast to engineering the MFN using only one PDMS elastomer having fillers (c), a high-grade optical MFN is prepared by molding a filler-free PDMS layer that is backed with a layer of Sylgard\textsuperscript{®} 184 (d). The lid is a hard material that reversibly seals the MFN and carries ports and tubings.
However, in specific circumstances, PDMS has drawbacks that can hinder its utilization as a material for microfluidic applications. Non-crosslinked oligomers in PDMS microfluidics challenge the long term survival of fragile cells such as primary neurons plated at low density\textsuperscript{14}. Thorough extraction of PDMS microfluidics using organic solvents remedies this situation. Many organic solvents swell PDMS\textsuperscript{15}. In this case, an option is to prepare solvent-resistant microfluidics by photocuring fluorinated pre-polymers\textsuperscript{16}. Permeability of PDMS to gases and evaporation of water through PDMS can make the osmolality and gas content (O\textsubscript{2} and CO\textsubscript{2}) of a medium for cell culture hard to maintain at optimal values\textsuperscript{17}. A microfluidic device assembled by sealing a hard plastic material, which is non-permeable to water or gases, with PDMS was developed by Takayama et al. (2009)\textsuperscript{18} to alleviate this problem.

A general drawback of PDMS that, to the best of our knowledge, has not yet been addressed is that it contains filler particles, which can severely impair optical observation of cells and other small objects in PDMS microfluidics. Filler particles are used to strengthen PDMS without making it brittle and are essential for formulating useful PDMS elastomers\textsuperscript{19}. Filler particles account for ~2 to 10\% of the weight of PDMS. Opaque or hard PDMS elastomers contain up to 40\% filler particles. Fillers are generally SiO\textsubscript{2} particles functionalized with vinyl groups and have diameters ranging from ~20 to 200 nm when formed by in situ precipitation. Fillers can be even larger when blended with PDMS elastomers\textsuperscript{20}. Other components in PDMS elastomers are the base elastomer (a siloxane with vinyl groups), which also contains a Pt-based catalyst in ppm quantities, and the curing agent (a siloxane with hydrosilyl functions). The base polymer typically represents 70\% or more of the weight of PDMS.

Owing to their size distribution and their tendency to swell with water, fillers can reach a significant size and become visible under an optical microscope, Figure 6.1.1(a). These drawbacks may result in severe confounding optical artifacts when small objects such as cells are proximal to PDMS microstructures and surfaces: not only can particulates in the PDMS appear like cells but their swelling with water also strongly diminishes the transparency of PDMS, Figure 6.1.1(b and c). Since PDMS prepolymer (such as the ones used for preparing Sylgard® 184) are viscous (3900 mPa s)\textsuperscript{21} they cannot be filtered or centrifuged to remove the larger aggregates. Here, we present a method to prepare PDMS microfluidics that are optically clear. The method is based on stacking two chemically-distinct layers of PDMS, one of which does not contain filler particles and is optically clear, Figure 6.1.1(d).

### 6.1.3 Experimental section

#### Chemicals, cells and laboratory equipment

Prepolymers and the catalyst for the bioclear siloxane as well as the anti-adhesion fluorinated silane used to prime the mold were from ABCR (GmbH, Karlsruhe, Germany). Solvents were from Sigma-Aldrich and of the highest purity available. Purified water was produced using a Simplicity 185 system (Millipore, Zug, Switzerland). The commercially available PDMS was Sylgard® 184 (Dow Corning, Midland, MI, USA).
N9 murine microglial cells were obtained and cultivated as previously reported\textsuperscript{22}. Trypsin-EDTA 0.25% solution from Sigma was used to prepare cell suspensions. Polystyrene beads having a diameter of 10 µm (solid content 0.2%) were purchased from Duke Scientific (Palo Alto, CA, USA) and were 10× diluted with water. Homogenous mixing of the PDMS prepolymer was achieved using a THINKY ARE-250 mixer (Thinky Corporation, Tokyo, Japan) at 2000 rpm for 1 minute. Custom-made PDMS mixtures were filtered prior to pouring them on molds using 0.45 µm syringe filters (Sartorius Stedim Systems GmbH, Dietikon, Switzerland). After pouring the PDMS (“bioclear” or Sylgard® 184) onto the molds, a vacuum (100 mbar) was applied for about 30 min to eliminate air bubbles and ensure proper filling of the microstructures.

**Mold and Si lid fabrication**

A Si mold having 150-µm-deep structures was fabricated from a 4-inch Si wafer (Siltronix, Geneva, Switzerland) using standard photolithography, photoplotted polymer masks (resolution of 64 000 dpi, Zitzmann GmbH, Eching, Germany) and deep reactive ion etching (STS ICP, Surface Technology Systems, Newport, UK). The mold was primed with a thin fluorinated material before pouring the PDMS: it was first cleaned using an air-based plasma (30 s, 300 W coil power) and then placed in a desiccator containing ~0.2 mL of 1H,1H,2H,2H-perfluorodecyltrichlorosilane (ABCR GmbH, Karlsruhe, Germany). Priming took place at 100 mbar for 30 min and was followed by a baking step at 80 °C under ambient conditions. Sylgard® 184 PDMS MFNs were cured at 60 °C for at least 12 h. The PDMS replica was peeled from the mold and individual MFNs were cut using a scalpel. The PDMS MFNs had a thickness of ~2.2 mm. The Si lids with vias were produced in a 4-inch Si wafer using the same microfabrication methods as for the Si mold.

**Device assembly and microfluidic setup**

Nanoport™ Assemblies (Upchurch Scientific, Ercatech, Switzerland) were aligned and mounted onto the backside of the Si lid using a temperature-activated adhesive film (3M™ Bonding Film 583, IBZ Industrie AG, Adliswil, Switzerland) cut in a circular shape. Bonding took place at 120 °C for 30 min. These ports provide a fast and simple interface to connect the device to high-precision syringe pumps (Nemesys, Cetoni GmbH, Korbussen, Germany) and shut-off valves (Upchurch) using standard tubing and fittings (Upchurch).

The microfluidic devices were assembled by aligning and placing a PDMS MFN onto a Si lid. The conformal contact between the PDMS and the Si sealed the device sufficiently due to spontaneous adhesion. The device was connected and prefilled with water and the cells or beads introduced using the appropriate pump/valve configuration\textsuperscript{22}. Cells were deposited for 15 min using a flow rate of 2 µL min\textsuperscript{-1} and a concentration of ~10\textsuperscript{5} cells mL\textsuperscript{-1} followed by a rinsing step (water) with the same flow rate. Suspended polystyrene beads were deposited in the PDMS MFNs by flowing a solution of beads for 2 min at a flow rate of 3 µL min\textsuperscript{-1}. 
Optical characterization

The MFNs were placed on the stage of an inverted microscope (Nikon Eclipse TE300, Egg, Switzerland) during the experiments. The microscope was equipped with a Camcorder (Sony CDR-SR100E, Schlieren, Switzerland) to take images or videos when needed. Micrographs of PDMS MFN cross-sections were taken using an upright microscope (Nikon, Eclipse 90i, Egg, Switzerland) equipped with a color CCD camera (Nikon DXM 1200C, Egg, Switzerland). The changes in optical density at 600 nm wavelength (OD600nm) were measured using an eppendorf BioPhotometer (vaudaux-eppendorf, Schoenenbuch, Switzerland). PDMS pieces of 1 mm thickness were placed vertically into 0.5 mL cuvettes which were then filled with deionized water. The calibration for zero extinction was set using deionized water.

