FluidFM: Combining AFM with micro-fluidics
for applications in lifesciences and
multiparameter surface characterization

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Science is organized knowledge. Wisdom is organized life.
— Immanuel Kant
I dedicate this thesis to my family.
For their love, endless support, and patience during my time between jobs.
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This thesis would not have been possible without the help and support of numerous people. This section is dedicated to all of them. Without their contribution, I would probably not be writing these words today.

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Pascal André Behr
Abstract

The glass micropipette technique is still considered the gold standard for many single cell manipulation tasks. This method was invented over a century ago, and has since found a multitude of applications in life science related research areas. Many established methods in modern medicine like in vitro fertilization are based on this technique.

This work reports on a novel kind of instrument, inspired by the micropipette. It is based on standard atomic force microscopy (AFM) in combination with specially modified, microfluidic sensing probes. This new approach is called fluidic force microscopy (FluidFM). FluidFM is essentially a special kind of micropipette with force feedback capabilities. Thanks to these novel attributes, FluidFM is able to remedy essential drawbacks of traditional glass micropipettes such as their limitation to optical positional control only. Such a device has the application scope of the glass micropipette technique, augmented with the precision and force control capabilities of AFM technology.

The goal of this work was to develop the first functional prototype of a FluidFM instrument. For this, it was necessary to fabricate suitable hollow sensing probes to enable microfluidic functionality on AFM.

The first part of this work introduces the basic principles of atomic force microscopy and explains how AFM probes can be modified to support FluidFM applications. Different strategies for producing such FluidFM probes are presented. We will show how it is possible to produce hollow AFM cantilevers based on silicon derived materials. Furthermore, multiple fabrication strategies are proposed in order to obtain probes made entirely from photoplastic structural materials.

The second part presents a typical FluidFM setup as developed within the scope of this work. The most important components of the instrument are explained in detail. We will describe how these components must be engineered in order to obtain a functional FluidFM instrument. The major properties of FluidFM such as cargo liquid...
Abstract

Flow rates are elaborated in detail.

In the final sections, the developed prototype is utilized for various proof of principle experiments within the field of life sciences and for the purpose of multi-parameter surface scanning. It will be shown how FluidFM can be employed as a microprinter to deposit minute amounts of substances on a surface within a liquid environment. Furthermore, the technology will be utilized to carry out different manipulation tasks on single cells. Different operational modes such as gentle contact and direct injection of individual cells are demonstrated. Last but not least, the system is extended with an electrochemical setup in order to measure the tiny ionic currents flowing through the aperture of the hollow FluidFM probe. Such a modified instrument is shown to be capable of acquiring multiple sample properties in parallel during a single scan of the substrate. The method is thereby applied to a substrate that is difficult to measure with alternative techniques in order to extract information about its topological, mechanical, and electrical properties.

In summary, this doctoral thesis introduces FluidFM, a novel kind of microfluidics enabled version of an atomic force microscope and explains how it can be used for several life science related applications and for the purpose of multi-parameter surface characterizations.
Zusammenfassung


Das Ziel der vorliegenden Arbeit war die Entwicklung eines funktionierenden FluidFM Prototyps. Für diesen Zweck war es nötig, kompatible hohle AFM Messsonden zu entwickeln und zu fabrizieren, um FluidFM Funktionalität auf der Basis eines klassischen Rasterkraftmikroskops zu ermöglichen.

Zusammenfassung

zu bauen welche vollständig aus photo-aktiviertem Epoxidharz bestehen.


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6.18 Microfluidic probe technology: Assessment of benefits and drawbacks. 152
In the three decades since its first invention, the atomic force microscope (AFM) [Binnig and Quate, 1986] has already found a multitude of applications in physics and material science: It offers atomic resolution imaging of surfaces [Giessibl, 2005] and can be used as a nano-fabrication and nano-manipulation tool. AFM has demonstrated the ability to position atoms and molecules [Sugimoto et al., 2005] as well as to deliver substances to a surface with high spatial resolution [Piner, 1999]. Over the last few years, AFM has further matured to become an important tool for biological research [Hörber and Miles, 2003]. It can be used to image biological entities such as single cells, bacteria, and viruses in their native environments. The technology has also been shown to provide insights into the mechanical properties, metabolism, and adhesion of individual cells [Müller and Dufrêne, 2011]. Furthermore, successful delivery of molecular cargo to the interior of a cell has been reported [Nishida et al., 2002, Chen et al., 2007]. However, these methods offer limited control and reproducibility. So far, most of the demonstrated applications of AFM in biology are still limited to relatively coarse mechanical interactions such as indentation, dilation, and compression. The technology therefore still lacks the essential functionality needed to make it a truly versatile and universal tool for research applications in the field of cell biology.

Until today, the undisputed gold standard for many cellular manipulation and analysis tasks has been glass micropipettes. Even though they were proposed over a century ago [Barber, 1904], they are still ubiquitous and enjoy wide acceptance among the research community. Applications such as injection [Hiramoto, 1974], transfection, collection, and dispensing of one or more cells [Anis et al., 2010] are feasible and well-established with this technique. Furthermore, glass micropipettes are relatively inexpensive and can be custom made to the specific requirements of an experiment. They can also be combined with electrodes to measure ionic current signals such
Chapter 1. Introduction

as those passing across the membrane of a cell via ion channels [Neher et al., 1978]. However, this application, known as patch-clamping, is difficult to master and highlights the most important drawbacks of micropipettes: They offer limited positional control and are mechanically fragile. Since only optical inspection via a microscope can be used to position a micropipette, operators are limited by the resolution limits of optical microscopy. Furthermore, glass micropipettes are typically quite long, and are therefore prone to unwanted mechanical vibrations and other perturbations.

Motivated by the inherent drawbacks of both AFM and glass micropipette based techniques in cell biology research, a novel kind of instrument is proposed that combines the best of both worlds. This novel device has been termed a fluidic force microscope (FluidFM) and is essentially a micropipette with force control capabilities. Such a device embraces both the broad applicability of the glass micropipette technique while harnessing the precision and force control capabilities of AFM technology.

1.1 Thesis objective and chapter summary

The main goal of this thesis is to develop a force controlled micropipette that can be used as a micro-manipulator with force feedback capabilities. The device is envisioned to provide a multitude of applications in the fields of life sciences and surface characterization. To achieve this goal, a first working setup of a fluidic force microscope was developed over the course of this work. The idea for FluidFM was originally conceived in the group of Prof. Dr. Janos Vörös and PD Dr. Tomaso Zambelli at ETH Zürich and is considered the groundwork for all developments achieved within the scope of this work. This novel type of instrument will be characterized and described in detail. The most important building blocks of FluidFM, such as the required microfluidics enabled sensing probes, are explained in the corresponding chapters. Furthermore, the versatility of the technology is demonstrated through various proof of principle experiments. In conclusion, the thesis will summarize the established novel instrumentation platform from both an engineering and an application centred perspective.

This thesis is divided into several chapters as follows: Chapter 2 provides an overview of the utilized material and methods as a reference. For reasons of comprehensibility and structure, a review of current state of the art techniques in the field and the associated scientific context of this work are only provided after introducing the general principles of FluidFM. Consequently, the materials and methods section is followed by a general introduction to atomic force microscopy in chapter 3, together with the attendant theoretical concepts. Next, chapter 4 will explain how a microfluidic chan-
nel can be integrated into a standard AFM cantilever from a microprocess engineering standpoint. Several fabrication strategies for creating completely hollow polymeric AFM cantilevers have been developed and are presented. Chapter 5 will introduce the concept of fluidic force microscopy from an instrumentation perspective. The first functional FluidFM setup as engineered within the scope of this thesis will be explained. The numerous optimizations carried out as the setup evolved over the time are also described. In particular, the main components of a typical FluidFM system are identified and elucidated. Additionally, the main properties of a FluidFM setup and its core modes of operation are explained in detail. Next, the scientific context of this work is reviewed in detail in chapter 6. An overview of the most important state of the art techniques offering similar functionality and features is provided and these are compared with FluidFM to see how it differs from existing instruments.

The subsequent chapters focus on proof of principle experiments carried out using the established FluidFM setup. The goal of these experiments was to investigate the different applications enabled by this novel technology. Chapter 7 reports how the instrument can be used as a tool to deposit minute amounts of a substance onto a suitable substrate in liquid environments. Next, chapter 8 investigates how FluidFM can be employed for applications in life sciences, specifically in the manipulation, perturbation, and handling of individual cells. Chapter 9 then elaborates how an electrochemical setup can be combined with a FluidFM probe to extend the technology for the measurement of ionic currents. Such a hybrid setup is shown to enable novel applications such as multi-parameter surface characterization. Chapter 10 provides an overall conclusion to this work together with an outlook on promising future research topics. Last but not least, supplementary information and illustrations are provided in the corresponding appendices as a reference.
2 Materials & Methods

This chapter summarizes all materials, substances, equipment, instruments, and processes employed within the course of this thesis. As explained in section 1.1, an overview of current state of the art techniques in the field and the associated scientific context of this work will be provided in chapter 6 after introducing the general principles of FluidFM. This arrangement has been chosen for reasons of comprehensibility and to ensure the logical structure of the entire work.

The beginning of this chapter provides a description of the various scientific instruments used and their basic working principles. Next, an overview of all the materials and processes for the microfabrication of hollow AFM sensing probes made of photo-plastic material is provided. A summary of the software tools used for the respective computer simulation tasks can be found in section 2.5. The information presented in this chapter is intended as a reference for the reader by providing a quick overview of all materials and methods employed. Actual experimental design, results, and associated theoretical background can be found in chapters 3 to 9.

2.1 Measurement and imaging instruments

2.1.1 Atomic force microscopy

Atomic force microscopes (AFMs) belong to the family of scanning probe microscopes (SPMs). Detailed theoretical information on atomic force microscopy can be found in chapter 3. Several different types of commercial AFM instruments have been utilized for this work.
Chapter 2. Materials & Methods

**JPK Nanowizard I**

A Nanowizard I BioAFM from JPK Instruments (Germany) was used as a platform to build the first FluidFM prototype as well as for general AFM imaging. The instrument was employed as a standalone AFM system on top of a JPK standard stage. In combination with optical microscopy techniques, the instrument was operated together with a JPK manual precision stage. For live cell imaging, a JPK BioCell, a cover-slip based liquid cell, was used to control the temperature of the imaged substrate. Figure 2.1 provides an overview of the Nanowizard I platform. The instrument was operated using JPK SPM Control Software (Version 2.x/3.x and 4.x) running on a Debian/Ubuntu Linux computer, as supplied by the instrument provider.

![Figure 2.1: Overview of the JPK Nanowizard I BioAFM platform.](http://www.jpk.com)

**Cytosurge FluidFM on Nanosurf FlexAFM platform**

FluidFM experiments involving electrode integration (see chapter 9) were mostly carried out using the commercially available FluidFM platform supplied by Cytosurge AG (Switzerland). The instrument builds on the FlexAFM platform from Nanosurf AG (Switzerland) and is operated either using a Nanosurf Easyscan 2 or C3000 controller. Figure 2.2 shows a FlexAFM scan-head with FluidFM modifications, as indicated by the blue lid.

FlexAFM employs a standard optical beam deflection system (see also section 3.1.2) to detect cantilever movements. The light source used is a near infra-red laser diode. Thus the system is compatible with most fluorescent dyes commonly used in biological research. To employ phase contrast imaging in combination with the instrument, an additional correction optics module is placed on top of the scan-head. When working with opaque samples a combined top- and side-view camera can be added to the setup to locate the desired measurement position on the substrate.

The instrument can be controlled via Easyscan v3.x control software as supplied by
2.1. Measurement and imaging instruments

Figure 2.2: Commercial FluidFM instrument based on the Nanosurf FlexAFM platform. **Left:** Close-up view of a Nanosurf FlexAFM Scan Head including FluidFM related modifications. **Right:** Nanosurf FlexAFM with FluidFM modifications on an inverted optical microscope.

Nanosurf AG. In order to carry out FluidFM experiments, the instrument can also be controlled using the CyUI control software supplied by Cytosurge AG. Detailed information on this advanced user interface is provided in section 5.2.6.

**AFM cantilevers**

For standard AFM experiments, different types of commercially available cantilevers were employed to acquire AFM images and force spectroscopy curves. Table 2.1 provides a list of all commercial AFM probe types used in this thesis. More detailed technical specifications can be obtained from the corresponding manufacturers.

<table>
<thead>
<tr>
<th>Model</th>
<th>Manufacturer</th>
<th>Stiffness Constant</th>
<th>Resonance (air)</th>
<th>Coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSC38/noAl</td>
<td>Mikromash</td>
<td>0.03 N/m</td>
<td>10 kHz</td>
<td>-</td>
</tr>
<tr>
<td>CSC38/Cr-Au</td>
<td>Mikromash</td>
<td>0.03 N/m</td>
<td>10 kHz</td>
<td>Cr / Au</td>
</tr>
<tr>
<td>NSC15/AlBS</td>
<td>Mikromash</td>
<td>40 N/m</td>
<td>325 kHz</td>
<td>Al</td>
</tr>
<tr>
<td>PPP-Contr</td>
<td>Nanosensors</td>
<td>0.2 N/m</td>
<td>13 kHz</td>
<td>Al</td>
</tr>
<tr>
<td>PPP-BSI</td>
<td>Nanosensors</td>
<td>0.1 N/m</td>
<td>28 kHz</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.1: List of all utilized standard AFM cantilevers.

**Software for AFM image analysis**

For advanced AFM data analysis and image processing, we used the open source software *Gwyddion x64 version 2.36*. Gwyddion offers complete control over all applied filters and data correction procedures. The software is maintained by a
dedicated community of software engineers and can be downloaded and used free of charge under the GNU General Public License version 2. Further information can be found on the project website at http://gwyddion.net/.

2.1.2 Optical fluorescence microscopy

Fluorescence is the emission of light by a physical entity (atom, molecule, or nanostucture) which has been excited to a higher quantum state through the absorption of light or another form of electro-magnetic radiation. Upon relaxing to its ground state, the substance itself emits light of a longer wavelength than the wavelength of the original exciting radiation. This quantum mechanical effect can be exploited to visualize specific targets within a microscopic image. Labelling a specimen with fluorescent molecules - known as fluorophores - that specifically bind to the desired target allows a high signal to noise ratio to be achieved. The high spatial resolution of the underlying optical microscope can thereby be maintained. However, prolonged exposure to the exciting light can lead to perdition of the fluorophores’ fluorescent properties. This effect is called photo-bleaching. Multiple fluorophores can be utilized to image more than one component within the same specimen. The individual signals must be acquired in serial and later overlaid to create a multi-channel fluorescent image of the sample.

Epifluorescence microscopy

When excitation and detection of the fluorophores are carried out through the same light path, this is known as epifluorescence microscopy. For this thesis, only inverted microscopes were used for epifluorescence applications. This is where the objective lens is always located underneath the specimen. Illumination of the specimen is thus achieved either through the sample or from below. The main advantage of this design is the reduced working distance, allowing it to use objectives with very high numerical apertures and immersion liquids. At the same time the top side of the specimen provides free access for other techniques such as an atomic force microscope. However, only transparent samples can be used this way. For opaque samples, the AFM cantilever and the surface of the sample cannot be visualized as the light path is blocked.

Confocal laser scanning microscopy

A more advanced type of fluorescence microscopy, known as confocal laser scanning microscopy (CLSM), provides superior features and performance for optical fluores-
2.1. Measurement and imaging instruments

cence microscopy imaging. In contrast to epifluorescence microscopy, with CLSM only part of the specimen is illuminated with light. A focused laser beam is passed over an area of interest with a specific wavelength. This way it is possible to reach higher excitation powers than with conventional bright-field illumination techniques. Similar to epifluorescence based instruments, the emitted fluorescence signal is separated from the laser light using emission filters. In addition, however, a pinhole is integrated into the light path. Thanks to this additional optical element, fluorescent signals from optical planes that are out of focus can be omitted. In this way secondary fluorescence signals from other areas of the sample do not deteriorate the acquired image information. A schematic illustration of a typical CLSM light path is shown on the left in figure 2.3.

CLSM allows the acquisition of much sharper images with higher signal to noise ratio than using standard epifluorescence based methods. Furthermore, the exact position of the current focal plane can be registered. This in turn makes it possible to acquire multiple images on different horizontal planes within the same sample. These images can subsequently be used to reconstruct a three dimensional representation of the specimen with high spatial resolution (so-called z-stacks). It is also possible to combine multiple fluorescent dyes in the same sample to investigate different components in parallel. CLSM technology has matured into an important tool for many biological research applications and offers pronounced advantages over conventional epifluorescence light microscopy [White et al., 1987].

For this work, an LSM510 confocal laser scanning microscope supplied by Carl Zeiss (Germany) was employed in combination with an ultra-sensitive CCD Camera from Hamamatsu Corporation (Japan), as illustrated in the right image of figure 2.3. Detailed information on the different combinations of the utilized setups can be found after the next section.

**Combined AFM and fluorescence microscopy**

For combined AFM/optical microscopy experiments as well as for standalone optical fluorescence microscopy, different optical microscopes were employed in combinations with various digital cameras. The following listing denominates the different combinations of cameras and microscopes that were utilized in combination with AFM. Each microscope was equipped with a standard set of emission filters for the most commonly used fluorescent dyes, such as FITC and Rhodamine.
2.1.3 Pressure regulation for microflow control

In order to control the microflow of liquid inside hollow AFM cantilevers, a pressure difference is applied to the attached liquid reservoir. A more elaborate overview of the most important methods for controlling the amount of liquid flowing through a microfluidic system is provided in section 5.2.3. The methods and instruments employed for this work are described in the following sections.

Hydrostatic pressure control

The simplest method for applying positive or negative differential pressures to the microfluidic components of a hollow cantilever is known as hydrostatic pressure generation. This method makes use of the pressure exerted by a column of liquid with a certain height. Hydrostatic pressure is governed by equation (2.1). In this expression, $\rho$ denotes the density of the liquid medium in the column, $h$ stands for the height of the medium, and $g$ is the acceleration due to gravity. In this work, liquid is loaded into an external reservoir (see figure 5.3) that is connected via flexible tubing to the

<table>
<thead>
<tr>
<th>Microscope</th>
<th>Camera</th>
<th>Microscope</th>
<th>Camera</th>
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</thead>
<tbody>
<tr>
<td>Zeiss Axiovert 200</td>
<td>Hamamatsu C9100-13</td>
<td>Zeiss Axiovert 100</td>
<td>Zeiss MRm Rev.3 Motic Moticam</td>
</tr>
<tr>
<td>Zeiss LSM510</td>
<td>Hamamatsu C9100-13</td>
<td>Zeiss Observer.Z1</td>
<td>Hamamatsu C9100-13</td>
</tr>
</tbody>
</table>

Table 2.2: Table of all used combinations of optical microscopes and digital cameras.
2.1. Measurement and imaging instruments

A hollow cantilever mounted on a suitable probeholder. Detailed information about the utilized probeholders can be found in section 5.2.2. The liquid is then backfilled via the reservoir and tubing into the hollow AFM probe. The height of the liquid column producing the hydrostatic pressure is simply achieved by modulating the position of the reservoir with respect to the aperture near the apex of the cantilever. It is important to note that hydrostatic pressure is not dependent on the shape of the liquid container and hence its volume. This effect is called the hydrostatic paradox.

\[ p = \rho g h \]  

Using this method it is possible to readily apply a pressure difference to a microfluidic system. The resolution is determined by the precision of the positioning mechanism employed to control the height of the liquid column, \( h \). For water, a height difference of 1 cm generates a pressure difference of 0.981 mbar. However, as the name suggests, this is only useful if a constant amount of pressure is needed over a long period of time. The method is not practicable for applications requiring fast and frequent switching of the applied pressure, as this would require complex motorized actuation of the position of the external reservoir. Such movements would introduce mechanical noise and pressure perturbations that are not desirable in any high precision experiment involving the measurement of tiny forces, such as an AFM experiment. Furthermore, it should be noted that the fluid density \( \rho \), local gravity, and other factors are likely to vary from one experiment to another. Automation of such a system is also cumbersome and introduces additional perturbations into the system.

Active pressure regulation instrumentation

Instead of using an indirect method to create a pressure difference in the microfluidic channel of a hollow AFM probe, it is also possible to use a system that actively regulates and controls the pressures applied to the reservoir. Such devices can be based on different techniques such as syringe pumps, peristaltic pumps, or even integrated micro-sized actuators. However, as explained in section 5.2.3, a method based on the use of a sophisticated semi-open pneumatic system inspired by an electronic Wheatstone Bridge configuration was identified as most suited to our requirements. The following instruments are all based on this technology and mainly differ in the available pressure ranges and ability to switch between positive and negative pressure.

Microfluidic flow control systems (MFCs)  To be able to apply stable pressure differences to the microfluidic component of a hollow AFM probe while maintaining the
Chapter 2. Materials & Methods

ability to dynamically switch between different pressures, an MFCS-4C-345mBar from Fluigent SA (France) was used. An example photograph of such a system is shown in figure 2.4. This device offers highly stable and accurate pressure control with a resolution of 2.5% of the full scale of the instrument. For example, for a positive pressure range of 25 mbar, the device would exhibit a minimal output pressure step of 23 μbar. It also provides reasonably fast settling times to reach steady state conditions after switching between different pressure values. However, the product was only available in either positive or negative pressure range configurations when the corresponding experiments were conducted. To use both positive and negative pressures differences, it would have been necessary to use two independent instruments and manually switch them according to the required pressure range. This system was mainly used in combination with the AFM platform from JPK.

Figure 2.4: Microfluidic flow control system MFCS-4C-345 with attached reservoir. The system is supplied by Fluigent SA (France). By controlling the pressure in the attached reservoir chambers, the liquid flow rate through an attached microfluidic system can be precisely regulated (Image source: www.fluigent.com).

Cytosurge pressure controller 1.0 For experiments carried out on the commercial FluidFM platform from Cytosurge AG (Switzerland), the supplied pressure control system included with the system was used. In contrast to the instrument from Fluigent, the Cytosurge Pressure Control system is optimized for FluidFM applications. The first generation of this instrument, shown in image 2.5, has all its components integrated into a single assembly: pumps, tubing, reservoir, and control unit. The device comes in the form of a compact tabletop unit and offers the advantage of using both positive and negative pressure as needed. Switching between the two regimes is carried out automatically without the need for explicit user intervention. Switching times and resolution of the instrument is comparable to the device from Fluigent. The resolution of the instrument is also set at 2.5% of the full scale of the instrument for both the negative and positive pressure ranges.
2.1. Measurement and imaging instruments

Figure 2.5: Hardware control panel of the first generation pressure control unit from Cytosurge AG (Switzerland). The device offers both vacuum and over pressure and integrates all the pumps within a compact sized tabletop unit.

Cytosurge pressure controller 2.0 For experiments carried out after 2012, a new generation of pressure control system from Cytosurge AG (Switzerland) was employed. The main difference between this system and the first generation iteration is the separation of the noisy mechanical parts from the actual control unit. Image 2.6 shows an overview of the instrument. The pumps and reservoirs have been moved into a separate housing, as shown on the right in figure 2.6. This unit is connected to the controller part via a combined pneumatic electrical cable. In order to minimize mechanical noise from being coupled into the sensitive AFM setup, the pumping unit can be conveniently placed away from the actual experimental setup (e.g., on the floor underneath the vibration isolation table). The pressure regulating parts of the instrument included in the control unit do not generate any mechanical noise and thus can be placed very close to the experimental setup. The regulating unit is pictured on the left in figure 2.6.

Figure 2.6: Cytosurge pressure controller 2.0. This instrument features two independent units. **Left:** The control unit holds the pneumatic regulation plant and provides the connection to the external microfluidic system **Right:** The pumping entity houses the mechanical pumps and pressure reservoirs and can be placed far away from the experimental setup. (Image source: www.cytosurge.com).

All components in the pressure regulation plant are implemented as milled aluminium channels instead of flexible tubing. This substantially improves the performance of the device with regard to pressure changes. As the pneumatic connections are now made from very rigid material, they do no longer negatively impact the settling time of
the system in response to pressure changes. Steady state pressure values can therefore be reached considerably faster than with the MFCS system from Fluigent and first generation controller from Cytosurge. Furthermore, there are explicit pressure/vacuum release valves integrated into the system, allowing it to directly release pressure/vacuum whenever needed and quickly switch between pressure values over the full range of the device with only limited lag times.

The Cytosurge Pressure Controller 2.0 offers an enhanced pressure range from -800 mbar up to +1000 mbar. The two subunits (pressure and vacuum) of the instrument each measure the applied pressure via a 10 bit sensor. This allows for a discretization of $2^{10} = 1024$ steps over the full range of both the vacuum and over-pressure ranges. The controller thus offers a resolution of 0.78 mbar for negative and 0.98 mbar for positive pressure, respectively.

The system can be operated via standalone software supplied by the manufacturer. When carrying out FluidFM experiments it can also be directly controlled from within the CyUI touchscreen interface, as explained in section 5.2.6.

### 2.1.4 Electrochemical impedance spectroscopy

Chapter 9 will show how adding an electrode inside the microfluidic reservoir extends the applicability of FluidFM technology to allow the measurement of electrical signals such as ion conductivity through the hollow cantilevers. In order to characterize the electrical properties of the resulting electrochemical setup, the system was characterized via impedance spectroscopy. For the purpose of these measurements, the entire probeholder assembly was mounted inside a custom-built Faraday cage in order to shield it from electromagnetic interference during measurements. The setup was characterized using a PGSTAT30, a high current potentiostat/galvanostat from Metrohm Autolab BV (The Netherlands). The instrument is shown in figure 2.7 and offers a compliance voltage of 30 V and a bandwidth of over 1 MHz. In combination with the optional FRA2 accessory module, it is well suited for high fidelity electrochemical impedance spectroscopy sweeps.

### 2.2 Microfabrication

A major part of this work was dedicated to the fabrication of custom-made hollow AFM cantilevers. All major microfabrication processing was performed in the FIRST-
CLA\textsuperscript{1} cleanroom facilities located in the ETH Zürich central campus. The left image of figure 2.8 shows the facility’s wet bench processing area. Minor process steps such as the acquisition of optical micrographs, resist development, and final release of the fabricated structures from the carrier wafer were also carried out in the cleanroom facilities of the Institute of Electromagnetic Fields in the Electrical Engineering department at ETH Zürich.

\textbf{Figure 2.8:} The ETH Zürich FIRST-CLA cleanroom facility offers a complete range of equipment supporting all essential processes required for the fabrication of state of the art MEMS devices. \textbf{Left:} Wet bench processing area of the FIRST-CLA cleanroom facility. \textbf{Right:} The Süss Microtech MA/BA6 Mask aligner can be used for near UV lithography on 3" and 4" wafers.

\section*{2.2.1 Wafers}

All processing was carried out on standard 4 inch sized wafers. During the development of the different processing strategies, both fully transparent glass wafers and opaque silicon wafers were utilized. Table 2.3 summarizes the types of wafers used

\textsuperscript{1}FIRST - Frontiers In Research: Space & Time - www.first.ethz.ch | http://www.zfm.ethz.ch/d/mems/
Chapter 2. Materials & Methods

during process development for the fabrication of custom-made hollow AFM probes.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Material</th>
<th>Orientation</th>
<th>Thickness</th>
<th>Finish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolog Semicor Ltd</td>
<td>Silicon (100)</td>
<td>(100)</td>
<td>500 μm</td>
<td>single side polish</td>
</tr>
<tr>
<td>Mark Optics Inc</td>
<td>Borofloat 33 glass</td>
<td>-</td>
<td>500 μm</td>
<td>double side polish</td>
</tr>
<tr>
<td>Mark Optics Inc</td>
<td>Borofloat 33 glass</td>
<td>-</td>
<td>700 μm</td>
<td>double side polish</td>
</tr>
</tbody>
</table>

Table 2.3: Summary of all substrate wafers used

2.2.2 Photolithography and photomasks

Standard optical lithography was used for micropattern transfer. All lithography processing steps were carried out using an MA/BA6 mask aligner from Süss Microtech (Germany), as depicted on the right of figure 2.8. The mask aligner features a high-power 350 W Hg light source with illumination spectra in the range between 250 nm and 400 nm. The instrument offers both soft and hard contact exposure operation. In hard contact mode, the typically attainable minimal feature size is around 1 μm. However, other processing parameters can have a significant influence on the effective attainable minimal resolution.

All photomasks were designed on a computer using the CAD layout software L-Edit 12.0 from Tanner EDA Inc (USA). Before release to manufacturing, all mask designs were design rule checked against a minimal feature size of 1.5 μm to ensure there were no structures below the mask supplier’s resolution limit. CAD design data from an example photo mask created for this work is depicted in figure 2.9.

Figure 2.9: Example photomask CAD Data of a photolithography mask used during fabrication of photoplastic SU-8 probes as described in chapter 4. Left image: overview of complete mask. Right image: Close-up view of magnified area indicated in red on the left hand image.
2.2. Microfabrication

All utilized photomasks were manufactured by Deltamask BV (The Netherlands). The masks were created using a DWL 200 Laser Beam Pattern Generator from Heidelberg (Germany) on a square chrome mask blank made from soda-lime glass with a side length of 5 inches. The masks were always stored inside the cleanroom. Before mounting them inside the mask aligner, they were cleaned in a two-step process using high purity organic solvents such as acetone and isopropyl alcohol. Detailed information on the exact layout of all applied photomasks is provided in appendix A.2.

2.2.3 Photoresists for pattern transfer and lift-off applications

Depending on the requirements of the individual microfabrication process steps, different kinds of photosensitive resists were used for pattern transfer via optical lithography. Table 2.4 lists the different types of resist applied in this work. All resists were kept at room temperature inside the cleanroom environment at least twenty four hours before use in small working containers for better handling. Stock containers were stored in a refrigerator at 5 °C at all times to ensure constant resist quality as long as the expiry date was not reached.

Resist application was always carried out using a spin-coating device. Control of the target layer thickness could thus be achieved by varying the spin speed and acceleration parameters. All baking steps (i.e., soft bake and post-exposure bake) were performed using hotplates with precise temperature ramping control. Development of exposed resist layers was carried out using the appropriate developer solution as recommended by the resist manufacturer.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Tone</th>
<th>Supplier</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>ma-N1405</td>
<td>Resist</td>
<td>Negative</td>
<td>micro resist technology GmbH</td>
<td>Lift-off and pattern transfer</td>
</tr>
<tr>
<td>AZ4533</td>
<td>Resist</td>
<td>Positive</td>
<td>AZ Electronic Materials</td>
<td>Lift-off and pattern transfer</td>
</tr>
<tr>
<td>AZ4562</td>
<td>Resist</td>
<td>Positive</td>
<td>AZ Electronic Materials</td>
<td>Sacrificial layer and pattern transfer</td>
</tr>
<tr>
<td>LOR-B</td>
<td>Ancillary</td>
<td>-</td>
<td>MicroChem Corp</td>
<td>Sacrificial layer and lift-off</td>
</tr>
<tr>
<td>MF319</td>
<td>Developer</td>
<td>-</td>
<td>Rohm and Haas</td>
<td>Resist development</td>
</tr>
<tr>
<td>ma-D 533S</td>
<td>Developer</td>
<td>-</td>
<td>micro resist technology GmbH</td>
<td>Resist development</td>
</tr>
<tr>
<td>mr-Dev 600</td>
<td>Developer</td>
<td>-</td>
<td>micro resist technology GmbH</td>
<td>SU-8 development</td>
</tr>
<tr>
<td>AZ400K</td>
<td>Developer</td>
<td>-</td>
<td>AZ Electronic Materials</td>
<td>Resist development</td>
</tr>
<tr>
<td>Microposit 351</td>
<td>Developer</td>
<td>-</td>
<td>Shipley Company</td>
<td>LOR development</td>
</tr>
</tbody>
</table>

Table 2.4: Overview of photoresists, ancillaries, and developers used during microfabrication of custom-made hollow AFM probes.
2.2.4 Sacrificial materials

During the production of polymeric hollow AFM probes, a technique commonly referred to as *sacrificial layer technique* was applied. These methods are used to create hollow cavities and channels during microfabrication by means of a temporary layer acting as a filling spacer. The method is increasingly used to create microfluidic devices using standard micromachining techniques [Peeni et al., 2006]. A sacrificial layer fills in the volume of the desired cavity until the fabrication is complete and is subsequently removed via selective etching in order to release a corresponding hollow structure embedded in the final device. In this work, different materials were employed as sacrificial layer materials.

**LOR lift-off resist**

Supplied by Microchem Corp (USA), LOR is an ancillary resist based on polydimethylglutarimide. It is optimized for lift-off and sacrificial layer applications. In this work, a formulation of LOR-10B was employed that is suitable for target layer thicknesses between 5 μm and 15 μm. LOR resists cannot be directly patterned via photolithography since they do not contain any photoinitiator substances. For this reason, patterns of LOR are always defined via a secondary imaging resist in the form of a soft mask and subsequent co-development of both layers. Special care needs to be taken to avoid pronounced undercut of the LOR layer compared to the secondary patterning layer when used as sacrificial material. The dissolution rate of LOR can be optimized by varying the applied soft bake temperature during pre-processing of the resist. Detailed information on LOR-10B processing can be found in [Golden et al., 2009]. Development and striping of LOR are achieved via a 20% and 100% solution of microposit 351 developer, respectively.

**AZ-4562 positive tone resist**

Another type of resist used as sacrificial layer material is AZ-4562, which belongs to the AZ 4500 series of thick resists featuring optimized adhesion properties. AZ-4562 is supplied by AZ Electronic Materials (Luxembourg). It is compatible with all major plating and wet etching processes commonly used in microfabrication. Resist thicknesses in the range of 5 μm or higher can be readily obtained using AZ-4562. Thanks to its high thickness compatibility, the resist is well-suited for applications requiring high-aspect ratio structures. While the resist is not officially endorsed for use as a sacrificial layer material, we found this application highly feasible. Like SU-8, this resist has been pioneered as a structural material for the fabrication of MEMS.
2.2. Microfabrication

2.2.5 SU-8 photoplastic resist

SU-8 is a common material used in modern microfabrication processes to create microstructures and MEMS devices made from photoplastic materials. SU-8 was invented by IBM Research [Lee, 1995, LaBianca and Gelorme, 1995]. The material was originally developed to satisfy the need for a resist film technology capable of creating high aspect ratio pattern transfer structures without the use of an x-ray source. However, thanks to its flexibility and compatibility with standard UV lithography, the material has since been increasingly used as a structural material within numerous MEMS devices [Despont et al., 1997].

General properties of SU-8

SU-8 photoplastic is a negative tone photoresist consisting of the epoxy-based EPON SU-8 resin from Shell Chemicals. The resist is rendered photosensitive by adding a commercial triaryl-sulfonium salt known as CYRACURE UVI from Union Carbide. EPON SU-8 is the resist with the highest commercially available epoxide functionality thanks to its eight epoxide functional groups per monomer component, as shown in figure 2.10. Due to the highly cross-linked nature of this photoplastic material, it exhibits very high thermal stability as well as excellent chemical resistance to a multitude of substances. Since the curing agent is not consumed in the cross-linking process, the material achieves a very high degree of networking. Cured SU-8 material features a relatively low Young's modulus of around 4 GPa. Furthermore, structures featuring thicknesses >1000 μm with aspect ratios as high as 18:1 can be achieved with this material [Lorenz et al., 1998]. This dramatically increased design space makes it possible to create novel and complex microstructures such as hinges, micro-gears and micro-mirror arrays that benefit from the relatively low Young's modulus of the material. In comparison, commonly used silicon-based structural materials such as silicon nitride ($Si_3N_4$) and silicon oxide ($SiO_2$) exhibit much higher Young's moduluses in the range of 250 GPa and 75 GPa, respectively. For these reasons, the material is well-suited to producing soft cantilevers for AFM/FluidFM and bio-sensor applications. Further information on SU-8 properties and applications for microfabrication can be found in [Genolet, 2001].
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Figure 2.10: Illustration of the molecular structure of SU-8. **Left:** The eight 3-membered rings forming the cyclic ethers of the epoxide functional group can be easily identified in the SU-8 monomer. **Right:** Cross-linked polymer material after polymerization by photoacid generation and subsequent heat activation. Figure by Iridium (own work) licensed under CC BY 3.0 via Wikimedia Commons.

SU-8 processing

A typical SU-8 workflow is similar to conventional photoresist processing. Image 2.11 illustrates the process flow used for this work. First the wafer surface is pre-treated in order to ensure good adhesion for the SU-8 layers. This step is optional, but highly recommended depending on the wafer material used. Next, a layer of raw SU-8 resist is spin coated onto the target wafer. Using specific formulations depending mainly on the weight percentage of solvent and effective spin-speed, it is possible to precisely tune the final thickness of the SU-8 layer. Immediately after spin coating, the wafer is soft-baked at a temperature of around 95°C in order to evaporate the remaining solvent components from the SU-8 layer. The desired thickness is only achieved after sufficient soft-bake times. Careful temperature ramping is advised to reduce intrinsic mechanical stress in the final device. After the soft-bake, the SU-8 layer is mechanically stable and solid. Only after reheating it above 55°C does the layer start to reflow, as this marks the glass transition temperature of the material. The layer is now ready to be exposed by means of a standard i-line photolithography mask aligner. For improved resolution and sidewall profiles, a high-pass filter that removes UV radiation below 350 nm wavelengths is recommended. The exposure doses will have to be increased accordingly in this case. UV light exposure generates acid catalyst components in the resist that will induce SU-8 cross-linking. However, since the molecular motion is almost entirely inhibited in the solid state layer, this process needs to be activated by heating the exposed material in a post-exposure bake step to a temperature of 95°C. Again, it is important to use slow temperature ramps for both the heating and cooling phases of this process step. Effective cross-linking
2.2. Microfabrication

activity is only observed for temperatures above the glass transition temperature of uncross-linked SU-8.

Figure 2.11: Overview of the typical processing flow used for microfabrication with SU-8 resist. The process flow is similar to conventional photoresists; however, there is an additional post-exposure bake step that needs to be carried out in order to initiate cross-linking of the monomers to create a matrix polymer structure with high mechanical and chemical stability.

After the post-exposure step, the structures can be developed and hard-baked at higher temperatures to increase their mechanical robustness. However, it is also possible to repeat the same process to create multi-layered structures made from SU-8. Additional layers can thereby be placed on developed or undeveloped structures from previous SU-8 layers. These potential additional processing steps are indicated in figure 2.11 via dotted line arrows. For multi-layer exposure it is recommended to apply the exposure dose that corresponds to the combined thickness of all present SU-8 layers to ensure proper interlayer cross-linking. The post-exposure parameters, however, are not changed.

2.2.6 Thin film deposition

As explained in section 4.3, the microfabrication process developed in this thesis for the creation of hollow photoplastic AFM cantilevers required the use of different metal layers.

An enhanced sacrificial layer technique as proposed by [Genolet, 2001] was employed to release the final probes from the substrate wafer. The required metal layers were created by means of a physical vapor deposition process based on an evaporation source heated via an electron beam. For this purpose a modified Edwards E306 evaporation system from HHV Ltd. (UK) was used. The resulting metal layer thickness was monitored in real-time via a quartz crystal microbalance located inside the machine’s
evaporation chamber. Pure chromium and gold pellets supplied by Kurt J. Lesker Company (USA) were used as target materials for the evaporation process.

To create metal layers for use as an embedded mask, the Univex 450 thermal evaporator system from Oerlikon Leybold Vacuum (Switzerland) was used. The chromium layer was evaporated from pure chromium pellets provided by Kur J. Lesker Company (USA) sitting in a radiation shielded crucible heater supplied by RD Mathis Company (USA).

### 2.3 Buffers and Solutions

#### 2.3.1 HEPES

(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is an organic chemical buffering agent commonly referred to as HEPES. It is widely used in cell culture and related experiments. HEPES exhibits superior properties with respect to maintaining pH in the face of changes in carbon dioxide concentration when compared to other solutions such as bicarbonate buffers. HEPES can be applied for buffering ranges between pH 6.8 and 8.2. Due to the temperature dependent dissociation constant, HEPES is a very effective agent to ensure long-term stability of the structure and function of dissolved enzymes when stored at lower temperatures.

#### 2.3.2 HEPES-2

HEPES-2 buffer is prepared from 10 mM HEPES complemented with 150 mM NaCl in ultrapure water (Merck-Millipore). The pH of the solution is adjusted to 7.4 using 6 M NaOH. It is also commonly referred to as HEPES buffered saline (HeBS).

HEPES-2 was used to prepare functional surface coatings required for spotting applications in a liquid environment, as demonstrated in chapter 7. It can also be used to directly cover FluidFM cantilevers with an anti-fouling coating, as described in detail in [Doerig, 2013, Guillaume-Gentil et al., 2014]. Furthermore, HEPES-2 was applied as a buffering and dilution agent for various cantilever cargo solutions employed in the different experiments carried out during this work.

#### 2.3.3 Phosphate-buffered saline (PBS)

PBS was prepared from 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate and 2 mM potassium hydrogen phosphate in ultrapure
2.3. Buffers and Solutions

water (Merck-Millipore). The pH was adjusted to 7.4. PBS was used as buffering agent for various cargo solutions.

2.3.4 Fluorescein stock solution - FITC tracer

In order to visualize fluid traces that flow from a FluidFM probe, the addition of fluorescein isothiocyanate (FITC) to the cargo solution has proven a very easy and effective technique (see also section 7.1). For this reason, a stock solution of FITC tracer was prepared. The stock solution can be readily added to the cargo solution in a suitable concentration to avoid negatively affecting overall experimental performance.

FITC is a highly fluorescent compound. It emits light at a wavelength of 520 nm when excited with blue light at a wavelength of 496 nm.

FITC stock solution was prepared from 1 mg/mL fluorescein sodium salt (Sigma Aldrich) in HEPES. The stock solution was kept refrigerated and was added to the experimental cargo solutions just before loading into the FludiFM probe.

2.3.5 PLL-g-PEG / PLL-g-PEG-Biotin

PLL(20)-g[3.5]-PEG(2) from SuSos AG (Switzerland) was used for various surface coating applications. The notation stands for the graft copolymer comprising a PLL backbone with a molecular weight of 20 kDa, a grafting ratio of lysine-mer/PEG side chain of 3.5 and PEG side chains of molecular weight 2 kDa [Huang et al., 2001]. Image 2.12 indicates the chemical structure of this copolymer molecule. The general short description PLL-g-PEG standing for poly(L-lysine)-grafted-poly(ethylene glycol) will be used throughout the remainder of this thesis.

PLL-g-PEG is a polycationic copolymer. Therefore it adsorbs spontaneously from aqueous solutions onto negatively charged surfaces where it forms a monolayer of densely packed PEG chains. Such a coating impedes unspecific binding of proteins that normally adsorb onto various surfaces [Huang et al., 2001]. It can therefore be used as a non-fouling coating.

PLL-g-PEG is also available with functionalized PEG such as PEG-Biotin. PLL (20)-g[3.5]-PEG (2)/PEG(3.4)-biotin(50%) from SuSos (Switzerland) is PLL-g-PEG with a ratio of 45% to 65% Biotin functionalized PEG. PLL-g-PEG-Biotin will be used as short notation in the text for this type of functionalized PLL-g-PEG.
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2.4 Electrochemical components

2.4.1 Ag/AgCl electrodes

The electrode integrated into the reservoir of the FluidFM setup consisted of a teflon insulated silver wire with a short portion of exposed bare silver at one end. The exposed silver was chlorinated in a 3 mol KCl solution via electrolysis for one minute to form a layer of AgCl salt on the bare silver surface. Ag/AgCl electrodes created in this way can be readily renewed or recreated using a new silver wire whenever needed. This way good electrode quality can be ensured for all experiments. The silver wire used was supplied by Science Products GmbH (Germany).

2.4.2 Wax for maximum ionic current impermeability

A commercial FluidFM probe has a small gap between the back-port enclosure and the rest of the probeholder cantilever assembly. A small leaking current is observed via this gap whenever the assembly is placed in an electrolytic environment. A layer of Paraplast Plus as supplied by Sigma-Aldrich (Switzerland) was therefore applied over
this gap to ensure proper electric sealing. Paraplast is a paraffin - polyisobutylene mixture with a melting point of 56 °C. The wax is melted and the back of the probeholder assembly with the attached connector is briefly dipped into the molten wax. This way a conformal layer of paraffin remains on the reverse of the probeholder. Due to the highly hydrophobic nature of the wax, this layer essentially acts as an excellent diffusion barrier that prohibits any transfer of electrolyte components between the reservoir inside the probeholder and the surrounding liquid.

### 2.4.3 Patch clamp amplifier - PicoAmp 2

In order to measure the tiny ionic currents flowing through the aperture of an electrode enabled FluidFM setup, a suitable amplifier needs to be employed. For this work, a PicoAmp 2 patch-clamp amplifier from Tecella Inc. (USA) was used. This instrument is very compact and could therefore be directly attached to the AFM head of the measurement setup to ensure an optimal signal to noise ratio. Since this amplifier is essentially an I/V converter with a high gain factor; all current signals are recorded as voltages. In order to determine the corresponding current values, the gain factor of the amplifier needs to be recorded for each experiment. Thanks to the built-in compensation circuitry of the amplifier, it is possible to counteract for the most disturbing parasitic electronic components of the system. Liquid junction potentials and the parasitic capacitance of the hollow cantilever could therefore be effectively compensated in order to ensure optimal signal fidelity.

