Doctoral Thesis

Fluid FM for Force-Controlled Electrophysiology

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FLUIDFM FOR FORCE-CONTROLLED ELECTROPHYSIOLOGY

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

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2014
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Finally yet importantly, I sincerely thank the Swiss National Science Foundation for the financial support and thanks to ETH Zurich for providing the prerequisites for a high quality research environment.
The FluidFM combines AFM and microfluidics, the latter being that branch of microtechnology specialized in the fabrication of micro- to nano-sized devices that allow to confine and manipulate volumes of liquids from μL to fL. This is achieved with the fabrication of microchanneled cantilevers that allow delivering of liquids from an external reservoir to a micro- or nanometer sized aperture located at the free-end of the cantilever. The FluidFM has demonstrated to be a valuable tool in single-cell manipulation and in local surface modification (e.g. surface patterning in liquid environment). It is correct to think about FluidFM probes as pipettes or syringes with reduced dimensions. Indeed micropipettes share with the FluidFM many application fields. However, the FluidFM probes offer the unique ability to sense interaction forces with AFM sensitivity.

Here I present FluidFM setup modified to enable measurement of ionic currents flowing through the embedded microchannel at picoampere scales. The setup is firstly applied in the electrophysiology field, in particular for force-controlled patch-clamp experiments in the whole-cell configuration. Patch-clamp is an electrophysiology recording technique that allows to control the transmembrane potential of single cells and thus to study the dynamics of voltage-gated ion-channels. Even though powerful and still considered to be the golden standard in the field, conventional patch-clamp suffers from low throughput and from the need of highly skilled operators. The FluidFM is used here to address these hurdles by automatizing the procedure of precise control and positioning of the probe onto the cell by means of force feedback. In addition, the system allows for simultaneous recording of transmembrane ionic current and force. This ability is exploited here to electrically stimulate isolated mice cardiomyocytes and to record the flow of ions through the membrane simultaneously with the generated transversal force. The FluidFM thus
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shows to be a valuable tool especially in the mechano-biology field, where forces and electrophysiology play in conjunction.

With the addition of a precise actuator, the same electronics and electrodes used for patch-clamp becomes capable to perform SICM. SICM is scanning probe imaging technique invented with the scope of imaging of living cells in physiological conditions and relies on the substrate-probe distance dependence of the ionic current flowing through the aperture of a pipette. The FluidFM demonstrated the ability of being used in this mode and we proved it by imaging both PDMS made calibration grids and living hippocampal neurons. Among other advantages, FluidFM probes can be modified with FIB to obtain apertures down to 50 nm diameter without increasing the electrical impedance to problematic levels, as it is the case in glass micropipettes. The force control of the AFM is then exploited to run simultaneously with the ionic current controller resulting in a novel imaging mode. In this mode, DC SICM can be carried faster and limiting the interaction force with the sample. It is demonstrated that when the SICM controller fails to keep the probe at a safe distance while scanning, the intervention of the force controller prevents excessive force to be exerted.

The application of the FluidFM on interesting biological systems are hindered by the lack of adequate aperture shape and size. The technique of Focused Ion Beam has proven to be a valuable tool for the fabrication of aperture with custom design. I fabricated application-driven apertures to pioneer the following fields: i) microbial and mammalian cell adhesion, ii) injection/extraction into/from mammalian cells, iii) patch-clamp and iv) SICM.

The thesis ends with a pilot study where the FluidFM is proposed as a balance for micro-objects. Single spherical borosilicate beads were immobilized using suction with a tipless FluidFM probe in air. Based on the resonance frequency shift, one is able to calculate the mass of the immobilized particle. The accuracy and precision of the mass measuring system is confirmed by mass calculation upon volume knowledge gathered with AFM imaging. The FluidFM brings two key advantage to the field: i) the non-complexity of the protocol showed to increase the throughput by an order of magnitude and ii) the particle position on the cantilever is well defined by the location of the microchannel aperture allowing thus direct comparison between different particles.
Il campo della microfluidica è specializzato nella fabbricazione di dispositivi su scala micro e nano per il confinamento e la manipolazione di volume fra µL e fL. Come peculiarità della tecnologia FluidFM, la microfluidica viene combinata con il microscopio a forza atomica (Atomic Force Microscope - AFM) attraverso l’integrazione di un micro canale nella microleva. In questo modo viene reso possibile il trasferimento di liquidi da un serbatoio esterno ad un’apertura di dimensioni micro- o nano-metriche situata all’estremità libera della microleva. Il FluidFM ha dimostrato di essere un valido strumento per la manipolazione di singole cellule e la modifica locali in superfici, come ad esempio litografia in liquido. Le microleve FluidFM sono analoghe a pipette e siringhe, ma di dimensioni molto ridotte. Infatti, le micro-pipette condividono con il FluidFM molti campi di applicazione ma con una fondamentale differenza: il FluidFM preserva l’abilità AFM di misurare forze d’interazione con elevata sensibilità.

In questo lavoro viene presentata una versione di FluidFM modificata per la misura di correnti ioniche su scala pico-amperometrica attraverso il microcanale integrato nella microleva. Lo strumento sviluppato è dapprima applicato al campo di elettrofisiologia, in particolare al monitoraggio di esperimenti di patch-clamp in configurazione “whole-cell”. Quella del patch-clamp è una tecnica di elettrofisiologia che permette il controllo del potenziale di membrana di una singola cellula e quindi lo studio delle dinamiche di apertura e chiusura di quei canali ionici che sono regolati dal voltaggio. Nonostante questa tecnica sia tutt’ora considerata la miglior scelta nel campo, soffre di basso rendimento e della necessità di un operatore altamente esperto. L’uso del FluidFM in questo campo risolve questo problema automatizzando la laboriosa procedura di avvicinamento della sonda – in maniera precisa e controllata – sulla membrana cellulara utilizzando il sensibile controllo
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retroattivo della forza. In aggiunta, il sistema permette l’acquisizione simultanea di correnti attraverso la membrana cellulare e della forza. Questa abilità viene nel nostro caso sfruttata per stimolare elettricamente cardiomiociti primari isolati da topi, per indurne la contrazione e misurarne simultaneamente la forza trasversale insieme alle correnti ioniche trans-membraniche. Il FluidFM è quindi uno strumento ideale in special modo nel campo della meccano-biologia, in cui forze ed elettrofisiologia lavorano all’unisono.

Con l’aggiunta di un attuatore di precisione, lo stesso tipo di elettronica usato per il patch-clamp può essere utilizzato come microscopio a scansione a conduzione ionica (Scanning Ion Conductance Microscope – SICM): una tecnica di produzione d’immagine inventata appositamente per la raffigurazione topografica di cellule viventi in condizioni fisiologiche. Questa tecnica si basa sulla dipendenza della corrente ionica che scorre attraverso l’apertura di una pipetta, dalla distanza di quest’ultima dal substrato. Il FluidFM ha dimostrato l’abilità di riprodurre questa tecnica di raffigurazione su griglie di calibrazione fatte in PDMS e su neuroni ippocampali in vita. Tra gli altri vantaggi, le levette del FluidFM possono essere modificate con l’aiuto della tecnica del fascio ionico focalizzato (Focused Ion Beam - FIB) ed ottenere così diametri inferiori ai 50 nm senza aumentare la resistenza elettrica delle levette a livelli problematici come invece avviene nel caso delle classiche micro pipette. Il controllo della forza retroattivo conferito dall’AFM viene qui usato simultaneamente al controllo retroattivo sulla corrente ionica di cui sopra, dando luogo a un nuovo metodo di imaging. In questa modalità è possibile accelerare la scansione in DC-SICM, limitando nel contempo le forze di interazione con il substrato. È qui dimostrato che durante la scansione, quando il controllo retroattivo della corrente ionica fallisce nel mantenere la levetta ad una distanza adeguata, l’intervento del controllo retroattivo della forza previene che le forze d’interazione diventino eccessive.

L’applicazione del FluidFM su interessanti sistemi biologici è limitata dalla mancanza di aperture del micro canale adeguate in termini di dimensione e forma. La tecnica FIB ha dimostrato di essere di aiuto per la fabbricazione di aperture con design personalizzato. Nell’ambito di questo lavoro sono state fabbricate aperture il cui design è stato appositamente studiato per esplorare i seguenti campi di applicazione: i) adesione di cellule mammifere e batteriche, ii) iniezione/estrazione in/da cellule mammifere, iii) patch-clamp e iv) SICM.