6.1.4 Results and discussion

Our strategy to achieve high-grade optical MFNs is to prepare composite MFNs by stacking a PDMS layer that does not contain fillers and a commercially available PDMS such as Sylgard® 184. The filler-free PDMS is here termed “bioclear”. As a result of not having fillers, the “bioclear” PDMS will be harder and more brittle than Sylgard 184®. For this reason, it is backed with a second layer of Sylgard® 184 to enable convenient handling of the MFNs. Figure 6.1.2 depicts the preparation of a composite PDMS MFN. The “bioclear” layer is first poured onto a microfabricated mold. After 45 min of curing at 60 °C, a layer of Sylgard® 184 is added and curing proceeds overnight. The microfluidics are released from the mold. Curing the first layer for only 45 min keeps it incompletely cured and ensures some degree of cross linking with the second layer. The “bioclear” PDMS layer replicates the microstructures from the mold and is in direct contact with the cells. This material and the cells both appear in the same focal plane during optical imaging and characterization of the cells. We prepared composite MFNs having a total thickness ranging from ~1 to 5 mm and kept the thickness of the “bioclear” layer at approximately 500 µm. The thickness requirements for the “bioclear” layer are defined by the depth of focus of the microscope setup. Because the supporting Sylgard® 184 layer can become slightly opaque during experiments with aqueous solutions, it should be sufficiently far away from the focal plane so that particles do not appear during microscopy. The thinnest composite MFNs were ~1 mm. This thickness permits cells to be viewed using standard 4×, 10× and 20× fluorescence and brightfield objectives as well as long working distance 40× (~3.7 mm) and 60× (~2.6 mm) objectives.

The material for the “bioclear” layer was developed to meet the following requirements: (i) it should not contain fillers that could agglomerate or cause haze upon contact with water, (ii) in the uncured state, the material should have a viscosity that allows filtration, (iii) in the cured state, it should have sufficient adhesion to seal a microfabricated lid and (iii) it has to be biocompatible. From earlier work by Schmid and Michel (2000)²³, it is known that mixtures of vinylmethysiloxane-dimethysiloxane and methylhydrosiloxane-dimethyl-
Figure 6.1.2 – Replication molding of a high-grade optical PDMS MFN. A Teflon-coated Si mold is placed in a clean plastic dish (a) and ~3 g of a “bioclear” mixture is poured onto the mold (b). After a 45 min curing step at 60°C (c), Sylgard® 184 (~25 g) is poured onto the mold (d) and cured overnight at 60°C (e) before separating the composite MFN from the mold (f, g).

siloxane copolymers, although having low viscosities as prepolymer, can form PDMS elastomers with good mechanical properties without containing a filler. However, these elastomers are much more brittle than commercial elastomers. The goal in formulating these elastomers was to fabricate PDMS stamps having submicrometer patterns. Here, fillers can be omitted because the mechanical stability is a lesser issue for MFNs, which have microstructures with dimensions in the range of tens of micrometers. The rather soft Sylgard® 184 performs well in terms of mechanical stability for most typical microfluidic applications. Therefore, we started with a prepolymer mixture similar to those described by Schmid et al. (2000)\textsuperscript{23} and stepwise reduced the crosslink density by using copolymers with a lower fraction of crosslink functionalities. Softer PDMS materials generally show a better conformal contact and better adhesion to flat surfaces. Whereas elastomers containing the higher functionalized VDT731 (7.5 mol% methylvinylsiloxane) component still did not show sufficient adhesion, mixtures with the low vinyl-content XG0677 (2 mol% methylvinylsiloxane), especially when combined with a low-functionality hydrosiloxane
component (HMS151), resulted in softer elastomer materials with adequate adhesion for sealing of MFNs. Best results were obtained by further adding a vinyl-terminated dimethylsiloxane (DMS V31) (“bioclear 1.5”, Table 6.1-1). Further reducing the crosslink density (“bioclear 1.6”, Table 6.1-1) resulted in a too soft jelly-like material, not suitable for molding. Detailed prepolymer formulations are given in Table 6.1-1. These prepolymers have a significantly lower viscosity than Sylgard 184® and particles (debris, dust) can be filtered using 0.45 µm pores, which presents another advantage.

Figure 6.1.3 shows the optical appearance of Sylgard® 184 and a “bioclear” PDMS that were exposed overnight to deionized water. The water uptake of silicate-based fillers present in Sylgard® is responsible for the haze of the material. The optical microscope images taken at the interface between Sylgard® (upper layer) and the “bioclear” layer reveal a smooth

![Figure 6.1.3](image.png)

**Figure 6.1.3** – Optical appearance and density (at 600 nm wavelength) of Sylgard® 184 and “bioclear” PDMS after exposure to deionized water. (a) Sylgard® 184 and “bioclear” cured side by side in a Petri dish and exposed to deionized water for 12 h. Swelling of the silicate fillers significantly reduces the transparency of Sylgard® 184 whereas in (b) the “bioclear” PDMS stacked with Sylgard® 184 on a mold remains transparent and free of microscopic particulates. (c) OD$_{600nm}$ of Sylgard® 184 and “bioclear” samples immersed in deionized water during 24 h. A strong increase in extinction is seen with Sylgard® 184 whereas no measurable change occurred in the “bioclear” samples.
Figure 6.1.4 – Brightfield optical microscope images of 10 µm polystyrene beads deposited in a MFN made solely from Sylgard® 184 (a-c) or made by stacking Sylgard® 184 with a “bioclear” PDMS layer (d-f).

Table 6.1-1 – Filler-free PDMS formulations for preparing optically clear microfluidics.

<table>
<thead>
<tr>
<th>PDMS “bioclear”a</th>
<th>Vinysiloxane component</th>
<th>Vinyl content [mol%]</th>
<th>Amount [g]</th>
<th>Hydrosiloxane component</th>
<th>Hydrosiloxane content [mol%]</th>
<th>Amount [g]</th>
</tr>
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<tr>
<td>1.1</td>
<td>VDT 731R³</td>
<td>7.5</td>
<td>45.4</td>
<td>HMS 301³</td>
<td>27.5</td>
<td>14.6</td>
</tr>
<tr>
<td>1.2</td>
<td>VDT 731R³</td>
<td>7.5</td>
<td>36.0</td>
<td>HMS 151³</td>
<td>16.5</td>
<td>24.0</td>
</tr>
<tr>
<td>1.3</td>
<td>XG0677³</td>
<td>2.0</td>
<td>55.2</td>
<td>HMS 301³</td>
<td>27.5</td>
<td>4.8</td>
</tr>
<tr>
<td>1.4</td>
<td>XG0677³</td>
<td>2.0</td>
<td>51.2</td>
<td>HMS 151³</td>
<td>16.5</td>
<td>8.8</td>
</tr>
<tr>
<td>1.5</td>
<td>XG0677³ / DMS V3³</td>
<td>2.0 / 0.5</td>
<td>36.1</td>
<td>HMS 151³</td>
<td>16.5</td>
<td>5.8</td>
</tr>
<tr>
<td>1.6</td>
<td>VDT 127³</td>
<td>1.0</td>
<td>45.0</td>
<td>HMS 03³</td>
<td>3.5</td>
<td>15.0</td>
</tr>
</tbody>
</table>

¹ generic formula  ² generic formula  ³ generic formula

All mixtures contained 20 µL catalyst (SIP6831.1) per 60 g of PDMS.
interface between both layers. In stark contrast to Sylgard®, the “bioclear” layer is free of particles. These differences become critical when observing small objects inside a MFN. The change in OD_{600nm} during 24 h exposure to water is shown in Figure 6.1.3(c). The extinction values of 14 pieces of Sylgard® 184 and 3 pieces of “bioclear” were measured, showing a strong difference in behavior of the two materials in contact with water. No measurable change in OD_{600nm} was observed with the “bioclear” samples whereas the Sylgard® 184 samples showed during the first hours a strong increase of extinction, which then saturated after 12 h.

Polystyrene beads that were deposited inside a MFN made from Sylgard® 184 are hard to discern and count, Figure 6.1.4(a-c). The auto focus of the digital camera mounted onto the optical microscope was not able to focus properly on the floor of the MFN where the beads were. This was obviously not the case when beads were deposited on the “bioclear” layer of a composite MFN, Figure 6.1.4(d-f).

6.1.5 Conclusions
A new PDMS formulation was developed that enabled the replication of bi-layer MFNs having high grade optical properties in the region of interest, while preserving good mechanical stability and softness of the MFNs. Numerous applications, such as droplet generation for the inclusion of chemicals, particle and cell sorting processes, toxicity assays, single cell studies and the alignment of nanowires and rods, can benefit from this method. Empirically developing an elastomer that meets the requirements for a specific application is often time consuming and requires good knowledge of the matter. Once the formulation is found, justification of the work is given by the easy reproducibility and gain in quality of the results. We believe that this method can help scientists in a broad range of scientific fields to eliminate issues caused by commercially available PDMS formulations containing fillers and improve the quality of optical investigations through such materials.