Using the amplifier, it is also possible to apply a potential between the two electrodes of the FluidFM setup. To test the probes after they were mounted on the instrument, a small voltage pulse was applied. The resulting ionic current response was analyzed in order to extract the electrical resistance of the DC current component. Typical resistance values were found to be in the range of 26 MΩ to 35 MΩ for the case of 150 mM KCl electrolyte for all cantilevers when far away from the substrate. Once these parameters are confirmed, the instrument is ready to begin the actual experiment.

### 2.5 Computer simulations

In order to assess different properties of FluidFM, especially with respect to the microfluidic probes, several computer simulation models were developed. All simulations were carried out using a specialized simulation environment available at ETH Zürich for academic use.
2.5.1 Mechanical simulations using ANSYS

Ansys software from Ansys Inc (USA) is a comprehensive suite of simulation tools that is widely used in industry and academia for product development to simulate different properties of an object before physically producing it. The software can be applied for simulation driven development of novel devices to identify and derive suitable specifications to obtain the desired properties in the final product. For this thesis, the Ansys 3D Design Simulation and Virtual Prototyping work suite Version 14.x was used to carry out structural mechanics studies of hollow photoplastic FluidFM probes.

The software utilizes the finite element method in order to transform partial differential equations into a set of linear algebraic equations. In this way it is possible to tackle complex and non-linear geometries that would not be feasible to solve analytically. Ansys features complete control over all model parameters such as CAD design of the device under test (DUT), meshing parameters, and the governing equations of the underlying physical model. The tool also allows perturbations of the DUT to simulate how the device reacts and to extract the desired properties from this response.

With the Tcl/Tk scripting language it is possible to easily create elegant user interfaces in Ansys so the user can input the different simulation parameters (e.g., DUT dimensions, material properties, etc.). For parametric analysis of multiple parameter sets, the tool can be further combined with Matlab from Mathworks Inc. (USA) to automatically supply parameter sets as a vector to the simulation. Matlab can also be used to carry out the necessary post-processing steps after each simulation run, such as the creation of suitable output graphs.

2.5.2 Multiphysics simulations via Comsol

For problems involving coupled physical phenomena (so called multi-physics problems), the finite element software Comsol Multiphysics from Comsol Inc. (USA) was used. The software environment offers similar functionality to Ansys. However, it is optimized to solve systems where multiple, conjugated physical effects need to be considered. CAD Data of the DUT can be directly created within the software environment or imported from a third party tool. The extensive post-processing capabilities of Comsol Multiphysics can be harnessed to analyze the simulation results.

For this work, Comsol Multiphysics Version 4.x licensed for academic use was used. Simulations were carried out using the heat transfer module and the MEMS module included in Comsol Multiphysics.
Atomic force microscopy (AFM) belongs to a superior branch of microscopy known as scanning probe microscopy (SPM). This large family of microscopy techniques encompasses a multitude of instruments that all generate spatially resolved images of a substrate by means of some kind of physical probe that is scanned over the specimen. Many types of SPM instruments can investigate different kinds of interactions at the same time. The way such interaction principles are used to obtain images of the specimen are utilized to categorize the different SPM devices into subgroups and are commonly referred to as imaging modes. Atomic force microscopy is one of the largest subgroups of general scanning probe microscopy.

The goal of this chapter is to provide the underlying theoretical principles behind AFM technology. The theoretical concepts governing the relevant sensing probe properties of atomic force microscopy - cantilevers - are also elaborated. A thorough understanding of these subjects is crucial, as AFM technology is one of the cornerstones of fluidic force microscopy (for more detail, see chapter 5).

3.1 Basic principles of AFM

Atomic force microscopy is a type of nano-scale imaging device and technology derived from scanning tunnelling microscopy (STM). Invented in the early 1980s by Gerd Binnig and Heinrich Rohrer [Binnig and Rohrer, 1983], STM marked a major milestone for the imaging of nano-sized surface structures by providing resolutions well beyond the optical diffraction limit down to individual atoms. STM also marks the first type of SPM microscope ever developed wherein a probe is systematically scanned over a surface in order to obtain an image. However, since STM relied on the quantum mechanical tunnelling current between the tip and surface as a feedback signal, the technology is only capable of imaging conductive samples. In 1986, Binnig,
Chapter 3. Atomic force microscopy

Quate, and Gerber invented a novel type of instrument derived from STM that relied on the usage of purely mechanical probes to scan the substrate in order to sense the forces exerted by the atomic structure of the sample on the probe tip. They called their new device an atomic force microscope [Binnig and Quate, 1986]. In contrast to STM, AFM does not require a substrate to have very specific properties in order to image it. The device is capable of working on both conductive and non-conductive samples and can be routinely operated in ambient and liquid environments [Hansma et al., 1994].

When an a substrate is imaged using AFM, a sharp tip - usually attached to the far end of a micromachined cantilever - is scanned over the surface of interest [Albrecht, 1990]. Image 3.1 shows an example of a typical microcantilever for use in modern commercial AFM systems. As can be seen from this image, the tip apex of an AFM sensing probe generally features a very small curvature radius on the order of a few nanometers. As the sensing probe is brought into close proximity with the substrate surface, both attractive and repulsive forces start to act on the sharp tip, resulting in a mechanical deflection of the cantilever beam. This deflection can be detected via a suitable sensing system (see also section 3.1.2). The deflection of the probe is thereby governed by Hooke’s Law, which describes how the deformation of a mechanical spring is dependent on the applied force. By rasterizing the sample over a rectangular area in a line by line fashion, it is thus possible to extract topographical information from the sample. In addition, AFM can also be used to probe the tiny forces involved in molecular processes, e.g., singular unfolding events in the case of complex protein structures.

![Figure 3.1: Example of a typical atomic force microscopy cantilever used to acquire high resolution topographical information from a substrate. Left: AFM cantilever with pyramidal tip structure at the free end. Right: Close-up view of an AFM tip apex. Both figures by SecretDisc licensed under CC BY 3.0 via Wikimedia Commons.](image-url)
3.1. Basic principles of AFM

As outlined above, the forces acting on the tip apex of the cantilever can be of both repulsive and attractive nature when the tip is in very close contact with the substrate. Attractive forces are mainly caused by Van der Waals interaction forces and are due to electron coupling effects of atoms and molecules that are very close to each other. Repulsive forces on the other hand stem mostly from interactions associated with the overlapping of electron orbitals in nearby atoms and molecules as described by the Pauli Exclusion Principle (Pauli repulsion) or from ionic repulsion [García and San Paulo, 1999].

The interaction between a pair of electrically neutral atoms can be approximately described using the Lennard-Jones potential model (also referred to as L-J potential or 6-12 potential) [Bhushan, 2007b]. The L-J potential describes how the repulsive and attractive components of the interaction change depending on the distance between the two atoms and the depth of the potential well induced by the two influential factors. Equation (3.1) shows the normalized form of this mathematical model, where \( r \) denotes the actual distance between the two atoms and \( \sigma \) stands for the distance at which the potential is zero. \( \epsilon \) is the depth of the potential well caused by the interaction of the two species.

\[
V = 4\epsilon \left( \frac{\sigma^12}{r^12} - \left( \frac{\sigma}{r} \right)^6 \right) \tag{3.1}
\]

The gradient of the L-J potential is equal to the magnitude and direction of the interaction forces. Figure 3.2 shows a normalized, graphical representation of the L-J potential dependent on the pair distance \( r \).

3.1.1 Piezoelectric actuation

The ability of AFM to image samples at high resolutions requires the use of an actuation system that can control the spatial positioning of the scanning probe cantilever with high precision. This is typically achieved using piezoelectric elements as actuators. Such devices rely on the use of the piezoelectric effect. First evidence for piezoelectric behaviour of certain materials was discovered in the mid-18th century by Carl Linnaeus and Franz Aepinus. Upon compression of a piezo active substance, a resulting separation of charges can be detected. Conversely, a change in spatial dimension can be induced by subjecting a piezo active material to a sufficiently high potential difference. Piezoelectrically driven changes in the size of such devices are typically limited to about 0.2% of the overall dimensions of the actuator. Therefore this technology is perfectly suited to precisely controlling the spatial movements of AFM probes in correspondence to the applied control voltage.
Modern piezoelectric actuators are typically made of ceramic multilayer stacks. Since they always exhibit pronounced hysteresis, such actuators are best operated in closed-loop mode where their actual elongation is constantly monitored and adjusted to the desired value. State of the art piezoelectric actuators can achieve repositioning accuracy in the sub-nanometer range.

### 3.1.2 AFM cantilever deflection detection

In order to measure the small deflections in the microcantilever that occur during AFM imaging, a suitable sensing principle needs to be employed. The following paragraphs will explain the most common sensing principles available for use in AFM technology.

**Optical beam deflection (OBD)** Optical beam deflection is by far the most commonly used technology for detecting the position dependent deflection of an AFM cantilever [Meyer and Amer, 1988]. Figure 3.3 depicts a typical OBD sensing setup...
3.1. Basic principles of AFM

used for AFM imaging. In OBD the deflection of the cantilever is monitored by reflecting a focused laser beam from the far end of the AFM microcantilever onto a suitable detector. This photosensitive device should then be capable of detecting the movement of the reflected laser light according to the force dependent angle of deflection of the cantilever. Typical OBD implementations rely on using four-segment photo diodes, which make it possible to register both vertical and lateral movement of the laser spot. In this way, both the vertical movement and torsional responses of the AFM probe during substrate imaging can be measured. OBD is compatible with ambient environmental conditions and liquid environments. OBD typically requires an additional correction prism to compensate for potential changes in the refractive index of the AFM probe holder assembly when completely immersed in liquid.

Most commercial implementations of AFMs currently available on the market are based on OBD technology. This is mainly due to its robustness, good price performance ratio, and relative ease of use. Furthermore, OBD does not require any special type of cantilevers and can hence be operated with AFM probes from third party sources without the need for cumbersome device customization. All AFM instruments used in this work are based on the OBD principle.

Interferometry Instead of measuring the angle dependent location of the reflected laser beam via a photodetector device, it is also possible to detect the position of the cantilever via optical interferometry [Erlandsson, 1988]. Interferometers are a well-established method for measuring small displacements. Interferometric distance measurement is achieved by superimposing the incident and reflected electromagnetic light waves onto the object of interest. Typical implementations of this principle for application in AFM use an optical fiber that is positioned close to the cantilever to emit and collect the reflected light of a suitable laser beam in order to measure the position of the probe [Rugar et al., 1989]. Due to the very small deflections that need to be detected, the interferometer is operated below the fringe range of the utilized laser light. For this reason, the technique suffers from overproportional sensitivity to parasitic mechanical noise and thermal drifts in the optical components. Furthermore, interferometric AFM systems need much longer settling times before the system is in a thermal equilibrium state when compared to OBD based systems. Hence despite its high precision, optical interferometry is rarely used in commercial AFM instruments.

Capacitive feedback Capacitive measurement of the cantilever deflection can be achieved by using a metal coated cantilever as an electrode. A second, fixed metal electrode in the vicinity of the cantilever can be used to form a parallel plate capaci-
Chapter 3. Atomic force microscopy

Figure 3.3: Schematic of an atomic force microscope with optical detection of microcantilever deflection (OBD). It clearly illustrates how the reflection of a laser beam can be used to track the movements of the microcantilever during imaging operation. Figure by GregorioW. This vector image was created with Inkscape and is licensed under CC BY 3.0 via Wikimedia Commons.

itor together with the electrode on the probe. As soon as the distance between the two electrodes changes, this can be detected as a change in capacitance with very high precision [Goddenhenrich, 1990]. However, this approach suffers from reduced compatibility when operated in liquid. DC components of the electric fields are readily screened by ions present in the liquid and hence reduce the applicability of this method. By using electrically isolated electrodes in conjunction with impedance measurement at high AC frequencies, it is possible to use capacitive readouts inside an electrolyte environment. However, the two capacitor electrodes can only be a few micrometers apart.

Tuning fork actuation In a tuning fork AFM setup, the microcantilever is typically attached to a quartz-based tuning fork device. Such components are readily available since they are a standard component in modern watches. These piezoelectric tuning forks can be precisely actuated and read out in parallel to detect shifts in frequency and amplitude of the excited vibration of the attached AFM cantilever in close proximity to the sample [Edwards et al., 1997]. A tuning fork setup is compatible with operation in
3.2. AFM Cantilevers: Micromachined sensing probes

Ambient air as well as in a liquid environment, provided the tuning fork is sufficiently insulated. However, it is only possible to carry out dynamic mode imaging with this approach, since the feedback mechanism relies entirely on the dynamic response of the tuning fork cantilever assembly and is not sensitive to steady state interactions. Thanks to their high eigenfrequencies, tuning fork AFMs can be operated at very high frequencies in the MHz range, making it possible to image the sample with potentially high frame rates for video like AFM imaging [Giessibl, 1998].

Piezoresistivity based self-sensing probes

Exploitation of piezoresistivity makes it possible to create self-sensing AFM probes, so named because they no longer depend on an external sensing principle in the AFM instrument. For example, it is possible to integrate piezoresistive materials directly into the cantilever structural material. Its electrical resistivity changes in response to strain, such as whenever the cantilever is deflected by the sample. A commonly used material exhibiting piezoresistive properties is silicon. It offers sufficient sensitivity and is readily compatible with the microfabrication processes used to produce standard AFM probes.

Since the approach requires specially adapted cantilevers, it is not very commonly employed in commercial AFM systems. However, for situations requiring the use of multiple AFM cantilevers in parallel, where each probe needs to be monitored independently, piezoresistive detection has clear advantages over other techniques such as OBD. The creation of rectangular arrays of cantilevers has been shown to be feasible [Indermühle et al., 1997]. This makes it possible to circumvent inherent serial scaling limitations of other AFM detection principles to allow massively parallel high-throughput operation of atomic force microscopes.

3.2  AFM Cantilevers: Micromachined sensing probes

3.2.1 Cantilever fabrication

Modern AFM microcantilevers for OBD-based operation are produced using techniques adapted from the microelectronics industry (integrated circuit fabrication). Microscaled devices exhibiting mechanical (and electrical) features are commonly known as microelectromechanical systems (MEMS). MEMS technology has seen immense technological advances over the last three decades. Nowadays MEMS are used as miniaturized building blocks in many kinds of products in the form of sensors [Bogue, 2007], actuators [Bell et al., 2005], and micromechanical components such as hinges for micro-mirrors [Van Kessel et al., 1998].
AFM cantilevers are typically fabricated on a wafer scale, allowing the production of multiple probes in parallel. By using photomasks exhibiting many identical copies of the device to be produced via lithographic pattern transfer, it is possible to achieve parallel manufacturing of multiple MEMS devices. Thanks to the associated economy of scale benefits, standard cantilevers can nowadays be produced at very little cost per unit. The actual microcantilever is usually attached to a larger handling chip. The handling chip makes it possible to manipulate the AFM cantilever using macroscopic tweezers in order to mount it on a compatible AFM microscope. The handling chip is typically fixed to a specific probeholder supplied by the AFM manufacturer, so that can in turn be attached to the macroscopic parts of the AFM microscope.

Cantilevers are commonly made from silicon, silicon oxide, and silicon nitride. To increase wear resistance of the sharp probe, diamond coatings are often applied to the sensing tip apex in order to improve its lifetime [Niedermann et al., 1998]. Cantilevers made purely from polymeric SU-8 have also been reported in literature [Genolet et al., 1999]. However, they are not currently regularly used with commercial instrumentation.

To improve the reflectivity of the cantilevered probes, a thin metallic layer made from gold or aluminium is evaporated on the reverse of the cantilever. For very thin cantilever beams or in the case of hollow probes, the use of a reflective layer is crucial to achieving good system performance in terms of force sensitivity and signal to noise ratio.

### 3.2.2 Cantilever geometry and properties

Commercially available AFM probes have either rectangular or V-shaped cantilevers. The second design is commonly used to reduce the susceptibility of the probe to lateral forces. However they are more complex to fabricate and therefore more expensive to buy. A direct comparison of the two probe geometries can be found in [Sader and Sader, 2003]. To obtain a sharp tip at the apex of the cantilevers, a pyramidal structure is created via sophisticated and usually proprietary fabrication techniques, relying on the combined use of selective etching of different silicon based materials. The shape of the sharpened structure can be adapted to the intended application. Typical curvature radii for such probes are in the range of a few nanometers for maximum imaging resolution and reduced convolution effects on the acquired topographical information. Modern AFM probes are also optimized to image vertical features within high aspect ratios by means of a hammer-shaped tip apex. Such probes are regularly used for quality control inspections during the fabrication of integrated circuits in the microelectronics industry, e.g., deep trench inspection during dynamic random
3.2. AFM Cantilevers: Micromachined sensing probes

Application specific probes are also available with a spherical colloid glued to the far end of the cantilever [Atkins and Pashley, 1993] or with specific chemical functionalization to probe molecular interactions in biochemical research (also known as chemical force microscopy) [Frisbie et al., 1994]. Investigation of the electrical properties of samples is also possible, such as in the case of conductive AFM (C-AFM) [Zhang et al., 1999]. This application typically uses probes with an apex that has been rendered conductive by coating it with a suitable metal layer. By applying a bias potential between sample and conductive probe, it is possible to detect areas of the substrate that exhibit conductive properties while at the same time acquiring its topography. However, C-AFM is only applicable to substrates that are imaged in a dry environment.

3.2.3 AFM cantilever spring constant

The vertical deflection of an AFM cantilever during imaging depends on the forces acting on the probe. However, the deflection is only detected by the optical beam deflection system as a voltage readout from the corresponding photodetector that first needs to be converted into an actual force magnitude using Hooke’s law. To be able to do this, it is necessary to know the cantilever’s vertical spring constant $k$ [N/m]. The spring constant depends on the dimensions and material properties of the probe and can be described via classical mechanical theory using equation (3.2) for the case of a cantilever clamped on one side. $E$ denotes the elastic modulus of the cantilever structural material, $I$ is the associated second moment of inertia, and $L$ is the total length of the cantilever beam. The equation is valid only for small deflections; however, for AFM this is mostly the case.

$$k = \frac{3EI}{L^3}$$  \hspace{1cm} (3.2)

For a cantilever with a rectangular cross section, the second moment of inertia can also be expressed as a function of the cantilever dimensions according to equation (3.3).

$$I = \frac{wt^3}{12}$$  \hspace{1cm} (3.3)

In this equation, $t$ denotes the thickness of the cantilever beam and $w$ is its width. By combining both equations, the spring constant for a rectangular cantilever with
dimensions $w, t, L$ can be expressed as:

$$k = \frac{E w t^3}{4 L^3}$$  \hspace{1cm} (3.4)$$

It soon becomes apparent that the spring constant of an AFM probe is most influenced by changing its thickness $t$ and/or its length $L$, assuming the material remains the same. Most commercially available rectangular AFM probes exhibit spring constants in the range between 0.01 N/m and 50 N/m. To obtain softer probes, they have to be made thin ($t \sim 1 \mu m$) and reasonably long ($L \sim 500 \mu m$).

Precise knowledge of all dimensional and material parameters is required to accurately calculate the spring constant of a specific AFM probe. Due to variations in thickness and elastic properties of the structural materials during the fabrication process, it is very difficult to accurately assess those parameters directly via process specifications. For this reason, the probe manufacturer typically only supplies a statistical range for the expected spring constant. In order to precisely determine the actual stiffness of an individual probe, it is necessary to determine it experimentally whenever a new cantilever is mounted.

### 3.2.4 Experimental determination of the cantilever spring constant

Several methods have been proposed in literature to determine the spring constant $k$ of an AFM probe experimentally. Some of them rely on using a second, known cantilever while others try to extract the information from the fundamental physical properties of the probe itself. A widely accepted and used method that does not rely on a secondary reference system is known as the Sader method and is based on John Sader’s theorem [Sader et al., 1999] describing the relationship between the spring constant of a cantilever beam and its fundamental eigenfrequency (resonance frequency in a vacuum). The method therefore no longer requires the knowledge of the cantilever thickness. According to the theorem, the fundamental eigenfrequency of the probe in a vacuum $\omega_{vac}$ is related to the material and dimensional properties of the probe via equation (3.5).

$$k = M_e \rho_c w t L \omega_{vac}^2$$  \hspace{1cm} (3.5)$$

where $M_e$ is the normalized effective mass, $\rho_c$ the density and $w, t, L$ are the spatial dimensions of the cantilever. For long rectangular cantilevers ($L/w > 5$), [Sader et al.,
3.2. AFM Cantilevers: Micromachined sensing probes

1995] reported that the effective mass can be approximated using equation (3.6).

\[ M_e = 0.2427 \] (3.6)

since it is very difficult to accurately measure the thickness of the cantilever, a method for determining the spring constant using only the top-view dimensions of the rectangular cantilever \((L, w)\) is desirable. In [Sader et al., 1999] it was shown that the fundamental resonance frequency \(\omega_f\) in a fluid is determined primarily by inertial effects stemming from the surrounding fluid. Therefore, the fundamental resonance frequency in a vacuum can be directly calculated from the corresponding resonance frequency in a fluidic environment such as ambient air, provided the quality factor \(Q_f\) of the cantilever in the fluid is much larger than 1 \((Q_f \gg 1)\). The spring constant \(k\) of a rectangular cantilever is dependent on the resonance frequency \(\omega_f\) in fluid according to equation (3.7) [Sader et al., 1999]:

\[ k = \omega_f \sqrt{1 + \frac{\pi \rho_f w}{4 \rho_c t} \Gamma_{re}(\omega_f)} \] (3.7)

\(\Gamma_{re}(\omega)\) represent the real components of the hydrodynamic function \(\Gamma\) of the surrounding fluid, which depends on its Reynolds number and is only influenced by the cantilever width \(w\). The areal mass density \(\rho_c t\) of the cantilever is further given by:

\[ \rho_c t = \frac{\pi \rho_f w}{4} [Q_f \Gamma_{im}(\omega_f) - \Gamma_{re}(\omega_f)] \] (3.8)

By substituting equations (3.7) and (3.8) into equation (3.5), it was shown that the spring constant \(k\) can be determined by only measuring the quality factor \(Q_f\) and resonance frequency \(\omega_f\) in fluid (typically air) according to expression (3.9).

\[ k = 0.1906 \rho_f w^2 L Q_f \Gamma_{im}(\omega) \omega_f^2 \] (3.9)

The resonance frequency and quality factor in air can be readily obtained by measuring the thermal noise spectrum of the cantilever. The dimensions \(L\) and \(w\) of the probe are usually supplied by the manufacturer of the probe or can be easily measured using a microscope. The main advantage of this method is that neither the density, elastic modulus, nor thickness of the cantilever need to be known. These parameters are both difficult to accurately measure and greatly influence the final result. Therefore,
this indirect method allows the actual cantilever spring constant to be determined much more accurately compared with other methods. It also has the advantage that it can be carried out in situ with the cantilever mounted on the AFM, just before starting the actual experiment.

3.3 Common AFM measurement modes for topography imaging

AFM imaging of a sample is typically carried out over a rectangular area that is scanned line by line using the AFM probe. Information on the substrate is extracted based on the lateral position of the probe on the sample. AFM imaging can be carried out using different imaging modes whereby static changes of the cantilever deflection (contact mode) or dynamic changes of the probe response when excited near its resonance frequency are recorded.

3.3.1 Contact mode operation

Contact mode imaging was the first imaging mode developed for AFM [Binnig and Quate, 1986]. The cantilever is brought into close proximity with the sample until it touches it. Contact mode imaging thus always operates in the repulsive force mode according to the Lennard-Jones potential as illustrated in figure 3.2. In contact mode the cantilever is typically operated in a steady state mode without any external oscillations imposed on the probe.

Constant height scanning In this imaging mode, the probe is fixed at a constant height in the repulsive force mode and remains unchanged during the entire scan. The resulting cantilever deflections are recorded in order to extract topographical information from the sample. Since no feedback mechanism is used, constant height imaging suffers from frequent damage to the scanning probe cantilever, as deflections on rough surfaces can become very large and the resulting forces can easily exceed the \( \mu \text{N} \) range. For these reasons, constant height imaging is rarely used when working with state of the art AFM microscopes.

Constant force imaging In constant force imaging mode, the AFM probe is still operated in repulsive force mode. However, in this case the vertical position of the AFM probe is constantly adjusted in order to maintain a constant force setpoint. This
3.3. Common AFM measurement modes for topography imaging

is achieved by means of a proportional integral differential (PID) control system, where the deflection value of the cantilever is used as a feedback signal. While the potentially damaging peak forces acting on the cantilever can be remedied using this approach, the method still suffers from the fact that soft samples can be damaged during the imaging process. Since the probe is still operated in repulsive force mode, soft samples can still be indented too much or even dragged along with the probe, making it impossible to image them. Even though the use of very soft micro cantilevers can circumvent this problem to a certain degree, they still operate in direct contact with the sample, making it impossible to avoid any unwanted later interaction between the substrate and the AFM tip.

3.3.2 Dynamic mode imaging

In dynamic mode imaging, the AFM probe is operated in so-called intermittent contact mode (see figure 3.2). The cantilever is excited to oscillate near one of its resonance frequencies with a small amplitude (typically below 100 nm). Thus the probe only touches the sample briefly in every oscillation cycle and thus reduces the normal and lateral forces acting on the substrate. The oscillatory behaviour of the cantilever is influenced by the interaction forces between the sample and the probe.

The amplitude, frequency, and phase of the cantilever can be used as a feedback signal to control the position of the probe with respect to the sample topography. In the most straightforward implementation, the piezo drive of the atomic force microscope adjusts the distance between the oscillating probe and the sample in order to maintain a set cantilever oscillation amplitude (setpoint). Dynamic mode imaging is the method of choice for imaging very soft samples such as biopolymers and lipid membranes. It is capable of working in both dry and liquid environments and is therefore frequently used for the imaging of biological samples.

3.3.3 Force modulation imaging

Another interesting imaging mode highlighting the force sensitivity of the AFM is known as force modulation mode. This mode represents a secondary imaging mode of AFM that can be used to identify and map differences in relative surface stiffness, as occur in heterogeneous substrates [Radmacher et al., 1993]. The sample topography can thus be recorded in parallel. The probe is modulated with a small vertical oscillation with a much higher frequency than the horizontal scan rate by means of a separate piezoelectric actuator. The mean force acting on the sample is subsequently modulated, such that it is always equal to what it would be in a corresponding contact
mode scan using a constant force setpoint. Softer areas of the sample yield relatively lower oscillatory amplitudes than stiffer areas, as they produce higher energy dissipation from the cantilever oscillation. The shift in magnitude of the probe's oscillation amplitude therefore provides a precise and convenient metric for the relative stiffness properties of the underlying substrate. The parallel recording of multiple substrate parameters using AFM and related SPM techniques is an ongoing area of research that is expected to gain further importance as newly developed techniques are integrated into commercial systems in the future.
4 Combining AFM cantilevers with microfluidics

As explained in the preceding chapter, atomic force microscopy has seen notable advances and novel developments in terms of imaging modes, force measurements, and micro-/nano-manipulation capabilities since its invention in the late 1980s. Thanks to this trend, the applicability of the technology has been extended to ever more areas of research that need to investigate the physical and chemical processes that occur at the micro- and nano-scales. This is especially true for the relatively novel field of using AFM for life sciences [Casuso et al., 2011]. As well as advances in signal processing and control systems, AFM has also benefited from innovations in sensing probe cantilevers. There are many ways to modify and extend scanning probes to change their properties and enable novel applications. However, only limited work has been reported in literature on extending AFM to allow direct sample manipulation. In light of this, this chapter will address a significant shortcoming of standard AFM scanning probe technology: The ability to handle liquid.

4.1 Implicated evolution: From bulk AFM cantilevers to hollow microfluidic probes

Traditional AFM techniques relying on cantilevers made from bulk materials already have limited liquid microhandling capabilities (see also chapter 6). However, they lag far behind the powers of competing devices such as classical glass micropipettes. Hollow AFM probes as developed and used in the scope of this thesis for the purpose of building a fluidic force microscope incorporate the capabilities of classical glass micropipettes into traditional bulk material AFM probes. This allows for superior liquid handling capabilities by combining the best of both worlds into a single device. The flexibility, precise force control, and positioning capability of the AFM is preserved, while a completely enclosed microfluidic channel in the form of a hermetically sealed
cavity within the cantilever beam is introduced. Finally, the interoperability with an external liquid reservoir and a well defined aperture at or near the apex of the AFM probe is assured. These modifications and additions complement this novel family of AFM cantilevers, wherein a completely new degree of freedom is essentially integrated into a conventional AFM setup: Hollow probes can be seen as a true evolution from completely inert AFM cantilevers made from bulk materials. These hollow probes can serve as a microfluidic system while maintaining all the major attributes of AFM operation. The integration of microfluidic functionality into atomic force microscopy cantilevers is beneficial for applications requiring the use and control of liquids at the micro- and nano-scales. Inspired by the ubiquitous glass micropipettes, as explained in section 6.1.8, these hollow probes have great potential to further push the application horizons of AFM. This is especially true for the field of life sciences. For these reasons the integration of microfluidic components into AFM cantilevers must be classified as an implicated evolution to create a force-controlled nano-pipette, as defined by the original problem statement that led to the development of FluidFM technology.

The following chapter will introduce the hollow AFM probe designs that have been used and developed for this thesis. These are a major cornerstone of fluidic force microscopy, as will be explained in chapter 5. The designs satisfy the following requirements:

- Completely enclosed microfluidic channel
- Essential compatibility with an external liquid reservoir
- Well defined micrometer- and nanometer-sized apertures
- Full interoperability with conventional AFM instrumentation

### 4.1.1 Defining the aperture - The critical process step in mass production

The most critical step when integrating a microfluidic system into an AFM cantilever is the formation of a suitable aperture (outlet opening) in the microfluidic channel at or near the apex of the AFM probe. The exact position is chosen depending on the planned application.

Especially for experiments requiring sub micrometer sized apertures, the production of such features poses significant technological challenges. It is not feasible to directly
create structures with sub micrometer sized features using conventional i-line UV photolithography processing.

The following sections explain the two main approaches used to form apertures during the production of hollow cantilevers for the probes used in this thesis:

- **Serial** Creation of the aperture for each individual probe in series after completing the fabrication of the hollow cantilever structure.
- **Parallel** In-line, wafer-level formation of the aperture during the actual fabrication process of the hollow cantilever.

Serial fabrication techniques, wherein an aperture is created only after the complete cantilever structure has been produced, offer maximum flexibility with respect to the position, shape, and size of the aperture. However, this also makes the production process expensive, since only one aperture can be created at a time. Hence, the processing of a complete 4" wafer with up to 850 hollow probes can be very labour and resource intensive. For this reason, the serial approach is only applied for prototyping purposes in novel applications where the optimal aperture design first needs to be determined experimentally. For commercial applications of hollow probes, however, only parallel fabrication of apertures offers the necessary economy of scale benefits associated with wafer level mass production of MEMS devices.

The following sections will show how hollow probes can be produced using different materials and microfabrication techniques. Probes featuring both serial and in-line (parallel) creation of apertures will be presented for this purpose.

### 4.2 Fabrication of silicon-based hollow cantilevers

The decision to fabricate MEMS devices using silicon-based materials has many advantages: First, they can be readily produced on a wafer scale using the same microfabrication techniques used in the microelectronics industry to create integrated circuits. Furthermore, they offer an extensive choice of substrate materials while providing excellent processing compliance, giving highly reproducible production results. For these reasons, silicon-based MEMS technology is a suitable choice for creating hollow atomic force microscopy cantilevers. This approach is further motivated by the fact that classical bulk AFM probes are also generally made from silicon-based materials.

All the silicon-based hollow probes used for this work were developed and fabricated in
close collaboration with external research partners and commercial suppliers. These parties already possessed valuable expertise in creating hollow MEMS structures that could be used as cantilevers for atomic force microscopy applications. Together with these partners, the specific requirements of hollow probes for the purpose of designing a fluidic force microscope were defined and implemented. The following section provides an overview of two approaches to producing silicon-based hollow AFM cantilevers which fulfil the specific requirements of fluidic force microscopy, as will be shown later in chapter 5.

4.2.1 Type A hollow probes: Bulk micromachining in combination with wafer level thermal fusion bonding and serial fabrication of the aperture

_Type A_ hollow probes are the first type of microchannelled AFM cantilever used during this work. They are based on an advancement of so-called NADIS probes, as first introduced by [Meister et al., 2003]. NADIS is a technique for nano-scale dispensing of liquids using AFM cantilevers (more information about NADIS technology is provided in section 6.1.3). Type A probes were developed in close collaboration with CSEM SA, a Swiss research and development company located in Neuchâtel. The type A hollow probes used during this work satisfy the requirements set forth in section 4.1. They are fabricated using bulk micromachining techniques [Kovacs et al., 1998] by means of a two wafer thermal fusion bonding process of silicon [Harendt et al., 1992]. This process was first proposed in 2005 by [Hug et al., 2005a]. However, it was not until three years later that the fabrication process was adapted to create hollow AFM probes for use in nano-scale dispensing of fluid in a liquid environment, as will be later shown in chapter 7.

**Fabrication process for type A probes - Bi-wafer process with thermal fusion bonding**

The fabrication process for type A hollow cantilevers uses thermal fusion bonding of two pre-processed silicon wafers. Figure 4.1 depicts the general process flow for the fabrication of such probes.

The reservoir and a V-shaped inlet grooves are structured on the first wafer as shown in figure 4.1a. The structures are defined via standard photolithography methods in combination with anisotropic wet etching using KOH\(^2\) etchant. The second silicon...
4.2. Fabrication of silicon-based hollow cantilevers

Figure 4.1: Schematic overview of the microfabrication process for producing hollow cantilevers of type A. Reprinted from reference [Meister et al., 2009b], copyright 2009, with permission from Elsevier.

wafer is pre-patterned to feature a pyramidal pit that will define the shape of the final cantilever tip. A 100 nm thin layer of silicon nitride ($\text{Si}_3\text{N}_4$) is subsequently deposited and structured to cover only the inside of the pyramidal pit. The silicon nitride layer is illustrated in green in image 4.1b. Next, the shape defining the microfluidic channel of the cantilever beam is defined via dry etching using a standard Bosch process [Laermer and Schilp, 1996]. Afterwards, the two prepared wafers are rinsed, aligned, and brought into close contact with each other, as depicted in figure 4.1c. Using thermal fusion bonding at temperatures between 800 °C and 1000 °C, the two wafers are bonded together and a thermal oxide layer ($\text{SiO}_2$) is grown at the same time inside the cavities formed by the two wafers. The thermal oxide layer is indicated in red in figure 4.1d. To release the silicon oxide cantilever, parts of the silicon wafer that are not covered by $\text{SiO}_2$ are removed via selective wet etching in KOH. The overall duration of this process needs to be precisely tuned in order to only partially release the V-shaped inlets in the handling chip, as seen in figure 4.1e. The fabricated structure is finally separated from the wafer by breaking the silicon oxide at predetermined fracturing points, as illustrated in figure 4.1f.

Thanks to the handling chip, the hollow cantilever can easily be manipulated using tweezers and mounted onto a standard AFM system. As the cantilevers themselves extend briefly beyond the edge of the handling chip, the laser beam of the optical beam deflection system of the AFM can be aligned well on the far end of the cantilever, where the pyramidal structure of the tip is located. This is essential to achieve optimal deflection sensitivity during AFM operation. Last but not least, the whole device is coated with a thin metallic layer on both sides, consisting of a 5 nm chromium...
adhesion layer and a 50 nm gold film. This metallization serves two purposes: The backside metal layer is used as a reflective layer to increase the reflectivity of the cantilever to the laser light of the AFM OBD system (see also section 3.1.2). The frontside layer will be needed to avoid parasitic charging effects during the milling process for the serial formation of the aperture by means of a focused ion beam.

Figure 4.2: Scanning electron microscopy image of an example type A hollow AFM cantilever. **Left:** Handling chip with single hollow cantilever extending from its base. **Middle:** Cross section of the hollow cantilever. **Right:** Overview of the pyramidal tip structure of the cantilever with a top aperture. Scale bars are 200 µm, 5 µm and 10 µm, respectively. Reprinted from reference [Meister et al., 2009b], copyright 2009, with permission from Elsevier.

Figure 4.2 depicts an example type A hollow probe, fabricated using the fabrication process shown in image 4.1. It should be noted that while the cantilever beam itself is made of silicon oxide, the pyramidal structure of the tip apex consists of silicon nitride. Using two different materials for the tip and the remaining structure of the probe has the advantage that it is possible to independently tune the wall thickness of these structures. While thicker walls are beneficial for improved mechanical robustness of the cantilever beam structure, it marks a limiting factor for the minimal achievable aperture size at the apex of the probe. Thinner pyramid wall thickness directly translates into smaller aperture sizes that can be realized. Furthermore, the use of different materials might also be beneficial for the envisioned application of the probe, as the surface properties of the pyramid could potentially play a crucial role.

Using this fabrication process it is also possible to create cantilevers with multiple microchannels, as depicted in figure 4.3. These could potentially be used as independent inlet and outlet channels in order to improve microflow control or to exploit multiple cargo solutions in a quasi parallel fashion without needing to change the probe first.

**Aperture fabrication using focused ion beam milling**

As with the first generation NADIS probes, type A hollow cantilevers depend on serial post-processing methods to create a suitable aperture for the microfluidic channel at the apex of the cantilever.
4.2. Fabrication of silicon-based hollow cantilevers

Focused ion beam (FIB) milling is an invasive technique that is often used for localized and maskless removal of material [Watkins et al., 1986]. Thanks to the precise and versatile imaging capabilities of the instrument, it is possible to accurately define the position, size, and shape of the aperture to be drilled. An FIB milling setup is generally constructed in combination with a scanning electron microscope column, allowing it to control and inspect the milling procedure in situ and in real-time. Since the milling process is most efficient when the surface of the substrate to be milled is normal to the ion beam axis, specially adapted holders need to be constructed that allow it to tilt the sample accordingly.

After fabrication of the complete cantilever including handling chip, the cantilevers were mounted on a suitable probe holder in order to be able to position them accordingly with respect to the axis of the incident ion beam used to mill the aperture. The probe's frontside metallization layer acts as a conducting path to reduce unwanted charge accumulation on the substrate. Charge build-up would otherwise result in the beam drifting, and hence in less exact positioning and definition of the aperture.

With FIB it has been shown that it is possible to create both circular and rectangular openings. In addition, the exact location of the aperture, whether it be on the very apex of the probe or on the face of one of the pyramid walls, can be precisely controlled using this technique. Figure 4.4 shows two different types of apertures that were created on type A hollow cantilevers using FIB. Depending on the desired location and type of aperture, different incidence angles of the ion beam with respect to the probe were found to be optimal in terms of yielding satisfying fabrication results [Bitterli,
Chapter 4. Combining AFM cantilevers with microfluidics

2012].

Figure 4.4: Nanometer sized tip apertures fabricated using focused ion beam milling. Left: Aperture located on a face of the pyramidal tip structure near the apex. This syringe-like configuration can be used for microinjection purposes, as demonstrated in chapter 8. Right: Pyramidal top aperture probe. This tip design is well-suited for nano-scale liquid dispensing applications, as carried out in chapter 7. Reprinted with permission from reference [Meister et al., 2009a], copyright 2009, American Chemical Society (ACS).

The flexibility of FIB milling is clearly advantageous for research purposes in order to rapidly determine the optimal tip and aperture design for a prototype. However, the serial nature of the process is a limitation in terms of the commercial feasibility of this approach.

4.2.2 Type B hollow probes: Surface micro-machining using sacrificial layer technology and in-line aperture creation

Type B hollow probes are made using an alternative micro-fabrication technique, based on the use of a surface micromachining process with a sacrificial layer. The entire process only requires one wafer. By combining this fabrication method with other techniques, it is possible to obtain hollow AFM probes with sub-micrometer apertures that are completely created in situ on a wafer level without the need for serial fabrication steps as in the case of type A hollow probes.

Sacrificial layer technique

A sacrificial layer is a layer in a MEMS device that is used as a placeholder during the fabrication of cavities in a surface micromachining process. The material of the sacrificial layer is chosen such that it can be selectively removed without harming the structural material of the actual MEMS component. Upon complete removal of the
sacrificial layer, a cavity defined by the dimensions of the ‘sacrificed’ material will be obtained in the final MEMS device.

The use of sacrificial layer techniques allows for a flexible approach to the fabrication of hollow AFM probes. Not only does it allow precise tuning of the dimensions of the microchannel inside the cantilever beam, it also makes it possible to create channels with heterogeneous structures. This is very helpful, since it allows the integration of pillars into the micro channel for improved mechanical stability during fabrication and operation while requiring only a thin overall wall thickness.

Since the sacrificial layer is only removed at the very end of the process, it also helps to protect the probes from any unwanted mechanical damage during production. When working with transparent structural materials, it is also possible to optically inspect whether the sacrificial material has been completely etched away or not. This simple quality control step can efficiently determine whether a cantilever beam is functional or not with respect to the integrated microfluidic channel. An efficient quality control process is very important for commercialization of such probes, as time consuming inspection steps would only render them more expensive.

**Fabrication process for type B hollow cantilevers**

The fabrication process of type B probes strongly depends on the type of aperture and tip structure being produced. The simplest types of probe that can be created using this process are tipless probes, as shown in figure 4.5. Tipless probes are simply hollow cantilevers that feature a micrometer-sized aperture at the far end of the cantilever beam. For other probe designs such as pyramidal probes with an aperture at or near the apex, the process needs to be extended accordingly. However, the principle of using sacrificial material to create the microfluidic channel inside the cantilever remains mostly unchanged.

The complete fabrication process for type B hollow cantilevers is illustrated in figure 4.6, as developed in collaboration with the commercial FluidFM providers Cytosurge AG (Switzerland) and SmartTip BV (Netherlands). The substrate wafer consists of a standard 4-inch silicon wafer and is indicated in gray in the process flow overview shown in image 4.6. In the case of type B probes, the structural material of the probe consists of silicon nitride $Si_3N_4$ (indicated in magenta). Silicon nitride has the advantage that the deposition process can be tuned to obtain structures that are virtually free from any intrinsic mechanical stress. The process uses poly-crystalline silicon (also called semi-crystalline silicon, shown in cyan) as sacrificial material. Poly-silicon is basically silicon; however, in contrast to amorphous or single-crystal silicon,
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Figure 4.5: Tipless type B hollow AFM cantilever. **Left:** This probe design does not have a tip structure at the far end of the cantilever. Instead, there is only a circular aperture. Openings with diameters of 2 μm, 4 μm and 8 μm have been created so far. Photograph provided by Cytosurge AG (Switzerland), copyright 2013. **Right:** Cross sectional view of a tipless type B hollow cantilever. The cavity formed using the poly-silicon sacrificial layer between the two silicon nitride walls is clearly visible.

it differs in the fact that it is made up of multiple small silicon crystals (so-called crystallites). Poly-silicon has been chosen because it can be selectively etched from the structural silicon nitride material. Furthermore, it is removed by the same etchant as the silicon substrate of the supporting wafer. Thus the sacrificial material can be cleared in the same process step used to etch away the non-essential parts of the silicon support wafer from the completely fabricated probes.

Figure 4.6: Illustration of the different process steps for the fabrication of type B tipless hollow cantilevers. Adapted from original image provided by SmartTip BV (the Netherlands), copyright 2010.

The fabrication process for type B tipless probes starts by depositing a layer of silicon nitride on a supporting silicon wafer. This layer will form the bottom half of the hollow cantilever in the final device. This initial step is shown in the process flow overview in figure 4.6I. The next step is to pattern the micrometer-sized apertures into the silicon nitride film as shown in figure 4.6II. For other types of tip structures,
4.2. Fabrication of silicon-based hollow cantilevers

additional processing steps will be required before continuing with the rest of the production procedure. As an example, a modified process flow for creating pyramidal probes with top apertures is explained later in this section. The corresponding extra fabrication steps are illustrated in figure 4.9. Next, as shown in figure 4.6III, a layer of poly-silicon material is deposited on the silicon nitride that will serve as the sacrificial layer. This poly-silicon sacrificial layer is subsequently patterned as shown in figure 4.6IV in order to define the exact dimensions of the final microfluidic channel inside the cantilever. Once the sacrificial layer has been defined, a second layer of silicon nitride is deposited on top of the existing structures (figure 4.6V), forming the upper part of the final cantilever assembly. Both the upper and lower silicon nitride layers will then be patterned so as to define the final dimensions of the cantilever beam. At the same time, an aperture for the inlet part of the final probe is created that will connect the hollow probe to the external liquid reservoir, as shown in image 4.6VI.

In-line creation of sub-micrometer apertures: Corner lithography

For the production of more advanced types of tip structures such as pyramidal probes with a top aperture at the apex, it is necessary to create apertures that are much smaller than the typical resolution limits of around 1.0 μm to 1.5 μm that can be achieved using standard UV lithography. In order to create such sub-micrometer sized apertures in-line, a process called corner lithography is used. The principle of corner lithography was first demonstrated by [Sarajlic et al., 2005].

Figure 4.7: Principle of corner lithography process used for the creation of sub-micrometer structures by means of standard photolithography processing. Reprinted with permission from reference [Berenschot et al., 2012], copyright 2012, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

In essence, the technique relies on a structure with concave corners such as the pit of a pyramidal groove, as shown in figure 4.7I. The concave structure is then conformally coated with a suitable material layer, as depicted in image 4.7II. The added layer is subsequently etched away isotropically, and due to the limited access of the etchant to the concave corner, the deposited film is removed from all surfaces except for the material located inside the concave corner. By precisely tuning the timing of the etching process, the desired amount of material remains in the corner that will later
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act as a localized sacrificial layer. This procedure is illustrated in figure 4.7III.