La tesi termina con uno studio pilota in cui il FluidFM viene proposto come bilancia per oggetti microscopici. Singole particelle di borosilicato con forma sferica sono state reversibilmente immobilizzate con l’applicazione di pressione negativa nel microcanale...
usando levette “Tipless” aventi un’apertura circolare di 2 µm, in aria. La massa della particella appesa può quindi essere stimata basandosi sulla riduzione della frequenza di risonanza. La precisione e l’accuratezza della stima è stata confermata dal calcolo della massa basato sull’esatta conoscenza del volume, acquisita con le immagini AFM. Il FluidFM apporta due vantaggi chiavi nel campo: i) la semplicità operativa aumenta la produttività di un ordine di grandezza e ii) la posizione di immobilizzazione sulla microlevetta è ben definita dall’apertura del microcanale, permettendo il confronto diretto tra le particelle misurate.
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CHAPTER 1

1. Introduction

The idea to combine Atomic Force Microscopy (Binnig et al., 1986) with microfluidics came up in our laboratory (Laboratory of Biosensors and Bioelectronics, ETH Zurich) back in 2006. The vision was to develop a force-sensitive nano-pipette to be used on one side for single-cell manipulation and on the other side for local surface modification. The inventors got inspired by the microchanneled cantilevers, i.e. AFM cantilevers having an embedded microchannel, developed at the CSEM SA in Neuchatel (Meister et al., 2008). About five years later of intense developing and cooperative work amid LBB, CYTOSURGE, NANOSURF and SMART TIP, the FluidFM became a commercial product.

The peculiarity of the FluidFM system is represented by the tight connection of the microchannel to an external reservoir, which triggered a concept transition from single AFM probe to indeed a force-controlled nano-pipette compatible with standard optical microscopy. This crucial difference opened a whole new range of applications because the system was compatible eventually both in liquid environment as well as in air. Targeted were in particular single-cell manipulation and local surface modification.

The advantages conferred by the FluidFM concept originally sparked off the idea of force-controlled patch-clamp because this application will take advantage of all the key aspects of the FluidFM. Standard patch-clamp is in fact a single cell technique, which requires precise positioning of a glass micropipette on the cell membrane, but is hampered by the not sufficient resolution of an optical microscope. As the FluidFM can operate in liquid, the force-control conferred by the AFM thus provides the necessary gentleness of the cantilever-membrane contact, while the electrolyte-filled microchannel is used to record
ionic currents or to deliver chemicals. To date, such innovation is not reported in the literature because only the FluidFM configuration opened such possibility.

Force-controlled patch-clamp in the whole-cell mode is the focus of this thesis. Therefore, I have structured this introductory Chapter as follows: the 1st paragraph is dedicated to the general importance of electrophysiology; the 2nd one to the voltage-gated ion channel; the 3rd to description of the patch-clamp technique (history, gigaseal, patch-clamp configuration and last developments); finally the 4th to the FluidFM (its demonstrated applications).

1.1. Importance of electrophysiology

Electrophysiology is the study of electrical properties of biological systems like cells and tissues. It refers to the recording techniques that allows such studies. In 1952 Hodgkin and Huxley described mathematically for the first time the propagation of electrical signals in the giant nerve fiber of a squid (Hodgkin and Huxley A F, 1952). Their work became of fundamental importance for the subsequent efforts to understand electrophysiological behavior and characteristics of biomembranes. Later, in the early 1970s, measurements of the electrical “noise” in experiments on synapses between neurons and muscles where interpreted to be “all-or-nothing” opening behavior of individual ion channels as acetylcholine repeatedly bound and unbound to them (Anderson and Stevens, 1973). “Noise” analysis has been the most popular technique to characterize gating kinetics and conductance of different channels as the individual study of ion channel was not possible yet. It was only with the advent of the patch-clamp technique in 1976, a technique optimized by Neher and Sakmann (Neher and Sakmann, 1976), that it became possible to record currents of single ion-channels. The impact of their work was so important, that they were awarded with the Nobel Prize in physiology or medicine in 1991.

The sequencing of the human genome revealed more than 400 ion channels, whereas only a fraction of these proteins have been studied in deep by cloning and by testing their functionalities. Ion channels play a fundamental role in physiology for the regulation of ions and molecules transport through the cell membrane. They are therefore interesting in terms of therapeutic targets: in fact, their central functional role in human physiology and pathophysiology, and their presence in diverse tissues makes them optimal drug targets. Indeed, ion channel mal-functions were associated with a growing number of human diseases called channelopathies (Bagal et al., 2013; Hoffman, 1995). Alone, mutations in ion channels have been identified for more than 60 channelopathies.
However, the potential of ion channels as drug targets was shown to be a double-edged sword since they may indeed interact with drugs that are not designed to interact with. A notable example are eight non-cardiovascular pharmaceuticals that have been withdrawn from the market\(^1\) during 1990 and 2001, because they were shown to cause Torsade de Pointes (TdP), a potentially life-threatening form of tachyarrhythmia (Fermini and Fossa, 2003). It is believed that TdP is linked with QT\(^2\) interval prolongation, even though not yet fully understood. In fact, prolongation of the QT interval and TdP seems to be very complex and affected by numerous factors (Roden, 2004). However, among others, inhibition or blockage of the hERG K\(^+\) channel has shown evidence to prolong the QT interval and thus predisposition to lethal arrhythmias (Curran et al., 1995; Roden et al., 1996). As a consequence, to date, more than 160 non-cardiac drugs have been reported to alter the normal ventricular action potential duration and/or induce TdP (CredibleMeds, 2014). Worldwide regulatory agencies responded to this risk of non-selectivity of drugs by changing their regulation: indeed, non-cardiovascular drugs that show to affect a normal electrocardiogram (ECG) are now considered to have a potential life-threatening fatal outcome, instead of listing it as a side-effect only, increasing the risks of late rejection of a new developed drug.

This introduction about the hERG channel serves to underline the complexity and unpredictability of the interaction that a compound might have with diverse targets following independent biological pathways. By adding to the picture the costs of development and discovery of new drugs in pharmaceutical industries (Kaitin, 2010), the importance to identify potential unwanted interaction in the early stages of drug discovery becomes obvious. Novartis, for example, states that for one drug to be successfully launched on the market 14 years and 2 billion US dollars are needed on average. In addition, only about 1 compound over 10000 makes it through this high risk and financially intensive journey (http://www.novartis.com).

For these reasons, pharmaceutical companies have the need to recognize as early as possible (i.e. in the pre-clinical stage) non-specific interaction of a potential drug candidate. In fact, given the wide range of compounds that inhibit the hERG K\(^+\) channel, screening of their interaction became standard procedure in the preclinical assessment of drug

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\(^1\) Ion channel modulators usually guarantees very high revenues to pharmaceutical industries. In 2002 they accounted for the third-best-selling group of prescription drugs (12 US$ billion estimated). E.g. in 2003 Pfizer’s antihypertensive generated around US$ 4 billion sales (Xie et al., 2004).

\(^2\) A defined portion of the electrocardiogram (ECG) signal during heart contraction.
development, thus reducing the hurdles in the subsequent regulatory approvability. In the different stages of the drug development cycle, the new compounds are tested in assays and animal models (DiMasi et al., 2003) to address different aspects such as safety and efficacy throughout the different development phases. As anticipated before, one phase is about screening and studying the electrophysiological implication of the new compounds. For this scope, electrophysiology provides the necessary electrical recording techniques needed to measure the flow and transportation of ions through biological membranes.