6.1.6 Acknowledgements
We thank R. Stutz for his help with the fabrication of the Si lids and molds for the PDMS microfluidics, L. Gervais for discussions, Neuro-Zone s.r.l. for a generous gift of microglia, and our colleagues W. Riess, M. Despont as well as Viola Vogel (ETHZ) for their continuous support.
6.1.7 References

6.2. Overflow microfluidic networks: application to the biochemical analysis of brain cell interactions in complex neuroinflammatory scenarios

The content of this chapter has been published:

Bianco, F., Tonna, N., Lovchik, R.D., Mastrangelo, R., Morini, R., Ruiz, A., Delamarche, E. and Matteoli, M.
Overflow microfluidic networks: application to the biochemical analysis of brain cell interactions in complex neuroinflammatory scenarios

Contributions – I designed and fabricated the microfluidic networks and built the setup for performing experiments. I optimized experimental procedures and trained NT, RM and AR in using two chamber oMFNs. I helped troubleshooting and technically supervised NT and AR during the experiments. I co-wrote the manuscript under the lead of FB, who conceived the biological experiments together with MM. The main parts of the manuscript, where I contributed were: the second half of the introduction, the device concept, the oMFN Fabrication and Setup, and the figure captions. I also reviewed the final manuscript and provided critical input. I collected all the biological data and micrographs from FB and NT and assembled the figures for the manuscript.

6.2.1 Abstract

Neuroinflammation plays a central role in neurodegenerative diseases and involves a large number of interactions between different brain cell types. Unraveling the complexity of cell-cell interaction in neuroinflammation is crucial for both clarifying the molecular mechanisms involved and increasing efficacy in drug development. Here, we provide a versatile analytical method for specifically addressing cell-to-cell communication, using primary brain cells, a microfluidic device and a multiparametric readout approach. Different cell types are plated in separate chambers of a microfluidic network so that culturing conditions can be independently controlled and single cell types can be selectively primed with different stimuli. When chambers are microfluidically connected, the specific contribution of each cell type can be finely monitored by analyzing morphology, vitality, calcium dynamics and electrophysiology parameters. We exemplify this approach by examining the role of astrocytes derived from two different brain regions (cortex and hippocampus) on neuronal viability in two types of neuroinflammatory insults, namely metabolic stress and exposure to amyloid beta fibrils, and demonstrate regional differences in glial control of neuronal physiopathology. In particular, we show that during metabolic stress, cortical but not hippocampal astrocytes play a neuroprotective role; also, in an exacerbated inflammatory
scenario consisting in the exposure to Aβ + IL-1β, hippocampal but not cortical astrocytes play a detrimental role on neurons. Aside from bringing novel insights into the glial role in neuroinflammation, the method presented here represents a promising tool for addressing a wide range of biological and biochemical phenomena, characterized by a complex interaction of multiple cell types.

6.2.2 Introduction

Neuroinflammation is a nonspecific immune reaction to tissue damage, neurodegeneration or pathogen invasion, which occurs via the combined action of resident cells such as microglia and astrocytes, and systemic cells like monocytes and macrophages.\(^1\) It is widely established that the inflammatory response promoted by tissue damage serves to further engage the immune system, thus initiating tissue repair. Whereas in most cases this response is self-limiting and induces beneficial effects (e.g., phagocytosis of debris and apoptotic cells), sustained inflammation may result in tissue pathology, favoring the production of neurotoxic factors that amplify underlying disease states. In the latter case, the sustained inflammatory responses that contribute to neurodegeneration, although initially triggered in a disease-specific manner, may end up in becoming independent of the original inflammation-inducing molecules, such as amyloid-beta (Aβ) in the case of Alzheimer’s disease or metabolic alteration in the case of ischemia.\(^2\)

Activation of astrocytes and microglia - the latter being the resident immune cells in the CNS- is one of the universal components of neuroinflammation and cross talk among these cells activate positive feedback loops, which may result in the amplification of inflammation. A large effort is therefore required to understand the cell-to-cell biochemical interactions underlying the neuroprotective or neurotoxic roles of microglia and astrocytes, and how these interactions are perturbed in chronic disease states.\(^3\)

Given the large number of possible combinatorial interactions between different brain cell types contributing to inflammations, and since cellular responses can be modulated by spatial and temporal signals from the surroundings\(^4,5\) there is a critical need for the identification of new strategies enabling cell-to-cell interactions to be addressed while manipulating cellular microenvironments. Microtechnology has provided tools to create microdevices for conducting many types of laboratory assays on a very small scale with cost and time savings and fine control of experimental setups. In particular, microfluidics have recently been used to study cellular ensembles as well as single cells.\(^6,7\) Advantages of microfluidics over larger cell culture systems include the fact that few cells can be seeded, cultured and studied in defined chemical and topographical environments.\(^8,9\) However, most microfluidic devices lack flexibility, require complex protocols for cell deposition and maintenance in vitro, and do not allow a simultaneous analysis of morphological and real-time functional parameters. For example, microfluidics having multiple compartments for studying neurons and neuritic insults have been developed but it is difficult to keep one compartment independently stimulated from the other because the compartments are isolated by carefully maintaining an appropriate hydrostatic pressure between them.\(^10\) In another example, a handheld
recirculation system was developed for culturing and imaging cells. This system includes many functionalities for seeding cells, pumping and cell perfusion but each cell culture requires one device and set of peripherals. Here, we use overflow microfluidic networks (oMFNs) for investigating various stress conditions on primary neurons, either in isolation or in microfluidic biochemical communication with glial cells. oMFNs have open cell chambers that are surrounded with array of wettable micropillars (i.e. “overflow zones”). These microfluidic chips therefore permit depositing and culturing cells for days, and afterward closing the chambers with a lid for circulating precise amounts of chemicals/solutions throughout the chambers (see below). The general concept of seeding cells and studying them in open or closed chambers using microfluidics is broadly pursued. Here, we specifically combine microfluidic-based experiments (i.e. culture and stimulation/stress of cells) with characterization methods such as immunocytochemical staining, quantitative intracellular calcium imaging and electrophysiological recordings, thus achieving a new method for efficiently dissecting specific cell type contribution. In addition, oMFNs are simple and disposable elements, which can be used with standard equipment such as cell incubators, syringe-based pumps and inverted microscopes. Through this novel approach, we demonstrate that astrocytes from distinct brain regions differentially affect hippocampal neurons (HNs) challenged with different types of inflammatory stimuli.

6.2.3 Results

Device concept

The oMFNs used for all experiments presented here are made in poly(dimethylsiloxane) (PDMS). The oMFNs have a footprint of 32 × 26 mm and the small separation distance of 1.6 mm between the chambers (edge-to-edge distance) allows visualization of both chambers simultaneously using a 4× microscope objective. The layout of the channels and corresponding ports permit liquids to be drawn sequentially or independently, if needed, through the chambers. The oMFNs are briefly treated with an oxygen-based plasma and coated with poly-L-lysine before a cell suspension is placed on each cell chamber. The overflow zones around the cell chambers have numerous wettable microstructures, which withdraw any excess liquid during the closing of the chip and ensure a sealing of the cells chambers. Figure 6.2.1(a) shows that different liquid droplets deposited on each cell chamber do not mix when the chip is closed. After sealing the oMFN with a Si lid, 6 ports are available for establishing specific culture or stimulating conditions by pumping media through the chambers with well-defined flow rates for the required time. For example, ports 1, 2 and 5 can be used to flush liquids through chamber A sequentially or in parallel so as to create a biochemical gradient in this chamber. For flushing a chemical in both chambers, port 2 can be used while keeping ports 1, 3, 5 and 6 closed and port 4 open. The volume of each chamber is approximately 0.5 µL, thus minimizing the amount of cells required. Moreover, by using high-precision pumps with flow rates ranging from 10 to 50 nL s⁻¹ very small amounts of reagents or culture media can be drawn through the cell chambers.
Figure 6.2.1 – Illustration of a oMFN having two cell chambers, functional analysis of the cells in the chambers, and protocols for probing cells interactions. (a) Cell cultures can be grown independently, each with its own culturing medium, on the vicinal cell chambers of a oMFN made in PDMS. The oMFN can subsequently be closed using a lid at the appropriate culturing time. The wettable microstructures around the chambers wick away excess culture medium, guaranteeing perfect sealing and avoiding mixing of liquids. The 6 ports glued on the lid are in regard with microchannels servicing the chambers and can be left open, closed with a threaded plug, or connected to a computer-controlled syringe. For example, injecting a solution via port 2 while leaving port 4 open and the other ports closed results in perfusing both chambers one after the other. (b) Before closing the oMFN with a lid, cells can be extensively monitored as shown in these representative images of HNs grown on oMFNs. Cells differentiate in culture and express late-stage specific markers (i.e. Vglut) and staining with βIII tubulin shows development of the neuronal network. Moreover, representative traces of mEPSCs on HNs grown either under standard culturing conditions on glass or on oMFNs are shown. These traces exhibit comparable amplitude and frequency (see text for details). (c) Table and sketch showing experimental combinations for culturing and stimulating different cell populations plated in the chambers.