Figure 4.8 shows how such a localized pit of sacrificial material can be used to create nanometer-sized apertures at the apex of a pyramid of a hollow AFM probe. Image 4.8A shows the pit of sacrificial material as obtained via corner lithography in red. Using local oxidation of silicon (LOCOS) or by depositing another suitable structural material, the pyramidal structure of the hollow cantilever is fabricated and the shaft of sacrificial material serves as a mask in the apex of the pyramid, as can be seen in image 4.8B. Subsequent selective removal of the sacrificial material followed by selective etching of the supporting wafer material results in a pyramidal structure featuring a sub-micrometer aperture, as depicted in figures 4.8C and 4.8CD, respectively. Image 4.8E shows an electron microscope image of an example hollow pyramid structure with a 240 nm aperture at the apex.

Figure 4.8: Process flow based on corner lithography for the creation of sub-micrometer sized apertures in hollow AFM cantilevers with pyramidal tips. Reprinted with permission from reference [Berenschot et al., 2012], copyright 2012, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

This process, as demonstrated in [Berenschot et al., 2012], allows the fabrication of apertures located on the apex of the pyramid. Using the same principle, it is also possible to create apertures located on the four side walls of the pyramid just beneath the apex [Berenschot et al., 2008]. By optimizing the technique accordingly, it is possible to completely integrate it into a wafer level process for massively parallel fabrication of sub-micrometer apertures in-line during the production of the hollow probes [Burouni et al., 2013].

Figure 4.9 shows how corner lithography process can be integrated into the process flow for creating type B hollow AFM probes, as shown in figure 4.6. Using a silicon {100} wafer it is possible to form pyramidal pits in the silicon wafer via KOH-based anisotropic wet etching. To define the size of the pyramid, a suitable region of the silicon wafer is exposed via a silicon oxide hard mask, as illustrated in figure 4.9A and
4.2. Fabrication of silicon-based hollow cantilevers

subsequently etched with KOH as shown in image 4.9B. Next, as illustrated in figures 4.9C and 4.9D, a small sacrificial pit is defined at the base of the pyramidal groove using the corner lithography method. Afterwards, the same techniques as used in the original tipless probe process are used to define a hollow channel using poly-silicon as a sacrificial layer material. The necessary process steps are depicted in figures 4.9E to 4.9K. Similar to type A hollow probes, the process can also be accomplished with two different materials for the structural parts of the cantilever beam and the pyramidal tip. Image 4.10 shows an example type B cantilever with a pyramidal tip structure encompassing a top aperture produced using corner lithography methods.

![Figure 4.9: Modified process flow for the fabrication of type B hollow cantilevers with pyramidal tip structures and sub-micron apertures. Adapted from original image provided by SmartTip BV (the Netherlands), copyright 2010.](image)

Glass carrier chip attachment via anodic bonding  In the last step of the fabrication process for type B probes, a suitable carrier chip needs to be attached to the produced cantilevers. This is done just before releasing the cantilever structures from the supporting silicon substrate. This carrier chip is necessary in order to be able to handle the hollow probes with tweezers. In contrast to type A probes, this handling chip is not made of silicon, but Pyrex glass. To be able to access the microfluidic
system of the hollow cantilever, the handling chip needs to have an access port that will serve as the inlet channel connecting an external liquid reservoir to the rest of the probe. For this reason, the Pyrex glass wafer is first treated using a technique known as micropowder blasting. Similar to sandblasting for macroscopic removal of material, micropowder blasting can be applied to mill small holes into a Pyrex glass wafer. The size and location of the hole needs to be chosen such that it will be exactly in line with the inlet port in the top silicon nitride layer of the cantilever assembly. The Pyrex glass wafer is attached to the cantilever assembly via anodic bonding, as illustrated in image 4.6VII.

**Final release of the probe** The final release of the fabricated probe is achieved by selectively removing the supporting silicon wafer using wet etching techniques. The poly-silicon material of the sacrificial layer is dissolved in the same process step. In order to structurally support the thin layer of silicon nitride sitting under the inlet part of the Pyrex glass wafer, a small fraction of silicon wafer is retained just underneath it. The size of this mechanical reinforcement needs to be carefully tuned in order to avoid touching the substrate with this part of the probe before the actual cantilever tip makes contact. However, without this mechanical reinforcement, the cantilever would not be functional, since the thin silicon nitride membrane could barely withstand any pressure changes occurring inside the microfluidic channel, which would result in a non-functional probe due to a fractured membrane.

To detach the finished cantilever from the wafers, suitable grooves are cut into the
4.3. Fabrication of hollow cantilevers using SU-8

Pyrex glass wafers using a high RPM diamond blade wafer saw that will later serve as a pull link to determine where the probes will break upon mechanical release. In order to increase the reflectivity of the probes for the optical beam deflection system of the AFM, the reverse is coated with a layer of 50 nm of Au sitting on an adhesion seed of 5 nm Cr or Ti. State of the art type B hollow probes are fabricated with a set of pillars in the hollow channel of the cantilever. These pillars have been demonstrated to not negatively impact the performance of the device in terms of AFM relevant properties [Doerig, 2013]. However, an improved production yield and increased mechanical robustness during routine handling can be achieved.

Type B probes can also be produced without any type of aperture, featuring only a closed pyramidal structure. Using serial techniques similar to those used for type A probes such as FIB, these probes can be individually processed in order to quickly create different kinds of aperture designs. Thanks to this flexibility, it is possible to quickly create prototypes to explore probes featuring novel types of apertures before establishing a corresponding fabrication process for massively parallel industrial production at the wafer scale.

4.3 Fabrication of hollow cantilevers made from photo-plastic SU-8

While CMOS compatible materials are used for the majority of MEMS devices, there has been considerable progress in the use of alternative materials as structural parts of such microdevices. Silicon and ceramics such as nitrides of silicon are well known and widely used due to their excellent compatibility with most aspects of modern microfabrication technology. The possibility to also integrate electronic functionality into MEMS devices is an additional advantage of this family of materials. Furthermore, there has been considerable effort in providing as many economy of scale benefits as possible to silicon-based microfabrication techniques. However, pure crystalline silicon is still a complex and expensive raw material. For such reasons, this work investigated an alternative method of producing hollow AFM probes that are not made from silicon derived materials but rather from a completely new family of substances: polymers.

Alternative substances for MEMS devices such as polymers offer many beneficial properties. Polymeric materials can be produced in huge quantities at low prices. Furthermore, they offer a much larger diversity of material properties than silicon-based materials and therefore represent a broader design space in terms of the physical properties of the resulting MEMS devices. The pronounced chemical stability of
suitable polymers also makes it possible to use the final MEMS device in combination with highly aggressive chemicals. In the case of a hollow AFM cantilevers, it would for example be feasible to use HF\(^3\) inside the hollow probe channel as a cargo fluid. Such a device could then be employed for direct in situ etching of suitable materials at the nanometer scale. Such an application scenario would not be possible using silicon and silicon-based oxides as structural materials for the probe.

Research into polymer-based microfabrication techniques has seen tremendous progress over the last few years. It is becoming more and more common among the microfabrication community to use polymeric materials, not only during temporary steps in the microfabrication process such as in the form of photoresists, but directly as a structural material for the MEMS device itself. Novel types of materials for the fabrication of functional microdevices have been heavily investigated over the last two decades in order to push MEMS processing beyond the limitations imposed by the use of silicon-based materials. Substances such as parylene, polyimide, and SU-8 are just the most prominent examples of materials that can be use to create polymer-based MEMS devices [Liu, 2007].

The general fabrication approach of type B silicon-based hollow probes as explained in section 4.2.2 has been used as a starting point to develop suitable fabrication strategies for producing completely polymeric hollow AFM probes. In particular, the concept of using a sacrificial layer to define a hollow channel within the framework of a standard surface micromachining process has proven to be a promising approach. The following section presents different microfabrication strategies for the purpose of creating hollow AFM probes made completely out of polymeric material as developed in the scope of this work. The photosensitive polymer SU-8 was identified as a suitable candidate substance and has therefore been chosen for this purpose.

### 4.3.1 SU-8 as a structural material for MEMS processing

SU-8 is a novel epoxy-based negative tone photoresist that is already widely used for the creation of structural MEMS components [Conradie and Moore, 2002]. Due to its distinct absorption properties in the UV range, SU-8 is compatible with standard photolithography processes, even for very thick layers of resist. SU-8’s high-aspect ratio compatibility is one of the main reasons this novel material has gained widespread use in the microfabrication community. In addition, it has a relatively low Young's modulus and pronounced chemical resistance, helping SU-8 become a standard material in MEMS fabrication over the last two decades. Today, SU-8 is also widely used for...
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the creation of microfluidic systems (e.g., lab-on-a-chip devices [Ruano-López et al., 2006]) and has been determined to exhibit excellent biocompatibility [Tuomikoski, 2007]. Due to its high tensile strength after cross linking, SU-8 is often used specifically for heavy duty mechanical parts of MEMS devices in order to improve their mechanical robustness.

One of the difficulties associated with SU-8 processing is that it exhibits negative tone\textsuperscript{4} photosensitivity. This makes it difficult to directly create structures exhibiting hollow channels or embedded cavities using only photolithographic process steps. In contrast to positive tone resists, the fabrication of encapsulated microchannels is only possible using materials other than SU-8 as a sacrificial layer or by protecting parts of the device during fabrication using embedded-mask [Guerin et al., 1997] or moving-mask techniques [Kim Sang-kon and Hye-Keun, 2010]. Furthermore, SU-8 processing is only compatible with temperature ranges up to about 100 °C, making it very difficult to integrate with materials requiring higher processing temperatures.

The use of SU-8 as a structural material for MEMS devices has already been successfully demonstrated for bulk material cantilevers. Such devices can be used, among others, as cantilever-based biosensors [Nordström et al., 2008] or even as fully functional AFM imaging probes [Genolet et al., 1999]. Negative properties such as low wear resistance during routine scanning can be improved by employing a cartridge-based system: Rapid in situ exchange of the probe whenever imaging fidelity falls below a certain threshold can be employed to mitigate such shortcomings in an innovative and elegant manner [Genolet et al., 2000].

For detailed information about SU-8 properties, refer to the material and methods section 2.2.5. Sections 4.3.4 to 4.3.6 will demonstrate the different strategies for the production of hollow SU-8 probes developed during this work.

4.3.2 Device design using numerical simulation techniques

In the age of computer and numerical simulation tools, it is increasingly important to investigate possible design feature of a new device using computer assisted methods. Before actually producing a hollow probe using SU-8, it was important to understand how the final stiffness was expected to turn out depending on parameters such as cantilever length, height, and wall thickness. Since SU-8 exhibits a much lower Young's modulus than silicon-based materials, it was expected that using the same cantilever

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\textsuperscript{4}A negative tone resist is a type of photosensitive material where the portion of the material that has been exposed to light becomes insoluble. Unexposed areas of the resist are thereby dissolved by the corresponding developer solution.
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designs would yield much lower stiffness constants when using SU-8 instead of silicon [Genolet et al., 1999].

In order to investigate how different cantilever dimensions influence the stiffness of the probe, a numerical simulation model has been created wherein it is possible to sweep different parameter combinations in order to quickly determine their influence on the relevant properties of the resulting device.

Modal analysis using ANSYS

The numerical simulation suite ANSYS 11 was used as a simulation platform (see also section 2.5.1). A simple three dimensional model for a rectangular hollow cantilever (see figure 4.11) was established with the following parameters:

- Cantilever length $L$
- Cantilever width $W$
- Wall thickness $d$
- Cantilever thickness $t$

![Cantilever CAD model](image)

**Figure 4.11:** Cantilever CAD model used during modal analysis in ANSYS. The simulation model considers the following parameters: cantilever length $L$, width $W$, wall thickness $d$, and thickness $t$.

As shown in figure 4.11, the CAD model used for the cantilever is based on a simple design. The hollow channel is modelled by cutting out a smaller portion of the bulk
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part of a rectangular cantilever beam. The resulting wall thickness $d$ is also included as a variable parameter in the simulation model to determine how strongly this property influences the stiffness characteristics of the hollow probe.

Using the ANSYS parametric design language (APDL), a simple program was created in order to simulate the modal (eigenfrequency and mode shape) behaviour of a hollow cantilever made from SU-8. Material parameters for SU-8 are defined within the APDL code and in accordance with the chosen element for the finite element (FEM) simulation. The actual FEM analysis was carried out using solid-187 element types in ANSYS. This particular type of FEM element was selected as it supports meshing of structures with high aspect ratios and irregular shapes. It also allows the possible pre-stress properties of the structural materials involved in the simulation to be taken into account. Since SU-8 structures usually exhibit intrinsic pre-stress caused by the different processing temperatures required for their fabrication, it was important to be able to incorporate these effects into the simulation.

In order to efficiently sweep different parameter combinations, the simulation was invoked from a custom written MATLAB script to provide variable input vectors to the simulator while automatically saving the corresponding output data in a structured fashion for post-processing.

By calculating the eigenfrequency values for the first mode of vibration of the hollow probe, the corresponding stiffness values and maximum displacement of the structure can be readily extracted from the solution to determine the desired mechanical properties of the structure. For visualization purposes, it is also possible to visualize the shape of the different modes via an animated sequence showing the model cantilever. An example frame of the visualization depicting the maximum displacement for the first fundamental mode of a typical model cantilever is shown in figure 4.12.

Simulations can only provide meaningful results within the limitations of the model created for the simulation. In the case of this study, the mechanical dimensions of the model can be considered quite accurate. However, stiffness properties obtained this way can only indicate a probable range of expected values for the actual stiffness properties. Exact values are strongly dependent on the true material properties of the structural material. Furthermore, the stiffness is highly dependent on the length and height of the cantilever beam structure ($\sim r^3$ and $\sim L^3$). Since these parameters can only be controlled with finite accuracy when actually producing such probes in the cleanroom, the actual stiffness values can differ substantially from the expected ones. Nevertheless, the results of these modelling efforts can provide valuable input into the general design process of such devices. In particular, it helps avoid creating too many different combinatorial sets of probe parameters to be used on the final lithography
Simulation results

To determine the necessary cantilever designs for achieving stiffness constants in the range 0.1 N/m to 3 N/m, a series of simulations was run using the model explained in the preceding section.

MATLAB plots simulation results as multi-dimensional graphs indicating how the stiffness constant changes as two of the dimensional parameters are varied while the other two are kept constant. Figure 4.13 shows a typical output from a simulation of a cantilever with total thickness $t$ of 8 μm and a wall thickness $d$ of 3 μm. It can be seen how the cantilever stiffness changes depending on the length and width of the cantilever beam.

From figure 4.13, it can be seen that typical cantilever lengths in the range from 150 μm to 250 μm produce expected stiffnesses between 2 N/m and 0.5 N/m. Cantilever stiffness values in this range would provide the anticipated reduction in the cantilever spring constant when compared to similar silicon-based specimens. For this reason a series of probe designs exhibiting cantilever lengths in the range between 150 μm and 400 μm and a wall thickness between 3 μm and 6 μm were determined as a suitable
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The simulation further revealed that it is possible to achieve substantially lower stiffness properties even while using relatively large structures when compared to silicon-based hollow probes. This is highly desirable, since it is expected to positively influence the fabrication complexity of such probes. The use of SU-8 promises to yield cantilevers with soft stiffness constants without the need to use process parameters near the resolution limits of classical photolithography.

The goal of the simulation was not to determine how a hollow probe differs from a corresponding bulk material probe with respect to their stiffness constant $k$, as this can be readily calculated using a simple analytical expression, as explained in section 5.3.2.

### 4.3.3 Mask set for hollow SU-8 probes

Based on the results from the numerical simulations outlined above, a set of photomasks was produced that contain a number of different cantilever designs. These masks are optimized for use with standard 4-inch wafers. Via a simple T-Cell script (parametrized cell generation language), as provided by the Mask CAD software L-Edit (see section 2.2.2), it was possible to quickly create many different kinds of probe.
designs. The established T-Cell code used for the fully dynamic auto-generation of the different masks provides four parameters that can be varied: Aperture size a, cantilever length l, cantilever width w, and wall thickness d. The thickness t of the probe is not controlled via the structures on the photomask. Instead, it is determined during the spin coating process of the corresponding SU-8 layer within the production process. A complete listing of the T-Cell code is provided as a reference in appendix A.1.

The decision to incorporate many different cantilever designs was strongly motivated by the fact that it could not be determined a priori how well each design could actually be produced. It is difficult to predict how cantilevers with different lengths will behave based on the amount of intrinsic mechanical stress in the produced polymer structures. Earlier studies working on the fabrication of standard bulk cantilevers made from SU-8 suggest that tight control of thermal processing parameters is necessary to tailor intrinsic stress properties with respect to cantilever length to avoid buckling and bending of the final structures [Genolet, 2001]. Furthermore, the overall mechanical stability of the hollow probe structure is expected to correlate strongly with dimensional properties of the cantilevers such as channel width, wall thickness, and channel length. While the simulated model system allows the identification of a suitable parameter range to achieve the desired stiffness criteria, it does not give any information on the feasibility of fabricating the corresponding probes. For this reason, the emphasis during the process development phase was unapologetically placed on the feasibility of the overall process rather than on finding the optimal probe geometry. All probe types generated using L-Edit Software therefore tried to avoid extreme cases where the design was expected to suffer from additional difficulties such as structural dimensions near the resolution limit of standard near-UV lithography or distinct aspect ratios.

Figure 4.14 gives an overview on a representative photomask designed during this work for the production of photoplastic AFM probes featuring hollow microfluidic channels. The magnified section of the figure clearly shows how the different parameters influence the shape of the cantilever. Depending on the fabrication strategy, a number of individual masks are required for the different lithography steps. Most fabrication strategies developed in the course of this work required the use of 4 to 6 six individual masks, as will be explained in the following sections of this chapter. All photomasks designed for this thesis can be found in the appendix as a compendium with corresponding close-up detail A.2.

The following sections will elucidate three fabrication strategies that were developed and investigated during the course of this thesis. Due to the difficulty associated with the formation of hollow structures using a negative tone resist, a number of different
4.3. Fabrication of hollow cantilevers using SU-8

Figure 4.14: Example photomask for producing hollow SU-8 probes. **Left:** General view of a typical photomask used during the production of hollow SU-8 cantilevers. It can be seen that 288 individual probes can be produced on a single 4 inch wafer using this mask set. **Right:** Detail view of a pair of probes. The cantilever will be attached to the left side of the handling chip in the final device.

approaches can be chosen to create such structures with SU-8. All strategies are therefore mainly centered around different methods that can be used to create the necessary microchannel.

### 4.3.4 Strategy 1: Direct sacrificial layer process

The first process developed during this thesis is based on the use of a sacrificial layer to both pattern and fill in the parts of the device that will later serve as the hollow channel of the cantilever. The process is inspired by the fabrication approach used to produce silicon-based type B hollow probes, as introduced in section 4.2.2. An ancillary resist known as LOR-10B from MicroChem Corp (USA) is used as a sacrificial layer material. More detailed information about all the resists used can be found in the materials and methods chapter in section 2.2.3. LOR resists are typically used for bi-layer lift-off techniques during metal film patterning. However, it has been reported in literature that LOR exhibits low intermixing behaviour with SU-8 and can be efficiently dissolved using potassium-based developers [Schmid et al., 2006]. It is therefore possible to selectively remove the LOR sacrificial layer material without harming the cross-linked, structural SU-8 parts of the final device. This strategy is therefore dependent on the adhesion between different SU-8 layers that will make up the final device. The fabrication process is purely based on surface micromachining using a single substrate wafer.
Figure 4.15: Microfabrication process flow for tipless hollow SU-8 probes according to strategy 1. A: Silicon substrate wafer (purple). The substrate wafer can also be coated with a suitable metal layer stack (Cr/Au/Cr) for efficient release of the produced probes, B: Coating of SU-8 bottom layer and subsequent patterning (orange), C: Spin-coating and patterning of sacrificial layer made of LOR-10B (green), D: Formation and patterning of SU-8 top layer (orange), D: Deposition and structuring of thick block layer for mechanical stability with a thickness >150 μm, F: Release of the final probe from the substrate wafer.

Process flow description

Figure 4.15 illustrates the process flow developed for this fabrication strategy. The process starts with a single side polished 4-inch silicon wafer, as seen in figure 4.15A. To enable the final release of the fabricated probes, an additional metal layer stack (5 nm Cr/ 50 nm Au/ 25 nm Cr, not shown in figure) is coated onto the silicon substrate wafer. This electrochemically enhanced sacrificial layer (ESL) significantly speeds up the release of the final probes from the substrate wafer upon completion of the microfabrication process [Genolet, 2001]. Next, the bottom layer of the probe is created by spin coating a film of SU-8 onto the substrate wafer. The bottom layer is adjusted to be 2 μm to 5 μm thick depending on the desired overall thickness of the
final cantilever. The bottom layer is subsequently patterned using the corresponding photomask to form a structure as shown in figure 4.15B (Mask SU-8 Bottom Layer, see appendix A.2.1). As pictured in figure 4.15C, a film of LOR-10B sacrificial material is spin coated onto the bottom layer and patterned in the shape of the planned microfluidic channel and liquid inlet port. The thickness of the LOR layer is selected based on the anticipated microfluidic channel height of the final device (Mask LOR Sacrificial Layer, see appendix A.2.2). Next, the top layer of the probe made from SU-8 is spin coated over the existing structures and patterned accordingly, as shown in figure 4.15D. The thickness of the top layer is typically chosen to be the same as the bottom layer to ensure constant wall thickness of the hollow cantilever (Mask SU-8 Top Layer, see appendix A.2.3). In the final lithography step, a thick block layer of SU-8 (>150 μm) is placed on the handling chip to improve the overall mechanical stability of the probe and make it suitable for macroscopic handling. This process step is shown in figure 4.15E (Mask SU-8 Block Layer, see appendix A.2.4). Last but not least, the fabricated probes are released from the carrier wafer and the LOR-based sacrificial layer is dissolved using a potassium-based developer solution. Thanks to the electrochemically enhanced sacrificial adhesion layer, the release step only takes a few minutes. The dissolution of the complete LOR layer inside the microchannel, however, can take up to 48 hours due to the diffusion limited nature of this process. An example list of all the processing steps required for this fabrication strategy is provided in appendix B.1.

Figure 4.16: Modified fabrication process flow for hollow SU-8 probes according to strategy 1 for the creation of probes with pyramidal tips

As an alternative to the process explained above, it is also possible to create probes featuring a pyramidal tip structure at the free end of the cantilever. Image 4.16 illustrates how the first two steps in the process flow need to be adapted to obtain such
pyramidal probes. This can be achieved by first patterning a substrate wafer made of single crystal silicon (100) using KOH to form a pyramidal groove, as depicted in image 4.16A. This shape will serve as a negative to form the tip structure when spin coating the SU-8 bottom layer of the probe in the next step 4.16B. All the remaining processing steps remain largely the same as for tipless probes. The feasibility of this approach for SU-8 has already been successfully demonstrated by producing bulk material cantilevers featuring sharp pyramidal scanning tips made purely from SU-8 resist [Genolet, 2001].

To ensure proper alignment between different photolithography steps, a set of custom-designed alignment structures was used. The corresponding complementary patterns are provided in appendix A.2.6 as a reference. All alignment structures were created on the substrate wafer either by etching the upper Cr layer from the ESL or by direct deposition of a patterned Cr/Au stack using a negative tone photoresist lift-off process (e.g., ma-N 405, see also section 2.2.3). The same alignment structures were used for all fabrication strategies to ensure proper inter-layer alignment during the different processing steps.

**Results**

For this thesis, only probes with tipless designs (no pyramidal structure at the free end of the cantilever) were produced to demonstrate the fundamental feasibility of the present approach. Functional probes could be obtained using this process strategy. Fabricated probes were successfully filled with liquid, as indicated in figure 4.17. However, since SU-8 exhibits pronounced hydrophobic properties, liquid filling is only possible after oxygen plasma treatment. Once the surface of the SU-8 has been activated in the plasma, the probe shows hydrophilic properties that only deteriorate slowly over time [Walther et al., 2007]. Furthermore, some specimens could be mounted on a commercial AFM microscope from JPK Instruments AG (Germany) and successfully put onto a glass microscopy slide. However, since there was not enough reflectivity for the IR-Laser, no active feedback signal could be established and recorded.

Dissolution of the LOR sacrificial layer was successfully achieved by immersing the processed probes in a solution of 100% Shipley 351 for 18 hours with continuous agitation. Since the dissolution process is diffusion limited, this procedure takes considerable time, as fresh stripping solution can only reach the reaction site via the microfluidic channel. The left side of figure 4.18 shows the status of LOR sacrificial layer dissolution after 6 hours. The same situation after 14 hours is depicted on the right side of figure 4.18. It can be seen that the layers of the probe start to delaminate.
4.3. Fabrication of hollow cantilevers using SU-8

Figure 4.17: Successful microfluidic actuation of an SU-8 probe fabricated using strategy 1. **Left:** Optical micrograph of an SU-8 cantilever specimen without any overpressure applied to the backport of the chip. **Right:** Liquid is ejected through the 2um aperture of the probe after applying a small overpressure. The process is completely reversible. The small agglomerates on the cantilever are salt crystals from dried cargo liquid which contains fluorescein.

from the each other. This is indicated with a white arrow for better clarity.

Figure 4.18: Micrograph of the LOR sacrificial layer dissolution process. These images were extracted from a time-lapse video that was used to record the whole operation. **Left:** Status after 6 hours. The white arrow indicates the current position where the sacrificial layer is being dissolved by the LOR stripping solution. **Right:** Situation after 14 hours in the stripping solution. The highlighted area shows how the SU-8 layers start to delaminate from each other.

Figure 4.19 shows an electron microscopy micrograph of an SU-8 hollow probe after completing all fabrication stages. In the left picture the cantilever can be seen to exhibit considerable bending due to mechanical pre-stress in the SU-8 structural material. The right hand side of figure 4.19 shows a close-up view of the inlet part of the probe. The hollow channel leading away from the inlet is clearly visible. However, in
this particular specimen, the SU-8 bottom layer has been entirely de-laminated from the device. While some specimens were found to be fully functional, the complete de-lamination of the SU-8 bottom layer was observed in the vast majority of probes produced with this processing strategy. The assumed reason for this phenomenon is discussed in detail in the following section.

Figure 4.19: Electron microscopy image of SU-8 Probe fabricated using strategy 1. Left: Cantilever attached to macroscopic handling chip. Due to strong intrinsic stress in the material, the long cantilever exhibits considerable bending in its equilibrium position. Right: Close up view of the inlet part of the probe.

Discussion

As explained at the beginning of this section, this microprocessing strategy is critically dependent on sufficient adhesion between the two structural layers of the device. However, during dissolution of the sacrificial layer material it was frequently observed that the different layers of SU-8 started to delaminate after prolonged exposure to the developing agent. Interestingly, this delamination always seemed to occur at similar locations on the probe. This could be an indication that these regions suffer from a preponderance of intrinsic mechanical stress. This problem could probably be mitigated by adapting the design of the probe to avoid any occurrence of peak stresses in the material caused by geometrical design attributes of the device. However, this approach was not further pursued in this work. The main reason for the unwanted effect is most likely the non-optimal processing conditions during the patterning of the sacrificial layer: Since LOR-10B requires temperatures above the recommended cross-linking temperature of the SU-8 polymer (>95 °C), it is suspected that the bottom layer of SU-8 only has limited amounts of uncross-linked epoxide functional groups remaining when the top layer is processed. For this reason, only a limited amount of cross-linking across the two layers of SU-8 can be achieved. In order to improve this, a prolonged hard bake can be carried out after the complete SU-8 process has finished.
and before starting to remove the sacrificial layer. Using this adapted process, more stable probes could be obtained; however, the overall yield of the process could not be improved to a level where efficient mass fabrication would become feasible.

Summary and conclusion

To sum up, it can be concluded that it is possible to produce functional hollow cantilevered probes made entirely of SU-8 material using the direct sacrificial layer process. However, the process suffers from several major and minor issues such as non-optimal material compatibility as well as prolonged processing times. However, the process is fairly easy to understand and does not require any special equipment apart from standard photolithography instruments. The process is also highly reproducible and could in principle be adapted to produce more advanced devices such as self-sensing cantilevers. Table 4.1 assesses the most important aspects of this fabrication strategy based on five important criteria.

<table>
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<th>Equipment availability</th>
<th>Process reproducibility</th>
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Table 4.1: Assessment of fabrication strategy 1 based on five criteria: 1) Complexity of the overall process, 2) Compatibility of the utilized materials with microfabrication, 3) Special equipment needed, 4) Repeatability of the process, 5) Extendibility to the creation of more advanced devices.

The use of LOR as a sacrificial material is not entirely compatible with the recommended processing temperatures of SU-8. For this reason, an alternative fabrication strategy that does not use a different and incompatible material as a sacrificial layer was sought. One example for such a process is presented in the next section.

4.3.5 Strategy 2: Sacrificial layer with embedded metal mask

Another way to create hollow channels within a negative tone structural resist can be achieved by using a protective masking layer embedded into the structural part of the device during fabrication. Such a strategy is commonly referred to as an embedded mask process. Using such an embedded mask, it is possible to protect parts of the non cross-linked structural SU-8 resist from UV light exposure during subsequent lithography steps. With this technique, a sacrificial layer can be created using only the structural resist itself rather than a different material. This has the advantage that processing compatibility between the structural and sacrificial layer materials is no
longer an issue.

A suitable protective mask layer can be created using a thin layer of metal that is just thick enough to block any unwanted UV radiation from reaching the underlying resist during the necessary lithography steps after the channel layer has been defined. Figure 4.20 illustrates this principle. A metal mask was chosen because a thin layer of several tens of nanometers is already enough to achieve a good UV light absorption compared to other materials. This way the extra layer of the embedded mask does not add noticeably to the final thickness of the cantilever and hence only has negligible influence on the resulting probe stiffness.

**Figure 4.20**: Illustration of embedded metal mask concept for the formation of a hollow microchannel using a negative tone photosensitive resist such as SU-8. **Left**: A thin film of a suitable metal is coated onto existing layers of SU-8 and patterned in order to protect the zone of the future microchannel from photo activation during subsequent UV light exposure. **Right**: Hollow channel after all non-cross-linked SU-8 has been removed by immersing the device in SU-8 developer solution.

**Process flow description**

Figure 4.21 depicts the complete process flow used for the fabrication of polymeric hollow cantilevers using an embedded mask technique. The beginning of the process is similar to the one shown in section 4.3.4. The complete process is carried out on a 4-inch silicon substrate wafer (not shown in figure 4.21). In the first step, as seen in figure 4.21A, the SU-8 bottom layer is prepared using standard photolithography (Mask SU-8 Bottom Layer, see appendix A.2.1). However, at this point, the exposed and cross-linked layer of SU-8 is left undeveloped. This is done in order to achieve a planar surface for the following lithography step (cross-linked SU-8 is shown in dark orange and non-cross-linked SU-8 in light orange). Next, a second layer of SU-8 is spin coated onto the bottom layer in order to define the walls of the probe, as shown in figure 4.21B. Again, this layer is completely exposed and post-exposure baked,
4.3. Fabrication of hollow cantilevers using SU-8

Figure 4.21: Fabrication process for hollow SU-8 probes according to strategy 2. The process is based on a 4-inch silicon substrate wafer coated with an ESL for fast release of the fabricated probes. A: Patterning of the SU-8 bottom layer via photo lithography. The SU-8 layer is not developed after the post-exposure baking step (cross-linked SU-8 is shown in dark orange, non-cross-linked SU-8 in light orange). B: Formation of the wall layer of the probe using SU-8. Once again the layer is not developed before the next step. C: Fabrication of the embedded metal mask made from Cr material. The patterning of the mask is achieved using Cr wet etching through a soft mask made from positive tone photoresist (not shown). D: Generation of the SU-8 top layer covering the microfluidic channel. No development occurs after the post-exposure step. E: To form the handling chip of the device, a thick layer of SU-8 is structured to form a thick block around the inlet port of the probe. All layers of SU-8 including the sacrificial layer inside the microfluidic channel are developed at the same time using SU-8 developer solution. After the SU-8 block layer is developed, a short Cr etching step is required to provide access to the sacrificial SU-8 located under the inlet port. F: Final release of the probe from the substrate wafer. The probe features three distinct layers of SU-8 that make up the hollow microchannel inside the cantilever structure.

but not developed (Mask SU-8 Wall Layer, see appendix A.2.5). The most critical part of this fabrication strategy is the formation of the embedded metal mask. This is achieved by evaporating a thin chromium layer onto the previously created S-8
bottom and wall layers to cover the whole wafer. Afterwards, the exact embedded mask layout is defined using a soft-mask made from positive tone photoresist that has been structured with the help of another lithography step (Mask SU-8 Wall Layer, see appendix A.2.5). Areas of the metal film that are not protected by this soft mask are then removed using chromium etchant, leaving the metal film only in regions where the SU-8 needs to be protected from subsequent UV radiation. Once the soft-mask has been stripped, the fabrication of the embedded metal mask is complete. This stage of the process is shown in image 4.21C with the metal mask illustrated in gray. After the embedded mask has been created, the process resumes analogous to strategy 1: Formation of the SU-8 top layer as shown in image 4.21D (Mask SU-8 Top Layer, see appendix A.2.3). This is followed by creation of the mechanical handling chip, as depicted in figure 4.21E (Mask SU-8 Block Layer, see appendix A.2.4). Figure 4.21F shows the complete probe after it has been released from the substrate wafer. The final release is again achieved using an enhanced sacrificial adhesion layer technique. An example list of all the processing steps required for this fabrication strategy is provided in appendix B.2.

Because the channel sacrificial material in this process is simply non-cross-linked SU-8, the final formation of the hollow channel structure can easily be achieved during the development of the entire SU-8 structure by means of standard SU-8 developer solution. The embedded metal layer is typically lost in this step, as it is only adherent to the surface of the sacrificial layer.

Results

In microfabrication, metal films are typically formed using evaporation equipment based on electron beam heating of the source metal (filament heating) or sputtering. For the first few fabrication runs, a filament heated evaporator was used for the formation of the embedded metal mask layer. The left image in figure 4.22 shows a micrograph of a finished embedded mask defining the cantilever of an example probe. The pattern transfer is precise and reproducible; the metal film is undamaged. However, the use of filament-based evaporators is unfortunately not feasible, as will be explained in the next section. For this reason, another film formation technique known as resistive heated evaporation was explored. An example of an embedded metal mask created with this technique is showcased on the right of figure 4.22. Using this type of metal film, similar pattern transfer fidelity as seen using a filament evaporator can be achieved. However, the film suffers from the presence of numerous micro-cracks. This was observed on most samples processed this way.

For the case of embedded masks fabricated using a resistively heated source, the
4.3. Fabrication of hollow cantilevers using SU-8

Figure 4.22: Result of embedded metal mask formation using filament and resistive heated thin film evaporators. **Left:** Metal layer deposited using e-beam (filament) heated evaporator equipment. The metal film is well-structured and exhibits uniform coverage over the whole sample. **Right:** Example of an embedded metal mask created using a resistive heated evaporator. Pattern transfer is satisfactory; however, the film exhibits many unwanted micro-cracks that could negatively impact the overall quality of the fabrication process.

formation of a hollow channel was successfully demonstrated on several probes. Figure 4.23 shows the resulting hollow channel structure as observed via optical and electron microscopy imaging. This channel has been formed by immersing the probe in SU-8 developer in order to remove the non-cross-linked portions of the SU-8 resist from the microchannel. In the optical image on the left of figure 4.23, it can be seen that the channel leading away from the inlet port is completely open, i.e., the sacrificial layer has been dissolved entirely. In the middle image, an overview of the situation on another probe from the same fabrication run displays the inlet port from a birds-eye view. A close-up detail of the channel leading away from the inlet is provided to the right of figure 4.23.

**Discussion**

The difficulties of this fabrication process are mainly associated with the formation of the embedded metal mask. While the definition of this structure via e-beam heated evaporation techniques produces nice results, as shown on the left in figure 4.22, subsequent processing proves difficult or even impossible. It transpires that the e-beam evaporation technique produces enough high energy photons in the UV and X-ray range that yields to unwanted exposure of the sacrificial layer portions of the SU-8. The sacrificial layer therefore exhibits cross-linking to an extent where it becomes impossible to dissolve it later using SU-8 developer or other solutions capable of removing non-cross-linked SU-8. This behaviour is also reported in literature [Eicher and Schlaak, 2007], and therefore an alternative method for the creation of a metallic
layer is needed.

For this reason, a resistive (thermal) evaporation system was used for all subsequent process runs. Since the two SU-8 layers are also sensitive to heat exposure, extra care is needed not to heat the sample too much. Otherwise, it was observed that the existing SU-8 layer exhibited extensive re-flow that resulted in low quality metal mask layers that could not be further processed. This is mainly due to the thermal shock induced by the vapor lobe when the first metal atoms reach the sample in the evaporator. In order to prevent the sample from heating up too much during this process, it was necessary to increase the heat capacity of the wafer by several orders of magnitude. This was achieved using a custom-made cooling stage made from pure copper. The wafer was attached to this cooler by means of a vacuum compatible thermally conductive paste. In this way, it was possible to keep the wafer temperature sufficiently low during the entire evaporation process. The process was carried out at low vacuum pressures in order to lower the temperature needed for evaporation of the chromium material.

Summary and conclusion

From the results, it is clear that the fabrication of polymeric hollow cantilevers using embedded masking techniques is in principle feasible. This is especially pleasing, since this method of buried channel formation in the case of SU-8 has previously only been demonstrated for thicker layers, in the range of several hundred micrometers [Ruano-López et al., 2006]. Several adjustments to the process as outlined in the previous section resulted in successful formation of a hollow channel, as depicted in

**Figure 4.23:** Example of a successfully fabricated hollow channel using the SU-8 embedded mask technique. **Left:** Optical microscopy image of a probe after SU-8 sacrificial layer dissolution. It can be seen that the entire channel has been cleared of sacrificial material. **Middle:** Electron microscopy image of the inlet part of a probe from the same batch. The channel leading away from the inlet port is clearly visible as a tunnel-like structure. **Right:** Close-up view of the beginning of the microchannel. It is clear that the hollow structure has been well-defined using the embedded mask technique.
image 4.23. However, there are a few caveats that render this strategy difficult and cumbersome to carry out. First, special equipment such as a resistive evaporator system is needed, which are normally not available in state-of-the-art microfabrication labs. Furthermore, the process suffers from reproducibility issues. A pronounced dependence on the environmental conditions during the embedded mask layer formation was observed. Exact control of all these parameters is sometimes difficult to ensure and leads to unwanted variances in processing results. Furthermore, the process is much more time consuming than the previously developed fabrication strategy.

In future, it would be nice to investigate combinations of resistive and e-beam heated evaporation techniques in order to create high quality embedded masks. Also, the use of an alternative metal boasting lower evaporation temperatures such as aluminium could provide better results for this type of microfabrication process.

Table 4.2 assesses the most important aspects of this fabrication strategy based on the same five criteria used in section 4.3.4.

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<th>Process complexity</th>
<th>Material compatibility</th>
<th>Equipment availability</th>
<th>Process reproducibility</th>
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Table 4.2: Assessment of fabrication strategy 1 based on five criteria: 1) Complexity of the overall process, 2) Compatibility of the utilized materials with microfabrication, 3) Special equipment needed, 4) Repeatability of the process, 5) Extendibility to the creation of more advanced devices.

4.3.6 Strategy 3: Sacrificial layer with pre-patterned mold

The third fabrication strategy investigated in the course of this work is based on the same approach as the first. However, instead of defining the shape of the sacrificial layer via photographic patterning as in strategy 1, an additional layer of SU-8 is introduced that is used to define the walls of the resulting cantilever.

Process flow description

Figure 4.24 illustrates the process flow for this strategy. Similar to the other fabrication strategies, this method starts with the formation of the SU-8 bottom layer as shown in figure 4.24A (Mask SU-8 Bottom Layer, see appendix A.2.1). Afterwards, the SU-8 wall layer is patterned and developed (Mask SU-8 Wall Layer, see appendix A.2.5).
Chapter 4. Combining AFM cantilevers with microfluidics

The status of the probe after this step is depicted in figure 4.24B. Together, these two layers form a mold for the future sacrificial layer that can easily be filled with a suitable placeholder material. The result of this next step is shown in image 4.24C, with the sacrificial layer material indicated in green. To achieve this, LOR-10B is spin coated onto the sample so as to barely cover the walls of the probes and completely fill the channel mold. This is followed by a layer of positive tone resist AZ-4562 (see section 2.2.3). Next, the sample is illuminated using the same mask used for the wall layer (Mask SU-8 Wall Layer, see appendix A.2.5) in order to render the photoresist located above the SU-8 wall structures soluble. Subsequent development of the photoresist using Shipley 351 developer solution removes all the AZ resist and LOR-10B from outside the channel mold. The rest of the photoresist is then stripped by briefly immersing the substrate in isopropanol solution. Thanks to the protective nature of the channel mold, the LOR material sitting there is not removed in this process. The remainder of the LOR-10B inside the channel then essentially forms a suitable sacrificial layer that can be selectively removed after the rest of the device has been processed. The remainder of the process follows the same steps as demonstrated in section 4.3.4. Formation of the SU-8 top and handling chip layers is shown in figures 4.24C and 4.24D. Dissolution of the sacrificial layer material is performed in a 80% solution of AZ-400K in water. This is followed by the final release of the probes by means of an enhanced sacrificial adhesion layer technique. The resulting probes feature cantilevers made from three distinct layers of SU-8, as illustrated in figure 4.24F. An example list of all the processing steps required for this fabrication strategy is provided in appendix B.3.

Results

The feasibility of this processing strategy has only been investigated with respect to the hollow channel formation. The left image of figure 4.25 shows an example of a sacrificial layer formed using this process moments after applying the SU-8 top layer. From an optical inspection, the pattern transfer and overall quality look promising. This is further encouraged by looking at the beginning of the dissolution process of the sacrificial material located inside the microchannel. The image on the right side of figure 4.25 depicts the situation during this process after the first 100 µm of the channel have been opened. The current location of the dissolution reaction inside the channel is indicated with a white arrow.
4.3. Fabrication of hollow cantilevers using SU-8

Figure 4.24: Fabrication process for hollow SU-8 probes according to strategy 3. The process is based on a 4-inch silicon substrate wafer coated with an ESL for fast release of the fabricated probes. **A:** Patterning of the SU-8 bottom layer via photolithography. The SU-8 layer is not developed after the post-exposure baking step (cross-linked SU-8 is shown in dark orange, non-cross-linked SU-8 in light orange). **B:** Formation of the wall layer of the probe using SU-8. Both SU-8 layers are developed in this step. **C:** Deposition of an LOR-B sacrificial layer inside the mold defined by the two SU-8 layers. **D:** Generation of the SU-8 top layer covering the microfluidic channel and subsequent development. **E:** To form the handling chip of the device, a thick layer of SU-8 is spin coated and structured in order to form a thick block around the probe’s inlet port. **F:** Final release of the probe from the substrate wafer. The probe features three distinct layers of SU-8 that make up the hollow microchannel inside the cantilever structure.

**Discussion and Conclusion**

This processing strategy was mainly investigated to find a way to overcome the difficulties associated with the material incompatibilities in the first strategy from section 4.3.4. Embedding the sacrificial layer material in a mold of pre-patterned SU-8 side walls greatly improves the reproducibility of the sacrificial layer patterning steps. Furthermore, the LOR-10B material can now be processed using lower temperatures, as dissolution rates are no longer as critical as before since the material inside the
Figure 4.25: Results of channel formation using pre-patterned mold. **Left:** Situation after formation of the sacrificial layer using LOR-10B and AZ4562 as patterning resist. The sacrificial layer is well-defined and the three SU-8 layers exhibit uniform quality over the complete device. Adhesion between different layers seems not to be an issue. **Right:** Situation after the first 100 μm of the LOR-10B sacrificial layer is dissolved by immersion in pure AZ-400K. The device shown in this image does not have an SU-8 block layer.

channel mold is protected from fast dissolution. This mitigates the adhesion issues observed in strategy 1. It is therefore expected that this strategy could be an improved version of the direct sacrificial layer approach. This is further supported by the fact that this novel strategy does not incur any new major drawbacks and hence has the potential to truly serve as an evolutionary method predicated on the first fabrication strategy.

To further improve reproducibility, the method could be combined with chemical-mechanical polishing techniques in order to obtain a perfectly filled channel with a co-planar surface for the subsequent fabrication steps. Unfortunately, this could not be attempted during this work, as no equipment for processing polymeric materials was available.

While the method has been proven to work in principle, further studies are needed to demonstrate complete feasibility of the approach for the fabrication of hollow cantilevers made entirely from SU-8 material. Table 4.3 summarizes the most important aspects of this approach based on the same five criteria evaluated in the context of the other two strategies.
4.4 Chapter summary and conclusion

Table 4.3: Assessment of fabrication strategy 1 based on five criteria: 1) Complexity of the overall process, 2) Compatibility of the utilized materials with microfabrication, 3) Special equipment needed, 4) Repeatability of the process, 5) Extendibility to the creation of more advanced devices.

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4.4 Chapter summary and conclusion

To summarize, this chapter has shown how microfluidics and AFM cantilevers can be combined to yield a completely new category of AFM probes. Such hollow probes are an essential part of fluidic force microscopy, as will be introduced in the upcoming chapter.

It was explained how this new family of microdevices should be seen as both an implicit and necessary evolution of standard AFM probe technology. The introduction of this novel degree of freedom to the realm of AFM has the potential to spur a multitude of completely new application areas for AFM.