Electrophysiological data can be measured indirectly, with the use of extracellular electrodes (e.g. ECG or electrode arrays) that commonly measure the field potential generated by many cells in slices or in vivo, or directly, where most commonly the patch-clamp technique is used (presented in detail afterwards). In particular, the pharmaceutical industry is waiting for electrophysiology techniques that help solving the above presented problem of new drug candidate screening. Therefore in the past years, intense research and development pushed to bring new technologies able to assess the demand of pharmaceutical industries for reliable, robust, reproducible and, more importantly, high throughput techniques that help to identify ideal compounds in the preclinical phase in a cost effective way.
1.2. Ion-channels: voltage-gated ion channels

From Galvani to Neher and Sakmann

The discovery of the bioelectricity effects dates back to the year 1792 where the Italian scientist Luigi Galvani firstly described what he called animal electricity (Galvani and Aldini, 1792), lately renamed with the term bioelectricity. With the experiments of Galvani the need of tools able to measure the bioelectricity raised and so electrophysiology started to exist. Today we know that coordinated ion fluxes through a special family of transmembrane proteins called ion channels are responsible for that phenomenon. It was only in the late 1970s that Neher and Sakmann introduced a technology able to directly measure the ion flow through single ion-channel: the so called patch-clamp technique, which is still considered the gold-standard nowadays. The patch-clamp technique allowed to understand how ions are exchanged across the cellular membrane and how action potentials are shaped by their dynamics.

Cellular membrane, membrane proteins and ion channels

Cell membranes play an essential role in a living organism since they provide the indispensable compartment structure needed to accomplish the majority of life-related functions: they form the boundaries between the intracellular and extracellular environment needed to maintain the gradients necessary to drive their vital functions, serving as energy reservoirs. However, a perfectly sealed barrier is not very useful in most cases, as no information can be exchanged. Evolution solved this with a wide variety of membrane proteins that regulate the traffic of different classes of molecules (sugars, ions, amino acids, etc.) that allows any process that involves crossing a biological membrane.
A particular class of pore-forming proteins is known as ion-channels. Their function is to allow specific ions to rapidly diffuse through the membrane down their electrochemical gradient. An ion channel can change conformation (opened, inactivated, or closed) in response of different stimuli and therefore impede or allow the passage of a determined ion. The most common stimuli type that can gate ion channels are ligand-gated and voltage-gated, but they can also be sensitive to mechanical inputs, chemical inputs and others. In this thesis, we will concentrate on the voltage-gated ion channels, which have their ion-conductance regulated by the voltage across the membrane (Bezanilla, 2005).

Voltage-dependent ion channels play a fundamental role in the generation and propagation of electrical signal in the nerves. They can be described as a protein having three main parts: the voltage sensor, the conducting pathway (or pore) and gates (Bezanilla, 2005). A movement of the sensor upon a change in the transmembrane potential initiates a conformational change of the conducting pathway, thus impeding or allowing the flow of the concerned ion(s).

**Membrane potential and Nernst potential**

There is a specific class of membrane proteins that are called transporters. Similarly to ion channels, their task is to mediate the transport of ion through the plasma membrane. However, they are structurally different from ion channels and the number of ions flowing is different: millions per second in the case of ion channels, thousands through...
transporters. The key difference is that transporters have the ability to \textit{pump} specific ions against their electrochemical gradient. One example is the so called Na⁺/K⁺-ATPase (Alberts et al., 1983). Their main task is to maintain the membrane polarization potential at a constant level. Indeed, in the intracellular and extracellular environments the concentration of every ion species is controlled. This forced, uneven ion distribution across the cell membrane gives rise to an electrochemical gradient which is measured as a membrane or resting potential and is specific for different cell types, some of which are summarized in Table 1-1. This concentration and voltage gradient across the membrane works analogously to a capacitor, and represents the source of energy for most of the cell’s activities.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Membrane Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td>-50 mV to -80 mV</td>
</tr>
<tr>
<td>Cardiomyocyte</td>
<td>-90 mV</td>
</tr>
<tr>
<td>Hepatocyte</td>
<td>-35 mV</td>
</tr>
<tr>
<td>Photoreceptors</td>
<td>-40 mV</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>-60 mV</td>
</tr>
<tr>
<td>Algae</td>
<td>-150 mV</td>
</tr>
</tbody>
</table>

Table 1-1: Examples of the membrane potential of different cell types.

As the membrane potential is generated from two well-known physical driving forces (i.e. electrical and chemical energy), their work can be calculated and equalized, resulting in the Nernst equation that relates the concentration of one particular ion species to its equilibrium potential, hereafter shortly presented:

Electrical work:

\[
W_{el} = z \times F \times E_M
\]  
Equation 1-1

Where \(z\) is the ion valence, \(F = 96485 \text{ C*mol}^{-1}\) the Faraday constant and \(E_M\) the potential difference exerted from the considered ion over the membrane. Whereas the chemical work is:

\[
\Delta G = R \times T \times \ln \left( \frac{C_o}{C_i} \right)
\]  
Equation 1-2

Where \(R = 8.3 \text{ J/(mol*K)}\) is the gas constant, \(T\) the temperature in Kelvin, \(C_i\) and \(C_o\) the concentration of the considered ion inside and outside the cell. Equalizing the two energies, one obtains the Nernst Equation:

\[
E_M = \frac{R \times T}{z \times F} \times \ln \left( \frac{C_o}{C_i} \right)
\]  
Equation 1-3

Goldman, in the 1940s, completed the work by introducing a new equation that describes the membrane potential resulting from the contribution of all the involved ions (Goldman, 1943). His equation is a weighted average of each contributing ion’s equilibrium potential.
(i.e. its Nernst’s potential), where the weighting factors represent the relative permeability of each ion. In the normal case, this is the Goldman’s equation:

\[
E_{M[K^+,Na^+,Cl^-]} = \frac{P_{K^+}}{P_{tot}} E_{K^+} + \frac{P_{Na^+}}{P_{tot}} E_{Na^+} + \frac{P_{Cl^-}}{P_{tot}} E_{Cl^-}
\]

Equation 1-4

Equation 4 combined with Equation 3 results in the following equation:

\[
E_{M[K^+,Na^+,Cl^-]} = -\frac{R * T}{F} \ln \left( \frac{P_{K^+}[K^+]_i + P_{Na^+}[Na^+]_i + P_{Cl^-}[Cl^-]_o}{P_{K^+}[K^+]_o + P_{Na^+}[Na^+]_o + P_{Cl^-}[Cl^-]_i} \right)
\]

Equation 1-5

The role of voltage-gated ion-channel in action potential generation

The coordinated activation of voltage-gated sodium, calcium and potassium channels are responsible for the generation of electrical signals in excitable cells. Such a coordination work is summarized in Figure 1.3 with a schema of the different phases of an action potential. Briefly, if the transmembrane potential exceeds a certain threshold, the voltage-gated Na\(^+\) channels open allowing a relatively small amount of sodium ions to enter the cell, following their electrochemical gradient. Sodium ion influx further depolarizes the membrane triggering an auto amplification process as more and more sodium channels open. This phase is called \textit{depolarization} and lasts a few milliseconds. When the potential gets close to the equilibrium potential of Na\(^+\) ions (~40 mV) the Na\(^+\) channels get inactivated until the resting potential is restored again. Simultaneously, the voltage gated K\(^+\) channels open allowing an efflux of potassium ions that overwhelms the influx of sodium. This is called the \textit{repolarizing phase}, where the membrane potential returns to its original state. The potassium channels remain opened beyond the reach of the resting potential, entering a \textit{hyperpolarization} phase, where the cell remains insensitive to further depolarizing stimuli, also known as \textit{refractory period}.

Figure 1.3 Typical action potential of a human nerve cell. The numbers indicate particular stages in the development of the action potential and illustration of the gating of the corresponding ion channel. Refer to the text for a detailed description of the different phases of the action potential (source: http://hyperphysics.phy-astr.gsu.edu/hbase/biology/actpot.html).
The action potential event is an all-or-none event, i.e. once the initial threshold is exceeded, the complete sequence is executed. This is the mechanism that auto-propagates the action potential along the plasma membrane. In addition, the refractory period and the inactivation of Na⁺ channels prevent from spreading the signal backwards. Like the above-mentioned ion channels, also the hERG channel is voltage-gated.

1.3. Patch-Clamp: the technique

The patch-clamp setup

In the previous paragraph, a definition of electrophysiology with the related review of its most important object of investigation, i.e. ion-channels, and their involvement in the generation of electrical signal was given. The patch-clamp is the most powerful technique for this scope. This paragraph aims to give an overview of what the patch-clamp technique encompasses. For an in-depth introduction to the topic, refer to the referenced books (Molleman, 2002; Sherman-Gold, 1993).

Many methods are currently widely used for ion-channel screening, such as ion flux measurement or the use of fluorescent indicator dyes, but none have the advantage to precisely control the voltage of the cellular membrane and most of them suffer from low temporal resolution (from seconds to minutes, compared to submilliseconds for patch-clamp) (Treherne, 2006). Control of the membrane potential is especially needed for voltage-gated ion channels, as it is the only way to control their “ligand” (the membrane potential).