For the experiments described in this study, primary HNs from E18 rat embryos were seeded in a cell chamber and cultured for up to 10 days in vitro (DIV) before seeding primary astrocytes from P2 pups in the other chamber. Cell-containing oMFNs can be placed in an incubator, and the medium can conveniently be exchanged by pipetting as much and often as required before performing experiments. Figure 6.2.1(b) shows the different combinations of distinct cell populations plated in the chambers, the cell chamber connectivity, and the modalities of cell stimulation used in the present study. Cells can be grown in chambers either separately (||) or while being in microfluidic communication (↔). One cell population
can be separately challenged with specific stimuli for a desired duration while the other cell population is kept perfused with culture medium (i.e., $A \parallel Bc$) and then put into microfluidic communication for the required experimental time (i.e., $A \leftrightarrow Bc$). Alternatively, cells can be challenged while in microfluidic communication, thus enabling analysis of functional effects produced by soluble factors and/or organelles released from cells cultured in chamber A on target cells in chamber B. Target cells can be regularly inspected and assessed in terms of morphological/functional properties using standard optical, fluorescence and electrophysiological characterization methods.

**Functional properties of neurons grown on oMFNs or in standard cultures**

Primary HNs plated on oMFNs develop normally and form wide networks of cells connected by synaptic contacts, as revealed by immunocytochemical staining with βIII tubulin, Figure 6.2.1(b). The ability of neurons plated on oMFN to form synaptic contacts (sites of communication among neurons) is demonstrated by Immunocytochemical staining for the synaptic vesicle glutamate transporter vGlut, which labels presynaptic boutons, Figure 6.2.1(b) (arrowheads). Quantitative evaluation of intracellular calcium concentrations shows basal intracellular calcium levels comparable to neurons maintained under standard culturing conditions on poly-L-lysine-coated glass ($F_{340/380}$, glass: $0.69 + 0.04$; PDMS: $0.56 + 0.03$; $n = 20$, $p = 0.35$, Student’s T-test) and a similar amplitude of the peak response following exposure to depolarizing stimuli with $50 \text{ mM KCl}$, ($F_{340/380}$, glass: $0.14 + 0.01$; PDMS: $0.15 + 0.01$; cells number = 15, 3 indep. experiments, Student’s T-test). Furthermore, HNs grown on glass or oMFNs show comparable amplitude (pA, glass: $92.67 + 10.01$; PDMS: $105.97 + 15.45$; cells number = 10, 3 indep. experiments, $p = 0.26$, Student’s T-test) and frequency (Hz, glass: $2.74 + 0.58$; PDMS: $2.41 + 0.73$; cells number = 10, 3 indep. experiments, $p = 0.97$, Student’s T-test) of miniature excitatory post-synaptic currents (mEPSCs) recorded using whole cell patch clamp. These data indicate that primary neuronal cultures from rat hippocampus grown on oMFNs exhibit morphological, functional and electrophysiological properties similar to those of neurons grown in standard culturing conditions on glass coverslips; hence, growth of cultures in the cell chambers of the chip neither influences neuronal viability nor the overall cellular phenotype and development.

**Hippocampal or cortical astrocytes differently protect hippocampal neurons during ischemic insult**

We interrogated the contribution of specific cell types to neuroinflammatory events that lead to neuronal degeneration in a well-characterized in vitro model of ischemia, namely, oxygen-glucose deprivation (OGD) protocol\textsuperscript{17}. To this end, primary HNs were grown in chamber B, while either cortical (CA) or hippocampal (HA) astrocytes were grown in chamber A, Figure 6.2.2(a). Cells were either cultured independently (Figure 6.2.2(a), protocols 1 and 2) or kept in microfluidic communication (Figure 6.2.2(a), protocols 3 and 4) in open microfluidic setting. Within these protocols, cells in the different oMFN chips were maintained either in
standard culturing conditions (Ac, Bc) or challenged with the OGD protocol (As, Bs). After 2 h of OGD, cells were allowed to recover for 24 h before being subjected to viability assays. The quantitative evaluation of cell viability, expressed as ratio of propidium iodide (PI) positive cells to the total number of cells (Figure 6.2.2(b), nuclear DAPI staining: blue; PI: red) revealed that whereas astrocytes (HA, CA) remain largely viable after the OGD challenge (PI/DAPI%: CA = 0.60 + 0.12%; HA = 2.24 + 0.77%), neurons (HN) maintained in isolation display a significant percentage of PI positive cells (19.18 + 4.65%, p < 0.01, Figure 6.2.2(c)). Notably, OGD challenge of HNs maintained in microfluidic communication with CAs results in a significant reduction of PI positive cells (2.90 + 2.08%, cells number = 30, 3 indep. experiments, p < 0.01) with respect to neurons maintained in microfluidic communication with HAs (19.58 + 3.80%, cells number = 30, 3 indep. experiments p = 0.95, Anova, post hoc Tukey’s method), suggesting a neuroprotective role played by CAs, but not by HAs.

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<tr>
<td>Exposure of cells in separate chambers or in microfluidic communication to OGD protocol</td>
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Figure 6.2.2 – Effect of hippocampal (HAs) and cortical (CAs) astrocytes on neuronal viability during ischemic insult. (a) Representation of the experiments in which hippocampal neurons (HNs), grown independently or in microfluidic communication with either CAs or HAs, are challenged with Oxygen-glucose deprivation (OGD) protocol. (b) Representative vitality images showing DAPI (blue) /PI (red) staining of HNs, grown either independently (protocols 1 and 2), or in microfluidic communication with either HAs (protocol 3) or CAs (protocol 4) and challenged with the OGD protocol. (c) Percentage of cell death following OGD protocol. Whereas challenged HAs and CAs do not exhibit increased cell death, challenged HNs show about 20% cell death. This number is significantly lower in OGD experiments that had HNs in microfluidic communication with CAs.
Hippocampal or cortical astrocytes differently affect neuronal viability during Aβ insult

To address whether the different effect on the neuronal viability exerted by astrocytes derived from different brain regions is limited to conditions of ischemic insult, we employed oMFNs to dissect cell-specific contributions in another neuroinflammatory context, namely, the exposure of brain cells to amyloid beta fibrils. HNs were grown in chamber B, while either CAs or HAs were grown in chamber A (Figure 6.2.3(a)) in open microfluidic setting. CAs and HAs in chamber A were independently exposed during 24 h to either Aβ alone or to Aβ + the pro-inflammatory cytokine IL-1β (Figure 6.2.3(a)), washed and put into closed microfluidic communication for 6 h with HNs (chamber B). HAs, CAs and HNs, independently challenged with either Aβ or Aβ + IL-1β do not show significant cell death (HN = 2.65 + 1.72; HA = 6.71 + 1.33; CA = 5.97 + 1.12; cells number = 15, 3 indep. experiments, post hoc Tukey’s method). Also, HNs, put into closed microfluidic