Hollow AFM cantilevers can be created with different microfabrication strategies and using different materials. It was demonstrated how silicon-based hollow AFM probes enable reliable fabrication results thanks to their high compatibility with existing MEMS fabrication facilities. The development of such probes was achieved in close collaboration with partner research groups. Two such probe designs was elucidated in detail.

Alternatively, the use of novel polymer-based materials such as SU-8 provides an interesting opportunity. Properties such as high chemical resistance, pronounced biocompatibility in combination with favourable mechanical qualities are strong arguments to motivate further development efforts.

A considerable amount of time was devoted to developing such microfluidic AFM probes during the course of this thesis. Several different microfabrication approaches were successfully envisioned and their feasibility proven. Improved production strategies for such probes inspired by the work shown in this thesis are interesting topics for future research projects in this field. One interesting approach is the use of a purely metallic sacrificial layer defined using galvanic deposition techniques such as electroplating. Other methods based on deep-UV lithography or polymeric sacrificial layers also offer interesting opportunities for further studies. Last but not least, the
introduction of nanoparticles modifying fundamental properties of the polymeric resist (e.g., magnetic nanoparticles [Suter et al., 2011, Suter, 2011]) promise to spur interesting new designs of hollow AFM probes with enhanced capabilities that would be difficult to achieve using classical silicon-based devices.
5 FluidFM: Fluidic Force Microscopy

The goal of this chapter is to introduce the Fluidic Force Microscope (FluidFM) and present the main properties of this recently invented research instrument. The first part of this chapter introduces the main concepts behind FluidFM. The hardware components and technologies involved are explained in detail. In the second part, the major properties of Fluidic Force Microscope systems are illustrated together with an overview of current developments in the software interface used to control FluidFM systems.

5.1 Introduction to FluidFM

FluidFM is an evolution of standard atomic force microscopy, as explained in section 3. Instead of using standard bulk scanning probes to carry out AFM experiments, FluidFM uses specially modified cantilevers with an integrated microfluidic channel. By combining such hollow cantilevered scanning probes as presented in chapter 4 with a standard AFM setup, it is possible to considerably extend the applicability of the technology in the field of life sciences and beyond. This is especially true when it comes to investigating and manipulating individual cells, as will be shown in chapter 8. The concept of recombining existing techniques into a novel device like FluidFM is represented in figure 5.1.

The idea to combine hollow scanning probes with an AFM was conceived at ETH Zürich in the Laboratory of Biosensors and Bioelectronics (LBB) back in 2007. PD Dr. Tomaso Zambelli, Prof. Janos Vörös and his co-inventors named the technology fluidic force microscope - or FluidFM.

The concept for FluidFM was first developed with the idea of a force-controlled micromanipulation device in mind. Specifically, the desire to create an automated,
Figure 5.1: FluidFM merges standard AFM with microfluidics technology to create a new type of device.

easy to use instrument for performing patch clamp experiments [Neher et al., 1978, Hamill et al., 1981] was one of the major driving forces for the development of this new technology. However, the possibility to dispense or sample tiny amounts of liquid at a well-defined spatial location in combination with the sophisticated force feedback properties of an AFM has already opened the door to a wealth of novel applications going well beyond patch clamping.

Figure 5.2 contains a schematic representation of all the major components in a typical FluidFM setup. It can immediately be seen that the setup can be operated inside a liquid environment, making it possible to work under physiological conditions with live biological samples. Integration with optical microscopy techniques is also denoted, and the components of the underlying standard AFM using optical beam deflection readout techniques are indicated. The remaining sections of this chapter will explain the functions and characteristics of these building blocks with respect to the overall FluidFM system.

5.2 Main components of a FluidFM setup

FluidFM is a complex technology with many components needed for it to work. For this reason, a major goal of this thesis was to find a way to integrate all the necessary modules into a single setup in order to create a practicable platform for conducting experiments using FluidFM technology. The following sections reports on all those components and how they have been integrated into a complete and functional FluidFM setup.

5.2.1 Atomic Force Microscope

One of the main constituents of FluidFM is the atomic force microscope. Figure 5.1 shows how AFM is one of the two supporting pillars of the FluidFM, both physically
5.2. Main components of a FluidFM setup

![Schematic overview of all components of a FluidFM system operating under physiological conditions in combination with an inverted optical microscope](image)

**Figure 5.2:** Schematic overview of all components of a FluidFM system operating under physiological conditions in combination with an inverted optical microscope

and conceptually. FluidFM relies on the features and capabilities of AFM to achieve high resolution operation with respect to parameters such as space, time, and force. The most important attributes of atomic force microscopy were explained in detail in chapter 3.

As will be shown in a later section of this chapter, the underlying atomic force microscope can ideally be operated in combination with optical light microscopy. To enable the complete applicability of FluidFM, the AFM should also support operation in a liquid environment in order to render it compatible with live specimens such as eukaryotic cells, yeast, or bacteria. Therefore, in order to maximize the utility of FluidFM, it was determined that the ideal platform to create a functional setup was a state-of-the-art BioAFM offering complete optical integration and operation under physiological conditions. The Nanowizard I system from JPK Instruments AG (Germany), as described in section 2.1.1, promised to be a suitable candidate upon which to build the first working FluidFM prototype.
5.2.2 Cantilever and probeholder with integrated microfluidics

The second pillar of FluidFM technology, as shown in figure 5.1, is the cantilevered scanning probes with integrated microfluidic channel. The combination of these probes with an atomic force microscope is the essential concept behind FluidFM technology. Hollow cantilevers are typically microfabricated using techniques and methods borrowed from standard MEMS technology. Detailed information on the different types of hollow probes developed for this thesis and that have been used in actual FluidFM experiments can be found in chapter 4.

Looking at hollow probes in the context of FluidFM system components, it is clear that one of the major challenges is the actual integration of such probes into the overall setup. The hollow cantilever needs to be physically attached to the system in a suitable way. The following prerequisites for the cantilever-probeholder assembly have been identified as crucial to successful FluidFM operation:

**Water tightness / Leak free attachment**

The attachment between cantilever and probeholder has to be watertight. This is the only way to operate the device in a liquid environment without any parasitic loss of liquid or gas from the microfluidic part of the setup into the surrounding media.

**Mechanical stability**

The cantilever-probeholder assembly should ideally behave like a rigid body (e.g., the cantilever should not move when pressure is applied to the microfluidic channel). If this is only partially the case, the system will be much more difficult to operate in a stable manner. This is especially true with respect to the precise force-control of the AFM.

**Mechanical interoperability**

The cantilever-probeholder assembly needs to be mechanically compatible with the atomic force microscope to which it is attached. This is important, as each AFM instrument has other requirements in that domain. Choosing a system which requires only minor adaptations to a FluidFM probeholder is therefore highly desirable.

**Optical interoperability**

The cantilever-probeholder assembly should not impinge on the light-path of the optical beam deflection system in the underlying AFM. If this condition is not met, force controlled operation of the hollow probe will not be possible.
5.2. Main components of a FluidFM setup

**Microfluidic access**
A suitable microfluidic access port needs to be integrated into the cantilever-probeholder assembly. In particular, a connection to an external actuation system needs to be provided to control the microflow of liquid through the cantilever. Additionally, there must be a way to connect an external reservoir to the hollow cantilever. To ensure fast system dynamics, the total volume of the microfluidic system should be reduced as much as possible.

**Contamination free**
Ideally, the whole cantilever-probeholder assembly comes in the form of a one-way consumable that is fully contamination free (e.g., sterile or RNase free) when first mounted. After the FluidFM experiment has concluded, the assembly can be discarded and a new, clean unit can be mounted for the next analysis. Ideally, in order to have complete protection from unwanted contamination, all other parts of the AFM system never come into contact with the sample and the surrounding environmental substances (e.g., cell culture media).

**Electrochemical compatibility - no leakage currents/low capacitance**
For applications involving the measurement of ionic currents through the microfluidic probe, the cantilever-probeholder assembly should be free from any parasitic current pathways. This places an additional criterion on the watertight attachment between the cantilever and the probeholder, because there must not be any leakage current through that interface. Additionally, an electrode should be integrated such that the resulting device does not suffer from large parasitic capacitances in order not to negatively impact the supported bandwidth of the system when measuring AC currents.

**Handling versatility**
One of the most important aspects of every piece of equipment is undoubtedly its operation and handling. For this reason, every probeholder design is also evaluated with respect to its handling versatility and ease of use. The required setup time as well as the complexity of this process are assessed under this criterion.

In the course of this work, several different types of cantilever-probeholder assemblies were developed and characterized. The different designs are presented in the following paragraphs and benchmarked against the criteria identified above. Most of these designs represent an evolutionary development from an initial prototype into an optimized piece of equipment meeting all or most of the requirements set forth above.
Chapter 5. FluidFM: Fluidic Force Microscopy

Design 1 - First probeholder prototype

The JPK NanoWizard I (see section 2.1.1) was the platform of choice to create the first functional FluidFM setup. The instrument features a robust and fully transparent probeholder that is entirely made out of glass. The probeholder can be immersed up to 8 mm into a fluid in order to operate the AFM under a liquid environment. The dimensions of the device turned out to be very favourable for the purpose of drilling a channel directly into it in order to create a suitable access port to the microfluidic inlet of the FluidFM cantilever.

![Figure 5.3: Overview of the first functional FluidFM probeholder implementation](image)

**Left:** Drilled probeholder block made from polycarbonate. The probeholder has the same dimensions as the original part from the AFM manufacturer. The metal conduit on the side of the assembly is used to connect a tube to the drilled probeholder. **Right:** Complete overview of the cantilever-probeholder assembly, connected to an external reservoir via suitable tubing.

The first generation FluidFM probeholder was CNC machined from a transparent polycarbonate block to have the same dimensions as the original probeholder supplied by the manufacturer of the instrument. Mechanical and optical compatibility was verified by mounting the drilled probeholder with a standard AFM cantilever. Next, a suitable method to fix and seal a hollow cantilever to this drilled probeholder had to be found: In the first generation design it was determined that using an o-ring made from a bio-compatible two component glue (JPK Instruments AG, Germany) form in situ would allow it to create a liquid proof connection between the cantilever and the drilled probeholder. By controlling the ratio of the two components, this glue could be tuned to form a flexible, yet still mechanically robust compound once fully cured. By placing a small droplet of glue on the inlet of the probeholder drilling followed by a swift application of nitrogen gas through the assembly, a well-defined o-ring-like structure could be reproducibly created on the probeholder. Afterwards,
5.2. Main components of a FluidFM setup

The microchannelled cantilever was mounted on top of this o-ring while making sure that the inlet of the cantilever was aligned with the center of the o-ring on the probeholder. The cantilever could then be easily fixed using the standard medical steel spring supplied by the AFM manufacturer. The left image of figure 5.3 depicts such a first generation probeholder with a mounted cantilever. The probeholder inlet can be connected to an external reservoir via tubing, as shown on the right of figure 5.3. The reservoir can subsequently be filled with the desired cargo liquid. By applying an overpressure to the external reservoir by means of a suitable pressure control device, the cargo liquid is forced through the tubing via the probeholder into the microfluidic part of the cantilever.

Table 5.1 shows how well this first design was judged to meet the criteria identified for an ideal FluidFM cantilever-probeholder assembly.

<table>
<thead>
<tr>
<th>water tightness</th>
<th>mechanical stability</th>
<th>mechanical interoperability</th>
<th>optical interoperability</th>
<th>microfluidic access</th>
<th>contamination free</th>
<th>electrode compatibility</th>
<th>handling versatility</th>
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<td>✓</td>
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</tr>
</tbody>
</table>

Table 5.1: Assessment of the major properties of probeholder design 1.

From table 5.1, it can be seen that the first probeholder design is still a long way from satisfying all the criteria identified for an ideal FluidFM cantilever-probeholder assembly. In particular, the requirement for mechanical stability of the cantilever when applying pressure pulses is not satisfied at all. As long as this issue is unsolved, the performance of the AFM will be negatively impacted by unwanted changes in deflection of the cantilever upon modifying the applied pressure. Furthermore, the design suffers from large volumes in the microfluidic channel and tubing that have a negative effect on the overall system dynamics with respect to pressure changes. Last but not least, the procedure of creating the sealing o-ring is cumbersome and can only be carried out by experienced users.

Design 2 - Probeholder with integrated reservoir and improved mechanical stability

In order to address some of the drawbacks present in the first probeholder model, a second design was created based on the same AFM platform from JPK Instruments AG (Germany). For the second generation probeholder design, the problem of mechanical stability of the cantilever was mitigated by improving the fixing mechanism. Since the fixture used in design 1 is based on a spring clamp, whenever a force is exerted on it, the spring will react to this force by changing its dimensions accordingly. Hence, as a pressure pulse is applied to the microfluidic system, the hollow cantilever moves in
response to the dimensional changes of the metallic spring.

To overcome this issue, a design featuring a clamp that can be fastened using two screws is used to keep the cantilever in place while reducing the amount of deformation when subjected to any pressure changes. This is improved by fixing the cantilever to the probeholder body by means of a U-shaped holding clamp. This clamp presses the probe firmly against the underlying sealing o-ring by means of two screws.

Another improvement is the placement of the reservoir: It is now directly integrated into the probeholder body. This way, less cargo solution (<50 μl) is required during operation. At the same time, a significant reduction in unwanted dead volume is achieved. The system will therefore react much faster to any pressure changes applied to the reservoir.

Figure 5.4 illustrates this improved probeholder. It can be seen how the clamping element works and that the reservoir is now completely integrated. Loading of the cargo liquid is carried out before the experiment and the reservoir is sealed using a backport closure that connects to an external pressure source.

Figure 5.4: FluidFM Probeholder design 2 - integrated reservoir and improved mechanical stability. **Left:** CAD explosion view of the 2nd FluidFM probeholder design. The clamping element is shown in anthracite. The backport closure that serves as a seal for the integrated reservoir is attached by means of two extra screws and a second o-ring sealing element. **Right:** Photograph of the probeholder with a mounted FluidFM type B probe. It can be seen that the mounting clamp is slightly bent when the screws are completely tightened.

Instead of using an o-ring made from two glue components as in design 1, a cavity was milled around the outlet port of the probeholder into which a commercial o-ring from Johannsen AG (Switzerland) could be permanently installed. This design allows the
sealing component to be changed very conveniently whenever required. Figure 5.5 shows how the o-ring is integrated into the probeholder. Upon mounting the probe, it is important to align it properly with respect to the sealing o-ring in order to ensure a continuous fluidic access channel. Thanks to the integrated o-ring, the overall setup complexity for each experiment is significantly reduced.

**Figure 5.5:** Probeholder with integrated o-ring sealing element. **Left:** Overhead view of the o-ring sealing element. The o-ring sits in a recess that is machined into the surface of the probeholder around the liquid access port. **Right:** View from below through the transparent probeholder block. It is important to precisely align the inlet of the hollow cantilever chip with the integrated o-ring to ensure a water-tight seal.

The major limiting factor with respect to the improved mechanical robustness of this design is the thickness constraint imposed on the clamping element. The clamp could not exceed a total thickness of 0.2 mm or the tip holder would reach the sample surface before the apex of the cantilever. The first prototypes, made from industry grade tooling steel using electric discharge machining (EDM), turned out to be too brittle and failed during the first mounting trials. PEEK CF\textsuperscript{1} was finally used to produce a functional holding clamp. Although less stiff than the metallic version, these clamps are much easier to fabricate and far less prone to breaking under loading conditions.

Thanks to the clamp, the second probeholder generation noticeably reduced the vertical displacement of the tip during pressure pulse load. However, it was still not fully compensated. The residual displacement turned out to be due to the fact that the cantilever was not fully seated on the flat surface of the probeholder body but rather floating on top of the o-ring used for sealing the cantilever to the probeholder. Another problem inherent in this design is caused by the holding clamp not being mounted in

\textsuperscript{1}PEEK - Polyether ether ketone is an organic thermoplastic polymer that is often used for industrial and engineering applications. PEEK CF is a compound material made from PEEK and carbon fibers (typically 30%). CF addition lowers the expansion rate of PEEK and enhances its strength and stiffness properties.
line with the focal point of the probe but rather offset to the back with respect to the cantilever. Similar constraints as for the thickness of the clamp apply to the minimal distance between the cantilever apex and the mounting clamp, in order to prevent the probeholder from touching the surface of the sample before the cantilever. Because of this offset, any force acting on the cantilever chip is not exerted in the same axis as the corresponding reaction force caused by the mounting clamp. As illustrated in figure 5.6, the resulting momentum manifests in a rotational movement upon any change in pressure in the cargo liquid reservoir. The non-rigid bearing of the probe chip in combination with the still somewhat flexible mounting clamps therefore does not satisfactorily solve the issue of mechanical stability.

![Figure 5.6: Visualisation of the momentum acting on the probe chip in the second probe holder design. Left: Illustration from the CAD design data. It can be seen that a moment will result from the two forces acting on the chip. Right: Photograph from the real situation. The inlet channel (behind blue arrow) is not co-axially arranged with the probe fixation clamp.](image)

The hypothesis that the flexible o-ring is one of the main reasons for the mechanical instability was confirmed by heating up an o-ring to make it thinner. Cantilevers mounted with these altered o-rings exhibited far less residual mechanical movements compared to when a standard o-ring was used [Ossola, 2010]. Compared to the first probeholder prototype, the second design fullfils the following criteria for an ideal FluidFM probeholder:

<table>
<thead>
<tr>
<th>Property</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water tightness</td>
<td>✔️</td>
</tr>
<tr>
<td>Mechanical stability</td>
<td>✔️</td>
</tr>
<tr>
<td>Mechanical interoperability</td>
<td>✔️</td>
</tr>
<tr>
<td>Optical interoperability</td>
<td>✔️</td>
</tr>
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<td>Optical access</td>
<td>✔️</td>
</tr>
<tr>
<td>Microfluidic access</td>
<td>✔️</td>
</tr>
<tr>
<td>Contamination free</td>
<td>✔️</td>
</tr>
<tr>
<td>Electrode compatibility</td>
<td>✔️</td>
</tr>
<tr>
<td>Handling versatility</td>
<td>✔️</td>
</tr>
</tbody>
</table>

Table 5.2: Assessment of the major properties of probeholder design 2.
Since the probe is still prone to undesired mechanical movements when using probeholder design 2, yet another design review cycle was necessary.

### Design 3 - Probeholder with integrated reservoir and rigid mechanical clamping

For the third generation FluidFM probeholder, the main goal was to completely eliminate the parasitic mechanical movements of the hollow probe upon pressure actuation of the integrated microfluidic system. For this reason, the new design takes advantage of the $10^\circ$ inclination of the cantilever when mounted on the probeholder in order to achieve a thicker and more rigid fixation clamp. The developed shape of this new clamping element features a simplified geometry with less pronounced aspect ratios. The new clamp is therefore also easier to manufacture using common CNC machining techniques. The clamp can be made from both plastic or metal. The first prototype was made out of industrial grade spring-steel for optimal mechanical properties.

![FluidFM Probeholder design 3 - integrated reservoir and rigid mechanical clamping.](image)

**Figure 5.7:** FluidFM Probeholder design 3 - integrated reservoir and rigid mechanical clamping. **Left:** CAD detail of the improved clamping element. This new component used the inclination of the probeholder assembly to achieve better mechanical stability. **Right:** CAD assembly illustration of the complete version 3 probeholder. The improved clamping element is still attached using two screws. The rest of the device is not changed with respect to version 2.

Figure 5.7 shows two CAD images of the third version of the probeholder design: On the left, the rigid clamping element is depicted in detail. It can be seen that the element features the same $10^\circ$ inclination as the probeholder. This way, it is possible to easily fix the cantilever with high compressive forces onto the sealing o-ring around the probeholder outlet. The force is exerted onto the cantilever chip in the same axis as the force resulting from the application of overpressure to the microfluidic channel. Thus there is no longer any unwanted momentum acting on the cantilever chip upon pressure actuation. The complete assembly with a mounted chip is shown on the
right of figure 5.7.

Unfortunately, contamination is still an open issue in this design, as the device is intended to be re-used multiple times. Furthermore, the o-ring sealing around the drilling of the probeholder block is still not an optimal solution. It was observed during experiments that little air bubbles often form around the o-ring and remain trapped there. These bubbles most likely result from non-optimal wetting during the liquid filling of the fluidic system. Air bubbles in the channel can be disturbing to the overall experimental performance of a FluidFM system, because changing pressures in the channel always leads to potentially disturbing expansion or compression of such bubbles. When measuring the ionic currents flowing through the aperture of the probe (see chapter 9), a completely expanded gas bubble can easily lead to an interrupted current pathway, making any further measurement impossible.

Probeholder design 3 was benchmarked against the criteria imposed on an ideal FluidFM probeholder, as shown in table 5.3.

<table>
<thead>
<tr>
<th>water tightness</th>
<th>mechanical stability</th>
<th>mechanical interoperability</th>
<th>optical interoperability</th>
<th>microfluidic access</th>
<th>contamination free</th>
<th>electrode compatibility</th>
<th>handling versatility</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓</td>
<td>✔️</td>
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<td>✔</td>
<td>✗</td>
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<td>✓</td>
</tr>
</tbody>
</table>

Table 5.3: Assessment of the major properties of probeholder design 3.

To overcome the problem of bubble formation around the o-ring, a completely new design was developed in collaboration with the commercial FluidFM provider Cytosurge AG (Switzerland). The goal of this new probeholder-cantilever assembly was to preserve all the desirable features of the original approaches, but improve on all remaining attributes of an ideal FluidFM probeholder.

**Design 4 - Disposable probeholder with integrated reservoir and direct probe gluing**

Design 4 is a completely new version of the probeholder-cantilever assembly. The objective of this design was to create a component that can be used in a one-time fashion as a disposable consumable. This was done because it is desirable to use a completely clean probeholder for each FluidFM experiment, free from any contamination from preceding studies. In order to achieve this, the probeholder assembly was engineered in the form of a mechanical clip. The left image of figure 5.8 shows a photograph of such a clip. It is designed so it can be attached to a specially adapted AFM holder nose and be automatically aligned correctly. The clip was made compatible with the FluidFM system from the commercial providers Cytosurge AG and Nanosurf AG (Switzerland), as described in section 2.1.1.
5.2. Main components of a FluidFM setup

Design 4 - the so-called CytoClip - no longer relies on a mechanical solution to attach the cantilever chip onto the probeholder. Instead, the two parts are already pre-assembled during the production process using an epoxy-based adhesive. When fully cross-linked, the adhesive produces a rigid entity consisting of both the carrier clip and the cantilever chip. This way, it is also no longer necessary to include a distinct o-ring for sealing purposes, thus avoiding any unwanted bubble formation. The carrier clip is machined from PMMA\(^2\) by Cytosurge AG (Switzerland).

When attaching the CytoClip to the holder nose of the AFM head, it is important that the clip is correctly aligned with the optical beam deflection system. Because of this, the probeholder is equipped with an alignment system based on a three point bearing mechanism. However, to ensure acceptable alignment tolerances, the carrier clip has the be machined with 20 μm precision.

Similar to earlier designs, the CytoClip features an integrated reservoir holding up to 50 μl of reagent solution for use inside the hollow channel of the FluidFM probe. The holder nose can contain further elements such as an excitation piezo in order to make the system compatible with the numerous dynamic modes of traditional atomic force microscopy.

On the back of the CytoClip, the holding reservoir can be closed by means of specially adapted bayonet mount for quick and easy fastening and release. This back connector

\(^2\)PMMA - Poly(methyl methacrylate) is a synthetic polymer of methacrylate better known under the tradename Plexiglas. PMMA is commonly used in medical technology, e.g., to produce micro-fluidic devices such as lab-on-a-chip systems.
also ensures the connection to an external pressure control device. An example of a CytoClip device mounted on a commercial FluidFM system is depicted in figure 5.9.

![CytoClip mounted on a commercial FluidFM system. The probeholder is connected at the back using a backport connector. This connector also provides an air-tight connection with an external pressure control instrument.](image)

Thanks to the pre-assembled nature of design 4, the hollow cantilevers can already be packaged under cleanroom conditions to avoid any unwanted contamination with microparticles or microbiological entities. Once packaged, the parts can be further sterilized via suitable radiation-based methods. The device can be stored for longer periods without breaking or degrading in quality. An example of the packaging container is shown as a CAD image on the right of figure 5.8.

Table 5.4 shows how well design 4 fulfils the criteria for an ideal FluidFM probeholder.

<table>
<thead>
<tr>
<th>water tightness</th>
<th>mechanical stability</th>
<th>mechanical interoperability</th>
<th>optical interoperability</th>
<th>microfluidic access</th>
<th>contamination free</th>
<th>electrode compatibility</th>
<th>handling versatility</th>
</tr>
</thead>
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<td>✓</td>
<td>✓</td>
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<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

**Table 5.4: Assessment of the major properties of probeholder design 4.**

To sum up, the device from design 4 fulfils most of the criteria imposed on an ideal FluidFM probeholder. However, there is still room for improvement with respect to electrode compatibility (see also chapter 9) and unwanted cross contamination.

**Recapitulation - FluidFM probeholder assembly**

For experiments conducted on the JPK platform, probeholder designs 1 to 3 were used and have proven feasible for many experiments, as will be shown in the following chap-
5.2. Main components of a FluidFM setup

ters. Investigations carried out using the FluidFM platform from Cytosurge/Nanosurf were all carried out using CytoClips (design 4), as explained in the preceding section. In collaboration with Cytosurge, the device was tested on our instruments and was found to perform very well. Current and future FluidFM experiments were therefore all carried out using commercial, pre-assembled probes as supplied by Cytosurge AG (Switzerland).

Although the latest design already meets all the criteria imposed on an ideal FluidFM cantilever-probeholder assembly, there is still room for further improvement. One example would be to have all parts that are in contact with the sample be disposable consumables. This would achieve true protection from any unwanted cross-contamination. Furthermore, a smaller dimensional footprint would be desirable in order to be able to work in small containers such as standard multi-well microscopy plates.

5.2.3 Microfluidic flow control

Another major cornerstone of FluidFM is the mechanism used to control whether liquid flows from the hollow probe aperture or is sucked into it. A suitable actuation method needed to be found to efficiently control the microflow inside the microfluidic channel of a hollow AFM probe. An acceptable mechanism would have to enable the user to reliably influence, control, record, and store what the flow inside the channel is at a specific point in time. In addition, it would be desirable to be able to synchronize flow control to external triggering events such as the attainment of a specific setpoint of AFM cantilever deflection. The typical flow rate through a FluidFM cantilever is expected to be small (~ fl/s, see also section 5.3.5). Therefore, it will be difficult to precisely and quantitatively assess the flow rate through the aperture of the cantilever.

There are only a handful of established methods to control the flow rate in a microfluidic system. Requirements such as settling time dynamics and stability of the attained flow rates define which technique is most suited to the application at hand. The following paragraphs give an overview of the most important techniques available to regulate microfluidic flow.

**Volumetric actuation - Syringe pumps and peristaltic pumps**

Both these methods rely on the use of some kind of mechanical actuation that alters a volume to generate a pressure that will control the movement of liquid in the system. Instruments such as syringe pumps or peristaltic pumps build on this technique to
control different flow rates in a microfluidic system. Due to the mechanical actuation underlying these methods, the occurrence of a pulse-like flow behaviour is often observed in such systems. Especially at low flow rates and in conjunction with the use of stiff tubing, this effect will likely disrupt overall experimental fidelity. Furthermore, the response and settling times are typically quite long compared to other methods. Because of these issues, volumetric actuation was not considered a suitable candidate for controlling the flow inside a hollow FluidFM probe.

**Electroosmotic actuation**

In the case of electroosmotic pumps, fluid flow is achieved via an electric field applied to an electric double layer [Zeng et al., 2001]. While this method is often used to control the flow in lab-on-a-chip devices, it requires the integration of suitable electrode structures directly within the microfluidic device. For this reason, this flow control method is not very easy to integrate into a typical FluidFM Setup. While not impossible, this actuation method is expected to still require considerable research and microsystems engineering effort in order to be integrated into hollow AFM probes [Hug et al., 2005b]. Furthermore, the achievable pressure values are typically limited to around 0.7 bar. For these reasons, this method was not considered in this work.

**Integrated MEMS pumps**

Directly integrating a pumping device into the microfluidic system is another option to control the flow of liquid inside it. There are several different implementations for integrated pumping systems based on microelectromechanical systems technology. Some of them are very similar to their macroscopic equivalents, e.g., membrane pumps and peristaltic pumps [Nguyen et al., 2002]. Other implementations rely on the use of physical properties such as the controlled evaporation of liquids from the microfluidic system. Such an approach has already been shown in combination with a hollow AFM cantilever [Heuck et al., 2008]. The main advantage of integrated MEMS pumping systems is the possibility to precisely control fluid flow down to the pl/s range. It is therefore especially suited for applications requiring small reagent volumes. However, the integration into a microfluidic system is highly challenging, both in terms of engineering complexity and the associated fabrication process. Additionally, the design of such a device has to be specifically adapted to the designated application and would likely require specific modifications for different experiments. Integrated MEMS pumping was therefore considered to be a topic for a prospective research project and is not further covered in this work.
5.2. Main components of a FluidFM setup

Direct pressure generation - Hydrostatic pressure and active pressure regulation

A practical implementation of a flow control system based on direct pressure generation from an external system is very straightforward. For example, it can easily be achieved using a simple setup based on hydrostatic pressure using height differences. Another approach involves the use of an active pressure pump in combination with a complex network of pressure and release valves in order to achieve maximum stability and fast switching of the applied pressure. This method is based on the pneumatic analogy to an electrical Wheatstone bridge configuration [Shaw, 1889]. Both approaches were used during this work. The devices employed are presented in detail in section 2.1.3.

Through the application of a pressure difference via hydrostatic means or an active pressure regulation device, the liquid inside a microfluidic system will flow in accordance with a simple relationship, similar to the Ohmic law in electrical circuits, as shown in equation (5.1). \( \Phi_V \) [m\(^3\)/s] denotes the flow rate, \( p \) [Pa] is the pressure and \( R \) [Pa s/m\(^3\)] stands for the microfluidic resistance of the overall system.

\[
\Phi_V = \frac{p}{R} \tag{5.1}
\]

In contrast to other flow control methods, this approach does not require any special adaptations to the microfluidic components to be controlled. As long as a suitable pneumatic connection can be created between the pressure regulating device and the microfluidic system, this method can be used to control the flow rate. Furthermore, there are virtually no restrictions on the liquids that can be used inside the system. It is even possible to use mercury in order to create a dropping mercury electrode Kahan [1942] within a FluidFM setup [Schön et al., 2013].

A major drawback of this method is the indirect nature of the actuation principle: In order to achieve true quantitative flow control, the exact pneumatic resistance of the microfluidic system needs to be known in order to quantitatively translate the applied pressure difference into a flow rate with the help of equation (5.1). As will be shown in section 5.3.5, it is possible to gain approximate knowledge of the flow rates with respect to the applied pressure by means of computer simulations and analytical approximations. However, a true quantitative determination of the flow rate is not easily achievable, because commercially available liquid mass-flow sensors do not yet offer the required resolution ranges necessary to measure the liquid transport rate through a FluidFM probe. At the time of writing, the best available sensor devices are capable of resolving flow rates in the nl/min range.
Chapter 5. FluidFM: Fluidic Force Microscopy

Summary and comparison of microfluidic flow control methods

To control the microflow in a FluidFM setup, all the methods outlined above would in principle be feasible. Table 5.5 provides an overview of how the different techniques compare against each other with respect to different attributes relevant for FluidFM. For this work, the goal was to find methods that offer both satisfying performance against quantitative benchmarks and qualitative characteristics such as implementation complexity, automation, and handling versatility.

<table>
<thead>
<tr>
<th>Type of Technology</th>
<th>Flow Stability</th>
<th>Achievable Resolution</th>
<th>Response Time</th>
<th>Settling Time</th>
<th>Automation Capability</th>
<th>Integration Complexity</th>
<th>Handling Versatility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe Pump</td>
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</table>

Table 5.5: Comparison of microflow control methods with respect to their applicability for FluidFM.

It can be seen from table 5.5 that technologies relying on pressure differences (hydrostatic pressure and active pressure regulation) exhibit desirable properties in all aspects relevant for FluidFM. These methods have therefore been chosen for all the experiments carried out during the course of this thesis. A detailed description of the practical implementation and the instrumentation used can be found in section 2.1.3. However, other methods such as integrated MEMS pumps and electroosmotic pumps are expected to offer superior properties in quantitatively controlling microflow in a hollow cantilever. The integration of such technologies into a FluidFM setup would therefore make for an interesting future research project.

Even though true quantitative control of the microflow present in a FluidFM system is difficult to achieve, the decision to opt for an implementation based on direct pressure regulation devices has proven sufficient for almost all FluidFM applications developed so far. Most of the assays and experiments carried out thus far were clearly feasible even without true quantitative knowledge of the volumes dispensed or sampled through the aperture of the FluidFM probe.
5.2.4 Optical microscope

As can be seen in figure 5.2, FluidFM is readily compatible with optical microscopy techniques. Both standard inverted microscopes and more advanced instrumentation such as confocal laser scanning devices (see section 2.1.2) can be combined with FluidFM.

During the course of this work, it became increasingly evident that compatibility with optical imaging techniques is an important aspect of FluidFM technology. While FluidFM would allow the acquisition of substrate images via standard AFM imaging techniques alone, the overall versatility of the instrument would suffer considerably. The compatibility with optical microscopy techniques offers a convenient way to gain a quick overview of the status of both the instrument and the substrate.

Figure 5.10 shows a typical view of a FluidFM experiment carried out on an inverted microscope. Both the hollow cantilever and the target cell can be observed in real-time. This allows for true in situ control of the FluidFM probe that would not be possible using AFM imaging alone, due to the long times needed to acquire such images.

![Figure 5.10: Example of a typical FluidFM experiment with optical microscope integration. Both the targeted cell and the FluidFM cantilever can be observed in real-time at all times during the experiment.](image)

Optical control of the experiment makes it possible to capture the complete context of all experimental procedures carried out during a FluidFM experiment. Both the spatial and dynamic properties of the sample and its immediate environment can be supervised at all times. As both methods can be utilized in a true parallel fashion, it is possible to readily observe the FluidFM during the experimental procedure in order
to dynamically react to any changes, malfunctions, or unexpected outcomes.

Especially in the field of life sciences, optical microscope techniques are virtually ubiquitous in state of the art research facilities. Researchers in these fields are very familiar with the technique and have already established numerous related assays and methodologies. The possibility to position the FluidFM technology on top of these platforms has been identified as an important success factor to catalyse its adoption as a standard research instrument.

5.2.5 Sample stage for positional control

Another vital component of a FluidFM setup that typically goes hand in hand with the optical microscope is the sample stage. This component can be used to position the substrate separately from the rest of the instrument. Ideally, such a stage allows sample displacement along all three spatial dimensions independently.

Actuation along the in-plane axes of the substrate (X and Y directions) can be used to change the lateral position of the sample with respect to the FluidFM cantilever. The AFM itself typically also features a certain actuation range for the cantilever in the X, Y, and Z directions. However, the maximum travel distances are usually limited due to the underlying piezocrystal-based actuation principle. In the Z-direction (normal to the sample surface), the AFM offers only limited travel ranges in the order of 10 μm. For more advanced experiments, such as single cell force spectroscopy, this range is not sufficient and therefore a separate Z-actuator needs to be integrated into the sample stage to enable positioning of the sample over larger vertical distances relative to the FluidFM probe. Figure 5.11 shows a photograph of the sample stage of a commercial FluidFM system from Cytosurge AG / Nanosurf AG (Switzerland).

It should be noted that both the sample stage and the AFM piezoactuators form two independent actuation systems that need to be operable in sync in order to carry out FluidFM experiments with maximum flexibility. A major difficulty stems from the overlay of the two coordinate systems. This is specially important when trying to automate FluidFM operation, where the operator should be able to control the instrument based on information from the light microscope's optical video feed. As both coordinate systems are observed through the imperfect optics of the inverted microscope, suitable methods to derive a precise transformation to translate positions in the video information to distinct coordinates for both the sample stage and AFM positioning system are needed. Several alternative solutions to mitigate this problem were investigated during the course of this work and are elaborated in detail in the corresponding Master's thesis [Wolf, 2013].
5.2. Main components of a FluidFM setup

Figure 5.11: Photograph of a commercial FluidFM stage. The stage can be used to position the sample with respect to the FluidFM probe over longer distances than is possible using only the intrinsic actuators of the AFM.

5.2.6 Control Software

Suitable software to control and execute complex experiments using fluidic force microscopy methods is the final major pillar in a typical FluidFM setup. Since FluidFM relies on many different instruments that need to be operated in a highly synchronized fashion, it is vital to have control software that can carry out all these tasks from within a single user interface. For the feasibility studies carried out in this work there was no optimized software available to control all the involved instruments comfortably using a single program. For the first prototype, each instrument was controlled either manually or using the software supplied by the manufacturer. However, for more advanced studies where experimental complexity is greatly increased, this approach is no longer feasible and new ideas are required.

A complete FluidFM setup consists of the following individual devices that all need to be operated in sync to carry out meaningful experiments:

- Atomic force microscope
- Optical microscope
- Pressure control device
- X-Y sample stage
- Long-range Z-actuator
- Additional signal acquisition systems (e.g., I/V amplifier)
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The need for optimized control software increases when including additional measuring instrumentation into the setup, such as when measuring ionic currents through the hollow FluidFM probes (see chapter 9). These challenges led to the development of novel user interfaces to be used as FluidFM control software. A completely integrated piece of user software based on an advanced touchscreen interface has been developed in collaboration with the commercial FluidFM provider Cytosurge AG (Switzerland).

Figure 5.12: The fully integrated CyUI software can be used to conduct FluidFM experiments. The software has been developed in collaboration with Cytosurge AG (Switzerland). **Left:** Example screenshot of the CyUI software. The user interface is centred around the video feed from the optical microscope. Measurement locations can be directly selected on the sample via overlaid graphical tools. **Right:** Photograph of a FluidFM setup controlled via the touch enabled CyUI user interface.

The left image of figure 5.12 shows an example screenshot of this interface. The software is primarily centred around the information provided via the optical video feed from the inverted microscope. This software, known as CyUI, also takes advantage of the fact that the complete status of the system is known at all times and hence allows the user to track and retrieve experimental data without losing any information about its spatial and temporal context. A complete account of the location and time of measurements can thus be provided to the user and displayed directly within the original video information from the optical microscope. The right of figure 5.12, illustrates how the software can be controlled via touch-input to directly interact with the sample.

The main advantages of this comprehensive approach also account for the major difficulties that need to be solved to create such software: Special care has to be given to the correct overlay of all involved coordinate systems, and advanced image recognition and positioning algorithms are required to ensure proper compliance in positioning the hollow cantilever with respect to the video feed from the optical microscope.

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5.3 General properties of fluidic force microscopy

The following section provides an overview of the most important aspects of FluidFM technology. The major qualities and functionalities of the technology are investigated and reviewed.

5.3.1 Universal delivery and sampling system with force control

Two major aspects of FluidFM technology differentiate it from other manipulation techniques such as standard AFM microscopy and micropipettes:

Closed microfluidic system

Thanks to the hermetically sealed microfluidic system integrated in the cantilever of a FluidFM probe, it is possible to deliver liquids within a fluidic environment without intermixing. Virtually any soluble substance can be used as a cargo solution.

Force controlled pipette

Thanks to the force feedback capabilities of the underlying AFM, FluidFM can essentially be seen as a micropipette device with full force feedback control. This is achieved by measuring the interaction forces with the specimen at all times during an experiment.

Both of these qualities are enabled by either one of the two fundamental components of FluidFM: Atomic force microscopy and microfluidics, as explained at the beginning of this chapter in section 5.1. Combining these two techniques into a unified setup makes FluidFM a versatile and broadly applicable technology for use in life sciences, material sciences, and beyond as a universal liquid delivery and sampling system with force control. The fundamental operational modes of FluidFM can be summarized as follows:

Operation using overpressure

The most straightforward way to take advantage of the novel properties inherent in FluidFM technology is to apply positive pressure to the microfluidic system of the cantilever. This will result in a flow of liquid out of the aperture onto the sample. Using the FluidFM device with positive differential pressure leads to the following modes of operation:
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Liquid delivery
Using overpressure, liquid can be delivered to a target surface, object, or secondary fluid. Liquid delivery can be used both in air and liquid environments. The delivery of fluid benefits from the high spatial accuracy of the AFM system as well as the fast dynamic properties of the pressure control system [Meister et al., 2009b].

Particle delivery/release
Particles attached to the cantilever aperture or immersed in the channel liquid can be dispensed to a target. The user thereby benefits from the same beneficial properties with respect to resolution in space and time as with standard liquid delivery [Grüter et al., 2013].

FluidFM operation using overpressure already has many applications. However, the system can also be actuated using negative pressure (vacuum).

Operation using negative pressure (vacuum)
FluidFM can also be operated using negative differential pressure applied to the microfluidic channel of the hollow probe. In this way, the cantilever can be used to sample fluid from a substrate, as negative pressure results in the inflow of liquid into the cantilever through the probe aperture. Operation in this pressure regime enhances the fundamental modes of operation of FluidFM, as shown by the following list:

Liquid sampling
The cantilever can be used as a probe with high spatial accuracy and force control to collect liquid samples from a substrate for subsequent analysis.

Particle collection
Using sufficiently large apertures, FluidFM can be used to collect micro- and nano-sized objects directly into the cantilever for further analysis or subsequent delivery to another target substrate [Stiefel et al., 2012, 2013].

Spatial manipulation
The FluidFM probe can be used to spatially manipulate micro-objects such as cells and micro-beads. These objects are engaged in a way similar to a suction cup to create a rigid attachment to the cantilever. Aspirated objects can subsequently be released by reversing the pressure applied to the probe's microfluidic system. The objects to be manipulated can also be considerably larger than the aperture of the probe itself [Doerig, 2013].
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Adhesion measurement

Similar to spatial manipulation, objects can be removed from a surface. Using the AFM’s force feedback system, it is possible to record the forces occurring during this procedure. This allows quantitative investigation of the adhesion forces occurring between the surface and the manipulated micro-object [Potthoff et al., 2012].

In summary, by combining operation in both negative and positive pressure regimes, FluidFM can be applied as a truly versatile delivery, sampling, and manipulation tool for the micro- and nano-domains.

5.3.2 FluidFM cantilever spring constant

As explained in section 3.2.3, exact knowledge of the spring constant of an AFM cantilever is needed to quantify the forces involved in the interactions with the sample. To determine the spring constant of hollow FluidFM probes, it was necessary to adapt the corresponding analytical expression and to investigate whether the Sader method introduced in section 3.2.4 can still be applied to such cantilevers.

Analytical and experimental determination

The spring constant of a hollow AFM probe can be calculated using equation (3.2), as explained in section 3.2.3. Compared to a bulk cantilever, a hollow probe exhibits a different bending moment of inertia $I_h$. This is due to the missing mass from the integrated micro-channel. The cross-section derived moment of inertia of a bulk material beam of width $w$ and thickness $t$ can be determined from equation (3.3). In order to calculate the modified bending moment of inertia $I_h$ for the case of an equal sized hollow beam featuring a concentric rectangular channel of height $t_h$ and width $w_h$, the reduced mass of the device needs to be included in the expression. This can be achieved by subtracting both dimensions from each other, as shown in equation (5.2).

\[
I_h = \frac{wt^3}{12} - \frac{w_h t_h^3}{12}
\]  

(5.2)

Combining equations (3.2) and (5.2), the spring constant for an ideal, rectangular FluidFM cantilever can be calculated using the following expression:

\[
k_h = \frac{E}{4L^3} (wt^3 - w_h t_h^3)
\]  

(5.3)
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As mentioned in chapter 3, the ability to calculate the spring constant analytically is only of limited use, since the necessary high precision assessment of the important cantilever dimensions is difficult to achieve. Furthermore, equation (5.3) is only valid for the case of an ideal rectangular cantilever. However, real FluidFM probes of type A and B do not exhibit fully rectangular channels. It is therefore necessary to be able to experimentally determine the spring constant of such probes.

Fortunately, the method introduced by Sader presented in section 3.2.4 still holds, even for the case of hollow cantilevers, as confirmed via finite element analysis by [Doerig, 2013]. The Sader method is not sensitive to the thickness and material properties of the probe. It is primarily influenced by the hydrodynamic function, which remains unchanged as long as the exterior dimension of the probe still exhibits a long and thin rectangular shape. Additionally, the interior structures of the cantilever do not influence the method, hence the attribution of the microchannel can be neglected.

The effective mass $M_e$ of commercial type B probes was also investigated numerically by [Doerig, 2013]. It was seen that the effective mass of FluidFM probes is equal to that of a similar sized bulk probe within the resolution limits of the simulation model.

To conclude, the spring constant of FluidFM probes can be approximated using the dimensions and material properties of the probe. Furthermore, experimental determination of the spring constant by means of the Sader Method explained in section 3.2.4 is still valid for the case of hollow cantilevers and can be achieved by measuring the thermal excitation spectrum of the probe.

Comparison: Empty vs. filled FluidFM cantilevers  To determine how the filling state of a hollow cantilever influences the mechanical properties of the probe, several cantilevers both with and without cargo liquid fillings were investigated under ambient environment conditions by measuring their frequency response.

As the main difference between a liquid-filled probe and an empty specimen is the added mass of the fluid inside the channel, this effect is expected to be most influential. The addition of this extra mass is expected to change the eigenfrequency of the system, similar to a micromechanical cantilever-based sensing device [Raiteri et al., 2001]. Such a shift in resonance frequency can be readily measured by recording the thermal noise spectrum.