The patch-clamp technique relies on clamping the voltage across the cell membrane. This is done by positioning a micrometer-sized glass pipette on the cell of interest with subsequent application of small negative pressure to achieve a configuration known as cell-attached (see Figure 1.4a).
The mentioned micropipettes (Figure 1.4b) are fabricated by pulling commercially available glass capillaries with a programmable pipette-puller. High quality pullers have a multitude of parameters that define particular aspects of the fabricated micropipette (time, heat, number of pulling steps, etc.). Aperture diameter, taper shape, end-wall thickness are only some of characteristic dimensions of a micropipette. Micropipettes for patch-clamping have a typical aperture diameter of 1-5 μm and an impedance of about 1-8 MΩ when filled with physiological solutions.

The transmembrane currents and potentials are recorded with the use of amplifiers designed for patch-clamp. These are specially designed amplifiers equipped with ultra-low-noise components so that sub-picoampere currents can be recorded. The most common type of electrodes used to interface the metallic wires of the amplifier to the electrochemical cell is a silver wire coated with a composite of silver-chloride (AgCl). Chloride ions present in the solution react with the silver to produce AgCl and an electron, according to the following chemical reaction:

\[ \text{Cl}^- + \text{Ag} \leftrightarrow \text{AgCl} + e^- \]

On the other side, an electron reacts with the AgCl to produce Cl\(^{-}\) and Ag.

*Patch-clamp configurations* - One facet that makes the patch-clamp technique still the most rated mean to record single-cell electrophysiology is the high degree of flexibility that it confers. Indeed, after achieving the cell-attached configuration, different protocols allow to record single-channel activity or the dynamics of a channel population. Hamill et al. optimized these protocols in 1981 and are re-proposed here in Figure 1.5. The different obtainable configurations have as a common starting state the cell-attached configuration and allow to address a wide variety of electrophysiological problems.
The most important and critical step is the first one presented in Figure 1.5: the attainment of a tightly sealed connection between cell and pipette. To obtain high quality recordings and to precisely control the transmembrane voltage potential, such seal needs to have impedance in the giga ohmic range. The work of Neher et al in 1981 showed that these seals can only be obtained by taking special precautions to ensure cleanliness of the pipette. Indeed, one of the most important rule to follow is to use a fresh pipette for every tested cell (Hamill et al., 1981). Four main configurations can be reached from the cell-attached one: i) whole-cell, ii) perforated configuration, iii) outside-out and iv) inside-out.

**Whole-cell configuration** – The whole-cell configuration is obtained by application of further suction when in cell-attached configuration, or by application of short, high-voltage pulses. The aim is to disrupt the patched portion of membrane. By doing this, access to the cytosol is granted and the electrode is electrically connected to the intracellular environment. This technique allows to precisely control the transmembrane potential, especially useful when studying the behavior of voltage-gated ion-channels. In this configuration, the measured
ionic currents are the result of the total ion-channel population present in the cell membrane.

**Perforated configuration** – The perforated configuration is a variation of the whole-cell configuration. However, bioactive molecules are used to form small perforations in the membrane instead of suction. Antibiotic or Antifungal agents are commonly used, which naturally form pores in the membrane. The advantage of this configuration is a reduced dialysis of the cell. Drawbacks are higher access resistance and increased difficulty in achieving gigaseal.

**Outside-out configuration** – By withdrawing the pipette from the whole-cell configuration with gentleness, the patch membrane is torn from the cell while the gigaseal is maintained. The portion of membrane that remains attached at the pipette’s tip quickly reshapes leaving the intracellular side of the membrane in contact with the pipette’s solution. This configuration is optimal to study the effects of extracellular factors on the activity of single channel.

**Inside-out configuration** – Starting from the cell-attached configuration, one can excise a portion of the patch by rapidly withdrawing the pipette from the cell. The remaining portion of membrane attached to the pipette will have the intracellular side of the membrane in contact with the bath solution. This configuration poses the ideal conditions to study the effects of cytosolic factors on single ion-channels.

In the framework of this thesis, the FluidFM will be tested in the whole-cell configuration.

### 1.3.1. The gigaseal

Gigaseal represents the most important breakthrough in electrophysiological recording. Neher Sakmann recognized that it was possible to obtain a tight connection link between the pipette and the cell that measured billions of Ohms by pressing the pipette against the cell membrane and by suction application (Hamill et al., 1981). In this way, they could reduce the electrical noise by one order of magnitude and increase the stability of the recordings (Sigworth and Neher, 1980). Maybe one of the most important milestone achieved by Neher et al. is the understanding of the importance of only using fresh glass micropipettes. In fact, due to the strong interactions between the plasma membrane and the glass, the membrane (or membrane proteins) sticks so strongly on it that they will stick on the glass after removal of the pipette at the end of an experiment. The formation of further gigaseals is prevented from those membrane residues in the pipette.
Even though the gigaseal is essential in patch-clamp procedure, its formation is still not completely understood yet. Reason for this is the complexity of a native biological membrane, which substantially differ from an artificial lipid bilayer. Indeed, a wide variety of membrane proteins and different lipids populate the membrane. In addition, seal formation is influenced by the cytoskeleton and the intracellular- and extracellular-matrix just to cite some factors. The composition and structure of the patch is especially important when dealing with single-channel recordings in the outside-out or inside-out patch-clamp configuration. In this case, it is a fundamental concern to understand if the patch of membrane isolated by gigaseal formation is a representative membrane of a normal, native membrane or rather not. In discussions are elements like the presence of proteins in the patch domain, or in parts of it; the mechanical stress in the patch, the roles of sub-domains etc. (Suchyna et al., 2009).

In particular, two articles (Milton and Caldwell, 1990; Suchyna et al., 2009) discussed this problem in detail, proposing two different models of seal formation. While Milton & Caldwell propose that the seal is formed by a rather clean bilayer (Figure 1.6a), Suchyna et al. are in favor of a more complex model where proteins and cytoskeleton are present in the sealing region (Figure 1.6).

Figure 1.6   a Native membrane model vs. lipid bleb model. Two models for the physical basis of tight patch single channel recording. On the left, the dome is assumed to stay intact and to maintain the structure and composition of the native membrane. On the right, the lipid bleb model, assumes that a semi-clean lipid bilayer is extracted from the cytoskeleton and extracellular matrix to eventually form a gigaseal with the pipette. From (Milton and Caldwell, 1990). b Schematized structure of a patch according to Suchyna et al. Three regions are distinguishable: the dome, i.e. the membrane that spans the pipette, the gigaseal (between membrane and pipette’s glass) and the dome (green). Lipid bilayer is shown in red. Proteins of various types emerge from the lipid bilayer and denature against the glass, sticking to it (black). (Suchyna et al., 2009).
The first model is known as the *lipid-bleb* model and assumes that suction produces a drop in pressure outside the membrane and is greater under the pipette rim. Together with a distension of the entire patch, tiny bulges are assumed to occur. The shear stress created by this bulging may trigger a separation of the phospholipids from the extracellular matrix and the cytoskeleton, forming a bleb that expands rapidly and eventually form a gigaseal in the pipette. The membrane bleb inspected with electron microscopy techniques revealed that they did not contain any discernible organelles and appeared smooth, lacking any discernible cytoskeletal structure or extracellular matrix, in contrast to the appearance of the adjacent surface membrane. In support to this model, Milton and Caldwell cite a list of observations that are rationalized by this behavior. Among others, an explanation is given on why the channel density estimated by means of binding or flux measurements result in 10 times higher values than those estimates based on single channel data (Auerbach and Sachs, 1985). Also, those blebs are electrically similar to seals obtained from liposomes (Tank et al., 1982) or from passing the pipette tip through a lipid film (Coronado and Latorre, 1983).