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<tr>
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<td>Wash of priming stimulus</td>
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<td>Coculture for 6 h</td>
<td>As modifications</td>
<td>HA</td>
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Figure 6.2.3 – Role of hippocampal (Has) and cortical (CAs) astrocytes on neuronal viability in the context of Aβ insult. (a) Protocols involved HNs that were cocultured with either CAs or HAs, previously primed with Aβ or with Aβ + IL-1β. (b) Representative vitality images showing DAPI (blue)/PI (red) and βIII tubulin (green) staining of HNs from the four protocols described in (a). (c) Comparison of death rates of HNs (unlabeled bar). HNs in microfluidic communication with HAs show a significantly increase of cell death. This is not observable when HNs are cocultured with CAs. The detrimental effect, which primed HAs have on HNs, seems to relate to glutamate excitotoxicity because the presence of APV significantly reduces cell death.
communication for 6 h with CAs and HAs, previously primed only with Aβ or IL-1β, show only a moderate increase in neuronal death (data not shown) thus confirming previously reported observation that IL-1β or Aβ fibrils alone do not heavily impact neuronal viability.\(^\text{18}\) However, when HNs were put into closed microfluidic communication with HAs previously challenged with Aβ + IL-1β, a significant increase in cell death was detected (Figure 6.2.3(b), \(\text{HN} = 16.20 \pm 2.44\); cells number = 15; 3 indep. experiments, \(p < 0.01\), post hoc Dunnett’s method), which was not observed in HNs co-cultured with CAs under the same conditions (Figure 6.2.3(c), \(\text{HN} = 6.48 \pm 1.47\); cells number = 15; 3 indep. experiments, \(p = 0.35\), post hoc Tukey’s method). The harmful effect of HAs on HNs following exposure to Aβ + IL-1β was abolished in the presence of APV, a selective antagonist of NMDA receptors, Figure 6.2.3(c).

To address whether HN damage may result from the release of soluble mediators by HAs when exposed to Aβ + IL-1β, we took advantage of the possibility to close the oMFN lid and flush the cells in chamber B with the medium from chamber A, while monitoring cellular response using single-cell calcium imaging. Astroglial cells, either HAs or CAs,

![Figure 6.2.4 – (a) Representation of the experiments in which hippocampal (HAs, protocol 1) and cortical (CAs, protocol 2) astrocytes are primed with Aβ + IL-1β and then either further cultured independently (upper panel) or immediately put into microfluidic communication with HNs (lower panel). (b) Two representative traces showing single-cell ratiometric calcium acquisitions of HNs in chamber B exposed to glial-conditioned medium from chamber A coming either from independent culturing (upper panel) or immediate microfluidic communication (lower panel) (PPh= post-priming medium from HAs; PPc = post-priming medium from CAs; CMh= co-culture medium from HAs; CMc = co-culture medium from CAs). (c) Quantification of either cell calcium influx in HNs following exposure to glial-conditioned medium either from HAs (PPh) or CAs (PPc) with respect to biochemical depolarization by exposure to 50mM KCl (upper panel) and quantification of basal intracellular calcium levels in HNs cultured either independently or in microfluidic communication with either HAs (1) or CAs (2) primed with Aβ + IL-1β (lower panel).]
independently cultured in chamber A of open oMFNs, were then primed with Aβ + IL-1β for 24 h, washed and further cultured for 6 h, Figure 6.2.4(a). Subsequently, the chambers were put into microfluidic communication using the closed microfluidic setting, and intracellular calcium dynamics of HNs in chamber 2 were acquired by time-lapse ratiometric single-cell calcium imaging acquisitions, Figure 6.2.4(b). A quantitative analysis of the response of HNs to either HA post-priming medium (PPh) or CA post priming medium (PPc) was then carried out. Figure 6.2.4(c) shows that HNs challenged with PPh exhibit higher calcium transients compared to HNs exposed to PPc. Neurons in both chambers show higher basal intracellular calcium levels when in microfluidic communication, Figure 6.2.4(c) (lower panel), but respond similarly to 50 mM KCl stimulation, thus excluding a different intrinsic neuronal responsiveness to depolarization, Figure 6.2.4(c) (upper panel). In both cases, the calcium elevation produced by the post priming solution was blocked by the NMDA receptor blocker APV, Figure 6.2.4(b).

6.2.4 Discussion

The analytical method shown here uses microfluidic networks in open and closed configurations to investigate the role played by astrocytes derived from different regions of the brain in controlling neuronal viability under two types of insults, ischemic insult and exposure to Aβ + IL-1β. In both situations, a clear difference was found between hippocampal and cortical astrocytes, with hippocampal astrocytes failing in supporting neurons after ischemic insult and playing a more detrimental role on neurons after exposure to Aβ + IL-1β.

Although astrocytes throughout the central nervous system share many common traits, a marked phenotypic diversity is detectable among astrocytes from different brain regions. Heterogeneity includes the different types and levels of neuropeptides and receptors and the specific expression of membrane transporters and channels. These phenotypic differences result in functional heterogeneity among cortical and hippocampal astrocytes which results in specific functional features, for example in an increased glutamate uptake capability by cortical rather than hippocampal astrocytes following injury.

Although ample data suggest that astrocytes play a role in both the initiation and propagation of ischemic injury, the contribution of specific astrocyte populations to this process has never been defined. Astrocytes are the principal housekeeping cells of the nervous system, playing multiple supportive tasks for neurons, including re-uptake of neurotransmitters released during synaptic activity, control of ion homeostasis and release of neurotrophic factors, shuttling of metabolites and waste products, and participation in the formation of the blood-brain barrier. Failure of any of these supportive functions of astrocytes can represent a threat for neuronal survival. Evidence is suggesting that astrocytes and factors released by astrocytes are differently modulating neuronal functionality according to regional localization and concentration of chemical mediators. In particular, it has been shown that hippocampal astrocytes are more sensitive than cortical astrocytes to OGD stimulation, resulting in an
increased hippocampal release of LDH, although reasons for the observed difference have to date not been determined\textsuperscript{20}. By indicating a selective neuroprotective action of CAs – but not of HAs – upon OGD challenge, our data suggest regional differences in astrocyte ability to support neuronal metabolic needs under ischemic insult. This is likely to result from different astroglial vulnerability to ischemia, due to both a minor metabolic suffering of CAs\textsuperscript{35} and a higher sensibility of HAs to reactive oxygen species\textsuperscript{36}.

A primary role of astrocytes in control of neuronal viability has also been demonstrated in neurodegenerative disorders, such as Alzheimer's disease (AD), in which neuroinflammatory events are heavily involved. The main inflammatory players in AD are the glial cells which initiate the inflammatory response. Indeed one of the earliest neuropathological changes in AD is the accumulation of astrocytes at sites of Aβ deposition. Several lines of evidence suggest that neurons are damaged by neurotoxic molecules elaborated from glial activation, and indeed Aβ induces inflammatory mediators, such as cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), IL-1β and tumor necrosis factor alpha\textsuperscript{37,38,39,40,41,42,43,44,45}.

Our data indicate that astrocytes from different brain regions differently affect neuronal viability upon exposure to inflammatory stimulus Aβ + IL-1β, with HAs contributing more heavily to neuronal death than CAs. The harmful effect of HAs could presumably be mediated by the higher levels of glutamate accumulated in the hippocampal relative to cortical astrocyte medium upon stimulus challenge, given that neuronal death is prevented by the NMDA blocker APV. However, it is very likely that also other inflammatory mediators contribute to the process.

\textbf{6.2.5 Concluding remarks}\

Dissecting the interactions between cell populations using a flexible microfluidic format and various signal readouts is a powerful method for investigating the specific dynamics of molecular mechanisms involved in the crosstalk among different cell populations during neuroinflammatory events. By the combined action of morphological and functional analysis on primary cell cultures, either cultured independently or in microfluidic communication, we were able to distinguish brain-region-specific glial contribution in two different in vitro models of neuroinflammation, presumably because of a glutamate-mediated excitotoxicity caused by a region-dependent alteration of metabolic activity. We suggest oMFNs and the method shown here to be a valuable, versatile and flexible analytical solution to unravel how different cell types of the brain contribute to the crosstalk events leading to neurodegeneration.
6.2.6 Materials and methods

**oMFNs fabrication and setup.** oMFNs were fabricated by casting PDMS (Sylgard® 184, Dow Corning, Midland, MI) onto microstructured 4-inch Si wafers (Siltronix, Geneva, Switzerland) used as molds. These Si molds were made using standard photolithography (64,000 dpi polymer masks, Zitzmann GmbH, Germany) and deep reactive ion etching (STS ICP, Surface Technology Systems, UK). The Si molds were coated with a fluorinated material (1H,1H,2H,2H-perfluorodecyltrichlorosilane from ABCR GmbH, Karlsruhe) at the end of the etching process for better release of the PDMS after curing overnight at 60 °C. To this end, the Si molds were cleaned using an oxygen-based plasma, exposed to vapors of the fluorinated silane using a dessicator (50-100 mbar and 30 min exposure time), and baked 2 hours at 80 °C. The PDMS oMFNs with 150-µm-deep structures were separated using a scalpel and stored in a plastic dish until used.