Image 5.13 shows the thermal spectrum of a type A hollow AFM probe (see section 4.2.1) for three different cases. It can be seen from this graph that the extra mass of the liquid cargo lowers the resonance frequency of the cantilever, as expected. However,
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The stiffness constant of the probe remains unchanged within the measurement accuracy of the Sader method.

![Thermal noise spectra of type A hollow cantilevers for different filling states](image)

**Figure 5.13:** Thermal noise spectra of type A hollow cantilevers for different filling states: The associated resonance frequency and Q-factors are indicated in the graph. The blue curve was obtained for an empty probe and the red curve for the same probe with cargo liquid filling. The green curve shows the spectra of the same, liquid-filled probe in water. Reprinted with permission from reference [Meister et al., 2009a] Copyright 2009, American Chemical Society (ACS).

This outcome was anticipated, since the effective Mass $M_e$ of a hollow FluidFM probe as defined in equation (3.6) remains almost unchanged when compared to a similar sized bulk material cantilever. Therefore, the filling state of the probe does not fundamentally influence its mechanical properties. It can therefore be concluded that FluidFM applications can be carried out both using empty and filled cantilevers without the risk of obtaining completely different results.

The frequency shift observed whenever the mass of the cantilever changes could even be utilized to create a setup capable of measuring tiny mass differences between particles and liquids. This could be achieved by precisely measuring the probe's frequency response with a lock-in amplifier as the system is subjected to a change in mass.
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5.3.3 Compatibility with AFM-based topography imaging

Since hollow FluidFM cantilevers are essentially still fully functional AFM scanning probes, they can also be used for standard topography acquisition. As shown in the preceding section, the stiffness constant of a hollow cantilever is governed by the same parameters as a bulk probe. In principle, it is possible to use FluidFM probes to acquire satisfactory images when operated in AFM contact mode. However, all currently available FluidFM probes exhibit quite high stiffness constants in the range $> 1 \text{ N/m}$. Such high stiffness constants have been shown to directly influence the applicability of such probes to imaging a broad range of samples. While hard surfaces were easily imaged in contact mode using FluidFM probes, any attempt to image softer samples such as viable cells has failed due to the relatively high interaction forces that occur when using a scanning probe with high spring constant [Meister et al., 2009b].

While there the filling state of a FluidFM hollow cantilever has a clear influence on its resonance properties, as demonstrated above, it was determined that this does not compromise the performance of the probe to a point where AFM imaging is no longer feasible. It was shown that by operating the AFM in dynamic mode (amplitude modulation mode), it is possible to use FluidFM cantilevers to image softer samples as well. In dynamic mode, the effective stiffness constant of the probe is determined by equation (5.4), where $k$ is the absolute spring constant of the probe and $Q$ is the quality factor. The quality factor is also not noticeably impacted by the filling state of the probe, so that a substantial reduction of the effective spring constant can be expected.

$$k_{eff} = \frac{k}{Q} \quad (5.4)$$

Figure 5.14 shows an image of a living fibroblast cell that was acquired using a type A FluidFM cantilever without any aperture, as shown in section 4.2.1. The achievable spatial resolution was determined to be in the range of about 200 nm.

Utilizing FluidFM probes with apertures at or near the apex can have a large influence on AFM imaging fidelity. The left image of figure 5.15 shows a micrograph of a type B probe with a pyramidal top-aperture structure. For microfabrication reasons, the probe exhibits four distinct sub-tips around the opening at the apex. This produces unwanted convolution effects during topography imaging, as explained on the right of figure 5.15. Careful judgement should therefore be exercised while analysing and interpreting AFM images acquired with such probes.
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Figure 5.14: **Left**: AFM topography image of a living fibroblast. The image was acquired with a type A hollow probe in collaboration with CSEM SA (Switzerland) using a commercial AFM from JPK Instruments (Germany). The AFM was operated in dynamic mode (z-range 4 μm). **Right**: Detail scan of the same cell shown on the left (z-range 1.4 μm). Reprinted from reference [Meister et al., 2009b], Copyright 2009, with permission from Elsevier.

Nevertheless, it can be concluded that FluidFM probes can also be used for standard AFM image acquisition in addition to more advanced applications involving their microfluidic properties. The compatibility of FluidFM with AFM-based imaging techniques is an important property of this technology. By taking advantage of the ultra-high spatial resolution offered by the AFM, it is possible to control the position of a FluidFM probe on the sample with high accuracy. This allows the operator to address samples with features below the optical resolution limit. Furthermore, it enables direct in situ high-resolution visualization of the result of a FluidFM experiment. For example, in a lithography study it would be possible to directly image the fabricated structures without needing to change the scanning probe or to use other imaging techniques. This is a major advantage of FluidFM compared to competing techniques such as micropipettes, microfluidic probes or optical tweezers.

### 5.3.4 Liquid loading of FluidFM probes

The correct loading of liquid into the microfluidic system of a FluidFM is crucial to achieving good experimental performance. The loading process should be carried out such as to avoid the formation or trapping of any gas in the channel. Gas bubbles were observed to have potentially damaging influence when positive and negative pressure
is applied. It often led to blocked channels, because the bubbles were repeatedly compressed and expanded in the course of the experiment.

To avoid any clogging of FluidFM probes due to the liquid in the channel, it is also necessary to filter the cargo liquid substance to remove any particles larger than the aperture of the probe. Furthermore, an initial degassing of the cargo liquid using negative differential pressure is advised. Parameters influencing the loading procedure of a FluidFM probe are mainly the same as in any other microfluidic system and are strongly influenced by the following phenomena:

**Surface tension** Attractive forces acting between molecules in a liquid solution are responsible for the formation of an apparent film at the interface between air and liquid: The molecules at the surface of a liquid do not experience the same cohesive forces equally from all sides as they do in bulk regions of the liquid. Instead, they bond more strongly to their surrounding neighbours on the surface. This effect, known as surface tension, makes it more difficult to move an object through the liquid surface than moving it around when fully submerged. Liquids adapt their surface arc until both internal and external forces have reached an equilibrium. Surface tension is typically measured in N/m. The relationship between surface tension, pressure, and surface curvature of a fluid is described by the Young-Laplace equation. The interface is regarded as a surface with zero thickness:

$$\Delta p = \gamma \nabla \cdot \hat{n}$$  

(5.5)

In equation (5.5), $\Delta p \ [N/m^2]$ denotes the pressure difference across the interface, $\gamma$ is the surface tension, and $\hat{n}$ is the unity normal vector pointing out of the interface. For
a sufficiently small tube of circular cross-section, as in the case of the aperture of a FluidFM probe, the interface forms a meniscus that can be approximated as a sphere of radius $R$. In this particular case, equation (5.5) can be express as follows:

$$\Delta p = \frac{2\gamma}{R}$$

(5.6)

As shown by [Doerig, 2013], this translates into a pressure of 9700 hPa that is required to push a droplet of de-ionized water through an aperture with a diameter of 300 nm in a gaseous environment. The ability to eject cargo fluid through the aperture of a FluidFM probe which is not immersed in liquid is thus highly dependent on the actual diameter of the opening.

There are two main options for loading cargo solution into a hollow cantilever:

**Back-loading through reservoir**

FluidFM probes are typically back-loaded by filling the desired cargo substance into the external reservoir of the cantilever-probeholder assembly. Using constant over-pressure of a few hundred mbar, the liquid is forced into the microfluidic system of the probe. The gas inside the hollow channel of the cantilever is thereby pushed out through the aperture at the probe apex. Pressure is maintained until the front of the liquid reaches the aperture. Correct filling of the cantilever can be conveniently observed in real-time via the inverted optical microscope.

Due to the high forces required to overcome the surface tension for smaller apertures, it is not possible to directly eject liquids from apertures with a diameter below 1 μm in ambient air environment. However, when fully submerged in liquid, the surface tension is no longer an issue and cargo liquid can be ejected through nano sized apertures. To assist visibility of successful liquid dispensing via the aperture, the use of a fluorescent tracer substance (see also section 2.3) added to the cargo fluid is strongly advised. As shown in image 7.1, this way any liquid emerging from the hollow FluidFM probe can easily be tracked by means of fluorescence microscopy. Initial filling of the hollow probes should always be carried out under ambient air, as this facilitates the ejection of gas. To avoid formation of salt crystals that often occur when using physiological buffers, it is advisable to keep filled FluidFM probes in liquid whenever possible.
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Front-loading via aperture

Another way to load substances into the microfluidic system of a hollow FluidFM probe is directly via the aperture at the apex. This method is especially useful when working with expensive reagents in order to minimize the volumes used. Front loading is carried out by immersing the aperture of the probe directly into the desired cargo solution. By applying a negative pressure difference at the opening of the probe, cargo liquid is sucked into the microchannel of the cantilever via the aperture. For smaller apertures below 1 µm, this procedure is only feasible when the cantilever is already pre-filled with a suitable dummy solution (e.g., buffer) via the normal back-loading procedure. Due to surface tension it is otherwise virtually impossible to suck any cargo substance into the empty probe through such small apertures. It is also very important that the dummy liquid is completely filled up to the aperture in order not to trap any gas bubbles inside the microfluidic channel of the probe.

The front-loading procedure has proven well-suited for cargo liquids containing micro- or nano-particles. This is especially true when the number of loaded particles needs to be controlled. Thanks to the in situ optical control, it is possible to load individual particles into the FluidFM probe for controlled release at a target location on the sample [Stiefel et al., 2012].

In principle, it would also be possible to use front-loading to load multiple reagent solutions into the same FluidFM probe. One way to avoid intermixing the individual phases is to use silicone oils as a spacer material. This has already been successfully demonstrated for related techniques (see section 6.2.4). The same goal can be achieved using fluorinated inert oils. These substances form a multi-phasic system and hence do not intermix with aqueous solutions used for physiological applications. Loading of multiple substances into the same probes was not investigated within this work and would therefore be an interesting topic for future research on FluidFM technology.

5.3.5 Flow rate analysis and simulation

Using FluidFM probes, it is possible to dispense and manipulate minute amounts of liquid via the well-defined aperture of the hollow cantilever. It is desirable to be able to control the amount of liquid dispensed depending the applied pressure. Since the resulting flow rates were estimated to lie in the fL/s range, there are no direct methods to measure such flow rates with absolute precision. For this reason, only a theoretical and numerical estimation of the pressure dependent flow rate could be established.
5.3. General properties of fluidic force microscopy

The study of liquid flow rate in a hollow FluidFM probe is based on the same assumptions commonly used to study microfluidic systems [Stone et al., 2004]:

1. **Newtonian fluid** The observed stress in the fluid is linearly dependent on the strain via the fluid's viscosity parameter.

2. **Incompressible liquid** The liquid in the microchannel has a constant density, is not compressible, and has homogeneous viscosity.

3. **Boundary conditions** Boundary conditions on the walls of the microchannel are such that no slipping of liquid molecules along the walls occurs.

4. **Continuum assumption** The cargo fluid behaves as a continuous medium where the individual molecules making up the fluid do not have any effect on the behaviour of the liquid as a continuous substance.

**Flow regime estimation**

Whenever a fluid is in motion, two distinct flow regimes can be observed: *Laminar flow* and *turbulent flow*. In the case of laminar flow, no intermixing between adjacent stream-lines occurs and the fluid flows smoothly along the walls of the container. Laminar flow can often be analytically described and simulated using suitable numerical models. Turbulent flow, on the other hand, is a type of flow where the fluid undergoes irregular fluctuations and mixing. The flow is thereby constantly changing both in direction and magnitude. Turbulent flow can typically not be described using analytical means and only a limited set of numerical methods exist to model such system.

In order to determine the flow regime inside a FluidFM probe, it is necessary to estimate the Reynolds number of the microfluidic system in the cantilever. The Reynolds number is defined as the ratio of inertial forces to viscous forces for a given geometry. Laminar flow occurs when the Reynolds number of a specific system (geometry and fluid) lies below a value of 2000 [Rhodes, 1989]. For the case of a closed pipe, the Reynolds number \( Re \) is defined as follows:

\[
Re = \frac{\rho \nu D_h}{\mu} \tag{5.7}
\]

where \( \rho \) is the density, \( \nu \) the mean velocity, and \( \mu \) the dynamic viscosity of the fluid in the pipe. \( D_h \) denotes the hydraulic diameter of the system.
Using this equation, it is possible to calculate the critical liquid flow rates through the cylindrically shaped aperture of a FluidFM probe for which the laminar flow regime will still apply. For the case of an aqueous liquid, the mean fluid velocity for a Reynolds number below 2000 was estimated to be around 100 nL/s through an aperture with a diameter of 100 nm. Since such a high flow has never been observed in any experiment, the flow through the aperture can be assumed to be laminar. For the actual microchannel of the cantilever, the critical flow rate was calculated to be much higher. Therefore the liquid flow in the channel is expected to be laminar as well.

**Flow rate estimation via hydrodynamic resistance**

In a microfluidic system, the liquid flow rate $Q$ is proportional to the applied differential pressure $\Delta P$. The flow rate can therefore be calculated as follows:

$$\Delta P = R_h Q$$  \hspace{1cm} (5.8)

where $R_h$ denotes the *hydrodynamic resistance* of the channel. Similar to Ohm's law for electricity, individual hydrodynamic resistances in a network of channels can be summed if the channels are connected in series. Therefore, the overall hydrodynamic resistance of a FluidFM cantilever can be approximately subdivided into the hydrodynamic resistance of the cantilever channel and aperture.

The hydrodynamic resistance of a channel with a circular cross-section of radius $R$ and length $L$ can be calculated using equation (5.9). For the case of a channel with a rectangular cross-section of width $w$, height $h$, and length $L$, the resistance can be obtained using equation (5.10).

$$R_h = \frac{8\mu L}{\pi R^4}$$  \hspace{1cm} (5.9)

$$R_h = \frac{12\mu L}{wh^3(1 - 0.630h/w)}$$  \hspace{1cm} (5.10)

The typical channel length of a commercial FluidFM type B probe is 1.4 mm with a height of 1000 nm. Assuming a wall thickness of 350 nm, the size of the cylindrical aperture can be used to calculate its hydrodynamic resistance. Assuming a tipless type B cantilever with an aperture size of 2 µm, the flow rate $Q$ can be calculated using equation (5.8). In response to a pressure difference of 20 mbar, a flow rate of 3.6 pL/s is expected. This translates to flow speeds in the range of a few mm/s inside the microfluidic channel of the cantilever.
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Numerical simulation of flow rate

The flow rate in a hollow AFM probe can also be modelled numerically using state of the art multi-physics simulations tools. Such models are typically based on a solution to the governing Navier-Stokes equation along with the corresponding boundary conditions. The geometry of the probe can thereby be precisely modelled by means of finite element analysis.

Detailed simulations of different probe types have been carried out in [Doerig, 2013]. From these simulations it can be concluded that the analytical approximation from the preceding section provides a good estimation of the actual flow rates in a real FluidFM probe. Furthermore, the tip structure of a pyramidal top aperture probe adds little to the overall hydrodynamic resistance of the device. The hydrodynamic resistance of the pyramid itself would only become a dominant parameter for aperture sizes below 200 nm. For apertures above 500 nm in diameter, however, the hydrodynamic resistance of the channel is the limiting factor for the overall flow rate in the probe.

5.3.6 Effect of pressure application on the z-feedback

One important aspect of FluidFM is how the application of pressure (or vacuum) influences the mechanical response of the system. For this reason, several experiments using type B probes were carried out. It could be observed that with respect to mechanical deformations of the cantilever, there is a reaction that can be measured whenever the applied pressure is changed. However, the measured, absolute movements were quite small. Nevertheless, since AFM strongly responds to even the slightest changes in force, it was investigated how the system handles variations in pressure when in contact with a substrate.

For this purpose, a series of different pressure values ranging from 0 mbar to 1000 mbar and back with steps of $\Delta p = 100$ mbar occurring every 3 s was applied to a filled FluidFM probe mounted on top of a commercial FluidFM system. The cantilever was approached to a hard surface and held at a constant force set-point of 22.9 mV during the entire experiment. Figure 5.16 illustrates how the system reacted to such changes. On the upper graph, the deflection signal and the applied differential pressure values are plotted against the same time axis. On the lower graph, the corresponding deflection signal measured by the AFM’s OBD system is again plotted on the same reference time axis. It can be seen that the deflection signal of the probe changes in response to the variations in pressure, as expected. However, thanks to the fast feedback control of the AFM, the resulting net force acting on the substrate is quickly adjusted using the $z$-piezo of the microscope. While the piezo control signal is always modified whenever
the applied pressure changes, the net deflection is quickly readjusted to meet the employed constant force setpoint.

From this experiment, it can be concluded that even though the hollow cantilever is influenced by small changes in pressure, the overall system can suppress this effect in order to ensure constant interaction forces with the specimen at all times during an experiment. This property of fluidic force microscopy is truly unique to this technique and can only be indirectly achieved when using alternative methods such as glass micropipettes.

### 5.3.7 Versatile integration platform

As explained in section 5.2.6, a typical fluidic force microscopy setup requires integration and synchronization of many different instruments and techniques, such as optical microscopy, positioning devices, and pressure controlling methods. Many of the interesting capabilities of this technology arise from the sophisticated unification of all these components.

However, FluidFM is not limited to the components explained so far. As indicated, the technology can be readily combined with advanced methods such as confocal laser scanning microscopy, ionic current measurement devices, and more. FluidFM has the potential to function as a true integration platform for further technology developments. It is expected that in future research projects FluidFM will be integrated and combined with other downstream techniques like mass-spectroscopy to enable advanced applications such as single cell metabolomics. Furthermore, FluidFM has the potential to miniaturize many standard assays used in biological research in order to significantly reduce the necessary reagent volumes while at the same time increasing their spatial and temporal resolution.

### 5.4 Chapter summary and conclusion

A functional FluidFM prototype was successfully created. The major building-blocks of the setup were explained and further enhancements of these components were elucidated where applicable. The most important properties and modes of operation of FluidFM were introduced and will serve as a reference for subsequent chapters. It was concluded that FluidFM is a universal and versatile liquid delivery system with force control and high spatial resolution. FluidFM can be used in both dry and liquid environments. Using this technique, it is possible to systematically utilize atomic force microscopy to manipulate micro-objects and access the inside of biological
entities (e.g., single eukaryotic cells, yeast, or even bacteria). Standard AFM-based techniques are typically restricted to mainly surface centered applications. This limitation of traditional scanning probe microscopy can be overcome with FluidFM. The novel technology therefore enables a whole new palette of applications that would be cumbersome to achieve or even impossible using existing methods and devices, as will be shown in the next chapter.
State of the art - From standard glass micropipettes to SPM

In order to assess how FluidFM compares with competing methods, the following chapter aims to give an overview of current state of the art techniques. As it transpires, devices with similar features and functionalities are not limited to the broad family of SPM derived techniques. For this reason, the following review also includes instruments and methods that are based on completely different technological concepts. However, what they all have in common is that they combine some type of scanning functionality with specific microfluidic features.

To provide a better overview, all the methods presented are divided into the following two categories. The same classification is also illustrated in figure 6.1 for better clarity:

**Figure 6.1:** Classification of state of the art exhibiting scanning functionality combined with microfluidic features. **Left:** Devices based on physical interaction principles. **Right:** Devices based on chemical interactions with a sample.

Techniques for microfluidic based physical interactions and manipulations
This category encompasses devices that employ microfluidic functionality primarily for physically interacting with a substrate. As shown on the left in figure 6.1, this group of instruments includes techniques for mechanical characterization, force measurements, and other kinds of direct physical manipulations and analysis of substrates. It also includes instruments capable of lithographically modifying samples for micro- and nano-fabrication applications.

**Techniques for highly localized micro(electro)chemical interactions**

The second category contains techniques focusing on localized micro(electro)chemical interactions with the substrate. The right picture in figure 6.1 illustrates the most important aspects of this group of devices. This category includes devices for microchemical and microelectrochemical interactions. Systems where compartmentalization is achieved via multiphase techniques and hydrodynamic confinement are also attributed to this second class of instruments. While many of the presented systems are in principle capable of carrying out physical interactions as well, the focus of those technologies is clearly on their capabilities for localized chemical interactions with the substrate.

This categorization has been chosen because the principal features of the presented techniques are focused on one or the other of these two categories. FluidFM, in comparison, seems to cover both application areas equally well. It can be employed both as a versatile micromanipulation and lithography tool, as well as for highly localized chemical and electrochemical stimulation (see also chapters 7, 8, and 9). However, it must be remembered that this division into two categories is not at all exclusive. The categorization is not always clear, as many of the presented techniques can be extended or integrated within other methods, and some of them are already a combination of different techniques. Nevertheless, this categorization seems reasonable, as it also coincides well with the historical development of FluidFM technology, wherein the techniques have matured to cover both categories since its initial introduction.

The following sections present the basic principles and applications areas of the most important competing technologies. All techniques will be benchmarked against FluidFM to better estimate the impact of this thesis on these various areas of research.

Last but not least, the concept of *open space microfluidics* as introduced by [Kaigala et al., 2012] is a different attempt to classify this broad selection of similar techniques and devices. The idea behind this approach is presented in section 6.3. It will also be explained how FluidFM is positioned within this emerging category of devices.
6.1 Devices based on physical interactions with the substrate

6.1.1 Atomic force microscope (AFM)

Among the wealth of scanning probe techniques, AFM is the most prominent. Especially in the field of life sciences, the technology has seen tremendous developments since its original inception in the late 1980s, as described by [Binnig and Quate, 1986]. While originally limited only to topographical imaging with high spatial resolution, the technology has since found many new application areas thanks to a wealth of novel probe types and operating modes that have been created over the last three decades. These developments also enabled novel approaches towards the study of single biological subjects such as eukaryotic cells, bacteria, viruses, and biomolecules. While the technique in its original conception does not explicitly include any microfluidic functionality, it is nevertheless of vital importance to FluidFM, as both one of the cornerstones of the technology and a major competitor in its own right. For this reason, it is also included in this review. The most important application areas within life sciences where AFM competes with FluidFM will be explained in the following paragraphs.

A comprehensive description of the basic principles and theory underlying AFM can be found in chapter 3.

Characterization of mechanical properties using AFM

When an AFM cantilever is scanned over the surface of a cell, it is possible to acquire a high resolution image of the membrane in order to investigate its topography and structure [Baro and Reifenberger, 2012]. However, thanks to the sensitive force detection, it is also possible to directly study mechanical properties of interest: By indenting the cantilever into the cellular membrane and thus deforming it, important mechanical properties of the cell and its membrane can be revealed [Radmacher, 1997]. Mechanical probing is therefore not limited to single cells, but has recently been extended to complete tissue sections [Loparic et al., 2010]. Recent studies even suggest that the mechanical properties of cells as determined by AFM can be used as a characteristic marker to distinguish cancerous cells from healthy tissue with high confidence [Plodinec et al., 2012]. Such applications could be used for diagnostic purposes, with AFM based mechanical investigation of patient samples routinely used in hospitals to reliably diagnose cancer or other diseases.
Pro Contra

only standard AFM required  
cheap consumables  
proven track record  
strong tip shape dependency  
limited pulling force range  
cantilever functionalization

Table 6.1: *AFM for mechanical property testing: Assessment of benefits and drawbacks.*

It is evident that these applications are not dependent on any microfluidic functionality. FluidFM is therefore not expected to provide major advantages for these types of applications. The only area wherein FluidFM provides an advantage over standard AFM is for studies employing ideal sensing probes based on colloidal tip structures, as will be explained in the next paragraph. Table 6.1 provides an overview of the main advantages and drawbacks of this technique.

**Colloidal probe AFM**

In order to have a well-defined and known contact area during mechanical probing of substrates, it is desirable to use AFM probes with a sphere attached at the end of the cantilever instead of a sharp pyramidal tip [Ducker et al., 1991, Butt, 1991]. This technique, known as colloidal probe AFM, has found use in biophysical research, for example to probe mechanical properties of individual bacteria [Vadillo-Rodriguez et al., 2008] and to study various adhesion forces [Kappl and Butt, 2002]. Fabrication of colloidal probes is very cumbersome, because suitable micro-spheres have to be glued to the cantilever in series. One example of such a probe is depicted in figure 6.2. There are commercial, ready-to-use colloidal probes available on the market; however, they are typically expensive. Table 6.2 summarizes the most important advantages and drawbacks of this technique.

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<tbody>
<tr>
<td>only standard AFM required</td>
<td>limited pulling force range</td>
</tr>
<tr>
<td>proven track record</td>
<td>expensive consumable</td>
</tr>
<tr>
<td>defined tip shape</td>
<td>cantilever functionalization</td>
</tr>
<tr>
<td></td>
<td>only fresh probe for first measurement</td>
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</tbody>
</table>

Table 6.2: *Colloidal probe AFM: Assessment of benefits and drawbacks.*

Unlike colloidal probe AFM, FluidFM allows a micro-sphere to be quickly affixed to the free end of the cantilever by means of an underpressure in the probe's microfluidic channel. In this way, it is possible to essentially exchange the colloidal probe for every measurement in order to have a fresh sensing tip. It has been shown by [Dorig
6.1. Devices based on physical interactions with the substrate

**Figure 6.2:** Example of a colloidal AFM probe. A micro-bead is glued to the free end of an AFM cantilever. Image reproduced from reference [Kappl and Butt, 2002], Copyright 2002, with permission from John Wiley and Sons.

...et al., 2013] that the primary contact is crucial when measuring interaction forces with colloidal AFM. It is thus expected that the novel possibilities enabled by FluidFM could further push the adoption of this method.

**AFM-based single cell injection**

When an AFM probe is approached to the surface of a cell using higher forces, it is possible to rupture the membrane and gain access to the interior of the cell. AFM can therefore also be used as a tool to deliver molecules directly into inner compartments of a cell with force control. The first implementation of such a system used specially coated cantilevers to deliver specific molecules into the cytosol, where they would be released by means of a chemical process [Nishida et al., 2002], as shown in figure 6.3.

The technique has since been further refined by attaching a carbon nanotube (CNT) directly to the apex of the AFM probe to form a nano needle injector[Chen et al., 2007]. The desired molecular cargo can be chemically grafted to the CNT tip and released once inside the cell. While this nanoneedle approach clearly minimizes any unwanted perturbations of the cell during the injection procedure, the chemical release process is still purely stochastic and therefore hard to reproducibly control. Furthermore, the amount of cargo is limited to a only a few molecules and considerable chemical modification of the CNT nanoneedle is needed. This also severely limits the applicability of the technology, because after each injection the nanoneedle needs to be reloaded...
Chapter 6. State of the art - From micro-pipettes to SPM

with fresh cargo molecules. Table 6.3 summarizes the advantages and drawbacks of this approach.

<table>
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<tr>
<td>only standard AFM required</td>
<td>no serial delivery</td>
</tr>
<tr>
<td>minimally invasive (CNT)</td>
<td>complex consumable (CNT)</td>
</tr>
<tr>
<td>force feedback</td>
<td>cantilever functionalization</td>
</tr>
<tr>
<td></td>
<td>stochastic process</td>
</tr>
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</table>

Table 6.3: AFM based single cell injection: Assessment of benefits and drawbacks.

In order to have a truly versatile AFM-based injection instrument, it is highly desirable to be able to inject not only individual molecules but any kind of soluble substances that might be of interest. Using standard AFM probes, it is not possible to directly control the delivery of fluids into single cells. In order to achieve this, a microfluidic system needs to be integrated into the probes, as is the case with FluidFM technology. This way, it is also possible to reuse the same probe to inject multiple cells with the same solution without having to change or reload the probe for each injection experiment. Thanks to the force feedback of the underlying AFM, FluidFM-based injection experiments can be carried out with minimal distress of the target cell. The development of a FluidFM-based single cell injection procedure is one of the major topics of this work. A detailed report on this topic is provided in chapter 8.

Figure 6.3: Illustration of an AFM-based single cell injection system. A functionalized AFM probe is pushed into the cytosol of the cell where it is held in place until the cargo molecules desorb from the probe surface. Image reproduced from reference [Nishida et al., 2002], Copyright 2002, with permission from Elsevier.
6.1. Devices based on physical interactions with the substrate

Single cell force spectroscopy using AFM

Using AFM, it is also possible to analyse how strongly an object adheres to a surface. It has been shown that cellular adhesion processes vital to the proliferation and viability of many different micro-organisms and eukaryotic cells [Thiery, 2003]. The ability to characterize adhesion of cells to different surfaces such as medical implants, surgical instruments, and other hygiene-sensitive objects is essential in order to further improve their performance [Stevens and George, 2005].

Single cell force spectroscopy (SCFS) is typically carried out by attaching a single cell to an AFM cantilever and subsequently pulling it away from the surface while simultaneously recording the resulting cantilever deflections. In this way, it is possible to both quantitatively and qualitatively assess the adhesion of the cell to any surface of interest down to single molecular events [Helenius et al., 2008]. Figure 6.4 shows the different steps of a typical AFM-based SCFS experiment.

Figure 6.4: Illustration of a typical AFM-based single cell force spectroscopy experiment. A: Target cell identification. B: The target cell is engaged and the cantilever is held in contact for several seconds until the cell binds to the probe surface. C: Detachment of the cell from the surface while recording the corresponding cantilever deflection. D: Phase contrast image of a cell attached to an AFM cantilever. Image reproduced from reference [Helenius et al., 2008], Copyright 2008, with permission from Company of Biologists Ltd.
Chapter 6. State of the art - From micro-pipettes to SPM

The method requires elaborate chemical functionalization of cantilevers in order to form a sufficiently strong bond between the probe and the cell. As the maximum achievable bonding forces are quite low < 100 nN [Friedrichs et al., 2013], it is often only possible to apply this method to suspended cells that are then brought into contact with a surface. Furthermore, it is only possible to apply short interaction times (< 20 min) between the cell and the surface. The technique also suffers from low throughput (1-10 cells/day), because a new cantilever cell assembly needs to be prepared for each datapoint, and the necessary functionalization steps can be quite time consuming. Table 6.4 summarizes the most important features of this technique.

<table>
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<th>Pro</th>
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<tr>
<td>only standard AFM required</td>
<td>limited pulling force range</td>
</tr>
<tr>
<td>established method</td>
<td>slow, serial procedure</td>
</tr>
<tr>
<td>quantitative method</td>
<td>expensive to get good statistics</td>
</tr>
<tr>
<td>single cell resolution</td>
<td>cantilever functionalization needed</td>
</tr>
<tr>
<td></td>
<td>only limited interaction times possible</td>
</tr>
</tbody>
</table>

Table 6.4: AFM-based single cell force spectroscopy: Assessment of benefits and drawbacks.

FluidFM promises to significantly increase the throughput of this technique, while at the same time allowing it to probe much longer interaction times between the cell and the surface. By using the microfluidic system as a kind of suction cup, it is possible to attach a cell to the cantilever reversibly within a short timeframe. Thanks to the significantly higher bonding forces at the cantilever-cell interface (> 1 \(\mu\)N), it is possible to detach almost any fully spread adherent cells from the underlying surface. Using a simple washing procedure, it is then possible to reuse the same cantilever for multiple cells. In this way, it is typically possible to measure up to 10 adherent cells per hour for the case of eukaryotic cells. For other micro-organisms such as yeast (Saccharomyces cerevisiae), the maximum achievable throughput was reported to be considerably higher, at up to 200 individual experiments per day [Potthoff et al., 2012].

Micro- and Nano-tribology

AFM can also be used to investigate nano-scale adhesion and friction forces of biomolecules on different surfaces by measuring the lateral deflection of the cantilever [Bhushan et al., 2006, Bhushan, 2007a]. These studies can be exploited to design surfaces and interfaces to which micro-organisms such as bacteria and yeast cannot adhere. It has been shown that surface properties play a particularly crucial role in the formation of bio-films. A method to investigate the corresponding
properties is therefore highly beneficial to further extend our knowledge of the underlying mechanisms of bio-film formation and cellular adhesion on different kinds of substrates.

Similar results can be obtained using FluidFM. The main advantage compared to standard AFM is the possibility to exchange the sensing element as in the case of colloidal probes. Thus far, no systematic nano-tribology experiments have been conducted with FluidFM. However, it is believed to offer interesting opportunities for a future research project wherein different kinds of sensing caps can be attached to a cantilever by means of underpressure in the microfluidic channel. Such an exchangeable nano-toolbox is also expected to find applications in other areas such as failure analysis in integrated microelectronic circuits.

6.1.2 Dip-pen nano-lithography (DPN)

Dip-pen nano lithography is an advancement of standard AFM that can be used to deposit soluble substances on a surface with high spatial resolution [Jaschke and Butt, 1995, Piner, 1999]. The basic implementation of DPN does not rely on specially modified cantilevers. In the case of DPN, the liquid to be deposited (ink) is coated onto the pyramid of a standard AFM probe by dipping it into a reservoir. The coated tip is then brought into contact with the target surface to be patterned and the ink is transferred via a liquid meniscus onto the surface. DPN can be seen as the nano-sized equivalent of a quill pen. The basic working principle of DPN is illustrated in figure 6.5.

The minimal feature size that can be attained using DPN technology is dependent on many different factors, such as vapor pressure in the deposition chamber, surface chemistry, and tip shape. Nevertheless, feature sizes in the range of 15 nm have been readily achieved using this method [Zhong et al., 2014]. Table 6.5 assesses the most important features of dip-pen nanolithography.

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<tbody>
<tr>
<td>only standard AFM required</td>
<td>environmental control needed</td>
</tr>
<tr>
<td>established method</td>
<td>serial procedure</td>
</tr>
<tr>
<td>nanometer resolution</td>
<td>limited ink choice</td>
</tr>
<tr>
<td>good reproducibility</td>
<td>mostly limited to dry environment</td>
</tr>
<tr>
<td></td>
<td>capillary driven</td>
</tr>
</tbody>
</table>

Table 6.5: Dip-pen nanolithography (DPN): Assessment of benefits and drawbacks.

DPN is currently used for many applications, ranging from simple surface patterning
Chapter 6. State of the art - From micro-pipettes to SPM

Figure 6.5: Illustration of basic DPN working principle. The transfer of substance to a surface is achieved via a water meniscus at the apex of an AFM probe. The pattern of the transferred substance is controlled by scanning the probe over the surface. Image reproduced from reference [Piner, 1999], Copyright 1999, with permission from The American Association for the Advancement of Science (AAAS).

of biomolecules [Wu et al., 2011] to the fabrication of completely integrated protein chips [Lee et al., 2006]. DPN also has interesting applications outside the field of life sciences. Due to the mask-less nature of the lithography process, the technique is often used for rapid prototyping of nanostructures during the development of novel integrated microelectronic circuits and MEMS devices [Ginger et al., 2004].

The main disadvantage of dip-pen nanolithography compared to FluidFM is that, except for the deposition of lipids [Lenhert et al., 2010], it cannot usually be operated within a liquid phase environment. Furthermore, there is only a limited ink reservoir available on the tip, so regular reloading of the dip pen structure is necessary to be able to carry out prolonged writing experiments. Commercially available systems have partly mitigated these issues by providing arrays of multiple cantilevers for parallel operation of DPN within a fully controlled climate chamber.

6.1.3 Nanodispensing (NADIS)

NADIS technology is another approach to nanolithographic patterning of surfaces using AFM techniques [Meister et al., 2003, 2004]. Instead of building on a working principle similar to that of a quill pen, NADIS deposits liquids through a well-defined nano-sized aperture located at the apex of a specifically modified AFM probe. As shown in image 6.6, the liquid can be loaded on top of the cantilever into the pyramid groove, essentially providing a liquid reservoir for the writing ink. The closest macroscopic analogue to this method of transferring material is screen printing.
6.1. Devices based on physical interactions with the substrate

![Illustration of the working principle behind nanodispensing (NADIS). Substances are transferred to a surface via a small aperture at the apex of an AFM cantilever. The cantilever is thereby actuated with force control. Image reproduced from reference [Meister et al., 2003], Copyright 2003, with permission from Elsevier.](image)

The main advantage of NADIS over DPN is the much larger stock of writing ink available. However, as the reservoir is still open to the environment, it is necessary to add glycerol to the writing liquid to prevent premature evaporation from both the substrate and reservoir. NADIS probes have been used to deposit liquids with feature sizes well below 100 nm. In contrast to DPN, it is also possible to deposit particles such as polystyrene beads with NADIS [Meister et al., 2004]. A major drawback of NADIS probes is that they rely on the usage of serial techniques such as focused ion beam milling in order to create the aperture at the apex of the probe. Table 6.6 summarizes these findings.

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<tr>
<td>force control</td>
<td>environmental control needed</td>
</tr>
<tr>
<td>nanometer resolution</td>
<td>expensive cantilevers</td>
</tr>
<tr>
<td>good reproducibility</td>
<td>limited ink choice</td>
</tr>
<tr>
<td></td>
<td>limited to dry environment</td>
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<tr>
<td></td>
<td>capillary driven</td>
</tr>
<tr>
<td></td>
<td>only with glycerol</td>
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Table 6.6: Nanodispensing (NADIS): Assessment of benefits and drawbacks.

NADIS probes can be seen as a precursor to the first FluidFM probes: By extending the microfabrication process of NADIS probes it is possible to create a closed microfluidic structure extending along the complete cantilever, connecting the aperture with an external reservoir. The fabrication of such probes, referred to as type A FluidFM cantilevers, is explained in detail in section 4.2.1. FluidFM has many advantages over conventional NADIS, such as the ability to work under liquid phase and without the addition of glycerol.
6.1.4 Fountain pen probe (FPP)

Another interesting device that can be used for liquid deposition is a fountain pen (FPP) probe [Deladi et al., 2004]. FPPs are essentially microchannelled AFM probes; however, the aperture of the microfluidic channel is not located at the apex of the pyramidal tip but at its base, as shown in figure 6.7.

![Figure 6.7: Schematic of a fountain pen probe (FPP). The device is essentially a hollow cantilever with a reservoir in the back of the handling chip. The aperture of the microchannel is located at the base of the pyramidal tip. Image reproduced from reference [Deladi et al., 2004], Copyright 2004, with permission from AIP Publishing LLC.](image)

Fountain pen probes can also be seen as an evolution of dip-pen lithography, with a continuous feeding of fresh ink to the writing pyramid of the probe. Thanks to the closed nature of the channel, the problem of evaporation of the ink stock is no longer an issue. FPPs have been shown to work with inks that form a self-assembled mono-layer when deposited on a gold surface. It is also possible to employ the device as a tool for highly localized chemical-mechanical surface etching. Sub-micrometer resolutions have been reported in literature for both these applications [Deladi et al., 2004]. A list of the major drawbacks and advantages of the FPP technique is given in table 6.7.

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<td>force control</td>
<td>only works in air</td>
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<tr>
<td>sub-micron resolution</td>
<td>capillary driven</td>
</tr>
<tr>
<td>good reproducibility</td>
<td></td>
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<tr>
<td>continuous ink feeding</td>
<td></td>
</tr>
<tr>
<td>reservoir located in handling chip</td>
<td></td>
</tr>
<tr>
<td>batch fabrication is possible</td>
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Table 6.7: Fountain pen (FPP) probe: Assessment of benefits and drawbacks.

Fountain pen probes are a direct precursor to type B FluidFM probes, as described in section 4.2.2. Commercial FluidFM cantilevers are produced with a similar fabrication process. Compared to FluidFM, FPPs suffer from similar drawbacks as NADIS probes and are typically limited to operation in ambient air environments. However, the
6.1. Devices based on physical interactions with the substrate

relocation of the ink reservoir away from the cantilever is acknowledged to improve the dynamic behaviour of such cantilevers, because the overall mass is only slightly changed, unlike in NADIS probes.

6.1.5 Nanofountain Probe (NFP)/Volcano Tip

Another type of microchannelled cantilever is the nanofountain probe (NFP) [Kim et al., 2003, 2005]. It is also commonly referred to as a volcano tip. Similar to FPPs, NFPs feature a completely enclosed microfluidic channel inside the cantilever connected to a reservoir located in the handling chip of the probe. The tip structure is quite different, however: A core tip is encapsulated by an outer shell structure as shown in figure 6.8. Depending on whether the core tip protrudes above the outer shell or not, the probe can be used for either DPN-like applications or as a tool for force-controlled interaction with micro-objects such as cells.

Figure 6.8: Overview of nanofountain probe technology (NFP). A: Detailed schematic view of the tip structure of an NFP. B: Schematic illustration of a complete NFP probe. C: Micrograph of a typical NFP tip structure. D: Scanning electron microscopy image of an NFP cantilever attached to a handling chip with integrated reservoir. Image reproduced from reference [Kim et al., 2005], Copyright 2005, with permission from John Wiley and Sons.

NFPs have been successfully used for DPN applications on different surfaces [Salaita et al., 2007]. The technology is also capable of delivering particles such as nanodiamonds into cells [Loh et al., 2009]. However, the actual injection step can only be demonstrated in ambient air environments: In order for this to work, the media
surrounding the target cells had to be briefly removed, resulting in additional perturbations as physiological conditions were temporarily suspended. Recent studies have shown that NFPs can also be employed as a device for local electroporation of individual cells: By integrating an electrode into the cantilever's liquid channel, it was possible to create pores in the cell membrane that could be used to deliver cargo substances across the cellular membrane [Kang et al., 2013]. However, this application was only carried out using a micromanipulator and hence without force control.

Table 6.8 lists the major positive and negative characteristics of NFP/volcano tip technology.

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<td>force control</td>
<td>only works in air</td>
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<tr>
<td>sub-micron resolution</td>
<td>capillary driven</td>
</tr>
<tr>
<td>good reproducibility</td>
<td>complex microfabrication</td>
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<tr>
<td>continuous ink feeding</td>
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<tr>
<td>reservoir located in handling chip</td>
<td></td>
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<tr>
<td>batch fabrication is possible</td>
<td></td>
</tr>
<tr>
<td>compatible with electrode integration</td>
<td></td>
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</table>

Table 6.8: Nanofountain probe (NFP) technology: Assessment of benefits and drawbacks.

Unlike FluidFM, nanofountain probes have already been successfully manufactured in arrays of multiple probes [Kang et al., 2013]. However, there is not yet a system that allows a true parallel operation with force feedback control for each individual cantilever. Such systems are only actuated by means of a standard micromanipulator under optical control via an inverted microscope.

Compared to NFP technology, FluidFM has the advantage that it is also fully capable of being operated when completely immersed in liquid. This is especially important when working with viable cells where it is critical to use minimally invasive techniques whenever possible.

6.1.6 BioProbe cantilever

Another type of microchannelled AFM probe known as BioProbe [Kato et al., 2010] has recently been reported. The BioProbe is fabricated by bonding a Si wafer with an embedded nanoneedle made of silicon oxide onto a prefabricated silicon-on-insulator (SOI) wafer encompassing a microfluidic channel. Upon successful bonding, the silicon oxide nanoneedle remains as a high aspect ratio structure at the free end.
of the hollow cantilever. This is achieved by selectively removing any surplus Si from the original carrier wafers. However, the fabricated nanoneedle is still completely closed at this point. The aperture at the needle apex must therefore be formed using a suitable method such as focused ion beam milling. Due to the serial nature of this process, economic mass fabrication of this type of device is expected to be difficult.

Figure 6.9 depicts the typical fabrication process of BioProbes.

BioProbes have successfully been used for single cell injection experiments [Shibata et al., 2013]. The delivery of the substance through the probe was not controlled via pressure, but rather using electrokinetic methods such as electroosmotic pumping (see section 5.2.3). The injection procedure was carried out under force-control by means of a standard AFM. Figure 6.10 shows a scanning electron microscopy micrograph of a typical BioProbe.

Table 6.9 provides an overview of the most important advantages and drawbacks associated with the BioProbe technology.

With the BioProbe, a setup very similar to a typical FluidFM system can be created. While the BioProbe exhibits many congruent properties when compared to FluidFM cantilevers, there are still some apparent differences: One impressive feature of BioProbes is the high aspect ratio of the tip structure. This enables the device to engage structures located in grooves or similar topographical cavities. However, a major drawback of BioProbes is the relatively high stiffness constants in the range between 50 N/m and 170 N/m [Kato et al., 2010]. This is clearly a limiting factor, because the
Figure 6.10: Scanning electron microscopy image of a typical BioProbe. **Left:** Overview image of the complete device. The handling chip including the liquid access ports are shown. **Right:** Close-up view of the high aspect ratio nanoneedle structure of a BioProbe. Image reproduced from reference [Shibata et al., 2013], Copyright 2013, with permission from Elsevier.

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<tr>
<td>force control</td>
<td>expensive aperture fabrication via FIB</td>
</tr>
<tr>
<td>continuous ink feeding</td>
<td>limited fabrication reproducibility</td>
</tr>
<tr>
<td>reservoir located in handling chip</td>
<td>complex microfabrication</td>
</tr>
<tr>
<td>high aspect ratio tip</td>
<td>high cantilever stiffness</td>
</tr>
<tr>
<td>compatible with electrode integration</td>
<td>poorly defined aperture edge</td>
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Table 6.9: BioProbe cantilever technology: Assessment of benefits and drawbacks.

_probe might not be able to engage gently enough with soft and fragile samples such as cells. It is hypothesized that this is why there was only limited change observed in the force-distance diagram recorded during an injection experiment, as reported in [Shibata et al., 2013]. This makes it difficult to determine the exact moment when the probe has reached the cytosol of the target cell. Furthermore, the edge of the aperture is typically poorly defined.