The other model accounted by Suchyna et al. suggests that the patch region is more like a sample of the cell cortex rather than a bilayer, but with substantial differences in terms of heterogeneity and mechanical stress. The basis of this model comes from two distinct patch dome features: first, that it is supported by the cortical cytoskeleton (Akinlaja and Sachs, 1998); second, that the biological membrane contains proteins that affect adhesion, as they are embedded in the bilayer and in the extracellular matrix. The raising question is: how can a tight seal form if the bilayer is prevented to closely adhere at the glass surface from protruding proteins? E.g., acetylcholine receptors (AChRs) protrude more than 5 nm above the bilayer (Unwin, 1995). The proposed mechanism remembers the progress of a zipper, where AChRs denature against the glass (Ruknudin et al., 1991) and can pull the adjacent membrane closer to the glass, helping the seal to progress. The presence in the seal of AChRs as well as other membrane proteins that have been fused with GFP has been confirmed by electron microscopy (Ruknudin et al., 1991). In addition, the study of Suchyna et al. discusses in detail other seal-forming dynamics as the patch creep\(^3\) in function of the applied electric field and the role of the pH.

\(^3\) Creep rate is the rate at which the seal region moves deeper into the pipette (faster for negative than for positive pipette potentials at pH 7.0).
Depending on the pipette size, the amount of pressure applied, cell type and cell condition, it is believed that a combination of the models may occur. Both the cases are evidence-supported and many behavior of patch-clamping can be explained with the use of the one or the other model (Merkel et al., 2000). Suchyna and others imaged the process of seal formation by means of Differential Interference Microscopy (DIC) and showed how the membrane is sucked into the glass micropipette for several micrometers (Figure 1.7), thus spawning the gigaohmic resistance. This observation is of particular importance to understand the difference in terms of seal formation between conventional pipettes and FluidFM probes; refer to Chapter 4.3.1.

1.3.2. The cell as an electrical component

Patch-clamp amplifiers also include dedicated electronics to deal with particular effects arising from the inclusion of a cell in the electrical circuit as capacitance or series-resistance compensation. In fact, when successfully patched, the cell becomes integrative part of the electronic circuit connected to the current amplifier. To interpret correctly the recordings in cell-attached or whole-cell configuration, one has to understand the electrical characteristics of a cell first. The plasma membrane of an intact cell can be understood as a resistor in parallel to a capacitor and a voltage source (Figure 1.8). The resistor emulates the membrane permeability to ions, whereas the capacitance arises from the accumulation of charged particles across the thin plasma membrane. The voltage source stands for the transmembrane resting potential.
INTRODUCTION

When patched, the electronic components of the cells are added to those of the glass micropipette. Figure 1.9a shows the equivalent circuit of the cell-attached configuration. The micropipette is modelled with a parallel capacitance and resistance. Again, the capacitance stands for the accumulation of charged particles on the glass surface and the resistance for the constrained ion mobility. In addition, the patch is modelled with two resistors in parallel: the leak resistance accounting for the ions escaping the gigaseal, and the patch resistance. The latter represents the permeability to ions of the patched portion of the membrane.

Figure 1.9a Equivalent circuit of the cell-attached configuration. (Molleman, 2002)

b Equivalent circuit of the whole-cell configuration: the integrity of the membrane is lost and the transmembrane potential is defined through the inserted electrode (i.e. glass micropipette). In both diagrams, the dot is represented as the bath electrode. (Molleman, 2002).

Figure 1.9b depicts the equivalent circuit after rupture of the patch, i.e. in whole-cell configuration. In this configuration, the patch resistance is substituted with the access resistance highlighting the concept of direct access to the cytosol. By disrupting the plasma membrane, the transmembrane potential is lost too. The electrode has direct electrical
contact with the intracellular environment, conferring the ability to externally control or follow\(^4\) the membrane potential.

### 1.3.3. Patch-clamp at a glance

The major drawback of this technique is its need of a highly skilled operator and the low throughput of data per day, which settles indicatively at max. 15 cells per day. Table 1-2 summarizes the advantages and drawbacks of conventional patch-clamp.

<table>
<thead>
<tr>
<th>Pro</th>
<th>Contra</th>
</tr>
</thead>
<tbody>
<tr>
<td>High degree of flexibility, applicable on many different cell types, different recording modes</td>
<td>Procedure requires highly skilled operator and is technically challenging</td>
</tr>
<tr>
<td>Selectable target cell</td>
<td>Low throughput (&lt;15 cells per day)</td>
</tr>
<tr>
<td>Best recording quality</td>
<td>Temporal instability (~30 min)</td>
</tr>
<tr>
<td>Cheap consumables</td>
<td>Expensive electronics and manipulators</td>
</tr>
</tbody>
</table>

Table 1-2: Advantages and drawbacks of the conventional patch-clamp technique.

### 1.3.4. State of the Art

Especially in the pharmaceutical industry, the need of more data points per day in the drug screening process has shown the lack of automated technologies that allow the delivery of reliable measurements several order of magnitude faster than the conventional patch-clamp method. The lack was not concerning the hardware only, but the need of powerful software capable to analyze that amount of data was also a hurdle. Indeed, many companies recognized this huge opportunity and entered the market of electrophysiology to address this bottleneck in pharmaceutical preclinical research. In fact, today the higher-throughput methodologies have become integral parts of ion-channel drug discovery process.

In this chapter, I will give an overview of the automated patch-clamp systems available on the market and on the state of the art of recent nano-inspired devices.

---

\(^4\) The membrane potential is *controlled* in the classical voltage-clamp configuration, whereas it is *followed* by using the current-clamp configuration. These two modes can be intuitively understood as the *potentiostatic* or *amperostatic* modes of a classical potentiostat.
1.3.4.1. Commercial systems

In short, the commercial landscape of automated patch-clamp consists of variants of three different concepts: i) robotized conventional patch-clamping, ii) flip-the-tip concept and iii) planar patch-clamp. The three of them are shortly presented hereafter. For the interested reader, the review of J. Funlop et al. published in 2008 contains a valuable comparison between the already commercialized high-throughput patch-clamp technologies that were at that time already used in the drug research process (Dunlop et al., 2008).

The firsts commercially available fully-automated systems (including patch-pipette handling, gigaseals etc.) showed up around the late 1990s and basically were mimicking the conventional patch-clamp in an automated fashion. One of the first was the NeuroPatch by Neurosearch, which was a robot that took over the tasks of the operator, automatizing the pipette’s approach, seal formation and the perfusion tasks. Others developed fully automated systems following the same principle as the RoboPatch (Vasilyev et al., 2006) or the RoboCyte (Schnizler et al., 2003) to cite some.

An important innovative step has been done with the Flip-the-tip concept introduced in 2003 (Lepple-Wienhues et al., 2003), where the manual handling has been removed from the process. They did this by “inverting” the side of the micropipette used to address the cell: the cells are loaded from the backside of the pipette in form of a suspension and one cell is then trapped in the micropipette’s tip by gentle suction. Besides its very elegant and simplifying solution, the major drawback of this technology is the limited access to the extracellular solution and therefore the perfusion performance of these systems is limited.

The so called planar patch-clamp approach has nowadays practically overcome the conventional one, because it is the only one that can cope with the real throughput needs of pharmaceutical industry, which is around 10'000 data points per day. Planar patch-clamp chips consists of a flat substrate with a micrometer-sized aperture where a cell is
trapped with the help of computer-controlled suction (Fertig et al., 2002) (Figure 1.10, c). Even though the concept of planar patch-clamp has been introduced by a Russian scientist back in 1975 (Kryshtal’ and Pidoplichko, 1975), only a few years ago the technology has successfully entered the market of high-throughput electrophysiology. Aspects like the high cost of the consumables (chips), lower recording quality and reduced flexibility have hindered the success of planar patch-clamp techniques, which are yet source of discussion in the field. For a chronological overview of the evolution of planar patch-clamp techniques, the reader is redirected to the planar patch-clamping Chapter of the referenced book (Walz et al., 2007).

The major hurdle of planar-patch is the need of high quality cell lines. Since the cells are selected blindly, a large number of them has to be healthy and dissociated. This is usually obtained using known cell-lines that are friendly-to-patch and with known patch characteristics making it difficult to be used with primary cells isolated from animals. For this reason, traditional laboratories carrying on fundamental research are still clearly in favor of conventional patch-clamp.

![Table 1-3: Pros and cons of robotic patch-clamp, flip-the-tip and planar patch-clamp (Farre et al., 2008).](image)

In conclusion, even though automated patch-clamp has found his way into pre-clinical drug research, available systems are considered to be only medium throughput compared to the real needs in pharmaceutical industry, and costs per data point is still considered high, mostly because of the consumables cost. Table 1-3 gives a summary of advantages and disadvantages of the above presented automated patch-clamp systems.