The Si lids were fabricated using similar lithography and etching processes as for the Si molds. NanoPort™ Assemblies (Upchurch Scientific®, Ercatech, Switzerland) were mounted to the backside of the lids, in alignment with the vias for liquid connection.

Prior to the seeding of cells, the oMFNs were treated with an oxygen-based plasma for 30 s (Technics Plasma 100-E, Florence, KY) at 200-W coil power, and then coated with a 0.5 mg mL⁻¹ solution of poly-L-lysine. The oMFNs were incubated with the poly-L-lysine overnight at room temperature. After a washing step with PBS and water, the oMFNs were dried under a stream of N₂, and cell suspensions were added onto the chambers. This was done by placing ~750 cells mm⁻² in each chamber of an oMFN. The oMFN was then placed in a Petri dish with a few mL of water next to it to prevent evaporation of the liquid in the cell chambers. The Petri dish with the oMFN was then kept in an incubator for up to 10 days. If necessary, a replacement of growth medium during incubation was done using a pipette.

The tubing and fittings needed for microfluidic experiments were purchased from Upchurch Scientific. Active pumping of liquids was done using high-precision syringe pumps (Cetoni GmbH, Korbussen, Germany), which were equipped with 50-µL glass syringes (Hamilton, Bonaduz, Switzerland). A custom-made aluminum holder facilitated the assembly of the oMFN with the lid. During this procedure, the lid was normally connected to pre-filled tubing to prevent air bubble formation in closed oMFNs.

**Chemicals and biomolecules.** Antibodies against βIII tubulin (Cat. Number G712A, 1:300 dilution) were from Promega (Milano, Italy). Aβ1-42 (Cat. Number 24224, 1 µM) was from Anaspect (Fremont, CA); DAPI and FURA 2 AM were from Invitrogen (Milano, Italy). Propidium Iodide (20 ng mL⁻¹), Abs against GFAP (1:100 dilution) and IB4 (1:200 dilution) and KCl were purchased from Sigma-Aldrich (Milano, Italy); IL-1β was from Peprotech (DBA, Italy) and APV was from Tocris (Bristol, UK). V-glut (Cat. Number 135311, 1:1000 dilution) was purchased from Synaptic Systems (Goettingen, Germany).

**Primary cultures of hippocampal/cortical neurons and astrocytes.** Primary neuronal cultures were prepared from the brains of 18-day-old rat embryos (Charles River, Milan, Italy) as previously described with minor modifications. Briefly, the hippocampi or cortices
were isolated from total brain, incubated with trypsin at 37 °C, and then dissociated to obtain separated cells, which were then plated at a density of ~750 cells mm⁻² in each chamber of the chip and grown in neurobasal medium supplemented with B27, 0.5 mM glutamine and 12.5 μM glutamate. Hippocampal/cortical astrocytic cultures from rat pups (P2) were obtained using previously described methods. Briefly, after dissection, the hippocampi/cortices were dissociated by treatment with trypsin (0.25% for 10 min at 37 °C) followed by fragmentation with a fire-polished Pasteur pipette. The dissociated cells were cultured for 14 days, trypsinized and plated in a cell chamber at a density of 2000 cells/chamber, and the cultures were grown in minimum essential medium (Invitrogen, Italy) supplemented with 20% fetal bovine serum (Euroclone Ltd, UK) and glucose at a final concentration of 5.5 g L⁻¹. Prior to trypsinization, astrocytes were shaken as previously described in order to detach microglia cells. Cell purity of astrocytic culture on PDMS was confirmed by positivity to immunocytochemical staining with glial fibrillar acidic protein GFAP and lack of signal to microglia marker IB4 as previously reported.

**Immunocytochemical staining.** Primary cells, both neurons and astrocytes, were fixed in 4% paraformaldehyde and 4% sucrose at room temperature (RT) for 10 min. Primary and secondary antibodies were applied in GDB buffer (30 mM phosphate buffer, pH 7.4, containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl) for 2 h at RT, or overnight at 4 °C. The confocal images were acquired with a Leica SPE confocal microscope, using a Nikon (Tokyo, Japan) 40x objective. Each image was a z-series projection taken at 0.8-μm-depth intervals.

**Preparation of fibrillar Aβ and cells treatment.** Fibrillar Aβ₁₋₄₂ was prepared as previously described by incubating freshly solubilized peptides at 50 μM in sterile water for 5 days at 37 °C. CAs and HAs were stimulated with fibrillar Aβ₁₋₄₂ (1 μM) alone or combined to IL-1β (10 nM). After 24 h, the cells were washed with Krebs–Ringer solution, fresh neuronal medium was added, and the cells were placed in coculture with HNs for 6 h.

**OGD protocol.** To mimic ischemic condition *in vitro*, cells were exposed to OGD following published methods. Culture medium was replaced with a solution containing 130 mM NaCl, 5mM KCl, 2mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, and 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES). Culture plates were placed in an airtight chamber (Billups-Rothenberg, Del Mar, CA, USA). The chamber was flushed with 95% N₂/5% CO₂ for 5 min with 20 L min⁻¹ gas flow, sealed, and placed in a 37 °C incubator for the appropriate duration. After the insult, OGD media were replaced with neuronal complete medium, and the cultures returned to a normoxic environment. To examine the effects of reperfusion, cells were analyzed either immediately or after 24 h of recovery.

**Quantitative evaluation of intracellular calcium dynamics.** Cultures were loaded for 35–40 min at 37 °C with 2 μM Fura-2-AM in Krebs–Ringer solution buffered with HEPES, 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose, and 25 mM HEPES (pH 7.4), and were washed twice with pre-warmed Krebs–Ringer solution before recordings were made. The recording setup comprised an inverted microscope (Axiovert 100, Zeiss, Germany) equipped with a Ca²⁺ imaging unit. Polychrome IV (TILL Photonics,
Germany) was used as a light source. Fura-2 fluorescence images were collected with a PCO Super VGA SensiCam (Axon Instruments, CA, USA) at 25°C and analyzed with TILL Vision Software (TILL Photonics, Germany). Single-cell 340/380 nm fluorescence ratios, acquired at a sampling frequency of 1–4 s⁻¹, were analyzed with Origin 6.0 (Microcal Software Inc., MA, USA).

**Electrophysiological recordings.** Whole-cell voltage-clamp recordings of spontaneous synaptic activity were performed on rat embryonic HNs maintained in culture for 10-14 DIV. Patch pipettes (2-4 MΩ) were pulled using a micropipette electrode puller (Sutter Instruments) and filled with internal recording solution containing (in mM): KGluc 130, EGTA 1, KCl 10, MgCl₂ 2, HEPES 10, Mg ATP 40, Tris-GTP 3 (pH 7.3). The cells plated on glass cover slips or in cell chambers of microfluidic chips were placed in a recording chamber perfused continuously with extracellular solution containing (in mM): NaCl 125, KCl 5, MgSO₄ 1.2, CaCl₂ 2, KHPO₄ 1.2, HEPES 25, Glu 6 (pH 7.3). Recordings were conducted at -70 mV. The series resistance ranged from 10 to 20 MΩ and was monitored for consistency during recordings. Cells in culture with leak currents > 100 pA were excluded from our analysis. Signals were recorded using Multiclamp 700B amplifiers and digitized with Digidata 1440 (Axon Instruments, Molecular devices). Signals were amplified, sampled at 10 kHz, filtered to 2 or 5 kHz, and analyzed using the pClamp 10 data acquisition and analysis program.