### 6.1.7 Femtopipette/hollow AFM cantilever pipette

Last but not least, there is on final type of microchannelled AFM cantilever probe. This family of devices is known as femto- [Garza et al., 2014] or cantilever pipettes
6.1. Devices based on physical interactions with the substrate

[Ghatkesar et al., 2014] and was first introduced in combination with novel approaches for controlling the fluid flow rate [Heuck et al., 2008, Hug et al., 2005b]. Cantilever pipettes are based on the same fabrication process used for type A hollow FluidFM probes, as explained in section 4.2.1. In this case, however, the focus was clearly on extending the versatility of the probes, by integrating further functional components directly into the device during microfabrication. One prominent example of such an extension is shown in figure 6.11, where a functional MEMS pumping system based on evaporation of liquid has been integrated [Heuck et al., 2008].

![Figure 6.11: A: Schematic overview of a hollow cantilever pipette probe with integrated evaporation pump. B: Fabrication process of hollow cantilever pipette probes. This is based on the same process used to produce type A FluidFM probes. Image reproduced from reference [Heuck et al., 2008], Copyright 2008, with permission from Elsevier.](image)

Cantilever (femto-)pipettes can be used for various applications. For example, they can be used to controllably deposit and synthesize gold nanoparticles [Garza et al., 2014]. Figure 6.12 depicts an example femtopipette probe used for such experiments. Furthermore, the probes have been proven to enable true pipetting operation, wherein the device can also be used to sample liquid from a surface. This has been achieved using a syringe pumping system [Ghatkesar et al., 2014]. It should be noted that these experiments have so far only been conducted in an ambient air environment.

For quick reference, table 6.10 assesses the most important advantages and drawbacks of this particular type of microchannelled cantilever technology.

Since cantilever pipette probes are essentially based on the same technology as type A FluidFM probes, they exhibit mostly the same properties. Nevertheless, FluidFM has already been shown to also allow similar applications in a liquid environment and under constant force-control from the underlying AFM. Furthermore, it should be highlighted that current state of the art FluidFM probes (type B probes, see section 4.2.2) rely on a different microfabrication approach, where it is no longer necessary to
employ expensive serial techniques for the definition of a suitable aperture at the apex of the cantilever. However, it is interesting to see how cantilever pipette probes have been extended with extra functionality such as MEMS pumping systems. It would be interesting to have such functionality integrated into current type B FluidFM probes to further extend their versatility.

6.1.8 Glass micro- and nanopipettes

Another major family of devices offering similar functionality to FluidFM for physical interactions with a substrate is based on pulled glass micro- or nanopipettes. In contrast to all competing techniques introduced so far, glass pipettes are not produced by means of microfabrication processes borrowed from MEMS technology. Glass micropipettes have been around for more than a century [Barber, 1904, 1914], and are therefore well-established in the biomedical research community. The original invention of the technique is attributed to Marshall A. Barber [Terreros and Grantham, 1982]. His original instrument paved the way for a multitude of applications in modern life sciences research.

![Figure 6.12: Scanning electron microscopy image of a femtopipette probe. A: Schematic overview of the device. B: Overview micrograph of the complete device including handling chip. The fluid reservoir is directly integrated. C: Close-up micrograph of a cantilever cross section and pyramidal aperture. Image reproduced from reference [Garza et al., 2014], Copyright 2014, with permission from IEEE.](image-url)
6.1. Devices based on physical interactions with the substrate

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<tr>
<td>continuous ink feeding</td>
<td>complex microfabrication</td>
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<tr>
<td>reservoir located in handling chip</td>
<td>aspiration only demonstrated in air</td>
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<tr>
<td>compatible with integrated MEMS pumps</td>
<td></td>
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<tr>
<td>compatible with electrode integration</td>
<td></td>
</tr>
<tr>
<td>deposition and aspiration</td>
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Table 6.10: Cantilever (femto-)pipette technology: Assessment of benefits and drawbacks.

State of the art micropipettes are prepared using a macroscopic pipette blank made from borosilicate\(^1\) glass with a suitable diameter and wall thickness. From this, a micropipette can be fabricated by heating and simultaneously pulling on one side of the pipette blank until the desired aperture size is obtained. By means of fire polishing or similar methods, the aperture of a pulled micropipette can be further optimized for the specific application at hand. With this method, it is possible to reproducibly obtain apertures with diameters down to several hundreds of nanometers. While it is in principle possible to also obtain smaller openings this way, it is very difficult to reproducibly control the formation of such apertures. Nanopipettes therefore need to be inspected and characterized after fabricating them via a scanning electron microscope in order to determine their exact dimensions. Figure 6.13 shows an example of a typical glass micropipette attached to a bacterial spheroblast\(^2\).

Micropipettes are typically used in conjunction with an inverted optical microscope. This is also the only way of accurately controlling their position during an experiment if they are used without any electrodes inserted at the back. Spatial actuation of the pipettes is carried out by means of micromanipulators. Depending on the required positional accuracy, they can be based on piezo-resistive elements or precision motors such as encoded stepper motors or DC motors. The optical microscope needs to be isolated from any unwanted mechanical vibration to ensure minimal perturbation during the experiment. In order to control the liquid flow rate inside the pipette, a suitable pressure generation device or volumetric pumping system, such as a syringe pump, can be employed. Table 6.11 lists the most important benefits and drawbacks of general glass micropipette technology.

Since FluidFM cantilevers are essentially a special kind of micro-pipette, their applicability is very similar. However, thanks to the force feedback capabilities of the

\(^1\)Borosilicate glass is a type of glass that mainly contains silica and boron trioxide. It exhibits low thermal expansion. It is therefore also much more resistant to thermal shocks than other glasses.

\(^2\)A spheroblast is a cell from which the cell wall has been almost completely removed.
Chapter 6. State of the art - From micro-pipettes to SPM

Figure 6.13: Example of a pulled glass micropipette. The small object attached to the pipette aperture is a bacterial spheroblast. Image by Vlad75 at en.wikipedia (Transferred from en.wikipedia) [Public domain], from Wikimedia Commons.

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<td>no force control</td>
</tr>
<tr>
<td>gold standard for many applications</td>
<td>mechanical stability</td>
</tr>
<tr>
<td>compatible with electrode integration</td>
<td>risk of cell damage</td>
</tr>
<tr>
<td>deposition and aspiration</td>
<td>difficult to operate</td>
</tr>
<tr>
<td></td>
<td>limited automation</td>
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</table>

Table 6.11: Basic pulled glass micropipette technology: Assessment of benefits and drawbacks.

underlying AFM, there is an additional way to control the exact interaction with the substrate. In the case of glass micropipettes, it is only possible to control their positioning by means of optical inspection via an inverted microscope. Due to the limited resolution of the optical inspection methods, it is therefore often difficult to determine whether the pipette is already in contact with the substrate or not. Furthermore, the limited control makes it difficult to apply the technique to spread cells. It is therefore not uncommon that cells are severely damaged during the manipulation process. Micropipette-based assays can therefore often only be carried out by well-trained and experienced operators.

Despite these clear disadvantages in terms of operation simplicity and online control versus FluidFM, micropipettes are still regarded as the gold standard for a wide range of applications involving single cells and microorganisms in general. In the following sections, the most important applications of glass micropipettes without any elec-
trodes inserted at the back will be described. For applications requiring the use of additional electrodes, refer to section 6.2.

**Single cell injection using pulled glass capillaries**

Single cell injection experiments are typically carried out using glass micropipettes. The method was invented over a decade ago [Barber, 1914] and has since matured to become the gold standard for many applications requiring the delivery of substances to the inner compartments of a cell. Introduction of plasmids [Gordon et al., 1980], DNA [Capecchi, 1980], proteins [Zhang et al., 2000], peptides [Zhang et al., 2002], antibodies [Mercer et al., 1984], and quantum dots [Dubertret et al., 2002] have been readily demonstrated using micropipette-based injection systems. The most prominent example for this type of technique is found in reproductive medicine: In vitro fertilization (IVF) is the process where a human ovum (egg cell) is fertilized by injecting a spermatozoon directly into it. Figure 6.14 depicts a typical IVF procedure. It can be seen that glass micropipettes are used both for holding the target cell in place and for penetrating its membrane in order to deliver the spermatozoon.

**Figure 6.14:** Example of an IVF-ICSI procedure using glass micropipettes. A human oocyte is held in place by means of a holding pipette. A sharp micropipette is subsequently used to inject a spermatozoon into the oocyte. Image by Eugene Ermolovich (CRMI) (Own work) licensed under CC BY-SA 3.0 or GFDL, via Wikimedia Commons.

While the transfection rate for micropipette-based injections of single cells can theoretically be as high as 100%, cell viability is still highly dependent on the actual procedure, equipment, and experience level of the experimenter. The main reason for this is the limited control possible through optical inspection alone. The technique is also very labour-intensive and difficult to automate.

There are many different systems on the market for glass pipette-based microinjec-
tion. They typically consist of a suitable micromanipulator setup situated on top of an inverted optical microscope. To control the delivery of substance upon penetration of the cell, a suitable pressure pulse generator is required. Commercially available injection systems often also provide pre-fabricated and quality controlled glass micropipettes in order to improve control and reproducibility of the procedure. Image 6.15 shows an example of a commercially available microinjection platform.

Table 6.12 gives an overview of the advantages and drawbacks of the glass micropipette-based microinjection technique.

Figure 6.15: Example of a commercial microinjection platform. The glass micropipettes are controlled via a micromanipulator mounted on top of an inverted optical microscope. Image from http://shop.eppendorfna.com, copyright Eppendorf North America.

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<tr>
<td>equipment availability</td>
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<tr>
<td>cheap consumables</td>
<td>no force control</td>
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<tr>
<td>well-established</td>
<td>mechanical stability</td>
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<tr>
<td>works for many different cell types</td>
<td>risk of cell damage</td>
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<tr>
<td>works on adherent and suspended cells</td>
<td>low success rate for untrained operators</td>
</tr>
<tr>
<td></td>
<td>limited automation capabilities</td>
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Table 6.12: Microinjection using glass micropipettes: Assessment of benefits and drawbacks.

In contrast to micropipette-based microinjection, FluidFM has the advantage that
6.1. Devices based on physical interactions with the substrate

the whole procedure can be carried out under force control. Thus the injection process can be carried out with full control, even below the resolution limits of optical microscopy. Exact detection of membrane penetration can be carried out in order to minimize any mechanical stress on the target cell. Thanks to its force-feedback capabilities, FluidFM also has the potential to considerably reduce the operational complexity of microinjection experiments. Until now, only well-trained and experienced experimenters were able to carry out the procedure with satisfying yield. Last but not least, this also opens the doors to advanced automation scenarios in order to speed up the throughput of this serial method, which is a clear disadvantage compared to other intracellular delivery techniques.

**Cellular adhesion studies by means of glass micropipettes**

Event though glass micropipettes cannot directly be used to measure forces quantitatively, it is possible to use them to indirectly assess adhesion properties of suspended cells. One way of doing this, is by observing the deformation of a cell in response to a known change in pressure applied through a micropipette in the vicinity of the target cell. Another approach involves the use of two pipettes of the right diameter: One is used to clamp a cell of interest that has a micro-bead attached to it. The second micropipette is used to suck away the micro-bead using an underpressure. Knowing the amount of underpressure, the corresponding forces acting on the micro-bead can be calculated. Table 6.13 summarizes the principal characteristics of this technique.

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<tbody>
<tr>
<td>equipment availability</td>
<td>high operation complexity</td>
</tr>
<tr>
<td>cheap consumables</td>
<td>only indirect method</td>
</tr>
<tr>
<td>pN resolution</td>
<td>maximum force limited (&lt;100 pN)</td>
</tr>
<tr>
<td></td>
<td>cells can stick to micropipette</td>
</tr>
<tr>
<td></td>
<td>difficult to operate</td>
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</table>

*Table 6.13: Adhesion studies with glass micropipettes: Assessment of benefits and drawbacks.*

In contrast to FluidFM, the method is not feasible for investigating adhesion forces in the case of adherent cells because the maximum force range is limited. Furthermore, the procedure requires multiple pipettes and suffers from pronounced operational complexity. It is only an indirect way of measuring cellular adhesion forces and only yields usable results if the cells do not stick to the glass micropipettes.
Combined AFM and glass nanopipette systems

Glass micropipettes have also been successfully combined with SPM derived instrumentation. The most straightforward example is to use a bent glass nanopipette with a mirror device in place of the cantilever in a standard AFM instrument. Such a setup has been demonstrated to allow for the direct printing of proteins on a surface with a resolution of around 200 nm [Taha et al., 2003]. The nanopipette used had an aperture of 100 nm.

Recently, glass micro-pipettes have also been demonstrated in combinations with tuning fork based atomic force microscopes. The commercially available Hydra BioAFM system from Nanonics Inc. (Israel) is just one example of such a system. By combining the advantages of AFM with the versatility of micropipettes, this system has a lot of potential to become a useful tool for many research applications in the field of life sciences. The open design of the Hydra platform enables straightforward integration with established optical microscopy instruments, while keeping the substrate accessible from the top. Furthermore, the system is capable of operating with multiple tuning fork assemblies, enabling true parallel actuation of up to four individual probes. However, despite being available on the market for over 5 years, no published results have been definitely obtained using this technology.

Other research groups have employed glass micropipettes in combination with tuning fork based SPM systems to carry out metal-plating deposition assays in liquid with subsequent topography imaging of the created structures [Ito et al., 2010, Ito and Iwata, 2011].

Compared to FluidFM, there is one important disadvantage of this technique: It is not well-suited for applications in liquid. Due to the increased damping acting on the tuning fork resonator when operated in a fluid of higher density such as water, the Q-factor of the system is considerably reduced. This reduction in quality has a negative impact on the overall system performance and diminishes the versatility of the technique for applications requiring operation in liquid. Nevertheless, it is an interesting instrument based on a completely different actuation principle than that used for FluidFM.
6.2 Techniques used for localized (electro-)chemical interactions with the substrate

6.2.1 Glass micro- and nanopipettes with integrated electrode

The application scope of glass micro- and nanopipettes can be substantially broadened if they are augmented with the ability to measure ionic currents running through the pipette aperture. This is typically achieved by inserting a suitable electrode at the macroscopic side of the pipette. The electrode is thereby in direct contact with the fluid located inside the pipette. Similar functionality can also be achieved by using pipettes where the cavity is completely filled with a suitable metal or other type of conductive material. Such pipettes are referred to as tapered electrode micropipettes.

The following sections illustrate the most important techniques based on the use of such electrochemically sensitive micro- and nanopipettes.

Electrophysiological characterization of single cells - Patch clamp

The patch clamp is a technique applied in electrophysiology to study the activity of single or multiple ion channels embedded in the membrane of a cell. While the method can in principle be used for many cell types, it is mostly applied for studying electrically excitable cells such as cardiomyocytes and neurons. The patch clamp was developed in the late 1970s by Neher and Sakmann [Neher et al., 1978, Hamill et al., 1981] and has since seen many new developments aimed at increased automation and increased throughput [Dunlop et al., 2008]. Nevertheless, the original approach based on the use of pulled glass micropipettes is still considered the gold standard for assessing ion channel activity.

In order to measure ion channel activity, a suitable micropipette is brought into gentle contact with the membrane of the target cell. The surrounding media is thereby contacted with a second electrode, forming an electric circuit running through the aperture of the micropipette. Once in contact with the target cell, a precisely tuned underpressure is applied to the pipette in order to firmly seal the cell to it. The principal objective here is to create an electrically tight seal between the two objects, the so-called gigaseal configuration, because of its high resistance to ionic species. Once the gigaseal is established, the only possible current path between the two electrode passes via the ion channels of the cell under the pipette patch. Therefore, any recorded current can be attributed to the activity of those channels.

There are several different configurations for the patch clamp, depending on whether
a single channel needs to be examined or the current response of the whole cell is of interest.

A major limitation of this approach is the high access resistance through the pulled micropipette and the associated high parasitic capacitances. Typical patch clamp setups therefore have a cut-off frequency in the range of 1 – 3 kHz.

Due to the limited control available in pipette-based manipulation techniques, it is difficult to establish the gigaseal configuration without destroying the cell in the process. Only well-trained operators are capable of carrying out patch clamp experiments with satisfactory success rates.

FluidFM promises to further push user adoption of patch clamp as it has the potential to automate and simplify many of the critical steps involved in the procedure thanks to its force feedback capabilities. This has recently been shown to even allow patch clamp experiments on beating cardiac cells over longer periods of time [Ossola et al., 2015].

**Electrochemical nanopipette**

The electrochemical nanopipette is another example of a pipette-based device with integrated electrodes. The device is operated in ionic solution and the potential applied to the pipette tip controls the flux of molecules from the pipette. This way, the delivery rate can be precisely tuned. The resulting ion current can also be used to control the distance of the pipette from the target surface. Such a device has been employed to deposit fluorescently labelled DNA and proteins on specially prepared surfaces [Bruckbauer et al., 2002]. An illustration of this technique is shown in figure 6.16.

Compared to FluidFM, the device offers comparable or even better performance with respect to deposition of materials on a surface in liquid. However, the versatility of the nanopipette is much reduced compared to FluidFM, since many other applications such as cellular adhesion measurements are not possible due to the lack of a force control mechanism. Furthermore, the nanoprobes used exhibit a high access resistance (> 100 MΩ) which limits their usability for applications requiring the measurement of ionic AC currents. Table 6.14 lists the most important properties of this technique.
6.2. Techniques used for localized (electro-)chemical interactions

Figure 6.16: Electrochemical nanopipette for material deposition in liquid. **Left:** Schematic setup of a nanopipette system. **Right:** Example of a pattern written in liquid using fluorescently labelled biotinylated DNA on a streptavidin surface. Reprinted with permission from reference [Bruckbauer et al., 2002]. Copyright 2002, American Chemical Society.

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<td>cheap consumable</td>
<td>no force control</td>
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<td>distance control</td>
<td>limited ink choice</td>
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<tr>
<td>electroosmotic actuation</td>
<td>high pipette access resistance</td>
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<td></td>
<td>limited aperture reproducibility</td>
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Table 6.14: Electrochemical nano-pipette: Assessment of benefits and drawbacks.

6.2.2 Scanning ion conductance microscopy (SICM)

Scanning ion conductance microscopy is an imaging technique based on the use of glass micro-pipettes with integrated electrodes [Hansma et al., 1989]. The system can be used in a true non-contact fashion to acquire topographical information about a substrate. Due to this minimally invasive scanning method, SICM is well-suited to imaging soft and fragile biological samples such as live cells and tissues.

SICM works by measuring the tiny ionic currents through the aperture of a nanopipette. When such a pipette is approached to a surface, the effective conductance and hence the measured ionic current is reduced as the space through which the ions can flow decreases closer to the surface. These variations in current can be used as an efficient feedback signal to control the normal position of the pipette with respect to the surface. By scanning an SICM system over the substrate, it is thus possible to reconstruct a digital representation of the sample topography without the need for any direct physical or chemical interaction with the substrate. The resolution achievable with SICM is mainly determined by the opening diameter of the pipette [Prater et al.,
Chapter 6. State of the art - From micro-pipettes to SPM

1991]. Figure 6.17 illustrates the basic measurement principle behind SICM.

Figure 6.17: Working principle of an SICM microscope. A nanopipette is approached to the sample of interest whilst measuring the ionic current through the pipette aperture. This quantity can be used to control the position of the pipette from the sample surface. By scanning a probe over the substrate with constant distance, its topography can be reconstructed. Reproduced from [www.ionscope.com]. Copyright 2015, ionscope Ltd.

Commercial implementations of SICM instruments are readily available on the market. SICM imaging modes have been extended to overcome some of the drawbacks of standard DC mode operation. For example, a novel operating method called hopping mode has been introduced that allows for non-contact image acquisition on samples with high aspect ratios [Novak et al., 2009]. The major advantages and drawbacks of SICM technology are summarized in table 6.15.

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<td>cheap consumable</td>
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<td>distance control</td>
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<td>localized patch clamp</td>
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<tr>
<td>true non-contact imaging</td>
<td>high pipette access resistance</td>
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<td></td>
<td>limited aperture reproducibility</td>
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Table 6.15: Scanning ion conductance microscopy: Assessment of benefits and drawbacks.

FluidFM can also be operated in SICM mode and has the possibility to switch between operation as an AFM with force feedback or as a pipette with ionic current feedback. The main difficulty is to avoid any unwanted snap-ins of the probe to the surface when performing SICM measurements. One way to overcome this is to use stiffer probes and to operate them in dynamic mode for both AFM and SICM image acquisition. A nice feature of FluidFM is the possibility to switch between non-contact imaging
using SICM and AFM imaging modes in situ during scanning. This would, for example, allow the improvement of SICM imaging speeds on high aspect ratio samples without the need to use slower SICM techniques such as hopping mode.

### 6.2.3 Scanning electrochemical microscopy (SECM)

Another type of micropipette-based imaging technology is called scanning electrochemical microscopy (SECM). It is useful for measuring applications where the microelectrochemical properties of the sample are of interest and was first introduced in 1989 [Bard et al., 1989]. SECM is nowadays considered an essential tool for observing and controlling electrochemical redox reactions with ultra high spatial resolution. The possibility to characterize microelectrochemical properties at liquid/solid and liquid/liquid interfaces is vital for many research applications in the fields of chemistry, biology, and material sciences. The possibility to obtain information about such systems with high spatial resolution has led to widespread acceptance of the technology since its original invention [Amemiya et al., 2008].

To obtain localized electrochemical information, SECM measures the faradaic currents involved in redox reactions by means of an ultra-microelectrode (UME) tip. UME tips are typically made from tapered metal conductors that are encapsulated in a glass nanopipette. An electric potential is applied at the UME in a bulk solution containing redox-active species. When a sufficiently high bias potential is applied, an electrochemical reaction is triggered and the resulting diffusion limited faradaic currents are measured at the UME. In feedback mode, the steady state current is characteristically modulated whenever the UME is approached to an insulating or conducting surface. If the tip signal is not due to the electrolysis of a mediator species but rather a species generated at the surface, SECM should operate in generator/collector mode. Figure 6.18 explains the feedback mode operation of SECM together with the corresponding approach curve.

SECM-based imaging is typically achieved by scanning the UME tip over the sample at a constant height while the resulting current is recorded. The acquired current image is a mapping of the electrochemical surface properties to their respective spatial coordinates. For samples with high aspect ratios, the constant height imaging mode only provides a convolution of the localized surface reactions with the sample topography. In order to ensure that the recorded current is only due to faradaic reactions on the sample, the probe has to be scanned in constant-distance mode to ensure a constant spatial gap between the UME tip and the substrate.

Constant distance scanning cannot be easily achieved with classical SECM as there is
no direct method to control the distance between the UME disk and the sample during the experiment. For this reason, UMEs used for SECM have been integrated with other techniques such as SICM [Comstock et al., 2010, Takahashi et al., 2010, 2012] or AFM [Macpherson and Unwin, 2000, Kueng et al., 2003] to enable topography compensated SECM imaging. SECM-based instruments have recently also been shown to support highly localized deposition and etching of samples. Such devices can, for example, be used as lithography tools to enable novel patterning approaches for advanced microfabrication purposes [Clausmeyer et al., 2014]. However, most of these combined approaches require complicated MEMS-based probes that are difficult to fabricate reproducibly. Table 6.16 provides an overview of the major benefits and drawbacks of scanning electrochemical microscopy technology.

By integrating electrodes near the apex of a FluidFM probe (e.g., a ring electrode
6.2. Techniques used for localized (electro-)chemical interactions

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<td>true non-contact operation</td>
<td>no reservoir</td>
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<td></td>
<td>no distance control</td>
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Table 6.16: Scanning electrochemical microscopy: Assessment of benefits and drawbacks.

around the aperture) it would be possible to extend the complete spectrum of SECM features into a FluidFM setup. Furthermore, it would even be feasible to locally supply specific chemical solutions such as mediators in order to enable advanced SECM experiments. This would only be possible in SECM by means of multi-barrel micropipettes, since SECM does not typically have a separate reservoir like FluidFM. Furthermore, thanks to the AFM force feedback of FluidFM, the typical tip-sample distance problem from SECM would no longer be an issue.

6.2.4 Multiphase-based systems

Another interesting family of instruments for local chemical interaction with samples is based on multiphase systems. Such devices are based on the localization or compartmentalization between multiple phases of substances, either by using immiscible liquids or via slow diffusion mass transfer.

Glass micropipette filled with immiscible liquids

The simplest implementation of such a system can be achieved using a glass micropipette for spatially defined delivery of reagents or particles. In this way, it is possible to expose only selected regions of a cell culture to specific reagents. This approach can be useful for many applications such as toxicity studies with in situ control or single cell transfection studies.

For example, it has for example been demonstrated that small droplets of a denser phase cell suspension can be patterned into an aqueous immersion layer using a micropipette [Tavana et al., 2009]. Due to their affinity for the denser phase liquids, the cells remained captured within the droplet volumes and only seeded onto the substrate underneath them.

The same approach could be readily implemented using FluidFM, as the device can
also be employed as a micropipette with force feedback. It could even be possible to refine the approach using FluidFM because the force feedback would allow it to discern denser phases of liquid from surrounding immersion phases with high precision. FluidFM-based multiphase systems could therefore be used for applications such as biosensor fabrication, tissue engineering, and single cell transfection experiments.

Chemistrode

Another approach based on multiphase systems is known as chemistrode [Chen et al., 2008]. The Chemistrode is a microfluidic device with scanning capabilities that uses immiscible liquids to generate spacers between liquids inside the microfluidic channel. It can be used to investigate samples that rely on molecular signalling such as the communication between neighbouring cells in a confluent layer. Spacing between different stimuli substances is achieved by using an immiscible fluid as spacer and carrier matrix inside the microfluidic channel of the chemistrode.

To interact with the sample, the device has a small opening at its base where the aqueous fillings can interact with a hydrophilic surface underneath. After delivery of the stimulus substance, the system's chemical response can be readily sampled in situ with the same device. The chemistrode can therefore interrogate samples and monitor their response with a library of molecular signals from multiple locations with high spatial resolve. Examination of insulin secretion in Langerhals cells from a single murine islet has been successfully demonstrated with this approach [Chen et al., 2008]. The following table assesses the most important properties of chemistrode technology.

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<tr>
<th>Pro</th>
<th>Contra</th>
</tr>
</thead>
<tbody>
<tr>
<td>works in liquid and air</td>
<td>requires custom instrumentation</td>
</tr>
<tr>
<td>supports multiple cargo liquids</td>
<td>not many publications</td>
</tr>
<tr>
<td>deliver and collect chemicals</td>
<td>lateral resolution reduced</td>
</tr>
<tr>
<td>compatible with down-river methods</td>
<td>complicated loading procedure</td>
</tr>
</tbody>
</table>

**Table 6.17:** Chemistrode technology: Assessment of benefits and drawbacks.

Multiphase separation techniques could also be applied to FluidFM. However, in its current form there is only one available channel. FluidFM-based chemistrode operation would therefore only support either stimulus deliver or response sampling. However, with a multichannel FluidFM probe, true chemistrode-like operation is feasible. The same partitioning approach used by the chemistrode technique could also be employed in a single channel FluidFM probe to enable the use of multiple
substances. This could be interesting when depositing arrays of small droplets such as proteins. Furthermore, thanks to the optically transparent nature of FluidFM probes, it is easy to monitor multiphase loading of FluidFM cantilevers using an inverted microscope.

### 6.2.5 Devices using hydrodynamic focusing of liquids

Hydrodynamic focusing is a method where a liquid is confined by spatially enclosing it using a secondary fluid exhibiting a different flow rate and density instead of rigid walls. Hydrodynamic flow confinement is regularly used in devices such as particle counters and flow cytometers. Using this technology, it is possible to limit liquids to narrow spaces over long distances, a process that would be difficult to achieve using standard microfluidic approaches.

**Microfluidic probe (MFP)**

In combination with scanning probe techniques, hydrodynamic focusing can be used to create devices capable of confining liquid flow with high spatial resolution. The microfluidic probe (MFP) was first reported by IBM in 2005 [Juncker et al., 2005]. The device achieves hydrodynamic flow confinement using a flat probe surface with two or more apertures. One aperture is used as an inlet channel while the other serves as an outlet channel. By injecting liquid via the inlet port and immediately aspirating it via the outlet channel, it is possible to confine liquid flow to a well-defined region. An illustration of the MFP working principle is shown in figure 6.19. By placing an MFP near a surface, it is possible to selectively expose it to specific reagents with micrometer precision. The scanning capability of the MFP head makes it possible to interact with the substrate over large ranges, providing broad experimental flexibility. Using MFP with multiple apertures, it is also feasible to deposit a gradient of biochemicals to a surface [Qasaimeh et al., 2011].

The technology can also be employed to carry out immunohistochemistry assays, where multiple antigens can be applied and detected on the same tissue section [Lovchik et al., 2012]. These methods greatly reduce the required reagent volumes. Additionally, the fluorescent labelling of the various antigens does not have to be different, as they can be identified directly via their spatial position on the substrate.

MFPs have also been shown to support integration of down-river analytical techniques within a single device in the form of a system capable of lysing individual cells [Sarkar et al., 2014]. The resulting lysate can be readily collected using the outlet port of the
Figure 6.19: Working principle of a microfluidic probe. Cross-section view of the device. A stream of liquid is injected through the inlet port \( Q_I \) and aspirated together with parts of the surrounding liquid via the second aperture \( Q_A \). Because of hydrodynamic pressure from the surrounding fluid, the injected liquid is confined to a limited area around the inlet port. Adapted by permission from Macmillan Publishers Ltd: Nature Materials [Juncker et al., 2005], copyright 2005.

MFP probe. A microfluidic system with heating chambers and valves integrated into the same device was subsequently used to perform in situ single cell PCR amplification right after collection of the cell lysate. Table 6.18 provides an overview of the most important features of microfluidic probe technology.

<table>
<thead>
<tr>
<th>Pro</th>
<th>Contra</th>
</tr>
</thead>
<tbody>
<tr>
<td>supports a multitude of cargo liquids</td>
<td>no feedback loop</td>
</tr>
<tr>
<td>depositon of gradients</td>
<td>requires custom instrumentation</td>
</tr>
<tr>
<td>deliver and collect chemicals</td>
<td>relatively large probe</td>
</tr>
<tr>
<td>compatible with down-river methods</td>
<td>lateral resolution limited</td>
</tr>
<tr>
<td>reduction of reagent volumes</td>
<td>no force control</td>
</tr>
</tbody>
</table>

Table 6.18: Microfluidic probe technology: Assessment of benefits and drawbacks.

MFPs require a similar setup to FluidFM. While it is in principle possible to create FluidFM probes with MFP functionality, this has yet to be done. The spatial dimensions at which MFP operates are also clearly above the corresponding length-scales of FluidFM. MFP is a suitable tool for addressing tissues or smaller groups of cells, while FluidFM can be used to interrogate and interact with individual cells or other micro objects. Nevertheless, combining both approaches into a single instrument could potentially leverage the versatility of both techniques.
6.3 Openspace microfluidics: Devices for localized chemistry

The concept of open space microfluidics has been recently introduced by [Kaigala et al., 2012]. There is an ever growing need for technologies capable of providing similar functionality to standard microfluidic systems, but avoiding the constraints associated with the closed reaction chambers of such systems. By removing the walls and ceiling of a microfluidic system, a completely novel family of devices that offer much more experimental flexibility is established. With such openspace microfluidic devices, it is possible to work directly on the relevant biological interfaces, ideally even within their native environment without the need for scaffolds or other complicated sample preparation steps. Openspace microfluidics are typically non-invasive and completely bio-compatible. They should work in liquid environments and enable flexible and interactive interaction with the sample.

FluidFM clearly shares most of the characteristics of openspace microfluidic devices. According to Kaigala et al., the potential of such devices for applications in life sciences research is manifold, and they will soon become ubiquitous in research labs around the world. FluidFM is sure to play a major role in these developments. As will be shown in the course of this thesis, there are many possible applications for the technology, and the major challenge will be to focus on the most promising ones in order to harness the full power of FluidFM technology in the future.

6.4 Chapter summary and conclusion

As seen from this review, there are many current state of the art devices for manipulations similar to FluidFM, and each approach has certain advantages and drawbacks for various applications. When comparing them with FluidFM, however, it is clear that very few can offer the same broad applicability and versatility as FluidFM. The next few chapters will show how FluidFM can be used for deposition (chapter 7), single cell manipulations (chapter 8), and multiparameter material characterization (chapter 9). It will be demonstrated that the technology is working well, although many technical challenges remain to be solved.

Another interesting feature that becomes evident when looking at competing methods is related to the underlying core technology of each device: Almost all the techniques presented here are based on either pulled glass micropipettes or on devices produced utilizing MEMS-inspired fabrication techniques. While micropipettes are already ubiquitous in many life sciences research labs, MEMS based devices are still relatively
novel and less widely adopted. They often suffer from a perceptional gap when competing with pipette-based techniques. Although we strongly believe that MEMS-inspired devices are going to reach the same level of widespread adoption in the future, considerable effort is still needed to fully establish them in the community.
FluidFM based localised liquid dispensing in a liquid environment

When comparing FluidFM with other AFM-derived methods, as explained in chapter 6, it is evident that a major advantage of FluidFM over most other delivery techniques is the completely enclosed microfluidic channel in the cantilever. While systems such as NADIS (see section 6.1.3) or DPN-inspired techniques (see section 6.1.2) have similar capabilities for dispensing and patterning substances under a controlled humidity atmosphere, they are usually not capable of operating in a liquid environment. However, this is a key requirement to be able to work under physiological ambience conditions, especially for applications involving biochemically relevant solutions and live cells.

The ability to dispense solutions directly within another immersion substance is not just interesting when working with live samples such as single cells. It can also be very helpful during fabrication of novel devices that can be used as biosensors (e.g., for functionalisation) or to create arrays for protein screening (e.g., spotting different proteins).

The simplest type of deposition process typically yields small spots of settled material on the target surface. It is therefore often also referred to as spotting.

7.1 Dispensing solutions via FluidFM in a liquid environment

Before starting to deposit substances to a surface within a liquid environment with FluidFM, it was important to determine whether it is possible to eject liquid through the aperture of the probe when immersed in a fluidic environment. For this reason, a simple experiment utilizing a solution that can easily be visualised by means of
fluorescence microscopy techniques (see section 2.1.2) was conducted. A suitable substance for this purpose is fluorescein isothiocyanate (FITC). Using cargo solutions containing FITC, it was possible to clearly distinguish the liquid originating from inside the cantilever in the context of the surrounding immersion solution.

As soon as a pressure difference was applied to the cantilever, cargo solution was ejected into the surrounding ultrapure water bath. The size of the resulting diffusion sphere around the aperture of the probe is highly dependent on the applied pressure as well as on the duration of the pulse. This was expected, as higher pressure produces an increased flow rate through the aperture and longer pulses allow the ejected liquid to diffuse away from the aperture for longer times. Figure 7.1 depicts an example of such an experiment for a type B FluidFM probe filled with a solution of 6.25 μg/mL FITC in ultrapure water (Merck-Millipore Corporation). The indicated time series shows that liquid is ejected on pressure application. The tipless probe had an opening size of 2 μm. Since the addition of FITC tracer has proven very effective, an FITC tracer stock solution in PBS buffer was prepared, as explained in section 2.3.4. This greatly simplifies the addition of FITC tracer to any kind of experimental cargo liquid solution.

![Figure 7.1: Testing FluidFM liquid ejection when immersed in a bath solution. The figure shows example images of a time testing series using a type B FluidFM probe with an aperture of 2 μm. The cargo liquid is ultrapure water with added FITC tracer to make it visible using fluorescence microscopy techniques. Left: No pressure applied, no cargo substance leaves the probe. Middle: Situation after a pulse of 250 ms with an applied pressure of 25 mbar. Right: Same probe after a 1 s long pulse, again using a pressure of 25 mbar. Adapted from [Doerig, 2009]](image-url)

This feasibility experiment confirms that it is possible to successfully eject liquids from FluidFM probes when immersed in liquid. Furthermore, the addition of FITC to the solutions has proven a convenient and effective way to visualize the behaviour of the experimental substance as it is ejected from the FluidFM cantilever. For all
7.2. Nanoscale spotting of streptavidin on a functionalized surface

Further studies, a suitable amount of FITC tracer was always added to better monitor the experiment and to determine whether any liquid was ejected or not. The addition of FITC proved highly important, as it was not uncommon for the probes to become clogged or suffer other issues with the hollow cantilevers during the early FluidFM experiments.

The most important thing to note, however, that constant live monitoring of the experiment via the optical microscope is possible at all times during the procedure. This is a valuable attribute of FluidFM and greatly improves the usability of the techniques.

### 7.2 Nanoscale spotting of streptavidin on a functionalized surface

To investigate how well FluidFM is able to locally dispense substances in a liquid environment, a suitable experimental model system was needed. In order to quantify the size of the spots produced, it is necessary to immobilize the dispensed substance on the target surface. Especially when working in a liquid environment, insufficient immobilization makes it impossible to correctly assess the performance of the procedure, as the dispensed solutions are quickly dispersed in the immersion liquid due to diffusion and other hydrodynamic perturbations in the system.

A platform featuring strong binding between the dispensed substance and the surface was created by taking advantage of the well-known interaction between streptavidin and biotin: Streptavidin exhibits an extremely high affinity to biotin, and their bond is one of strongest non-covalent interactions known in nature. Streptavidin-biotin interactions have already been extensively studied using the force measurement capabilities of standard AFM technology [Lee et al., 1994].

By functionalizing a target surface with biotin and subsequently using a spotting solution containing streptavidin, it is possible to dispense small amounts of liquid via a FluidFM probe that are readily immobilized on the substrate. To create a suitable target surface, a custom flow cell was prepared on top of a common glass microscopy slide. The flow cell was plasma cleaned in ambient air plasma for two minutes before being immersed in a solution of 0.1 mg/mL PLL-\(g\)-PEG-Biotin in HEPES (For details on PLL-\(g\)-PEG, see section 2.3). After immersing the flow cell for 30 min, a suitable mono-layer of PLL-\(g\)-PEG-Biotin had formed on the glass bottom surface of the flow cell. The formation of this layer could be catalysed by pre-treating the flow cell in an ambient air plasma, as this procedure yields negatively charged glass surfaces. The redundant PLL-\(g\)-PEG-Biotin can be replaced by another immersion liquid of choice.
Chapter 7. FluidFM based localised liquid dispensing in a liquid environment

if required for the experiment.

Labelled streptavidin (different fluorophores) in HEPES supplemented with FITC tracer was utilized as cargo solution for the spotting experiment using FluidFM probes. The fluorescently labelled streptavidin in the solution leads to an exact and immobilized deposition of the cargo liquid on the substrate, while the FITC tracer allows the whole process to be observed without bleaching the fluorescence signal from the labelled streptavidin. Upon ejection from the FluidFM probe, the labelled streptavidin readily binds to the immobilized biotin on the glass substrate of the flow cell. The deposits created in this way can later be inspected by means of standard fluorescence microscopy or confocal laser scanning techniques (see also section 2.1.2).

All spotting experiments were carried out with type A FluidFM probes (see also section 4.2.1) with an aperture size of 1 µm at the top of a pyramidal tip structure as defined by FIB milling.

7.2.1 Spotting in PLL-g-PEG solution

The first spotting experiment was carried out with the FluidFM Probe immersed in a flow cell filled with a solution of 0.1 mg/mL PLL-g-PEG in HEPES. A solution of labelled streptavidin (Alexa 488, 20 µg/mL) in HEPES amended with FITC tracer was used. The experimental setup is illustrated in figure 7.2.

Figure 7.2: Experimental setup: Spotting in PLL-g-PEG solution. The FluidFM cantilever was filled with labelled streptavidin solution (Alexa 488, 20 µg/mL). Deposition of the cargo solution was carried out in a solution of 0.1 mg/mL PLL-g-PEG in HEPES. The figure is purely illustrative and not drawn to scale.

Optical inspection confirmed successful ejection of spotting liquid upon applying an
7.2. Nanoscale spotting of streptavidin on a functionalized surface

overpressure. For the actual experiment, the pressure applied to the FluidFM probe was controlled using hydrostatic pressure control with a height difference of 5 cm (see also section 2.1.3).

The deposited streptavidin spots were clearly distinguishable when printed with a pre-set spacing of 20 µm between subsequent spotting sites. To deposit material to the surface, the probe tip was kept in force-controlled contact with the surface for 30 s before being retracted and moved to the next spotting site. During the whole procedure a constant pressure of 5 mbar was applied to the FluidFM probe. The pressure was achieved by maintaining a height difference of 5 cm between the reservoir and FluidFM probe aperture during active spotting.

The left-hand picture in figure 7.3 illustrates the results of this first spotting experiment when inspected by means of a confocal laser scanning microscope. The right-hand image of figure 7.3 depicts the intensity plot corresponding to the deposited spots in the left image as determined with the CLSM. The average spot size was \( \sim 8 \) µm FWHM (Full Width at Half Maximum).

Unfortunately, it was not possible to carry out a time series to determine the optimal spotting time due to clogging of the microchannel in the cantilever. Binding to the labelled streptavidin of the cargo solution occurred as PLL-g-PEG-Biotin molecules from the immersion liquid diffused through the aperture into the channel, forming a thick gel at the aperture of the probe within a few minutes.
7.2.2 Liquid dispensing in HEPES-2 buffer

In order to avoid premature clogging of the FluidFM probes, another experiment using HEPES-2 buffer as immersion liquid was carried out. The buffer essentially replaced the remaining PLL-g-PEG-Biotin solution that was used to functionalize the substrate. The spotting was carried out using slightly different parameters from the first experiment, with a reduced height difference of 2.5 cm to obtain a value of 2.5 mbar as the applied pressure in the FluidFM probe. The contact time was also considerably reduced to 3 s. For the spotting liquid, labelled streptavidin (Oregon Green 488, 50 µg/mL) in HEPES was supplemented with FITC tracer in order to obtain a final concentration of 20 µg/mL streptavidin. All other parameters remained unchanged compared to the first experiment. Figure 7.4 illustrates the setup of this experiment.

![Experimental setup: Liquid dispensing in HEPES-2 buffer. The FluidFM cantilever is filled with labelled streptavidin solution (Oregon Green 488 20 µg/mL) in HEPES with FITC tracer. Deposition of the cargo solution is carried out in HEPES-2 as immersion liquid. The figure is purely illustrative and not drawn to scale.](image)

The results were quite different from the first experiment, as shown in figure 7.5. In HEPES-2 it was observed that the cargo liquid exhibited much stronger parasitic diffusion than before. The minimal achievable spot size was around 15 µm FWHM, much larger than in the first experiment. The spacing between individual spots was again set to be 20 µm. To emphasize the versatility of the technique, two different patterns of spots were created on the same surface: A matrix-like arrangement as shown on the left-hand side of figure 7.5 and a cross-like layout as depicted on the right-hand side.

To conclude, using HEPES-2 as immersion fluid, the microchannel in the cantilever was still functional even after several hours of operation. However, this came at the
7.2. Nanoscale spotting of streptavidin on a functionalized surface

Figure 7.5: Results from dispensing in HEPES-2 buffer solution. Parameters for all deposited spots: 3 s contact time with a pressure of 2.5 mbar under force control. **Left:** Confocal microscope image of a matrix-like arrangement of spots deposited from labelled streptavidin (Oregon Green 488) in HEPES. The distance between individual spotting sites is set to 20 µm. **Right:** Same experiment on another location of the same sample. In this case the spots have been placed in a cross-like layout. The pre-set spacing distance was again 20 µm.

cost of a much reduced spotting resolution. A system offering the same long-term stability of the probe functionality combined with an improved spotting resolution is therefore highly desirable.

7.2.3 Improving localization with D-Biotin

In order to determine the minimal achievable spot size for FluidFM-based microdispensing of streptavidin onto a biotinylated surface, a more suitable immersion liquid was needed. To address the problem of too much diffusion when spotting in HEPES-2 buffer solution, the immersion fluid was supplemented with D-Biotin\(^1\) in order to obtain a solution of 1 µmol D-Biotin in HEPES-2. This way, most of the streptavidin cargo solution that flowed from the aperture of the probe was instantly bound to biotin either on the surface or directly within the immersion media. The fraction of the spotting liquid that was not bound to the immobilized biotin on the surface could easily be rinsed away afterwards by exchanging the immersion solution. Figure 7.6 shows the system used to improve the resolution of the dispensing procedure.

First, a series of spotting experiments was carried out in order to determine the optimal deposition time. However, in contrast to the other two immersion solutions,

\(^1\)Eight possible stereoisomers of biotin \(C_{10}H_{16}N_2O_3S_1\) exist. D-Biotin denotes the (D+)-Biotin stereoisomer. It is the only stereoisomer of biotin found in nature. Furthermore, it is the only biotin stereoisomer that is enzymatically active.
only a negligible correlation between the spotting time and the corresponding spot size could be observed, as shown on the left in figure 7.7. This indicates that the chosen system with D-Biotin in HEPES-2 as immersion liquid is ideally suited to conducting spotting experiments with high accuracy and reproducibility.

From the time series it was determined that a spotting time of 5 s yielded the best results. In order to verify that the observed fluorescent signals were really due to the
Nanoscale spotting of streptavidin on a functionalized surface

immobilization of streptavidin, a photo-bleaching control experiment was conducted. The resulting data is shown on the right hand side of figure 7.7.

With the modified immersion liquid, the deposition resolution was much better than before: Spots with a diameter of not more than 3 μm FWHM could be created. Figure 7.8 shows a matrix pattern of spots that were deposited in series using the same probe. It should be noted how reproducibly the spot size could be controlled. Using a pyramidal FluidFM probe with an opening of less than 1 μm, it is expected that the minimal achievable spot size can be even further reduced.