**1.3.4.2. Recent nano-inspired developments**

The need for approaches that allow high throughput cell’s electrical activity examination together with high quality recordings has pushed the field of nanotechnology to propose novel technologies and methods. Both the potential of intracellular and extracellular recordings have been investigated. The first, notably being the most used and accounted for (e.g. whole-cell patch-clamp), has the advantage to produce high quality recordings
and usually allows precise positioning relative to the cell. In addition, the transmembrane potential so far can only be precisely controlled with intracellular recordings methods together with electrolyte-filled glass micropipettes. They however have the disadvantage of being invasive. Indeed, the contact between the cell and the electrode is difficult to be maintained for more than several hours (Sakmann and Neher, 1995). Extracellular recordings, in contrast, have a rather low signal to noise ratio compared to intracellular recordings. The reason for this is mainly the poor electrical connection at the cell/electrode interface (Prohaska et al., 1986). However, they have the advantage not to be invasive, so that recordings over prolonged period of time are possible. In addition, extracellular recordings allow for parallelization allowing high resolution recordings of single cell activity (Hierlemann et al., 2011) or from complete networks (Li et al., 2003; Wirth and Lüscher, 2004).

Hereafter I give a brief overview of the most innovative approaches that nanotechnology has produced. For a comprehensive review on the topic, the reader is redirected to the following review articles: Nanotechnology meets electrophysiology (Kwiat et al., 2013) and multi-electrode array technologies for neuroscience and cardiology (Spira and Hai, 2013).

Importantly, none of the hereafter proposed technologies have the ability to control the transmembrane potential, which prevents their use to assess voltage-gated ion channels.

MEAs vs. FETs

Two substantially different methods can be distinguished when discussing about electrical recordings at the nanoscale: with the use of conventional, metal-based, nano-sized electrodes, or with the use of field-effect-transistors (FETs). The first ones include the already spread field of micro electrodes arrays (MEAs). Although they solve the problem of the invasiveness, the studies so far did not show to improve substantially the spatial resolution. The reason of this limit lies in the nature of the devices: in fact, their miniaturization imply an increase in electrode impedance and a decrease of the seal resistances. Both account for a reduced signal-to-noise ratio. The impedance (and therefore the performance) of FETs, on the contrary, does not deteriorate with the miniaturization (Sze and Ng, 2006) and are therefore suitable for the fabrication of highly dense chips. It is however difficult to fabricate state of the art FETs which are sufficiently low-noise to allow recordings from single neurons (Voelker and Fromherz, 2005).
Intracellular recordings

The technologies presented in this paragraph are the result of the effort to merge the advantages of substrate-integrated electrodes array with the advantages of intracellular electrodes. An interesting example is the work of Xie et al. (Xie et al., 2012) that developed a device made of vertical Pt nanopillar electrodes. In this case, the cells are seeded on the electrodes that are engulfed during the growth process. They demonstrated that they could perform intracellular recording by nanoscale electroporation. The measurement can be repeated over long periods of time and with astonishing sensitivity, even though the recorded amplitude is still 10 times lower than conventional patch-clamp (Figure 1.11).

A similar work with similar results was made by Robinson et al. using silicon nanopillars instead of platinum (Robinson et al., 2012). Interestingly, they were able to show that many nanopillars spontaneously penetrated the cell membrane (Figure 1.11c). Another interesting approach that led to improved electrical coupling between cell and electrodes is represented by the work of Shappir et al. where mushroom-like electrodes are being engulfed via phagocytosis (Hai et al., 2010, 2009). By this approach they show “intracellular-like” recordings.

Figure 1.11: a SEM image of a vertical nanopillar-electrodes array. The pillars have a diameter of 150 nm. (Xie et al., 2012) b Section and magnification of the cell-nanopillar electrode interface. The electrode is engulfed by the cell. (Xie et al., 2012) c False-colors SEM of an array of nine silicon nanowires. The insulation silicon oxide layer is colored in blue, whereas the metal coated nanowire probes are colored in grey. (Robinson et al., 2012) d Rat cortical cell engulfing the nano-pillars electrode array. The inset shows a particular of the interface between a nano-pillar electrode and cell. (Robinson et al., 2012).
FETs based probes have demonstrated to be promising tools for recording and stimulation of excitable cells. Indeed, the approach, amplitude and noise of the recordings are very similar to those of conventional patch-clamping obtained with glass micropipettes. Tian et al. showed electrical recordings from cardiomyocytes with their novel 3D FET device designed as an out-of-plane bended silicon nano-wire. Their FET-based probe is capable of entering the cells through endocytic pathways, being therefore minimally invasive (Tian et al., 2010). In a follow up study and with a modified probe, they demonstrated high spatial resolution sensing together with intracellular recording (Jiang et al., 2012).

**Extracellular recordings**

Substrate-integrated microelectrode arrays (MEAs) represent the leading methodology for studying the electrical activity of large populations of cells in vitro. They can be simple miniaturized metal electrodes (Nam and Wheeler, 2011) (Figure 1.2), transistor based, i.e. sensitive on extracellular field potential changes (Hutzler et al., 2006) or based on other technologies like CMOS\(^5\) (Hutzler et al., 2006).

The first MEA appeared in the 1972 (Thomas et al., 1972) and proposed 30 electrodes arranged in two rows 50 μm apart. Today, MEAs may contain over 10'000 electrodes for in vitro applications (Hutzler et al. 2006, to cite an example) and over a hundred for in vivo applications (Hochberg et al. 2006 among many). Researchers started to combine planar microelectrodes array with microfluidics (Pearce et al., 2005). This innovative step is of particular interest when compared to the FluidFM system because the application fields become similar. Whereas patch-clamp has the advantage to provide highest recording quality on single cell level, microelectrode arrays can simultaneously address an entire cell population.

\(^5\) CMOS: Complementary metal-oxide-semiconductor. It refers to widely spread particular style of digital circuitry design, as well as a family of processes used to fabricate certain field-effect transistors.
The use of substrate-embedded microelectrodes array allows for simultaneous extracellular recording and stimulation of large populations of cells over a time period that spans over months, as no damage is inflicted to the cell of interest. However, the greatest drawback of extracellular sensing is the limited information gained compared to intracellular recordings (Buzsáki et al., 2012). In addition, big arrays become computationally intensive and data analysis may be a problem.

It is to overcome these limitations that development of MEAs is transitioning from a 2D design to 3D. The examples presented in the previous paragraph (intracellular recordings), are the first successful attempts to achieve intracellular-like recordings with substrate-integrated microelectrode arrays.
1.4. FluidFM and single-cell manipulation

Scope of this chapter is to introduce the FluidFM to its principal characteristics and functions. Refer to the review article *Force-controlled manipulation of single cells: from AFM to FluidFM* (Guillaume-Gentil et al., 2014a) for a critical discussion of the already carried out experiments and to Pablo Dörig’s dissertation (Dörig, 2013) for additional technical details.

1.4.1. The FluidFM

The FluidFM is an AFM configuration, the general concept of Scanning Probe Microscopy (SPM) has therefore to be introduced first. The SPM family comprehends all the techniques that produce an image of a sample, *pixel by pixel*, in a scanning fashion: a *probe* scans a specimen and interacts with it through highly localized physical phenomena that are separation-dependent. A finite number of datapoints is acquired during the scan, each corresponding to a pixel of the image. The resolution of these techniques varies considerably from technique to technique, with many of them reaching atomic resolution.

1.4.1.1. The Atomic Force Microscope (AFM)

For a comprehensive description of the AFM, especially about deflection detection methods, interaction forces and modes of operation, the manuscript by Ernst Meyer is a recommended reading (Meyer, 1992).

The AFM comes as an evolution of the scanning tunneling microscope (STM) (Binnig et al., 1982), for which the inventors were awarded with the Nobel prize in physics in 1986. They were among the first to recognize the potential of piezoelectric actuators, whose precision is affected from the electrical noise in the driving signal only, allowing them to reach sub-nm resolution. The STM is based on the tunneling current that arises between two conductors biased by a voltage that are brought at sub-nanometer distance. This current is highly dependent on the separation between specimen and scanning probe and is used to feed a feedback controller that drives the actuator to maintain said current at a constant value.
The biggest limitation of the STM is that its use is restricted to conductive substrates. With the STM as a starting point, the atomic force microscope was only a step further away. Indeed the first AFM demonstration was carried out with an STM sensor to monitor the deflection. The difference lies in the interaction type between substrate and probe. The AFM tip no longer interacts via tunneling current with the specimen, but via forces. Figure 1.14 shows a simplification of the involved forces that can be divided into repulsive and attractive forces. The summation of those two forces categories define the force felt by the AFM probe.