**Data analysis.** The data are presented as means ± SE. Statistical significance was evaluated by the Student’s test or one-way ANOVA. Differences were considered significant if p = 0.05 and are indicated by an asterisk in all figures, whereas those at p < 0.01 are indicated by double asterisks.

**6.2.7 Acknowledgments**

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6.2.8 References

6.3. Hydrodynamic levitation of a microfluidic Probe for sample-head distance control

The content of this chapter has been published:

Lovchik, R.D., Kaigala, G.V. and Delamarche, E.
Hydrodynamic levitation of a microfluidic Probe for sample-head distance control

Contributions – I conceived the concept of hydrodynamic levitation for controlling the distance of MFP heads to samples and planned the experiments together with the co-authors. I performed all the experiments and assembled the figures for the manuscript. I lead the writing of the manuscript and did the submission.

6.3.1 Abstract
We report on a self-regulating, simple and precise approach to control the distance between a scanning microfluidic probe and a surface. The approach is based on hydrodynamic levitation of the probe over a surface. We demonstrate this distance control in the context of life-sciences, where the surfaces are most often immersed in liquid, and typical variations of the surface topography are in the tens to hundreds of micrometers.

6.3.2 Introduction
Microscale patterning of curved and corrugated surfaces using scanning probe devices remains challenging, in particular for biological surfaces where contact between the probe and the sample must be avoided. Distance control is central to probes such as nanopipettes, microfluidic probes (MFPs) and atomic force microscopes. Generally, force, current, voltage or optical signals are used as feedback for distance control. These signals and feedback approaches are suitable for the μm range but not for operation within tens of micrometers and in liquid environments. One recent demonstration of locally processing surfaces on liquid environment is the cantilever-based approach, e.g. the FluidFM. In such systems, it is difficult to focus the laser in a liquid environment, and with multiple interfaces (air-liquid). This results in low Q-factor, and air bubbles tend to change refractive index, in turn disturbing the laser reflection. There is a critical need to develop strategies for regulating the distance between a scanning probe and a biological surface. We here report a simple and efficient distance control principle for the MFP.
The MFP is a scanning microfluidic technology, which hydrodynamically confines picoliters of a processing liquid on substrates immersed in liquid. It operates 10-50 µm above the surface. The MFP has been applied to flat substrates with roughnesses smaller than 5 µm to pattern proteins, stain tissues and cells.\textsuperscript{4,5} The main component of the MFP is a rhombus-shaped head made of Si and glass comprising microchannels terminating at an apex, Figure 6.3.1. So far, the sample-head distance was set manually based on a sample-head contact observed using an inverted microscope. This required transparency of the sample and its carrier (e.g. glass slide) and corrugated and curved surfaces could not be processed without human interaction. The head was leveled relative to the glass slide comprising a biological sample (e.g. tissue section) and the zero position established. Another approach was the \textit{a priori} calibration to specific topographies of the surfaces. This was time consuming, and some liquid-surface interactions were not easy or possible to visualize and often dust particles resulted in problems.

6.3.3 Principle

Here, we use hydrodynamic levitation for automated distance control. This requires only an additional pump and extra channels within the MFP head. A liquid flowing through levitation apertures generates a pressure underneath the head, thereby lifting it. At steady state, the lifting height ($d$) of the head depends on the weight ($F_{\text{weight}}$) as set with a balanced rocker.

![Figure 6.3.1 - Principle of the hydrodynamically levitated MFP.](image)

(a) A lifting force ($F_{\text{lift}}$) acts on the MFP head due to a liquid flow (D) through the levitation apertures, resulting in a lifting height ($d$). (b) Photograph of a MFP head comprising apertures for levitation, flow confinement and aspiration of immersion liquid. (c) Representation of flow lines between the apertures. The green arrows show the confinement of processing liquid and the black arrows represent the liquid injected through the levitation apertures. (d) Microscope image showing the confinement of a fluorescein solution and the injection of a suspension, containing 1 µm fluorescent beads, through the levitation apertures.
arm, the flow rate ($D$) through the levitation apertures, and the area of the apex. When the MFP head encounters a topographical variation, the distance is self-corrected due to the dependence of the lifting height ($d$) on the lifting force ($F_{\text{lift}}$) altering the hydraulic resistance ($R(d)$). The placement of the apertures on the apex is critical. Placing them near the injection/aspiration apertures perturbs the confined processing liquid whereas close placement to the periphery of the apex reduces $F_{\text{lift}}$. Design rules have been established for optimal apex size, aperture placement and flow confinement, and the head can easily be redesigned accordingly.\(^6\)

6.3.4 vMFP head fabrication and platform

The vMFP head is a two layer (Si/glass) microfluidic device comprising microchannels, vias for fluidic connection and a polished edge (apex) where the microchannels exit into open space (apertures). The apex physically supports the flow confinement, and has an area of approximately 1 mm\(^2\). The microchannels are typically 50 μm deep and taper from 200 μm width (starting from a via) to 50 μm (at the apertures). This design can easily be changed as needed and several variants of heads were described by Meister et al.\(^3\)

\[\text{Figure 6.3.2} – \text{Setup for local staining of an egg shell using a levitating MFP. (a) The setup consists of a motorized stage, a MFP head clamped in a holder, which was mounted on a record player arm to balance the weight. (b) Close-up of the holder with the levitating MFP head interacting with the egg shell.}\]
Fabrication of vMFP heads was done using standard microfabrication techniques such as photolithography, deep reactive ion etching and anodic bonding. The microchannels and vias were etched in a 500-μm-thick 4 inch Si wafer (Siltronix, Geneva, Switzerland). The microstructured Si wafer, with 33 vMFP heads was then anodically bonded to a 500-μm-thick glass wafer (Borofloat® 33, SCHOTT AG, Germany). The microstructures were filled with 80 °C molten wax (OCON 199, Logitech GmbH, Germany) for protection during dicing and polishing of the heads. Heptane was subsequently used to remove the wax from the microstructures of the polished heads.

The vMFP head was mounted in a custom aluminum holder prior to performing an experiment. The syringe pumps (not shown) were connected to the holder through capillary tubing. O-rings were used to seal the connection between the vias of the vMFP head and the ports in the holder.

### 6.3.5 Experimental section

We used the levitating MFP to pattern "challenging" surfaces, for example, a 35-μm-thick Cu substrate used for circuit boards and the shell of an egg. Etching Cu was performed using a Na$_2$S$_2$O$_8$ solution (20% w/v), while logwood brew (used to color Easter eggs) was applied to stain the egg shell. Rotating the egg around its axis resulted in 75 to 100 μm-wide stained lines, Figure 6.3.2 and Figure 6.3.3.

![Figure 6.3.3](image)

*Figure 6.3.3 – Local processing of "challenging" surfaces. (a) A 35-μm-thick Cu surface was locally etched using a confined Na$_2$S$_2$O$_8$ solution (20% w/v). (b) and (c) Lines of 75 to 100 μm width stained with logwood brew on the shell of an egg using the levitating MFP.*

### 6.3.6 Outlook

Using hydrodynamic levitation, a MFP can now easily process a large range of surfaces and not only biological specimens on glass slides. In addition, the MFP is now compatible with opaque surfaces and does not need complex sensing and actuation systems in the z-axis. This new distance control principle may spur the use of MFPs for microscale processing of surfaces in many fields. This approach is not limited to the MFP, but could broadly be used for other scanning probe approaches.
6.3.7 Acknowledgements

The authors thank U. Drechsler for assistance in microfabrication and M. Hitzbleck for discussions. V. Vogel (ETH Zurich), M. Despont and W. Riess are acknowledged for their continuous support.

6.3.8 References

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6.4. Drawbacks, solutions and practical tips

Publications can give the impression that everything in the laboratory worked just fine. In this section, I would like to provide some insight into difficulties we faced during the experimental work, describe solutions and practical tips that helped me in the lab.

6.4.1 Primary cells vs. cell lines

Cell lines are a convenient model system to test microfluidic devices, such as the "Brain Chip". When we developed the first generation of MFNs to study cellular signaling, we were not aware that the transition to experiments with primary cells would be a big challenge. The concept of using flow velocity boundaries to deposit cell lines in MFNs was useful and with this system, we were able to explore the performance of PDMS MFNs sealed with a Si lid. It gave us the opportunity to troubleshoot various problems, from the formation of air bubbles to leaking connections or delamination, as well as optimizing channel and chamber geometries. The system, however, did not work for experiments with primary cells for the reasons listed below.