![Image of spotted matrix with intensity graph](image)

**Figure 7.8:** Results for streptavidin spotting in a HEPES-2 buffered D-Biotin environment. Deposition parameters: 3 s contact time with a pressure of 2.5 mbar under AFM force control. **Left:** Confocal microscope image of a matrix-like arrangement of spots deposited from labelled streptavidin (Oregon Green 488) in HEPES. The reproducibility of the spot sized is worth noting. The distance between individual spotting sites is set to 20 μm. **Right:** Intensity graph along the white line in the upper right streptavidin spot. All measured spots exhibited diameters in the range of 3 μm FWHM. Image adapted from reference [Meister et al., 2009a], Copyright 2009, with permission from Elsevier.

These results were further confirmed using a type B FluidFM probe with a tipless aperture of 2 μm, as demonstrated in [Doerig, 2009]. When compared to pyramidal probes, tipless probes showed a much higher spot-size variation depending on the applied pressure and the overall contact time. This difference in influence of both parameters on the resulting spot size is likely caused by the fact that the aperture did not directly make contact with the target surface as the probe was approached to it. Due to the design of these probes, their aperture is always slightly away from the surface, especially for low force setpoint interactions. Nevertheless, a tipless FluidFM cantilever can also be used to deliver substances with high spatial resolution and
confinement to a target substrate in a liquid environment.

7.2.4 Lithographic dispensing of continuous lines in a liquid environment

With FluidFM it is also possible to directly write line patterns made from cargo solution onto a surface immersed in liquid. This mode of operation is a kind of lithography that could be used for many different applications, such as targeted functionalization of an area in a biosensor or to connect microelectronic circuits in situ to repair broken electronic ICs.

Image 7.9 shows an example pattern written using labelled streptavidin (Alexa Fluor 532) cargo solution on a biotinylated surface immersed in D-Biotin amended HEPES-2 buffer solution. The path was travelled with a constant velocity of 0.2 μm/s while applying a constant pressure of 25 mbar. The shape of the drawing could thus be freely programmed.

![Image of a path pattern](image)

**Figure 7.9:** Example of a path pattern written on a biotinylated surface using labelled streptavidin. The shape has a dimension of about 30 μm x 30 μm.

The idea to use FluidFM as a tool for in situ patterning has since been further explored in other research projects and has shown promising results for applications in microfabrication [Grüter et al., 2013] and for directed growth of neural networks under physiological conditions [Dermutz et al., 2014].
7.3 Chapter summary and conclusion

To summarize, it was shown that FluidFM is a powerful tool to achieve highly localized dispensing of dissolved substances when working in a liquid environment. The demonstrated minimal feature sizes are very promising and are expected to be further improved using optimized probes. However, further reduction of the deposition resolution is always prone to premature probe clogging. This has to be avoided using carefully balanced chemistry throughout the entire experiment. FluidFM has been shown to have great potential when utilized as a lithography tool for microfabrication purposes and applications involving local surface modifications. Last but not least, the highly localized delivery of substances can be further exploited to apply chemical stimuli with very high spatial accuracy to a variety of samples, such as individual living cells and even sub-cellular structures.
The goal of this chapter is to demonstrate how FluidFM can be used as a versatile tool in biological research applications to handle, manipulate, and analyse individual cells.

A critical factor when working with viable biological objects such as tissue section and single cells is the requirement to carry out the complete experiment under physiological conditions. This implies tight control over environmental factors like temperature and atmospheric composition. However, the most important factor is the ability to keep the sensitive substrates in a physiological buffer environment at all times to avoid any unwanted perturbations or even cell deaths during the assay. Another critical factor is the ability to precisely control the interaction forces between the fragile substrate and the probes during an experiment. The current gold standard for direct manipulation of single cells is glass micropipettes in combination with a multi-dimensional micromanipulation system. A major drawback of such systems is their restriction to optical monitoring, as explained in chapter 6. In order to ensure complete control of the experiment, even below the resolution limits of optical methods, novel technologies to address these shortcomings are needed.

The preceding chapter showed that it is feasible to dispense and pattern fluids within an aqueous environment by means of FluidFM technology. The high degree of functionality and reproducibility of those experiments is mainly due to the device’s ability to function perfectly within a liquid environment without any loss of force control efficiency, spatial resolution, and instrument handling. For these reasons, FluidFM has been identified as an ideal tool for handling and manipulating individual cells while minimizing any unwanted parasitic inductions of the investigated objects due to limited experimental control.

Current state of the art techniques for interacting with single cells are typically unable to precisely tune the interaction forces occurring between the probe and the sample.
This often leads to failure or low yield of the experiment. Thanks to the sensitive AFM force feedback mechanism inherent in FluidFM, it is possible to completely control how strongly the probe interacts with the sample. On the one hand, it is feasible to limit the interaction to a low force regime, enabling gentle and impeding interplay with fragile samples such as a single cell. On the other hand, the interaction forces can be tuned to result in a controlled puncturing of the cellular membrane in order to gain access to its interior structures. The resulting forces can thus always be monitored and controlled in situ and in real-time. The left image of figure 8.1 shows a typical force-distance curve where the two FluidFM contact regimes can be clearly discriminated. All these characteristics of FluidFM allow for a variety of applications such as spatial manipulation, injection, extraction, or even transfection of viable cells.

**Figure 8.1:** FluidFM force regimes for single cell interactions. **Left:** Force-distance spectrum obtained during a single cell injection experiment. The gentle contact and membrane perforation regimes can be clearly identified. **Right:** (A) depicts a syringe-like type A FluidFM probe that is well-suited to perforating cellular membranes. (B) shows a corresponding pyramidal top aperture type A FluidFM tip. Adapted with permission from [Meister et al., 2009a]. Copyright 2009 American Chemical Society.

As will be shown in this chapter, not all applications can be carried out using the same type of FluidFM probe. For applications requiring only gentle interaction forces, cantilevers with apertures at the apex of a pyramid or plain tip-less probes are best. For assays requiring the puncturing of the cellular membrane, a syringe-like tip structure with the aperture on the side of an sharp pyramidal tip apex is beneficial. While injection is in theory also possible with the other types of probes, the best results in terms of both efficiency and viability of the cells were only obtained when using syringe-like FluidFM cantilevers. The two probe types used for the different force regimes are depicted on the right hand side of figure 8.1. Thanks to the versatility of the FIB-based aperture formation, the ideal aperture design could be efficiently determined.
8.1 Perforation regime - force controlled single cell injection

The injection procedure in FluidFM is very similar to injection using pulled glass micropipettes; however, FluidFM has a number of important properties that differentiate it from the classical technique. Instead of relying solely on optical control to direct the position of the injection probe, FluidFM takes advantage of the high resolution imaging and force control capabilities of the underlying AFM. In the case of glass micropipettes, it is often not possible to precisely discriminate between gentle contact and penetration of the cell. Because of this drawback, cells are often fatally damaged during injection assays, resulting in low success rates or even cell death. Furthermore, the procedure requires highly trained and experienced operators in order to be successful.

Conversely, using FluidFM, the successful penetration of the cellular membrane can be readily detected as an indentation jump in the force distance spectrum in real-time during the procedure. An example of a force distance curve obtained during an injection experiment is depicted on the left in figure 8.1. Using specially adapted cantilever geometries featuring a sharp pyramidal structure with an aperture near the apex, it is possible to minimize any potential damage to the cell. A close-up micrograph of such a probe is shown on the upper right of figure 8.1. Thanks to these syringe-like FluidFM probes, both the normal forces and unwanted lateral movements of the injector can be precisely controlled and reduced to an absolute minimum. Due to the sharp pyramid of these probes, it is even possible to use them to acquire a high resolution AFM image of the target cell in order to identify the exact location for the injection.

8.1.1 FluidFM-based single cell injection procedure

To demonstrate the injection capabilities of FluidFM, a sample consisting of a non-confluent layer of C2C12 mouse myoblast cells was used as a model system. A mixture of FITC and phosphate buffered saline (PBS) was employed as injection cargo liquid inside a syringe-like type A FluidFM probe. This dye was chosen because it is not permeable to the cellular membrane and does not exhibit any binding affinity to it. First, a high resolution AFM topography scan of the target cell was acquired in dynamic mode. The corresponding AFM topography and optical interference contrast images are shown on the left of figure 8.2. The FluidFM probe was already filled with the injection cargo liquid before performing the topography acquisition.
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Figure 8.2: FluidFM-based single cell injection. Left: AFM scan and corresponding phase contrast micrograph of the target cell. Middle: Fluorescent microscopy image and z-stack of the same cell after injection, as obtained via CLSM microscopy. The clear localization of the fluorescent dye indicates a successful delivery to the cytosol. Right: Illustration of the injection procedure with syringe-like FluidFM probes (not drawn to scale). All images adapted with permission from [Meister et al., 2009a], Copyright 2009 American Chemical Society.

A suitable location for the injection procedure was identified using the topography image, and the AFM was changed to static operation mode. Using the AFM’s high precision piezoelectric actuator, the probe was then positioned over the cell at the target location, followed by a force controlled approach to the cell surface. Upon reaching the membrane, the forces acting on the cell were slowly increased until a characteristic spike was detected in the force distance spectrum, indicating that the probe had successfully penetrated the membrane of the target cell. The ability to precisely and conveniently detect the successful penetration of the cellular membrane is a truly remarkable advantage over glass micropipette-based injection systems.

After puncturing the membrane, the probe’s approach was immediately halted and the AFM control electronics maintained the current force setpoint to keep the probe stably inside the cell. Using hydrostatic pressure control, a pressure pulse of a few seconds was applied to the microfluidic cantilever, resulting in ejection of the fluorescent cargo liquid from the probe aperture into the cytosol of the myoblast cell. Upon retraction of the probe, the results were immediately inspected using confocal scanning laser microscopy. It was determined that the introduction of the liquid resulted in the appearance of a clear fluorescent signal limited by the shape of the injected cell, as shown in the middle of figure 8.2. The cell volume did not appear to have changed noticeably, and from flow rate simulations it was estimated that the injected volume did not exceed 10 fL. In order to verify that the dye had really been injected into the cytosol of the cell, fluorescent images at different focal planes were also acquired. The resulting z-stack profiles confirmed that the fluorescent dye was not limited to the
8.1. Perforation regime - force controlled single cell injection

The surface of the injected cell, but extended across the entire cytosol, as shown in the lower middle image of figure 8.2. As the fluorescent signal of the cell was stable over longer periods of time and no significant background signal from FITC was detected in the immersion liquid, it was further concluded that the pinched membrane had recovered and resealed well after removing the injection probe. Subsequent AFM imaging of the injected cell indicated no structural and morphological changes in the cellular surfaces either, a clear indication that membrane recovery occurs at a much shorter timescale than that needed to acquire an AFM image.

8.1.2 Serial injection of individual cells using FluidFM

FluidFM-based single cell injection has proved to be highly reproducible and robust. This observation was further strengthened by injecting multiple cells in series using the same probe. Figure 8.3 depicts three neurons that were injected with FITC. The injection probe did not suffer any clogging, and a success rate greater than 90% was observed for similar experiments. The procedure has recently been extended by Guillaume-Gentil et al. towards direct intra-nuclear injection, where the penetration of the nuclear membrane could readily be observed in real-time from the force-distance spectrum [Guillaume-Gentil et al., 2013]. Transfection efficiency and post-procedure cell viability were determined to be equally high as when injecting into the cytosol alone.

Figure 8.3: Serial injection of viable cells via FluidFM. **Left:** Optical DIC micrograph of viable neuroblastoma cells upon intracellular FITC injection of the three centred cells. **Right:** Corresponding fluorescent signal image showing the three injected cells containing FITC. Adapted with permission from [Meister et al., 2009a], Copyright 2009 American Chemical Society.

Compared to traditional glass micropipette techniques, FluidFM offers a considerably higher success rate and improved cell viability. Furthermore, thanks to the precise
positioning capabilities of FluidFM, injection of sub-cellular structures is feasible without the need for highly trained operators. Of course the same method can also be applied to extract the contents of a cell in order to isolate it for further down-river analysis. Such extraction assays have the potential to become an important tool for omics and quantitative drug development studies that benefit from true single cell resolution.

8.2 Gentle contact regime - force controlled superficial interactions with cellular structures

In contrast to glass micropipette techniques, FluidFM also makes it very easy to engage with cells on their surface. Gentle contact interaction with cells without penetrating their cellular membrane further broadens the application portfolio offered by FluidFM: Gentle contact makes it possible to deliver membrane permeable dyes and particles (e.g., single virions [Stiefel et al., 2012]) to the surface of a cell with utmost positional accuracy. Potential applications range from spotting different reagents to specific surface areas to targeted infection of a cell with a controlled amount of pathogens. These capabilities enable causality studies that would be otherwise difficult or even impossible to achieve.

8.2.1 Force controlled staining of fixed cells

In order to demonstrate the feasibility of delivering a membrane permeable dye to an individual cell without compromising its integrity, a model system of NG108 (DIV2) neuroblastoma cells grown on a poly-L-lysine (PLL) coated glass surface was employed. Prior to contacting the cells with FluidFM, they were fixated using a solution of 4% formaldehyde for ten minutes. The fixed cells were subsequently washed and then re-immersed in pure HEPES-2 buffer to carry out the experiment. A solution of ethidium bromide\(^1\) (EtBr) 10 \(\mu\)g/mL in HEPES-2 was used as a staining agent inside the FluidFM probe. EtBr is a fluorescent dye that is commonly used to stain dead microbiological entities as they exhibit a strong red fluorescence signal when EtBr intercalates with the DNA and RNA present in such samples. The probes used for the experiment were pyramidal top aperture type A FluidFM probes.

The right of figure 8.4 shows how two neuroblastoma cells were stained using EtBr. The probe was approached to the cell surface and kept at a constant force setpoint, while an overpressure of 2 mbar was applied to the FluidFM probe via hydrostatic

\(^1\)3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide
8.2. Gentle contact regime - superficial interactions with cellular structures

**Figure 8.4:** Staining single fixed cells using FluidFM. **Left:** Schematic illustration of the staining procedure using gentle force. The FluidFM probe is approached to the cell surface, where a small overpressure is applied to drive the staining agent towards the cell surface. **Right:** Fluorescent microscopy image of two neuroblastoma cells that have been stained with EtBr in series using an overpressure of 2 mbar.

pressure control. In this way the staining dye was driven toward the membrane under the aperture of the pyramid, where it further diffused into the cytosol. Since only the engaged cells exhibited fluorescent signals and no background signals were found in the surrounding buffer media, it was determined that the seal between the FluidFM probe and the cell surface was sufficiently good to avoid any unwanted leakage of staining solution.

### 8.2.2 Minimally invasive staining of viable cells

In order to verify that gentle contact staining is also applicable to live samples, another experiment was carried out using CellTracker Green\(^2\) as staining agent. CellTracker Green is a membrane permeable, non-fluorescent dye that is metabolized into non-permeable reaction products by the target cell. The impermeable species exhibit fluorescent properties and can therefore be used to probe both successful delivery and viability of the engaged cells.

Image 8.5 shows a non-confluent layer of neuroblastoma cells grown on a glass slide. A FluidFM cantilever filled with CellTracker green was immersed in physiological buffer and gently approached to the cell marked with a red circle and left in constant force contact for ten minutes to allow the staining dye to diffuse into the cell under the cantilever before being retracted. After an incubation time of 15 min the cell was imaged using fluorescent microscopy techniques. As can be seen in figure 8.5, the manipulated cell successfully became fluorescent while the surrounding cells did not exhibit any fluorescent properties. This experiment therefore clearly demonstrates the ability

\(^2\)CMFDA - 5-chloromethylfluoresceindiacetate
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Figure 8.5: Gentle staining of single live cell via FluidFM technology. **Left:** Schematic illustration of the gentle contact procedure (not drawn to scale). **Right:** Situation after an incubation time of 15 min. The stained cell starts to exhibit fluorescent properties. This indicates that the staining procedure has been successful and the cell is still viable. Adapted with permission from [Meister et al., 2009a], copyright 2009, American Chemical Society.

of FluidFM to selectively target single cells with force control in the non-perforation regime. The processed cell was not fatally damaged and the staining procedure was only limited to the selected target cell without influencing any neighbouring entities.

8.2.3 Addressing sub-cellular structures

Thanks to the excellent high resolution imaging capabilities of FluidFM, it is further possible to target sub-cellular structures that are difficult or impossible to address using only optical control. First, using a type A FluidFM cantilever with a sufficiently sharp tip, an AFM image of the region of interest of the destination cell is acquired. Next, the high resolution image information is used to guide the FluidFM probe exactly onto the sub-cellular target structure. Once the desired location on the sample has been identified, it is possible to carry out the desired manipulation experiment.

In order to demonstrate these high precision capabilities of FluidFM, a sample with two neuroblastoma cells connected via a neurite exhibiting an axonal varicosity at their center junction was adducted. The upper left image in figure 8.6 shows a differential interference contrast micrograph of the described model system. Using the AFM scanning capabilities of FluidFM, an AFM profile of the sample was acquired and used to precisely position the probe above the varicosity, as shown on the lower left of figure 8.6. A solution of membrane permeable acridine orange\(^3\) was delivered directly to the target structure in a force controlled fashion and without damaging

\(^3\)3-N,3-N,6-N,6-N-Tetramethylacridine-3,6-diamine
8.3 Chapter summary and conclusion

Acridine orange is commonly used to stain nucleic acids such as DNA and RNA. It was suspected that such molecules are present in the targeted axonal varicosity structure. The right of figure 8.6 shows the resulting fluorescence signals after staining the target structure for 1 min with a pressure of 2 mbar. A strongly stained varicosity and an apparent fluorescent intensity that decreased along the neurite away from the varicosity was observed. It should be noted that the fluorescent signal is limited to the sub-cellular structure only and no fluorescent signal in the surrounding cells was observed. This confirms that FluidFM not only allows the targeting of very small structures, but also provides sufficient control over the spatial confinement of the delivered substances during the complete experiment.

![Figure 8.6: Staining sub-cellular structures with FluidFM. Left: Optical microscopy overview of the model system and corresponding AFM topography cross-section graph of the target structure. Right: Situation after staining the sub-cellular structure for 1 min using acridin orange dye. Adapted with permission from [Meister et al., 2009a], Copyright 2009 American Chemical Society.](image)

8.3 Chapter summary and conclusion

This chapter showed how FluidFM can serve as a highly versatile tool for use in several different manipulations of individual biological subjects such as eukaryotic cells and even sub-cellular structures.

Injection of single cells with live monitoring of the cellular membrane penetration was successfully demonstrated with FluidFM. AFM-based methods have already been successfully used to introduce various materials into the cytosol of a cell. However, FluidFM offers distinct advantages over these techniques, as there is a virtually infinite supply of cargo substance available. This also makes it possible to use the same probe...
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repeatedly for multiple deliveries without the need to refresh or renew the delivery probe.

Operation in a gentle contact fashion enables a wealth of novel applications, such as the study of viral infection mechanisms, that benefit from the minimally invasive and highly reproducible delivery mechanisms to the cellular membrane [Stiefel et al., 2012]. The ability to carry out all these manipulations under physiological conditions is a cornerstone of all FluidFM applications related to life sciences. Along with the precise force control and liquid delivery regulation, the technology has the potential to open the doors to many more original experiments in life sciences and related fields such as physics, chemistry, and material sciences.
The preceding chapters showed how FluidFM can be used for single cell manipulations and for the deposition of materials in liquid environments. All these applications benefit from the ability of the technology to work with fluids in a controlled manner. As highlighted in chapter 6, FluidFM has the potential to become an important tool for many other applications that have so far been carried out using techniques based on pulled glass micropipettes. The main benefits of FluidFM are that it offers similar functionality, while at the same providing additional value in the form a precise force feedback control from the underlying AFM.

Glass micropipettes are often also used for applications involving conductive species in solution, such as ions in the case of electrolytes. Techniques like scanning ion conductance microscopy (see section 6.2.2), scanning electrochemical microscopy (see section 6.2.3), and patch clamp (see section 6.2.1) are all based on some type of electrochemical setup integrated into a glass micropipette. For this reason, the ability to measure and induce ionic currents through the aperture of a microchannelled FluidFM probe is a highly interesting avenue to further broaden the application horizon of the technique.

This chapter will explore how suitable electrodes can be integrated into a FluidFM setup to enable the measurement of ionic currents. Furthermore, it will be demonstrated how this added degree of freedom can be exploited in the investigation of material properties on a heterogeneous sample.

9.1 Integrating Ag/AgCl electrodes into FluidFM

In order to be able to induce and measure ionic currents through the micro-channel and aperture of a FluidFM probe it is necessary to introduce a suitable electrochemical
setup into the overall FluidFM instrument composition. The most straightforward approach for this is to place an electrode inside the cantilever in order to form an electrochemical half cell. A secondary electrode can subsequently be placed outside of the probe in the form of a reference bath electrode or as a working electrode via a suitable substrate in order to complement the electrochemical cell and hence the electric circuit. Figure 9.1 illustrates this approach for the case of a reference electrode located in the electrolyte solution surrounding the FluidFM probe.

![Diagram](image)

**Figure 9.1:** Schematic illustration of a FluidFM setup with integrated electrodes. By placing an electrode into the reservoir of the microchannel and a second electrode into the surrounding liquid bath, an electric circuit is established. By applying a suitable electric potential between these two electrodes, an ionic current can be prompted to flow through the aperture of the probe.

For this thesis, an Ag/AgCl electrode was placed inside the FluidFM probe (see also section 2.4). This well-known type of electrode is often used as a reference electrode in electrochemical studies. Properties such as fast electrode kinetics and high stability independent of the directionality of the current flow accounts for the widespread use of this type of device. Furthermore, Ag/AgCl electrodes can be readily produced and renewed as necessary in order to maintain experimental quality.

### 9.1.1 FluidFM probeholder with conjugated electrode

The main problem with integrating electrochemical functionality into a FluidFM probe is the small size of the microfluidic channel. This makes it very difficult to place an electrode directly inside the cantilever without using microfabrication methods during the actual production of the probe.
9.1. Integrating Ag/AgCl electrodes into FluidFM

Electrode junction block

A first attempt to include an electrode in a FluidFM setup was achieved by placing a custom made electrode junction block into a version 1 probeholder, as explained in section 5.2.2. The left picture of figure 9.2 shows the electrode junction block that was placed between the external reservoir and the probeholder. The main problem with this approach is the placement of the electrode, which is far from the apex of the probe. When filled with electrolyte, the long tubing essentially acts as an antenna, picking up electromagnetic interference from the environment. In addition mechanical vibrations (e.g., from an air conditioning system) introduce small perturbations in the electrolyte filled tubing. These disturbances directly induce disturbing ionic currents and negatively impact the overall signal to noise ratio of the setup. Furthermore, this system suffers from extensive parasitic electronic components as shown via impedance spectroscopy measurements inside a Faraday cage. By fitting the curve to an R(RC) model circuit, an overall serial resistance of 2.5 MΩ and a parasitic capacitance of 47 pF was extracted in the case of a type B tipless cantilever. The parasitic capacitance of the system should be kept as low as possible to allow measurement of fast changing current signals. More details about the electrical properties of this electrode setup are provided in reference [Ossola, 2010].

Figure 9.2: Probeholder designs with integrated electrode. **Left:** Electrode junction block used for the first generation FluidFM probeholder. The electrode is placed between the probeholder and the external reservoir. **Middle and Right:** Modified commercial FluidFM probeholder. The electrode is integrated into the back connector. When the connector is attached to the probeholder, the electrode is in contact with the cargo fluid inside the reservoir.

Back connector with electrode access port

In order to improve the performance of the combined FluidFM electrochemistry setup, a novel design based on a commercial FluidFM probeholder (see section 5.2.2) was created. In this case, the electrode was placed into the liquid reservoir integrated
into the probeholder clips. To achieve this, a thin silver wire was used to form a custom made Ag/AgCl electrode (for details, see section 2.4). This electrode was subsequently integrated into the pneumatic back connector used to join and seal the reservoir of the probeholder to an external pressure control device. Figure 9.2 illustrates this approach. The wire electrode is placed such that it is always fully immersed in the fluid inside the reservoir. By isolating the silver wire from the actual Ag/AgCl surface, the area in contact with the liquid always remains constant, even when the space occupied by the reservoir cargo liquid is slightly changed during an experiment. This is an important feature, since a varying electrode area would result in hard to interpret non-linear behaviour of the electrochemical setup. In such a configuration, the general performance of the system as an electrochemical probe proved to be high enough to carry out experiments involving tiny ionic currents. By means of impedance spectroscopy, it was revealed that the parasitic capacitance of this configuration could even be reduced further by only coating a small portion of the reverse of the backside with a reflective metal layer.

To ensure an electrically tight seal, an additional layer of melted paraffin wax was applied over the back connector in order to hermetically close off the reservoir chamber from the surrounding bath electrolyte. This coating can be easily removed and re-applied for every experiment as required and allows it to operate the tool without any leaking currents, even when fully immersed in liquid.

Numerical simulation of the probe further revealed that the majority of the remaining serial resistance of the setup is caused by the macroscopic length of the microfluidic channel leading from the reservoir to the aperture and not from the aperture itself. A more optimized electrode configuration is therefore still considered feasible via direct integration of the electrode into the cantilever during microfabrication. Nevertheless, the setup used proved adequate to carry out the original experiments, as will be shown in the following sections.

### 9.2 Concurrent AFM imaging and local ion conductance mapping

AFM with simultaneous mapping of the local ion-conductance is the simplest application of FluidFM with integrated electrodes. For this purpose, commercial type B FluidFM probes with a pyramidal top aperture of 300 nm diameter were used in combination with an electrode enabled back connector. The ionic current signals were measured with a PicoAmp 2 patch clamp amplifier from Tecella Inc. (USA). They were recorded together with the AFM topography by connecting the current
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signal to an auxiliary signal input of an easyscan 2 AFM controller from Nanosurf AG (Switzerland). AFM imaging was carried out using the software supplied by the instrument provider.

The FluidFM probes were back filled with a suitable electrolyte cargo liquid before each experiment. Proper electrochemical operation and admissibility was confirmed by immersing the probe in a liquid bath and testing it using suitable excitation signals, as described in section 2.4. Once the probes were confirmed to be functional within the expected ranges, the setup was ready for actual engagement of the sample via the AFM measurement modes. AFM scanning could thus be performed in both contact and dynamic mode imaging, depending on the experiment. The measured ionic currents were only used as a passive drag-along signal and never as a physical quantity for direct feedback control of the cantilever during imaging.

9.2.1 Static mode AFM topography with ionic current mapping

To goal of the first experiment was to demonstrate that an electrode enabled FluidFM setup could be used to map ion currents depending on the conductivity properties exhibited by the substrate material. In order to have a clear contrast, a system using both a conducting and isolating surface was determined to be ideal. Hence, the first model system consisted of a patterned layer of semi-conducting indium-tin oxide (ITO) evaporated on a glass microscopy coverslip, as shown in figure 9.3A.

![Figure 9.3: Static mode AFM topography with ionic current mapping. A: Schematic illustration of the experimental setup. B: AFM topography image acquired in contact mode. C: Corresponding ionic current map image. The regions of the insulator and ITO can be clearly distinguished. D: Histogram of the current map image. Three distinct levels of ion conductivity are present.](image)

The substrate was immersed in HEPES-2 buffer and the same solution was used as cargo fluid inside the hollow cantilever to avoid any unwanted liquid junction potentials. Except for a small region in the middle of the sample (floating electrode), the ITO coating itself was used as an electrode and held at ground potential. The
Ag/AgCl electrode placed inside the reservoir of the FluidFM probe was biased at 100 mV with respect to the grounded part of the ITO. The cumulative serial resistance of the solution, ITO, and pipette was measured at 33 MΩ far away from the substrate. AFM imaging was carried out in contact mode with a force setpoint of 4 nN. A square image of 60 μm x 60 μm was acquired with a tip velocity of 20 μm/s over a raster of 128 lines while recording 1024 samples/line. The resulting AFM topography image is presented in figure 9.3B along with the recorded current map as shown in image 9.3C. This data was acquired during the forward trace of the AFM imaging. The location of the ITO electrode layer is clearly visible from the topographical information recorded by the AFM. They are also consistent with the pattern observed in the ionic conductivity map. It can be seen that conducting areas of the sample yielded a different DC current through the aperture of the probe than the non-conducting domains. In addition, it should be noted that the current signal recorded on the floating ITO electrode exhibited a current difference with respect to the glass surface of 12 pA. The grounded ITO, on the other hand, exhibited a difference of 30 pA for the corresponding current magnitude. This clear distinction in current levels is also confirmed by the histogram of the ionic conductivity image, as shown in figure 9.3D. Three distinct levels of current responses could be identified that correspond to the three different regions of the sample (glass, floating ITO, grounded ITO).

The current response may be influenced by the dynamic rearrangement of the electric double layer underneath the probe aperture and other non-linear effects. Because of the small size of the aperture of the FluidFM probe, a substantial share of the overall potential drop between the two electrodes happens in this small section of the cantilever [Ying et al., 2004]. Therefore, the associated electric field gradients near the aperture are expected to be much larger than in the microfluidic channel and in the surrounding bath solution. Since the probe is moved over the sample at a continuous velocity, dynamic charging currents due to capacitive effects are expected to occur in the vicinity of a conductive substrate. The acquired current signal must therefore be interpreted in the context of a transient phase response of the sample to a highly localized perturbation of the equilibrium state. An influence of the measured current depending on the applied potential and the type of surface is therefore expected when measuring with nanopipettes [Donnermeyer, 2007, Lipson et al., 2011]. This effect could even be exploited towards extended functional imaging of surface charge effects [McKelvey et al., 2014].

Upon comparing both the forward- and the backward-trace measurements of the ionic current signal, it was observed that the ion current response was strongly dependent on the direction of movement of the probe. This effect is due to the lateral forces acting on the cantilever during contact mode imaging, as confirmed via lateral force
9.2. Concurrent AFM imaging and local ion conductance mapping

imaging. These lateral perturbations result in small torsional deformations of the FluidFM probe that has a direct influence on the gap size between the sample and the aperture. Since the diffusion of ionic species is directly influenced by the size of this gap, a strong directionality of the measured ionic current response is observed.

9.2.2 Force modulation and concurrent ion conductivity imaging

Because of the pronounced directional dependence observed in the ionic current map signal when imaging in AFM contact mode, only limited lateral resolution could be attributed to the measured current maps. For this reason, the acquisition of the AFM topography information was carried out using dynamic mode operation, namely force modulation mode imaging. Force modulation mode is a secondary AFM imaging mode that can be used to identify and map differences in material elasticity that would occur in heterogeneous substrates (see also section 3.3.3).

Force modulation mode imaging was applied to another model system which consisted of a 130 nm thick gold electrode fabricated on a Borofloat 33 glass wafer substrate from University Wafer Inc. (USA) that was uniformly coated with 12 nm niobium pentoxide (\( \text{Nb}_2\text{O}_5 \)). The gold electrode was partially passivated using SU-8. For this experiment, the substrate was immersed in a solution of 150 mM KCl. The same solution was used inside the hollow AFM probe as before. The gold layer of the substrate was used as an electrode and held at ground potential. The other electrode inside the hollow probe was biased at 100 mV with respect to the gold electrode. AFM imaging was performed over a 100 \( \mu \text{m} \times 100 \mu \text{m} \) area with a force set point of 10 mV. The excitation frequency of the cantilever for the local force modulation was set near the cantilever resonance frequency in liquid at 35.9 kHz using an excitation amplitude of 40 mV.

Figure 9.4 shows the acquired AFM images. Image 9.4A depicts the sample topography. The three distinct regions of the sample, namely the SU-8 passivation, gold electrode and glass substrate wafer can be clearly distinguished. 9.4D shows an example line scan along the red line of figure 9.4A. Because the sample has been scanned using force modulation mode, additional information on the relative stiffness properties of the sample could also be recorded. Image 9.4B shows the corresponding amplitude image. Regions exhibiting higher relative stiffness properties (higher Young's modulus) result in relatively larger amplitude responses. Again, the three distinct regions of the sample are nicely resolved. This is further confirmed when looking at the corresponding line scan in figure 9.4E. Gold exhibits the highest Young's modulus of the three materials, whereas SU-8 is known to be much softer than gold and glass. Last but not least, a mapping of local ionic conductivity was also measured and is shown in figure 9.4C.
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Figure 9.4: Concurrent force modulation and ion conductance imaging by means of FluidFM. A: AFM topography scan of a model sample consisting of three distinct materials: SU-8 passivation and gold electrode sitting on top of a niobium pentoxide coated glass wafer. B: AFM amplitude signal from force modulation mode imaging. C: Corresponding ionic current map image. The three distinct material areas are clearly resolved. D-F: Trace and retrace signal of a single line scan along the red line in the corresponding 2D maps for all three parameters: topography, elasticity, and local ionic current.

Along with the associated line scan graph in figure 9.4F. This data also nicely resolves the three different materials. As expected, the highest ionic conductivity was measured over the gold electrode, followed by the glass layer which was coated with thin layer of semiconducting niobium pentoxide. The smallest ionic conductivity was observed on the insulating SU-8 layer. In figures 9.4C and F, lower voltages correspond to higher currents because of the inverting current to voltage amplifier used.

Looking at both the forward and backward trace signals of the ionic current response, good compliance between the two signals was observed. Compared to contact mode AFM imaging, the lateral resolution of the current map is therefore much improved when operating the AFM in dynamic mode. In addition, the stiffness map is consistent with the elastic modulus values reported in literature for the three different materials. Therefore it is concluded that a true multiparameter imaging of different substrate properties is feasible using a hybrid FluidFM/ion-conductance instrument setup.
9.3 Multiparameter surface imaging using FluidFM

After validating the instrument for the acquisition of multiple material properties on two known model substrates, the technique was applied to investigate a more complex sample: Examination of the surface properties of conductive polydimethylsiloxan (PDMS) electrodes. The electrodes were obtained by integrating a ratio of 23 wt% silver (Ag) colloids into a PDMS matrix (Ag-PDMS). By embedding an optimal ratio of silver colloids into a PDMS matrix, the materials start to become electrically conductive. This composite material has already been successfully used in flexible neuroprosthetic implants for epidural electrical stimulation of the spinal cords in animal models [Larmagnac et al., 2012, 2014]. However, the exact structure of the electrode surface and how this translates into electrical properties of such electrodes is still being investigated. Figure 9.5A shows a scanning electron microscopy image of an example Ag-PDMS electrode surface. From the topographical information in the SEM image, it is clear that parts of the silver microparticles are present on the electrode surface where they often form small clusters. However, it cannot be determined from this image whether these clusters are still covered by PDMS or not. To understand how such electrodes function, it is important to be able to distinguish whether the particles are exposed on the surface or still embedded in the PDMS matrix. As such electrodes are typically applied in an aqueous environment, their properties have to be investigated under similar conditions. Established methods such as conductive AFM are therefore not feasible, since the surrounding liquid would effectively short-circuit the entire measurement setup.

For this reason, the FluidFM with integrated electrodes was applied for the inspection of different locations on the electrode surface in order to better understand the
working principles of such Ag-PDMS devices. All measurements were carried out using a solution of 150 mM KCl, both as the bulk electrolyte and cargo substance inside the hollow probe. The Ag-PDMS served as electrode and was held at ground potential. The Ag/AgCl electrode inside the FluidFM probe was biased at 100 mV with respect to ground. Imaging was always carried out in force modulation mode in order to simultaneously collect topographic, elastic, and conductivity information from the sample, as explained in the preceding section. A schematic representation of the measurement setup is shown in figure 9.5B.

**Figure 9.6:** Multiparameter overview scan of an Ag-PDMS electrode. A: Topography image of the Ag-PDMS electrode surface. The silver particles on the surface mostly occur in clustered groups. B: Stiffness map obtained from force modulation mode imaging. Exposed particles exhibit higher relative stiffness than particles covered by PDMS. C: Corresponding ionic conductivity map. Higher conductivity is mostly observed in areas where silver particles are present.

Image 9.6 depicts the topography of an Ag-PDMS electrode (9.6A) along with the relative stiffness map (9.6B) and corresponding local ion current signal (9.6C). It can be seen that not all topographic features of similar height exhibit the same conductive and elastic properties. By taking a closer look at individual particles it becomes clear that there is a distinct correlation between the local stiffness of the sample and the corresponding conductivity of the material. As can be seen from a close-up scan as shown in image 9.7A-C, particles of similar height are more likely to feature ionic current signatures corresponding to conducting surfaces if they show higher relative stiffness. Softer areas of the sample typically do not exhibit any elevated conductivity signatures. The same behaviour was observed in other sample locations.

One possible interpretation of these results could be that the exposed silver particles on the surface of the electrode are sometimes still partially or even completely embedded in the PDMS matrix. Therefore, only a limited part of the total surface area exhibits conducting properties while the rest is highly insulating, as is the case for pure PDMS. To further highlight this, the current map was converted into a grayscale image and the stiffness map transformed into a binary image via thresholding. By subsequently applying a pixel-wise logical conjunction operation on these modified
9.3. Multiparameter surface imaging using FluidFM

Figure 9.7: Analysis of Ag-PDMS multiparameter scanning. A: Detail view of the Ag-PDMS surface. Two particles of similar height are marked with circles. B: Relative stiffness map of the same region. The two particles are seen to exhibit different stiffness properties. The particle in the white circle is softer than the particle marked with a red circle. C: Corresponding ionic conductivity image. Again the two particles are marked. It can observed that stiffer particles in the red circle exhibit an elevated ionic conductance signature corresponding to a conducting surface. D: Overview image with overlaid heat map. Regions of both high relative stiffness and elevated ionic conductivity are overlaid on the topographical image of the sample. It can be seen that regions where both conductivity and stiffness are elevated are almost entirely limited to areas where particles are clustered on the surface.

images, it was possible to find areas of the sample with both higher relative current signals and higher relative stiffness values. Figure 9.7D shows the results obtained with these image processing operations overlaid onto the topography image of the electrode surface. From this image it can be seen that areas featuring both conductive behaviour and high relative stiffness are almost entirely limited to areas with pronounced silver particle agglomerates. At the same time, areas with predominantly softer material properties are seen to exhibit only limited conductive signatures. These results provide a strong indication that the electric performance of the custom made Ag-PDMS electrodes is mainly due to the partially exposed silver particles protruding through the PDMS matrix, rather than due to cracks in its surface. It is also apparent that these regions are all found on the upper right edges of such structures. This could be due to the highly directional stencil- and screen-printing techniques that were used to fabricate these Ag-PDMS electrodes [Larmagnac et al., 2014].

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9.4 Chapter conclusion and outlook

By integrating a suitable electrode setup into a standard FluidFM system, the application scope of the technique can be greatly extended. By applying such electrodes to the acquisition of ionic current signals during normal AFM imaging, true multi-parameter imaging can be achieved. It was demonstrated how FluidFM be used in this way to inspect topographical, mechanical, and electrical properties of a sample simultaneously within a single scan.

Comparing an electrode enabled FluidFM setup with similar techniques based on glass micropipettes, it is clear the technique can be further exploited to cover special applications such as force controlled patch clamp [Ossola et al., 2015], SICM, and scanning electrochemical microscopy within the same device. Hybrid systems that can dynamically switch between different imaging techniques in order to work with the most suitable method for the sample at hand are envisioned. One possible application of such a setup would be AFM-assisted hopping mode SICM to enable faster image acquisition without the risk of damaging the sample.
The primary goal of this thesis was to develop a working fluidic force microscope setup and to explore its potential by means of multiple feasibility studies. For this purpose, the functional building blocks needed to build such an instrument were elaborated. The evolution from the initial prototypes towards a disposable, easy to use cantilever probeholder assembly was described in detail, along with how this impacts the performance of the overall instrument. At the same time, the main properties of FluidFM technology along with its major operational modes were elaborated in chapter 5. Hollow AFM probes were identified as a core component of FluidFM technology. In chapter 4, different approaches to the fabrication of such probes using silicon-based materials were explained and several strategies for the definition of sub-micrometer sized apertures were discussed. Considerable effort was put into the development of FluidFM probes made completely from photoplastic SU-8 material. For this purpose, several fabrication strategies for producing such probes were explored.

In the second part of this work, several feasibility studies were carried out to explore different application centered features of fluidic force microscopy. It was shown how FluidFM can be employed as a powerful tool for localized dispensing of substances in a liquid environment (chapter 7). Furthermore, the technology was applied for different manipulations on individual, viable cells. Applications such as force-controlled microinjection and gentle staining of biological entities were successfully demonstrated for this purpose (chapter 8). In order to complement the application portfolio of FluidFM technology and to bring it on a par with classical glass micropipettes, an electrochemical measurement setup was integrated into a FluidFM setup. Such a hybrid system was shown to enable measurements of tiny ionic currents when immersed in a suitable electrolyte. The properties of this electrochemical FluidFM instrument were investigated on a pair of well-known model substrates. From these results, the method could be applied for the investigation of a custom-made PDMS-based electrode that...
has proven to be very hard to image with established techniques such as conductive AFM (chapter 9).

In summary, it was concluded that FluidFM technology is a powerful and versatile tool that is expected to spur a multitude of novel applications based on AFM technology in the field of life sciences and beyond. The integration of liquid handling capabilities directly into the sensing element of an atomic force microscope adds a whole new degree of freedom to the ever-growing variety of AFM inspired instrumentation. At the time of writing, FluidFM technology has already resulted in a number of additional research projects that are focused on novel applications and implementations of this promising tool.

With respect to the fabrication of hollow AFM probes made entirely from SU-8, the demonstrated fabrication strategies can be further improved. Alternative methods for defining the microfluidic channel based on a fully metallic sacrificial layers made from electroplating are envisioned. Such probes could, for example, be used in future to allow for the exploitation of cargo substances such as hydrofluoric acid that are incompatible with FluidFM probes made from silicon derived oxide materials.

The potential for FluidFM technology to be used as a utility in maskless lithography processes has been readily demonstrated by [Grüter et al., 2013]. It is now further investigated for its ability to direct the growth of biological cells towards the realization of specific neural network structures [Dermutz et al., 2014]. Using FluidFM as a tool for direct chemical and mechanical stimulation of neurons enables novel research vectors towards a better understanding of how neural networks process information on both a physical and mechanistic level.

The ability to dispense liquid droplets with high precision renders the technique interesting for research applications in the field of arraying technologies. The capability of the device to work in liquid has the potential to leverage the creation of high density protein arrays within their native environment. Furthermore, the study of the basic physical properties behind the formation of droplets is an interesting field for future research.

Microbiological applications of FluidFM have seen much effort for continuing studies. Since its invention, FluidFM has proven a highly flexible tool for spatial manipulations of biological objects such as adherent cells [Guillaume-Gentil et al., 2014], viruses [Stiefel et al., 2012], and yeast [Dorig et al., 2010]. Ongoing research in the areas of single cell force spectroscopy [Potthoff et al., 2012], bacterial adhesion [Potthoff et al., 2015], and single cell metabolomics are just some examples for such advanced studies.
The possibility to integrate an electrode into a FluidFM setup is expected to further enable a multitude of novel applications. For example, the technology could be modified to function as a scanning ion conductance microscope (SICM) with the capability for real-time in situ switching between SICM and AFM based imaging modes. The integration of electrodes further enables the tool to function similarly to scanning electrochemical microscopy. With such an imaging technique, it is possible to investigate local chemical properties of heterogeneous substrates via specific redox reactions. Using a similar approach, it is also expected to be feasible to carry out electrodeposition experiments using FluidFM with high spatial resolution. Last but not least, the creation of a force controlled patch clamp setup is also a highly interesting field for further studies with FluidFM [Ossola et al., 2015]. It shows great potential to simplify the cumbersome handling of this complex experimental technique.
Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Y. Zhang, R. McLaughlin, C. Goodyer, and A. LeBlanc. Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary

List of publications & student projects

This section denotes all publications and student projects contributed by the author. The results presented in this thesis are mostly based on these reports.

Papers

- **Multiparameter surface imaging using micro channeled hollow AFM probes with conjugated Ag/AgCl electrode**
  
P. Behr, D. Ossola, A. Larmagnac, J. Vörös, T. Zambelli
  
  *Manuscript in preparation*

- **Force-controlled patch clamp of beating cardiac cells**
  
  D. Ossola, M.-Y. Amarouch, P. Behr, J. Vörös, H. Abriel and T. Zambelli
  
  *Nano Letters, February 2015, doi: 10.1021/nl504438z*

- **Cooperative Vaccinia Infection Demonstrated at the Single-Cell Level Using FluidFM**
  
P. Stiefel, F. I. Schmidt, P. Dörig, P. Behr, T. Zambelli, J. A. Vorholt, J. Mercer
  
  *Nano Letters, 2012, 12 (8), 4219–4227*

- **Force-controlled spatial manipulation of viable mammalian cells and microorganisms by means of hollow AFM cantilevers**
  
P. Doerig, P. Stiefel, P. Behr, E. Sarajlic, D. Bijl, M. Gabi, J. Vörös, J. Vorholt, T. Zambelli
  
  *Applied Physics Letters 97, 023701 (2010)*

- **FluidFM: Combining Atomic Force Microscopy and Nanofluidics in a Universal Liquid Delivery System for Single Cell Applications and Beyond**
  
  
  *Nano Letters, 2009, 9 (6), 2501–2507*

- **Nanoscale dispensing in liquid environment of streptavidin on a biotin functionalized surface using hollow atomic force microscopy probes**
Bibliography

Microelectronic Engineering, Volume 86, Issues 4-6, April-June 2009, Pages 1481-1484

Patents

• Method for spatially manipulating a microscopic object and device for conducting said method
Gabi, M. and Vörös, J. and Doerig, S.P. and Behr, P. and Stiefel, P. and Zambelli, T. and Vorholt-Zambelli, J.