Repulsive forces are mainly due to the overlap of the electronic orbitals and appear at atomic distances. This repulsive behavior is also known as Pauli repulsion. It is in this regime that the probe is considered to be in contact. The biggest source of attractive forces arise from Van der Waals interaction between atoms, which are always present. On the long range, electrostatic forces are dominant. These forces arise from the localized
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charges and from the polarization of the substrate due to potential difference between the probe and the substrate. In air or in vacuum, these forces can be rather big and are usually compensated applying a potential between the probe and the substrate. In water, in the presence of ions, the electric fields are shielded and the long-range effect disappears. The most common method used to measure these weak forces (often in the sub-picoNewton range) is the leverage method: the probe is fabricated at the free end of a cantilever, and a laser is reflected off its back. A minute change in deflection is amplified by the laser that bounces off the cantilever into a photodetector, enabling very accurate measurement of the deflection. This detection method is called Optical Beam Detection (OBD) (Meyer and Amer, 1988) and is nowadays still the standard detection method (Figure 1.13).

Basic operation modes:

Two main operation modes can be distinguished. i) the static mode (or DC-Mode) and ii) the dynamic mode (or AC-Mode).

In the static mode, the cantilever is either kept at a fixed height position, or its height is controlled by feedback controller in order to maintain the deflection constant. In the first case, the probe is scanned in contact with the substrate and the recorded deflection contains topography information. In the second case, it is the piezo driving signal that contains the topography information, as the probe is supposed to follow the profile of the substrate.

The dynamic mode is characterized by the probe oscillating close to the resonance frequency. The probe-sample interaction force shifts the resonance frequency and influences the amplitude of the oscillation. This mode offers the possibility to obtain topography information in non-contact regime (compare with Figure 1.14).
1.4.1.2. FluidFM: the concept

The wish to actively deliver precise amounts of (bio)molecules through nanosized probes for experiments for local surface patterning stimulated the development of novel AFM-based strategies to dispense liquids, thus leading to the invention of microchanneled AFM cantilevers, i.e. cantilevers with an embedded microchannel (Guillaume-Gentil et al., 2014a).

OBD microchanneled cantilevers

This field was pioneered with the nanodispensing (NADIS) system (Meister et al., 2004) consisting of a standard AFM cantilever bearing a hollow pyramid with a nanosized aperture at the apex: a microdroplet of solution was placed into the hollow pyramid with the help of a glass micropipette, and after bringing the probe in contact with a substrate, nanodroplets of liquid were deposited by capillarity on the sample. Glycerol had to be mixed to the solution to limit the too quick water evaporation. Having the same evaporation issue in mind, a Dutch group proposed the first AFM cantilevers with an incorporated microchannel connecting on one side the hollow pyramidal probe and on the other side a macro reservoir (Deladi et al., 2004). The microchannel was obtained by etching of a sacrificial layer between two layers of Si₃N₄ (becoming eventually the cantilever walls), a strategy later also followed by a US group (Kim et al., 2005).

Another approach was to thermally bond two layers (one with the already etched channel) together (Hug et al., 2005; Meister et al., 2008). More recently, the two methods have been combined together by a Japanese group (Kato et al., 2010). The nanosized aperture at
the apex of the pyramid or close to it are produced either lithographically or drilled with focused ion beam (FIB). Such microchanneled cantilevers were used for deposition experiments only in air where capillarity is the ruling mechanism.

The transformation of a microchanneled AFM cantilever (see Chapter 3 for a detailed presentation) into a force-controlled nanopipette to be operated in liquid environment was achieved by connecting the microchanneled cantilever to a pressure controller via a fluidic circuit: a novel configuration that we called “FluidFM” (Figure 1.16a) (Meister et al., 2009). Micro-channeled cantilevers are fixed in a watertight way on a drilled AFM probeholder. Over- and underpressure can thus be applied in the nanofluidic channel making the FluidFM a versatile tool for single-cell manipulation (Guillaume-Gentil et al., 2014a) and surface patterning (Grüter et al., 2013).

Both the technologies underlying the FluidFM, i.e. microfluidics and the atomic force microscope (AFM) have marked the past decade of the field of micro-electro-mechanical system technology. Whereas AFM is a standard tool in surface science now entering the field of biology, microfluidics has been used to create all kinds of devices and systems, especially in the biomedical field, ranging from micropumps to a variety of micro- and nano-needles (Ashraf et al., 2011). Hereafter I give a short overview on the applications that have demonstrated the validity of combining microfluidics with AFM.

Figure 1.16  

**a** Diagram showing a microchanneled cantilever chip fixed to a drilled AFM probeholder. The FluidFM can be operated in air or with the whole system (probeholder and chip) immersed in a liquid. The external liquid or bath and the liquid in the microchannel may be identical or different. During liquid dispensing, the substrate can be simultaneously observed with an optical microscope either through the transparent probe holder or through the glass substrate. (Meister et al., 2009).  

**b** Detailed schema showing the components of a typical FluidFM setup composed of a FlexAFM (Nanosurf), a pressure controller and an inverted optical microscope. Source: www.nanosurf.ch.
1.4.1.3. **FluidFM selected applications**

After an initial validation phase at LBB, The FluidFM technology has been successfully transferred to the institute of Microbiology (ETH D-BIOL, Prof. Vorholt), to focus on single-cell studies. New protocols for injection and extraction have been established in this framework. Refer to the referenced review (Guillaume-Gentil et al., 2014a). I report here a summary of the applications where the FluidFM has been used.

**Controlled delivery of bioactive agents**

Past research proved that the ability to deliver an accurate amount of bioactive substances on, or into a biological system is of great interest. Meister and others were the first to use the FluidFM in this sense (Meister et al., 2009).

The simplest method to deliver fluid locally is to use the FluidFM similarly to a micropipette, dispensing liquid in gentle contact with the cell membrane. Following this strategy, the probe is brought above the target location or on top of a selected cell exploiting the force control capabilities of the AFM. Then, by careful control of the applied pressure, the bioactive substance is released. This approach has been firstly used by Meister and others to deliver membrane-permeable fluorescent molecules into a neuroblastoma cell (Meister et al., 2009). The same technique has been used from Stiefel and others to deliver a controlled number of virions (from 1 to 12) on single HeLa cells (Stiefel et al., 2012) (Figure 1.17a). Later, Guillaume-Gentil and others achieved selective isolation of single cells from confluent layers through localized trypsinization (Guillaume-Gentil et al., 2014b) (Figure 1.17b).

Dispensing into cells is achieved after membrane perforation. In this case the probe is pushed into the cell by monitoring the force which contains information on mechanical penetration events. The probe can therefore be placed in a controlled way into the cytosolic environment and thus allow local dispensing of agents into the biological system (Figure 1.17c). This injection procedure was demonstrated using FIB-modified FluidFM probes by Guillaume-Gentil and others (Guillaume-Gentil et al., 2013).
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Figure 1.17: Delivery of bioactive agents with the FluidFM. a Illustration of single vaccinia virus deposited on a single HeLa cell. (Stiefel et al., 2012). b Local cell detachment followed by trypsin release. (Guillaume-Gentil et al., 2014b). c Illustration of an injection into cell nucleus experiment with the FluidFM. (Guillaume-Gentil et al., 2013).

Spatial manipulation

Commonly referred to as pick-and-place, the FluidFM can be used to precisely, stably and gently manipulate micro-objects that are reversibly immobilized at the aperture of the cantilever by means of suction. The trapped object then can be repositioned on the desired substrate and released by application of overpressure (Figure 1.18).

Figure 1.18: Pick-and-place with the FluidFM. a With active force control, the aperture is positioned on top of the object that has to be immobilized. b Suction is applied and the cantilever is withdrawn. c While maintaining negative pressure, the FluidFM probe is moved to the new position. d The force-controlled approach is repeated and overpressure is applied to release the object onto the new position. (Dörig et al., 2010).