- Primary brain cells (neurons, astrocytes, microglia) need longer incubation times than cell lines. Our closed MFNs were not well suited to cultivate cells for several days. Gas exchange, nutrient supply and temperature control would have required a complicated setup, which would have made the system bulky and inconvenient to use. In particular, since our plan was to develop an easy-to-use system for biologists.

- Cell density: working on a microscale determines a crucial step in neuronal seeding density. A high density yields fasciculated cells (with axons clogged) and this is a signal of stressed cells. A low density is on the other hand problematic, given that primary cells need paracrine stimulation from neighboring cells in order to correctly differentiate. This is a key issue, and has taken our partner over one year to optimize. Although we showed that we can deposit precise numbers of cells in our closed MFNs, such optimization procedures would have been cumbersome without the "open" oMFNs.

- To have a quality control of the cells used for experiments in microfluidic systems, calcium imaging, patch clamp measurements etc. should be performed a priori. Cells seeded in closed systems are not as accessible as in open systems. Also, microscopy can be difficult in closed MFNs, for example, if transmission illumination is required or high magnification observations requires access from the upper side of the MFN.

- Primary microglia are sensitive to flow. They get activated, which would influence biological data. The oMFNs allow for cultivation without moving liquids through the chambers and is better suited for studies with these cells.
6.4.2 PMDS as a base material for microfluidic networks

PMDS is a great material. It allows for fast molding of precise, transparent, flexible, gas permeable and robust microfluidics. The most used PDMS for microfluidic applications is Sylgard 184®. The mechanical behavior of this commercially available PMDS is excellent with no auto-fluorescence in the standard range of wavelengths. During our work, we experienced some drawbacks with this material that can have a considerable influence on the quality of the experiments:

- PDMS can be used to seal microfluidic structures by adhesion, if the upper surface of the microfluidics is clean and of low roughness (e.g. polished silicon, plastic, glass). This bonding is weak and can quickly lead to delamination of the sealing PDMS layer when liquids are pushed through the channels at higher flow rates. We faced this problem during our initial experiments with the "Brain Chip". Besides reducing the flow rates and redesigning the structures to avoid high pressures, many microfluidics can be driven by pulling the liquids rather than pushing them through the devices.

- Due to the above mentioned delamination problems we changed our system to pulling of liquids. The next problem occurred: the formation of gas bubbles in the microfluidic channels, most likely due to degassing of the PDMS and/or gas transport through the PDMS. This occurs more so when liquids need to be pulled through long channels having a high hydraulic resistance. Again, the flow could be reduced to solve the problem, but this is not always possible. We noticed a drastic improvement when we placed a cover slip on the PDMS. For further improvement, the PDMS can be degassed using vacuum prior to use.

- If pulling liquids through the devices is not an option, I noticed an increase in adhesion of the PDMS to glass and silicon after a heat treatment of the assembled device at 140 °C overnight. The PDMS adheres much stronger to the silicon and withstands higher flow rates when liquids are pushed through the system.

- Air bubbles in closed PDMS MFNs are often hard to remove. They stick somewhere in the channels and cannot be removed using reasonable flow velocities. To prevent air bubbles, it turned out to be useful to pre-fill the system, when needed even with 5% ethanol. A filled system is easily purged subsequently with a buffer solution to remove the ethanol. An alternative is to use centrifugation to remove air bubbles, which is explained in chapter 2.

- Another property of PDMS is that it contaminates surfaces. Low molecular weight substances originating from the PDMS can change the properties of microchannels in contact. For example, a Si channel closed with PDMS can become more hydrophobic within only a few hours.

- I addressed the issue of filler particles present in Sylgard 184® already in chapter 6.1, where I propose a solution based on a hybrid PDMS for MFNs. When I realized that the swelling of filler particles and the presence of dust particles has a strong influence
on the quality of brightfield imaging, my first attempt was to filter the material before curing. It did not work. To remove the particles with filters having pore sizes below 2 µm was almost impossible. I tried various materials of filter membranes and was only able to push a few mL of PDMS through standard size syringe filters. One additional problem was that electrostatic forces deflected the PDMS exiting the filter cartridge and the PDMS would end up on the outside of the tube or flask. I also tried to centrifuge the PDMS, which did not improve the optical quality of the PDMS. Still, the small amounts of clean PDMS I yielded through filtration, triggered the idea for the hybrid PDMS, which I strongly recommend for applications that need to be imaged in the brightfield.

6.4.3 Connecting microfluidics to pumps

Interfacing microfluidic devices to peripheral pumps, valves or other equipment can be challenging. The following points address this topic.

- We often used Nanoport™ Assemblies (Upchurch Scientific) to connect our microfluidics to pumps or valves. They work well but the footprint of the ports that are glued onto the microfluidic chip is comparably big. The space requirement for one port is at least a circle of 8 mm diameter. In addition, the height of the assembled port is more than 1 cm. This was especially a problem when we wanted to do observations with an upright microscope and transparent lids (instead of Si). The adhesive rings, which are supplied with the ports did not always work well. Tip: If a connection has a leakage between the port and the chip, nail polish proved to be an efficient sealing agent. It is applied easily, dries quickly and can be removed with acetone.

- The fittings of Nanoport™ Assemblies are thought to be used only once. When the tubing is inserted and the fitting screwed into the port, the front conical part of the fitting is squeezed and the tubing sealed and locked in place. This is inconvenient when the fitting has to be removed and fitted again. Due to the strong locking, the tubing is twisted with every turn. We regularly cut the front part (Figure 6.4.1) of the fittings and replaced it with separate ferrules from other suppliers. This allows free turning of the fitting without twisting the tubing. And, the fitting is reusable, just by replacing the ferrule.

![Figure 6.4.1— Original fitting with integrated ferrule for Nanoport™ Assemblies (left) and modified fitting with separate ferrule (right).](image)
Because Nanoport™ Assemblies are expensive, it makes sense to reuse them. With the adhesive rings supplied, the device needs to be heated in order to remove the ports. I replaced the adhesive ring with rings I punched out of 3M™ Thermal Bonding Film 583. This adhesive film is cheap and bonding can be initiated either thermally or with a solvent (e.g. acetone). The removal of a port can be done using acetone.

In the MFP project, the connecting of tubing from and to the pumps and valves was a challenge. The first generation of MFP heads used a PDMS block with punched holes, where the capillaries were inserted. This system, although not suited for high pressures, allows the connection of many channels to tubing on a small footprint. For this work, I used either Nanoport™ Assemblies for the second generation heads or a custom made connection system (third generation heads) with O-rings and clamping of the heads. The later was a fragile system and several MFP heads were broken due to stress. An alternative to the system we used, is to use connectors from Dolomite-microfluidics. We tested a circular version of their connectors successfully. The connection is reliable, the dead volumes low and the usage convenient, Figure 6.4.2.

![Diagram of MFP head connection](image)

**Figure 6.4.2** – A new concept for connecting MFP heads using a circular Dolomite connector. Figure taken from Cors et al., under review.
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11) Tissue Microprocessing

10) Hydrodynamic Levitation of a Microfluidic Probe for Sample-Head Distance Control

9) Microfluidic probe for advanced staining of human tissue sections

8) Investigating neuroprotective effects of primary glial cells using overflow microfluidic networks

7) Overflow Microfluidic Networks

6) Vertical Microfluidic Probe Heads

5) A Method to Characterize Pattern Density Effects: Chemical Flare and Develop Loading

4) Multiparametric microfluidic chips for studying cellular pathways

3) Microfluidic selection of library elements

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**Talks and posters**

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12) Hydrodynamic Levitation of a Microfluidic Probe for Sample-Head Distance Control
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11) Local Immunohistochemistry on Tissue Sections using the Microfluidic Probe

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8) Overflow Microfluidic Networks
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7) Vertical Microfluidic Probe Heads
   Lovchik, R.D., Drechsler, U. and Delamarche, E. *Poster at µTAS 2010*, Groningen

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2) Cultivation of insect parasitic nematodes in an airlift reactor
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1) Cultivation of insect parasitic nematodes in an airlift reactor;