• A probe arrangement for exchanging in a controllable way liquids with micro sized samples of material like biological cells
Gabi, M. and Vörös, J. and Zambelli, T. and Behr, P.

• Touch-screen based scanning probe microscopy (SPM)
Gabi, M., Behr, P., Jud, S., Wolf, J., Weber, C.

Conference talks

• Combining AFM with hollow cantilevers for electrophysiological measurements
  P. Behr, D. Ossola, P. Doerig, P. Stiefel, M. Gabi, E. Sarajlic, D. Bijl, J. Vörös, T. Zambelli
  ISPM 2010, Sapporo, Japan, May 12, 2010

• FluidFM: A novel AFM-based tool for single cell experiments
  WACBE World Congress on Bioengineering 2009, 26 – 29 July 2009; The Hong Kong Polytechnic University, Hong Kong

• AFM-controlled injection of labelled molecules into single living cells
  P. Behr, M. Gabi, P. Studer, A. Meister, M. Liley, J. Przybylska, H. Heinzelmann, J. Vörös, T. Zambelli
  ESF Conference on Nanomedicine, San Feliu de Guixols, Spain, September 2008
Conference poster presentations

• **Combining AFM with hollow cantilevers for electrophysiological measurements**
  
  
  *AFM BioMed 2010, 12-15.05.2010, Rovinj, Croatia*

• **Controlled displacement of mammalian cells and microorganisms by FluidFM technology**
  
  P. Dörig, P. Stiefel, E. Sarajlic, D. Bijl, P. Behr, M. Gabi, J. Vörös, J. Vorholt, T. Zambelli
  
  *AFM BioMed 2010, 12-15.05.2010, Rovinj, Croatia*

• **FluidFM Technology: A novel AFM-based tool for single cell experiments and beyond**
  
  
  *ESB 2009 - 22nd European Conference on Biomaterials, 07-11.09.2009, Lausanne, Switzerland*

• **FluidFM: A novel AFM-based tool for single-cell experiments**
  
  
  *7th International Symposium on Scanning Probe Microscopy in Life Sciences, Oct. 8-9 2008, Berlin, Germany*

• **AFM-controlled injection of labelled molecules into single living cells**
  
  

• **AFM-controlled injection of labelled molecules into single living cells under physiological conditions**
  
  
  *3rd International Workshop on Approaches to Single-Cell Analysis, September 11th & 12th 2008, ETH Zurich*
Awards & prizes

• **ZKB Pionierpreis Technopark Zürich 2012** Cytosurge AG, ausgezeichnet für ihre wegweisende Entwicklung und pionierhafte Markteinführung der FluidFM Technologie. Der Preis ist mit dem 10'000fachen Wert der Zahl Pi Quadrat dotiert: CHF 98’696.04. Der Pionierpreis ist einer der wichtigsten Innovationspreise der Schweiz. (Source: www.pionierpreis.ch)

• **MRC Poster Award 2010**, Controlled displacement of mammalian cells and microorganisms by FluidFM technology, *AFM BioMed 2010*, 12.5.-15.05.2010, Rovinj, Croatia

• **Best Poster Award**, Hollow AFM Probes Designed for Nanoscale Dispensing of Material in a Liquid Environment for Applications in Life Science, *7th International Symposium on Scanning Probe Microscopy in Life Sciences*, Berlin, October 2008, poster in collaboration with CSEM AG (Switzerland).

Student projects

For the duration of this thesis, several student projects have been supervised by the author. The results obtained in those works are also included in this thesis.

**Johann Wolf**, Masterthesis
Robust SPM Probe Tracking, 2013

**Dario Ossola**, Masterthesis
Development of a force-controlled patch-clamp setup, 2010

**Pablo Döring**, Masterthesis
SkeletonFM: A custom low-cost AFM for FluidFM routines, 2009
A Photolithographic processing of SU-8

In this appendix chapter, all the relevant information regarding the photo-lithographic processing of SU-8 as utilized in this work is provided as a reference to the interested reader.

A.1 T-Cell code for parametric photomask generation

In order to be able to create the geometrical information for the different photomasks, a custom T-Cell script has been elaborated. This T-Cell script can be seen as a parametric description language, capable of producing geometrical information for the creation of a suitable photo-mask based on one or more parameters. For the purpose of this work, a script with four parameters has been programmed: Aperture size a, Cantilever width W, Cantilever length L and wall thickness d. Using this script, many different variations of probe design were generated for the final photo-mask. A complete listing of the script is provided below. The script has been written using the integrated editor of L-Edit Software (see also section 2.2.2).

```c
/***********************
* Cell Name: Cell1 - Parametric SU-8 Probe T-Cell
* Creator : Pascal Behr (behr@biomed.ee.ethz.ch)
* Date: October 2006
*
***************************************************************************/

module Cell1_code
{

#include <stdlib.h>
#include <nath.h>
#include <string.h>
#include <stdio.h>
#include <1data.h>
#include <lcomp.h>

```
Appendix A. Photolithographic processing of SU-8

```c
void Cell1_main(void)
{

  LCell  cellCurrent = (LCell)LMacro_GetNextTCell();
  int   a = (int)LCell_GetParameter(cellCurrent, "a")*1000;
  int   w = (int)LCell_GetParameter(cellCurrent, "w")*1000;
  int   L = (int)LCell_GetParameter(cellCurrent, "L")*1000;
  int   d = (int)LCell_GetParameter(cellCurrent, "d")*1000;

  LC_InitializeState();
  LC_CurrentCell = cellCurrent;
  LFile pFile = LCell_GetFile( LC_CurrentCell );

  /* Clear out existing elements */
  LLayer player;
  for ( player = LLayer_GetList( pFile ); player; player = LLayer_GetNext( player ) )
  {
    LObject pObj;
    LObject pObj_next;
    for ( pObj = LObject_GetList( cellCurrent, player ); pObj; pObj = pObj_next )
    {
      pObj_next = LObject_GetNext( pObj );
      LObject_Delete( cellCurrent, pObj );
    }
  }
  /* Clear out existing ports */
  LPort pPort, pPort_next;
  for ( pPort = LPort_GetList( cellCurrent ); pPort; pPort = pPort_next )
  {
    pPort_next = LPort_GetNext( pPort );
    LPort_Delete( cellCurrent, pPort );
  }
  /* Clear out existing instances */
  LInstance pInst;
  LInstance pInst_next;
  for ( pInst = LInstance_GetList( cellCurrent ); pInst; pInst = pInst_next )
  {
    pInst_next = LInstance_GetNext( pInst );
    LInstance_Delete( cellCurrent, pInst );
  }

  /* TODO: Begin custom generator code.*/
  LCoord x;
  LCoord y;
  LCoord xcount;
  LCoord ycount;
  pLayer = LLayer_Find( pFile, "marks B SUB Bottom" );
  {
    LPoint ptArray[16];
    ptArray[0].x = -1000000; ptArray[0].y = -1250000;
    ptArray[1].x = 600000; ptArray[1].y = -1250000;
    ptArray[2].x = 600000; ptArray[2].y = -800000;
  }
```

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A.1. T-Cell code for parametric photomask generation

```c
                ptArray[3].x = 0; ptArray[3].y = -W/2;
                ptArray[4].x = -L; ptArray[4].y = -W/2;
                ptArray[5].x = -L; ptArray[5].y = W/2;
                ptArray[6].x = 0; ptArray[6].y = W/2;
                ptArray[7].x = 600000; ptArray[7].y = 800000;
                ptArray[8].x = 3800000; ptArray[8].y = 800000;
                ptArray[9].x = 3800000; ptArray[9].y = -800000;
                ptArray[10].x = 600000; ptArray[10].y = -800000;
                ptArray[12].x = 4000000; ptArray[12].y = -1250000;
                ptArray[13].x = 4000000; ptArray[13].y = 1250000;
                ptArray[14].x = -1000000; ptArray[14].y = 1250000;
                LPolygon_New( LC_CurrentCell, pLayer, ptArray, 16 );
            }
            LBox_New( LC_CurrentCell, pLayer, (-L+d+100000, -a/2, -L+d+100000+a, a/2 );
            pLayer = LLayer_Find( pFile, "marks B LUR" );
        }
        }
        LPoint ptArray[563]; // create circular shape
        ptArray[0].x = 1000000; ptArray[0].y = -1250000;
        ptArray[1].x = 4000000; ptArray[1].y = -2500000;
        ptArray[2].x = 4000000; ptArray[2].y = 2500000;
        ptArray[3].x = -1000000; ptArray[3].y = 2500000;
        ptArray[4].x = -1000000; ptArray[4].y = W/2-d;
        ptArray[5].x = 2000000; ptArray[5].y = W/2-d;
        ptArray[6].x = 2200000; ptArray[6].y = 2000000;
        ptArray[7].x = 1401014; ptArray[7].y = 2000000;
        ptArray[8].x = 1401200; ptArray[8].y = 218400;
        ptArray[9].x = 1401440; ptArray[9].y = 238200;
        ptArray[10].x = 1401680; ptArray[10].y = 257580;
        ptArray[12].x = 1402220; ptArray[12].y = 287800;
        ptArray[13].x = 1403260; ptArray[13].y = 336600;
        ptArray[14].x = 1403230; ptArray[14].y = 356600;
        ptArray[15].x = 1403560; ptArray[15].y = 376600;
        ptArray[16].x = 1403940; ptArray[16].y = 396000;
        ptArray[17].x = 1404360; ptArray[17].y = 414600;
        ptArray[18].x = 1404780; ptArray[18].y = 434000;
        ptArray[19].x = 1405220; ptArray[19].y = 453400;
        ptArray[20].x = 1405680; ptArray[20].y = 472800;
        [...]; //not all point shown for space saving reasons
        ptArray[564].x = 1401680; ptArray[564].y = -267800;
        ptArray[565].x = 1401440; ptArray[565].y = -239200;
        ptArray[566].x = 1401200; ptArray[566].y = -210400;
        ptArray[567].x = 1401014; ptArray[567].y = -200000;
        ptArray[568].x = 2200000; ptArray[568].y = -200000;
        ptArray[569].x = 2000000; ptArray[569].y = -W/2+d;
        ptArray[570].x = -L/4; ptArray[570].y = -W/2+d;
        ptArray[571].x = -L/4; ptArray[571].y = W/2-d;
        ptArray[572].x = -1000000; ptArray[572].y = W/2-d;
        LPolygon_New( LC_CurrentCell, pLayer, ptArray, 563 );
        }
        pLayer = LLayer_Find( pFile, "marks B SUB Top" );
        }
        LPoint ptArray[15];
        ptArray[0].x = -1000000; ptArray[0].y = -1250000;
        ptArray[1].x = 6000000; ptArray[1].y = -1250000;
        ptArray[2].x = 6000000; ptArray[2].y = -800000;
```
ptArray[3].x = 0; ptArray[3].y = -W/2;
ptArray[4].x = -L; ptArray[4].y = -W/2;
ptArray[5].x = -L; ptArray[5].y = W/2;
ptArray[6].x = 0; ptArray[6].y = W/2;
ptArray[7].x = 600000; ptArray[7].y = 800000;
ptArray[8].x = 3800000; ptArray[8].y = 800000;
ptArray[9].x = 3800000; ptArray[9].y = -800000;
ptArray[10].x = 600000; ptArray[10].y = -800000;
ptArray[12].x = 4000000; ptArray[12].y = -1260000;
ptArray[13].x = 4000000; ptArray[13].y = 1250000;
ptArray[14].x = -1000000; ptArray[14].y = 1250000;
LPolygon_Nev( LC_CurrentCell, pLayer, ptArray, 15 );
}

LPolygon_Nev( LC_CurrentCell, pLayer, ptArray, 192 );

LPolygon_Nev( LC_CurrentCell, pLayer, ptArray, 192 );
A.2. Listing of all used photo-lithography masks

```c
ptArray[0].x = 1400000; ptArray[0].y = 0;
ptArray[1].x = 1400200; ptArray[1].y = -6400;
ptArray[2].x = 1400400; ptArray[2].y = -12600;
ptArray[3].x = 1401000; ptArray[3].y = -19000;
ptArray[4].x = 1401600; ptArray[4].y = -25200;
ptArray[5].x = 1403600; ptArray[5].y = -37600;
ptArray[6].x = 1406000; ptArray[6].y = -43800;
ptArray[7].x = 1406400; ptArray[7].y = -49800;
ptArray[8].x = 1408000; ptArray[8].y = -55800;
ptArray[9].x = 1409800; ptArray[9].y = -61800;

[...] // not all point shown for space saving reasons

ptArray[183].x = 1409800; ptArray[183].y = 61800;
ptArray[184].x = 1408000; ptArray[184].y = 55800;
ptArray[185].x = 1406400; ptArray[185].y = 49800;
ptArray[186].x = 1405000; ptArray[186].y = 43800;
ptArray[187].x = 1403600; ptArray[187].y = 37600;
ptArray[188].x = 1401600; ptArray[188].y = 25200;
ptArray[189].x = 1401000; ptArray[189].y = 19000;
ptArray[190].x = 1400400; ptArray[190].y = 12600;
ptArray[191].x = 1400200; ptArray[191].y = 6400;
LPolygon_Navi( LC_CurrentCell, pLayer, ptArray, 192 );

L Magnification mag; mag.num = 50; mag.denom = 26;
LTransform_Ext99 trans = LTransform_Set_Ext99( 3400000, 700000, 0, mag );
LPoint repeat_cnt = LPoint_Set( 1, 1 );
LPoint delta = LPoint_Set( 319231, 17308 );
LCell pInstCell = LCell_Find( pFile, "type_single_600_10_2" );
LInstance_New_Ext99( LC_CurrentCell, pInstCell, trans, repeat_cnt, delta );

L Magnification mag; mag.num = 5; mag.denom = 1;
LTransform_Ext99 trans = LTransform_Set_Ext99( 2200000, 175000, 0, mag );
LPoint repeat_cnt = LPoint_Set( 1, 1 );
LPoint delta = LPoint_Set( 251520, 70000 );
LCell pInstCell = LCell_Find( pFile, "Branding_Cell" );
LInstance_New_Ext99( LC_CurrentCell, pInstCell, trans, repeat_cnt, delta );

/* End custom generator code.*/
```

A.2 Complete Overview of all utilized photo-mask for the fabrication of hollow SU-8 cantilevers

In this section a complete listing of the different photo-masks that were employed in the presented fabrication strategies are provided.
Appendix A. Photolithographic processing of SU-8

A.2.1 Photolithography mask: SU-8 Bottom Layer

The SU-8 Bottom Layer mask defines the basic shape of the cantilever and handling chip. It also includes apertures and alignment structures for subsequent levels of SU-8.

Figure A.1: SU-8 Bottom Layer mask. **Left**: Overview of the overall mask. There are 4 different cantilever lengths on the mask with varying widths. **Right**: Detail view of two cantilever designs. The upper probe mask is used for 150 µm long probes whereas the lower design yields an device with a 500 µm long cantilever.

A.2.2 Photolithography mask: LOR Sacrificial Layer

The LOR Sacrificial Layer mask defines basic shape of the sacrificial layer and hence the final micro-fluidic channel of the hollow AFM probe. It is also used to specify the inlet to connect the probe with an external reservoir.

A.2.3 Photolithography mask: SU-8 Top Layer

The SU-8 Top Layer mask defines the upper half of the hollow cantilever. It is also used to define the inlet for connecting the micro-fluidic channel to an external reservoir.

A.2.4 Photolithography mask: SU-8 Block Layer

The SU-8 Block Layer mask defines the bulk part of the handling chip. It also includes the inlet for connecting the micro-fluidic channel to an external reservoir.
A.2. Listing of all used photo-lithography masks

Figure A.2: LOR Sacrificial Layer mask. Left: Overview of the overall mask. There are 4 different cantilever lengths on the mask with varying widths. Right: Detail view of two cantilever designs. The upper probe mask is used for 150 μm long probes whereas the lower design yields a device with a 500 μm long cantilever.

Figure A.3: SU-8 Top Layer mask. Left: Overview of the overall mask. There are 4 different cantilever lengths on the mask with varying widths. Right: Detail view of two cantilever designs. The upper probe mask is used for 150 μm long probes whereas the lower design yields a device with a 500 μm long cantilever.

A.2.5 Photolithography mask: SU-8 Wall Layer

The SU-8 Wall Layer mask defines the micro-fluidic channels via the wall thickness on an intermediate layer of SU-8. The mask is utilized for fabrication strategies 2 and 3 as shown in sections 4.3.5 and 4.3.6. It further includes the inlet for connecting the micro-fluidic channel to an external reservoir.
Appendix A. Photolithographic processing of SU-8

Figure A.4: SU-8 Block Layer mask. **Left:** Overview of the overall mask. There are 4 different cantilever lengths on the mask with varying widths. **Right:** Detail view of two cantilever designs. The upper probe mask is used for 150 μm long probes whereas the lower design yields a device with a 500 μm long cantilever.

Figure A.5: SU-8 Wall Layer mask. **Left:** Overview of the overall mask. There are 4 different cantilever lengths on the mask with varying widths. **Right:** Detail view of two cantilever designs. The upper probe mask is used for 150 μm long probes whereas the lower design yields a device with a 500 μm long cantilever.

A.2.6 Photolithography mask: Alignment structures

Photo-masks are aligned to previous layer via suitable alignment structures. A set of complementary alignment patterns is placed on all photo-masks. This way, precise inter-layer alignment is ensured even in extended multi-layer processing.
A.2. Listing of all used photo-lithography masks

Figure A.6: Photo-lithography mask for the alignment structures. **Left:** Alignment structures placed on the substrate wafer for subsequent mask alignment. **Right:** Corresponding negative alignment structures that are placed on all photo-masks for precise inter-layer alignment.
The following sections provide exemplary runsheets for the three different SU-8 cantilever fabrication strategies as explained in section 4.3. The runsheets are purely informative and are provided as is. They were continuously extended between the different iterations during the development of the respective fabrication strategies.

B.1 Runsheet for strategy 1: Direct sacrificial layer
[Date]
Run sheet: SU-8 Hollow Bar Cantilever

>>> Process development: LOR Sacrificial Layer <<<

[No of Samples, Sample Numbers]

Preparation and cleaning of Wafers

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Cleaning</td>
<td>O2 Plasma cleaning: 10min @ 600W</td>
<td></td>
</tr>
</tbody>
</table>

Evaporation of ESL

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr Evaporation</td>
<td>Program: Cr V NSR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 5.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 50 mA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition Rate: ~1.23 Å/s</td>
<td></td>
</tr>
<tr>
<td>Au Evaporation</td>
<td>Program: Au V SR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 50.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 19.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 70 mA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition Rate: 1~2 Å/s</td>
<td></td>
</tr>
<tr>
<td>Cr Evaporation</td>
<td>Program: Cr V NSR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 25.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 55 mA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition Rate: ~1.23 Å/s</td>
<td></td>
</tr>
</tbody>
</table>

Etching of metal alignment structures

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet chemistry cleaning</td>
<td>5min Acetone, Ultrasonic: YES, 50C</td>
<td>Power 9!</td>
</tr>
<tr>
<td></td>
<td>5min Isopropanol, Ultrasonic: YES, 50C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QDR rinse, CMOS QDR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spinrinse dry, program 2</td>
<td></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydration bake</td>
<td>5min @ 200C on hotplate</td>
<td>N2 blow dry</td>
</tr>
<tr>
<td>Adhesion Promoter HMDS</td>
<td>120s purge N2, 30s HMDS, 120s purge N2</td>
<td>In HMDS Vaporizer</td>
</tr>
<tr>
<td>Spincoat maN1410</td>
<td>5s 500rpm, 100/s 30s 4000rpm, 1500/s</td>
<td>0.7um film thickness</td>
</tr>
<tr>
<td>Softbake maN1410</td>
<td>60s @ 100C</td>
<td></td>
</tr>
<tr>
<td>Exposure</td>
<td>Mask 1 – Metal alignment marks, vacuum cont.</td>
<td>Increased exposure because of Cr layer (480mJ x 1.2)</td>
</tr>
<tr>
<td>Development</td>
<td>570mJ: 60-70s</td>
<td>ma-D533S, check with Microscope</td>
</tr>
<tr>
<td>Rinse and dry</td>
<td>QDR &amp; spin rinse dryer, P3</td>
<td></td>
</tr>
<tr>
<td>Chrome etching</td>
<td>Max 10s einlegen. Change between etch &amp; H2O: 5s etch, 10s H2O, 3s etch und fertig!</td>
<td></td>
</tr>
<tr>
<td>Rinse and dry</td>
<td>DI Water rinse! QDR &amp; spin rinse dryer</td>
<td>Be quick, use DI gun</td>
</tr>
<tr>
<td>Strip resist etch mask</td>
<td>5min Acetone, Ultrasonic: yes 5min IPA, Ultrasonic: yes</td>
<td></td>
</tr>
<tr>
<td>Rinse and dry</td>
<td>QDR &amp; spin rinse dryer</td>
<td></td>
</tr>
</tbody>
</table>

### PhotoLitho of SU8 bottom layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydration bake</td>
<td>15min on Hotplate at 200C cool down to RT</td>
<td>Immediately spincoat SU8 layer to prevent wetting</td>
</tr>
<tr>
<td>Spincoat SU8 2002</td>
<td>Ramp (100 rpm/sec) to 500 rpm for 5 sec Ramp (300 rpm/sec) to 2000 rpm for 30 sec</td>
<td>4.4ml</td>
</tr>
<tr>
<td>Softbake</td>
<td>Ramp from RT to 95 °C at 60% HP1 2min @ 95 °C cool down on glassplate</td>
<td></td>
</tr>
<tr>
<td>Exposure</td>
<td>SU8 W=400mJ, 5x 12.2s @ 8.9mW, 20s break WEC-TYPE: contact Exposure: hard contact AL-GAP 20um</td>
<td>227</td>
</tr>
</tbody>
</table>
PEB SU8  | Ramp from RT to 95 °C at 60% HP1  
2min @ 95 °C cool down on glassplate
Develop SU8 Bottom layer  | 1min in mrDEV600, 10s fresh, 10s IPA, QDR  
Microscope inspection  
(Fotos vorher nacher)

### PhotoLitho sacrificial Layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| Spin coat LOR 20B    | Ramp (100 rpm/sec) to 500 rpm for 5 sec  
Ramp (1000 rpm/sec) to 2000 rpm for 25 sec  
**Rest samples for 10mins at RT for reflow!** | 5.1ml  
Fully covered, some stuff.  
But ok! |
| Softbake LOR        | All samples at 95C for 1min                                               |                                              |
| Remove LOR residues | With ultra clean towel on a glass ceramic plate                           |                                              |
| Spin coat nLOF2070   | Ramp (100 rpm/sec) to 500 rpm for 5 sec  
Ramp (1000 rpm/sec) to 4000 rpm for 30 sec | 5ml  
Fully covered |
| Softbake nLOF       | Heat up to 90s at 95 °C from RT!!  
Cool down on hotplate                                                     | Slow cool down on glassplate                |
| Exposure             | nLOF W=180mJ  
WEC-TYPE: contact  
Exposure: hard contact  
AL-GAP 30um                                                        |                                              |
| PEB                  | nLOF: 90s at 95C, heat up from 80C!!!                                       |                                              |
| Develop              | 30s in 100ml 351/100ml DI H2O  
Di Inse  
15s in 50ml 351/150ml DI H2O  
Di Rinse und nochmals  
15s in 50ml 351/150ml DI H2O  
DI RINSE IN QDR |                                              |
| Rinse in QDR         | QDR                                                                       |                                              |
| Strip nLOF           | 10s in acetone, Ultra Sonic Power 1, RoomTemp!!  
2s IPA DIP und rinsen                                                   | Strips away perfectly                       |
| QDR & dry 228        | DI H2O rinse, dann dry program 3 in SRD                                   |                                              |
### PhotoLitho SU8 Cover Layer

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
</table>
| Spincoat SU8 | SU8 2005 5ml Fully covered  
Ramp (100 rpm/sec) to 500 rpm for 10 sec  
Ramp (300 rpm/sec) to 2000 rpm for 30 sec  
ALTERNATIV: SU8 100 6ml Fully covered  
Ramp (100 rpm/sec) to 500 rpm for 10 sec  
Ramp (300 rpm/sec) to 12000 rpm for 30 sec |
| Softbake | Ramp from RT to 95 °C at 60% HP1  
2.5min @ 95 °C, 1min at 65C  
Cool down on Hotplate |
| Exposure | W=400 -> 4*10s @10mW/cm2, 20s break  
Multiple exposure!  
HARD CONTACT! |
| PE Resting | 90 min at RT  
Less stress! |
| PEB | 3 min @ 97 °C ramp up and down  
haben ca 10min gemacht => besseres crosslinking und alles solvent weg!  
Cool down ON HP |

### PhotoLitho SU8 Block Layer

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
</table>
| Spincoat SU8 | SU8 2050 6ml Fully covered  
Ramp (100 rpm/sec) to 500 rpm for 10 sec  
Ramp (300 rpm/sec) to 10000 rpm for 30 sec  
ALTERNATIV: SU8 100 6ml Fully covered  
Ramp (100 rpm/sec) to 500 rpm for 10 sec  
Ramp (300 rpm/sec) to 12000 rpm for 30 sec |
| Softbake | SU8 2050  
Ramp from RT to 65 °C at 60% HP1  
8min @ 65 °C  
45min @ 95 °C  
SU8 100  
Ramp from RT to 65 °C at 60% HP1  
25min @ 65 °C  
80min @ 95 °C  
Cool down on Hotplate |
| Exposure | W=1400 -> 14*12.3s @5.7mW/cm2, 20s break  
Multiple exposure!  
SOFT Contact, left right alignment!!! |
| PEB | 5min at 65C  
15min at 95C  
Cool down ON HP |
<table>
<thead>
<tr>
<th>Process</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Develop</td>
<td>SU8 development Mr-DEV600</td>
</tr>
<tr>
<td></td>
<td>180mum =&gt; 16min in developer, 1min pure, 30s IPA rinse</td>
</tr>
<tr>
<td></td>
<td>Kein DI Water rinse!!!</td>
</tr>
<tr>
<td>Dry</td>
<td>Spinrinse dryer Programme 3</td>
</tr>
<tr>
<td></td>
<td>Optical inspection very good!!</td>
</tr>
</tbody>
</table>

**Remove Sacrificial Layer**

<table>
<thead>
<tr>
<th>Process</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolve LOR</td>
<td>Immerse in AZ-400k 1:3 DI H2O until channel is completely etched free from LOR</td>
</tr>
<tr>
<td></td>
<td>With constant agitation!</td>
</tr>
<tr>
<td></td>
<td>Other recipes possible to improve speed and adhesion between layers</td>
</tr>
</tbody>
</table>
B.2 Runsheet for strategy 2: Sacrificial layer using embedded metal mask
[Date]
Run sheet: SU-8 Hollow Bar Cantilever
>>> Process development: Embedded Metal Mask <<<

[No of Samples, Sample Numbers]

Preparation and cleaning of Wafers

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Cleaning</td>
<td>O2 Plasma cleaning: 10min @ 600W</td>
<td></td>
</tr>
</tbody>
</table>

Evaporation of Enhances Sacrificial Layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr Evaporation</td>
<td>Program: Cr V NSR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 5.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 50 mA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition Rate: ~1.23 Å/s</td>
<td></td>
</tr>
<tr>
<td>Au Evaporation</td>
<td>Program: Au V SR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 50.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 19.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 70 mA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition Rate: 1~2 Å/s</td>
<td></td>
</tr>
<tr>
<td>Cr Evaporation</td>
<td>Program: Cr V NSR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 25.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 55 mA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition Rate: ~1.23 Å/s</td>
<td></td>
</tr>
</tbody>
</table>

Etching of metal alignment structures

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet chemistry cleaning</td>
<td>5min Acetone, Ultrasonic: YES, 50C 5min Isopropanol, Ultrasonic: YES, 50C QDR rinse, CMOS QDR Spinrinse dry, program 2</td>
<td>Power 9!</td>
</tr>
</tbody>
</table>
### Dehydration bake
5min @ 200C on hotplate
N2 blow dry

### Adhesion Promoter HMDS
120s purge N2, 30s HMDS, 120s purge N2
In HMDS Vaporizer

### Spincoat maN1410
5s 500rpm, 100/s
30s 4000rpm, 1500/s
0.7um film thickness

### Softbake maN1410
60s @ 100C

### Exposure
Mask 1 – Metal alignment marks, vacuum cont.
Dose: W=570mJ/cm2

### Development
570mJ: 60-70s
ma-D533S, check with Microscope

### Rinse and dry
QDR & spin rinse dryer, P3

### Chrome etching
Max 10, Change between etchant & H2O: 5s etch, 10s H2O, 3s etch and done!

### Rinse and dry
DI Water rinse!
QDR & spin rinse dryer
Be quick, use DI gun

### Strip resist etch mask
5min Acetone, Ultrasonic: yes
5min IPA, Ultrasonic: yes

### Rinse and dry
QDR & spin rinse dryer

---

## PhotoLitho of SU8 bottom layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spincoat SU8 2005</td>
<td>Ramp (100 rpm/sec) to 500 rpm for 5 sec Ramp (300 rpm/sec) to 3000 rpm for 30 sec</td>
<td></td>
</tr>
<tr>
<td>Softbake</td>
<td>Ramp from RT to 95 °C at 60% HP1 1min @ 65 2min @ 95 °C</td>
<td>cool down on ceramic plate (with metal beaker)</td>
</tr>
</tbody>
</table>
### Photolitho of SU8 wall-layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spincoat SU8 2005</td>
<td>Ramp (100 rpm/sec) to 500 rpm for 5 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ramp (300 rpm/sec) to 3000 rpm for 30 sec</td>
<td></td>
</tr>
<tr>
<td>Softbake</td>
<td>Ramp from RT to 95 °C at 60% HP1</td>
<td>cool down on ceramic plate (with metal beaker)</td>
</tr>
<tr>
<td></td>
<td>1min @ 65 °C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2min @ 95 °C</td>
<td></td>
</tr>
<tr>
<td>Exposure SU-8 Wall Layer Mask</td>
<td>1) Glass Samples: W=420mJ (@405), 7x 9.0@ 6.7mW,10s pause</td>
<td>Expose for double layer thickness to improve interlinking (10um)</td>
</tr>
<tr>
<td></td>
<td>Power @ 405nm: 6.7mW</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) Si Samples: W=210mJ (@405), 4x 8.2s@ 6.7mW,10s pause</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Power @ 405nm: 6.7mW</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WEC-TYPE: contact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exposure: hard contact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AL-GAP 20um</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) 3min @ 95 °C</td>
<td></td>
</tr>
<tr>
<td>PEB SU8</td>
<td>Ramp from RT to 65 °C at 60% HP1</td>
<td>Normal PEB 5um layers</td>
</tr>
<tr>
<td></td>
<td>1) 1min @ 65 °C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ramp from 65 °C to 95 °C at 60% HP1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) 3min @ 95 °C</td>
<td></td>
</tr>
</tbody>
</table>
## Evaporation of EML

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr Evaporation</td>
<td>Program: Deniz</td>
<td>around 30nm Cr can be obtained with two rods</td>
</tr>
<tr>
<td></td>
<td>Evaporation source: Cr rods</td>
<td></td>
</tr>
<tr>
<td></td>
<td>time in between the 2 rods: 30min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 50 mA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deposition Rate: (~0.20\ \text{Å/s at } \sim13%\text{ power})</td>
<td></td>
</tr>
</tbody>
</table>

## Patterning of embedded Mask Layer EML

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion Promoter HMDS</td>
<td>120s purge N2, 30s HMDS, 120s purge N2</td>
<td>In HMDS Vaporizer</td>
</tr>
<tr>
<td>Spincoat AZ 4562</td>
<td>2s 500rpm, 300/s</td>
<td>4mL</td>
</tr>
<tr>
<td></td>
<td>30s 4000rpm, 500/s</td>
<td>6.2um thickness for good etch resistance</td>
</tr>
<tr>
<td>Softbake AZ 4562</td>
<td>Overnight Room Temp from Thursday to Friday</td>
<td>Remove edge bead and residues on glassplate</td>
</tr>
<tr>
<td>Exposure of AZ 4562</td>
<td>On Si Wafer : 300mJ, 54.5s @ 5.5mW, 27.3s x 2</td>
<td>6.2um thickness</td>
</tr>
<tr>
<td>Develop AZ 4562</td>
<td>AZ 400K 1:4 DI H2O, ca 45-60s (check)</td>
<td>PR is leaving after 40s</td>
</tr>
<tr>
<td>Chrome etch</td>
<td>30sec chromium etching in total. Wash with with H2O every 30min.</td>
<td>Works, but slowly since PR is still somewhat protecting</td>
</tr>
<tr>
<td>Strip PR AZ 4562</td>
<td>1min in AZ 400K pure</td>
<td></td>
</tr>
<tr>
<td>QDR rinse</td>
<td>QDR rinser cleaning</td>
<td></td>
</tr>
<tr>
<td>Trocknen</td>
<td>Spin Rinse Dryer, Prgrm 3</td>
<td></td>
</tr>
</tbody>
</table>
### SU8 Top Layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| Spincoat SU8 2005  | Ramp (100 rpm/sec) to 500 rpm for 5 sec  
                    Ramp (300 rpm/sec) to 3000 rpm for 30 sec  
                    For 111/039, 111/041, 111/042 and to 3000rpm (200rpm/sec) for 111/040 (35s) | 4.4ml                                                                                     |
| Softbake           | Room temp softbake for 1h15min  
                    NO Hotplate!!                                                            | With this time for the soft bake, wafer not stuck to the mask anymore                      |
| Exposure           | 1) SU8 W=560mJ (140*2*2), 11x 9.3s@5.5mW, 10s pause, (Power @ 405nm: 5.5mW)  
                    WEC-TYPE: contact  
                    Exposure: hard contact  
                    AL-GAP 20um                                                            | 15um exposure times (multilayer)                                                         |
| PEB SU8            | Ramp from RT to 65 °C at 60% HP1  
                    1) 1min @ 65 °C  
                    Ramp from 65 °C to 95 °C at 60% HP1  
                    1) 3min @ 95 °C | 5um bake times                                                                                     |
| Develop            | SU8 development Mr-DEV600  
                    5um => 1min, 10s fresh, 10s IPA                                         | Remove top SU8 to to chrome layer in inlets nur top layer bis EMM                           |
| Chrome etch        | 10-15s in Chrome etchant                                                 | Etch Chrome in inlets (adapt Mask!)                                                        |
| Rinse and Dry      | QDR rinse and Spinrinse dry Prgm 3                                       | Optical inspection                                                                        |

### PhotoLitho SU8 Block Layer (07.01.2011)

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| Spincoat SU8       | SU8 100  
                    6ml Fully covered  
                    Ramp (100 rpm/sec) to 500 rpm for 10 sec  
                    Ramp (300 rpm/sec) to 1000 rpm for 30 sec |                                                                                              |
| Softbake           | SU8 100  
                    Ramp from RT to 65 °C at 50% HP1  
                    30min @ 65 °C  
                    90min @ 95 °C                                               |                                                                                              |
| Exposure           | W=2400 -> 33*10.1s @7.2mW/cm2, 10s break  
                    Multiple exposure!                                           | HARD Contact, left right alignment!                                                         |
<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEB</td>
<td>10min relaxation after Exposure at RT!!!</td>
<td>Cool down ON HP</td>
</tr>
<tr>
<td></td>
<td>1min at 65C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20min at 95C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Develop</td>
<td>SU8 development Mr-DEV600</td>
<td>No DI Water rinse!!</td>
</tr>
<tr>
<td></td>
<td>180mum =&gt; 16min in developer, 1min pure, 30s IPA rinse</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>Spinrinse dryer Programme 3</td>
<td>Optical inspection very</td>
</tr>
<tr>
<td></td>
<td></td>
<td>good!!</td>
</tr>
</tbody>
</table>
B.3  Runsheet for strategy 3: Sacrificial layer using pre-patterned mold
Run sheet: SU-8 Hollow Bar Cantilever

>> Process development: Sacrificial layer with mold <<

Preparation and cleaning of Wafers

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Cleaning</td>
<td>O2 Plasma cleaning: 10min @ 600W</td>
<td></td>
</tr>
</tbody>
</table>

Evaporation of Enhanced Sacrificial Layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr Evaporation</td>
<td>Program: Cr V NSR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 5.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 50 mA</td>
<td>Deposition Rate: ~1.23 Å/s</td>
</tr>
<tr>
<td>Au Evaporation</td>
<td>Program: Au V SR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 50.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 19.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 70 mA</td>
<td>Deposition Rate: 1~2 Å/s</td>
</tr>
<tr>
<td>Cr Evaporation</td>
<td>Program: Cr V NSR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thickness: 25.0 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tooling factor: 85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z-factor: 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Density: 7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emission Current: 55 mA</td>
<td>Deposition Rate: ~1.23 Å/s</td>
</tr>
</tbody>
</table>

Etching of metal alignment structures

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet chemistry cleaning</td>
<td>5min Acetone, Ultrasonic: YES, 50C 5min Isopropanol, Ultrasonic: YES, 50C QDR rinse, CMOS QDR Spinrinse dry, program 2</td>
<td>Power 9! 239</td>
</tr>
<tr>
<td>Process Step</td>
<td>Parameter</td>
<td>Remarks</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Dehydration bake</td>
<td>5min @ 200C on hotplate</td>
<td>N2 blow dry</td>
</tr>
<tr>
<td>Adhesion Promoter HMDS</td>
<td>120s purge N2, 30s HMDS, 120s purge N2</td>
<td>In HMDS Vaporizer</td>
</tr>
<tr>
<td>Spincoat maN1410</td>
<td>5s 500rpm, 100/s 30s 4000rpm, 1500/s</td>
<td>0.7um film thickness</td>
</tr>
<tr>
<td>Softbake maN1410</td>
<td>60s @ 100C</td>
<td></td>
</tr>
<tr>
<td>Exposure</td>
<td>Mask 1 – Metal alignment marks, vacuum cont.</td>
<td>Dose: W=570mJ/cm²</td>
</tr>
<tr>
<td>Development</td>
<td>570mJ: 60-70s</td>
<td>ma-D533S, check with Microscope</td>
</tr>
<tr>
<td>Rinse and dry</td>
<td>QDR &amp; spin rinse dryer, P3</td>
<td></td>
</tr>
<tr>
<td>Chrome etching</td>
<td>Max 10, Change between etchant &amp; H2O: 5s etch, 10s H2O, 3s etch and done!</td>
<td></td>
</tr>
<tr>
<td>Rinse and dry</td>
<td>DI Water rinse! QDR &amp; spin rinse dryer</td>
<td>Be quick, use DI gun</td>
</tr>
<tr>
<td>Strip resist etch mask</td>
<td>5min Acetone, Ultrasonic: yes 5min IPA, Ultrasonic: yes</td>
<td></td>
</tr>
<tr>
<td>Rinse and dry</td>
<td>QDR &amp; spin rinse dryer</td>
<td></td>
</tr>
</tbody>
</table>

**PhotoLitho of SU8 bottom layer**

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spincoat SU8 2005</td>
<td>Ramp (100 rpm/sec) to 500 rpm for 5 sec Ramp (300 rpm/sec) to 3000 rpm for 30 sec</td>
<td>4.4ml</td>
</tr>
<tr>
<td>Softbake</td>
<td>Ramp from RT to 95 °C at 60% HP1 1min @ 65 2min @ 95 °C</td>
<td>cool down on ceramic plate (with metal beaker)</td>
</tr>
</tbody>
</table>
| Exposure                          | 1) SU8 W=210mJ (@405), 3x 9.9s@ 7.1mW,10s pause Power @ 405nm: 7.1mW | 240
| SU-8 Bottom Layer Mask            | WEC-TYPE: contact Exposure: hard contact AL-GAP 20um |                                        |
### SU8 wall-layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| Spincoat SU8 2005 | Ramp (100 rpm/sec) to 500 rpm for 5 sec  
Ramp (300 rpm/sec) to 3000 rpm for 30 sec | 4.4ml                                  |
| Softbake      | Ramp from RT to 95 °C at 60% HP1  
1min @ 65  
2min @ 95 °C cool down on metal beaker | Cool down slowly                        |
| Exposure      | 1) SU8 W=500mJ (125*2*2), 7x 9.9s@ 7.2mW, 10s pause  
WEC-TYPE: contact  
Exposure: hard contact  
AL-GAP 20um | 10um exposure times (multilayer)                                   |
| PEB SU8       | Ramp from RT to 65 °C at 60% HP1  
1) 1min @ 65 °C  
Ramp from 65 °C to 95 °C at 60% HP1  
1) 3min @ 95 °C | 5um bake times                                              |
| Develop       | SU8 development Mr-DEV600  
180mum => 2min in developer, 1min pure, 30s IPA rinse | Kein DI Water rinse!!!                 |
| Dry           | Spinrinse dryer Programme 3 | Optical inspection very good!!         |

### Pattern sacrificial layer

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Parameter</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| Spin coat LOR 10B  
110/176: 1500rpm  
110/177: 1200rpm  
110/178: 1000rpm | Ramp (200 rpm/sec) to 500 rpm for 5 sec  
Ramp (300 rpm/sec) to 1500 rpm for 45 sec | 8ml Fully covered                        |
|               | **Rest samples for 10mins at RT for reflow!** |                                        |
| Softbake LOR  | All samples at 55C for 60min | 241                                    |
Spincoat AZ 4562

<table>
<thead>
<tr>
<th>Duration</th>
<th>Speed</th>
<th>RPM</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2s 500rpm, 300/s</td>
<td>35s 4000rpm, 1000/s</td>
<td>(110/178 1700rpm only for 10um thickness)</td>
<td>this is probably too thick, channel is embedded, so normal 6um should be fine</td>
</tr>
</tbody>
</table>

| Thickness recommendation | 6.2um thickness for good etch resistance |

Softbake AZ 4562

<table>
<thead>
<tr>
<th>Duration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>60s @ 100C</td>
<td>Both samples, looks ok</td>
</tr>
</tbody>
</table>

| Remarks | Remove edge bead and residues on glassplate |

Exposure of AZ 4562 Wall Layer Mask

<table>
<thead>
<tr>
<th>Duration</th>
<th>Intensity</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>340mJ5mJ, 50s @ 6.7mW, single exp. (für 6um is ratsam, schnelles entwickeln). Eher höhere Dosis nehmen für 10um</td>
<td>6.2um thickness</td>
<td></td>
</tr>
</tbody>
</table>

| Remarks | Undercut optimieren, alignment optimieren |

Develop AZ 4562

<table>
<thead>
<tr>
<th>Duration</th>
<th>Solution</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF 351 1:4 DI H2O, 120s (check)</td>
<td>Clean MIF 351 1:4 DI H2O, 30s (check)</td>
<td>Hier prozess noch optimieren, damit ein gutter pattern transfer stattfindet</td>
</tr>
</tbody>
</table>

| Remarks | Undercut optimieren, alignment optimieren |

Strip AZ 4562 for 110/178 to only have LOR in the channel

<table>
<thead>
<tr>
<th>Duration</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aceton pure 30s in US power 1 at RT</td>
<td>Eventuell nur mit IPA ohne US probieren zum schauen.</td>
</tr>
</tbody>
</table>

PhotoLitho SU8 Top Layer

Spincoat SU8

<table>
<thead>
<tr>
<th>Duration</th>
<th>Solution</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU8 2005 5ml Fully covered</td>
<td>Ramp (100 rpm/sec) to 500 rpm for 10 sec Ramp (300 rpm/sec) to 2000 rpm for 30 sec</td>
<td>Check profile on alpha stepper =&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undercut optimieren, alignment optimieren</td>
</tr>
</tbody>
</table>

Softbake

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramp from RT to 95 °C at 60% HP1</td>
<td>2.5min @ 95 °C, 1min at 65C</td>
</tr>
</tbody>
</table>

| Remarks | Cool down on GP |

Exposure

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Break</th>
</tr>
</thead>
<tbody>
<tr>
<td>W=400 -&gt; 4*10s @10mW/cm2, 20s break</td>
<td>HARD CONTACT!</td>
</tr>
</tbody>
</table>

| Remarks | Multiple exposure! For 7um SU8 ohn 110/176 it was NOT enough, SU8 did not fully crosslink and delaminated, especially in Aceton after development |

Develop

<table>
<thead>
<tr>
<th>Duration</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>60s in mrDev600, 10s pure, 10s aceton</td>
<td></td>
</tr>
</tbody>
</table>
## PhotoLitho SU8 Block Layer

<table>
<thead>
<tr>
<th>Step</th>
<th>SU8 100</th>
</tr>
</thead>
</table>
| Spincoat SU8              | SU8 100
6ml Fully covered
Ramp (100 rpm/sec) to 500 rpm for 10 sec
Ramp (300 rpm/sec) to 1000 rpm for 30 sec |
| Softbake                  | SU8 100
Ramp from RT to 65 °C at 50% HP1
30min @ 65 °C
90min @ 95 °C |
| Exposure                  | W=2400 -> 33*10.1s @7.2mW/cm2, 10s break
Multiple exposure!          |
| PEB                       | 10min relaxation after Exposure at RT!!
1min at 65C
20min at 95C                |
| Develop                   | SU8 development Mr-DEV600
180 mum => 16min in developer, 1min pure, 30s IPA rinse |
| Dry                       | Spinrinse dryer Programme 3                                            |

## Remove Sacrificial Layer

| Step                      | Immerse in AZ-400k 1:3 DI H2O until channel is completely etched free from LOR
With constant agitation! |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolve LOR</td>
<td>Other recipes possible to improve speed and adhesion between layers</td>
</tr>
</tbody>
</table>

Optical inspection very good!!
Curriculum Vitae