This method has been demonstrated first to manipulate living bacteria on a solid substrate (Dörig et al., 2010) and to isolate bacteria from complex mixtures with optical interrogation (Stiefel et al., 2013). Guillaume and others extended the pick-and-place technique to mammalian cell (Guillaume-Gentil et al., 2014b). In addition, colloidal beads have been manipulated in the very similar way and used as exchangeable probes (Dörig et al., 2013).

Adhesion quantification

Similarly to the procedure described above, the FluidFM probe can be positioned onto an object of interest, which can be immobilized by application of negative pressure inside the microchannel. During the lifting process, the adhesion forces can be measured by means of AFM force spectroscopy.
Potthoff and others demonstrated this technique by measuring adhesion forces of both yeasts and mammalian cells (Potthoff et al., 2012) and used it to measure mammalian cell adhesion forces in function of the substrate topography (Potthoff et al., 2014).

The adhesive force measurement protocol of the FluidFM offers two unique advantages: i) higher throughput. In fact, in contrast to conventional single cell force spectroscopy techniques (SCFS), the fast and reversible immobilization offered by the FluidFM system allows for 10 times more measurements per day (Potthoff et al., 2012), ii) dynamic adhesion force studies are favored, as the cell can be cultured under standard conditions and only immobilized at the cantilever at the very moment of data acquisition, again, in contrast to protocols proposed by conventional SCFS.

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6 Commonly, one probe is needed for every adhesion measurement.
1.4.2. Competing technologies

In this Chapter an overview on the technologies directly competing with the FluidFM is given.

1.4.2.1. AFM-based lithography systems

NADIS, Dip-pen and Fountain pen

The first of such methods is known as the Dip pen nanolithography (Jaschke and Butt, 1995; Piner, 1999). An “ink” containing the desired molecules is firstly deposited on the scanning probe. By scanning the coated probe on a substrate, the ink is transferred by means of capillarity which depends on air humidity, temperature and surface tension (Figure 1.20a). As it is a capillary-driven method, it only works in a dry environment.

![Figure 1.20: AFM based lithography systems. a Dip pen nanolithography. (Jaschke and Butt, 1995). b NAnoscale DISpensing (NADIS) principle. A hollow pyramid functions both as nozzle and reservoir. (Meister et al., 2004). c Fountain pen design (Deladi et al., 2004).](image)

The NADIS (Meister et al., 2004) probe and the fountain pen design (Deladi et al., 2004) tried to solve the evaporation problem that was affecting the Dip pen nanolithography method by integrating a reservoir on the cantilever. In the NADIS case a hollow pyramid acted both as a reservoir and as a dispenser (Figure 1.20b), whereas the fountain pen had the reservoirs positioned back on the chip (Figure 1.20c). A microchannel connected to this reservoir pulled the liquid to the aperture in the pyramidal probe by capillary forces. Both systems are designed to work in air. Mixtures with glycerol are used to avoid evaporation issues. They cannot be used in a liquid environment because of the open reservoir.

Interestingly, the NADIS and the fountain pen are both precursors of the hollow cantilever used in FluidFM systems.

Volcano tip

Similar to the previously presented fountain pen, the volcano tip (or nanofountain probe) features a hollow cantilever and a reservoir positioned back onto the chip (Kim et al., 2005). As the fountain pen, its use was mainly in air where molecules were deposited on a substrate by means of capillary forces. Only recently, the authors released an upgraded
version with closed microfluidics and an integrated electrode (Kang et al., 2013). This probe was used in fully-liquid environment for successful single-cell electroporation. Even though they did not couple the probes with an AFM system, the Volcano probes are very similar in design and function to the FluidFM.

Figure 1.21: Functioning and design of the volcano-probe. a Illustration of the capillary-driven writing mechanism. b The liquid reservoir is positioned in the body of the chip. Liquid is drawn to the probe aperture via capillary effect. c SEM view of the volcano probe aperture. d SEM view of the whole chip body. (Kim et al., 2005).

The Bioprobe

The Bioprobe closely resembles the FluidFM. However, the microfabrication of such probes is more challenging. On the one side, the bonding of two wafers is difficult in terms of alignment. On the other side, FIB milling is needed to open the channel. The latter makes the mass production of such probes unlikely because of the low throughput and high costs of FIB. In addition, compared to FluidFM, the Bioprobe is stiffer (150 N/m), which is a drawback when operated in static mode (as it is the case in most of single-cell applications). However, they successfully operated the probe in full-liquid environment and showed in-liquid deposition and injection into mammalian cells by electroporation (Shibata et al., 2011).

Figure 1.22: SEM images of a bioprobe. Inset shows a zoom of the aperture. (Shibata et al., 2013).
1.4.2.2. Glass micropipettes

One of the major competitors of the FluidFM is the classical glass micropipette. Glass micropipettes are widely used in a broad range of applications: from electrophysiological recordings and single cell manipulation to high resolution imaging of cells (SICM, see Chapter 6). Glass micropipettes are the most used tool in biomedical research since decades as they are easy to produce and cheap. Their opening diameter can be well controlled by the adequate choice of parameters of the pipette puller. Sizes from several micrometers down to tens of nanometers are achievable and can be qualitatively determined by measuring its electrical impedance.

![Figure 1.23 Macroscopic image of a pulled glass micropipette. Source: (http://www.medicine.mcgill.ca/physio/vlab/rmp/record_circuit_n.htm).](http://www.medicine.mcgill.ca/physio/vlab/rmp/record_circuit_n.htm)

The major drawback of glass micropipettes is the lack of control on their positioning. This is usually solved by controlling the position with expensive micro- or nano-manipulators, under optical control.

Glass micropipettes can be seen as direct competitors to the FluidFM system especially in the following fields: i) patch-clamp, or more in general, for single-cell electrophysiological recordings, ii) cell injection experiments (Zhang and Yu, 2008) and iii) cell adhesion measurements (Shao and Hochmuth, 1996).

1.4.2.3. Optical tweezers

The invention of optical tweezers dates back to the year 1986 (Ashkin et al., 1986) and were firstly presented as single-beam gradient force optical trap. A highly focused laser beam provides forces in the order of piconewtons that traps dielectric objects at the focus spot.
It has been shown that this technique can successfully manipulate cells and particles (Grier, 2003). Although the exerted force cannot be measured directly, based on the displacement of trapped objects from the focus’ center a resolution in the range of femtonewtons can be achieved. The drawback of this technology is the very low force that can be exerted and the potential damage to tissues and molecules because of overheating and high light radiation.
2. Scope of the Thesis

2.1. Electrophysiology and the FluidFM: technical challenges

In the framework of my thesis, I envisioned to merge the FluidFM with the patch-clamp, the most widespread recording method among the electrophysiology techniques. In particular, I aimed at performing force-controlled patch-clamp in whole-cell configuration with the FluidFM to address voltage-gated ion-channels.

First milestone of the project was to develop the hardware and software necessary to integrate the electronics and the equipment related to the patch-clamp technique with the FluidFM (i.e. AFM controller). Hardware included the design and manufacturing of metal cages and holders for electronic noise protection and connectors to combine patch-clamp amplifier with the AFM. On the software side, LabView has been chosen to provide simultaneous data acquisition (ionic current and deflection) as well as the analysis environment.

2.2. Focused Ion Beam to enable pilot studies

Together with Prof. Vorholt of the Institute of Microbiology (ETH Zurich), we envisioned different applications where the use of the FluidFM had the opportunity to answer original questions in the field of biology. These applications are shortly described in Chapter 4. The FIB was the appropriate tool to manufacture customized FluidFM probes with apertures specially designed for the selected applications and to address problems related to the manufacturing quality of the probes. FIB fabrication of FluidFM probes has constituted an important part of my work and allowed to employ the FluidFM for i) adhesion
force measurement of mammalian and microbial cells, ii) injection and extraction from mammalian cells, iii) patch-clamp and iv) SICM.

The protocols for the fabrication of the above mentioned FluidFM probes are reported in Chapter 4.

2.3. Force-controlled electrophysiology

After the developmental phase of the FluidFM for electrophysiology, two different application scenarios were envisioned:

- Assessment of advantages and limitations of the FluidFM for force controlled patch-clamp experiments
- Utilization of the system for gentle imaging purposes, merging scanning ion conductance microscopy and AFM together.

Finally, the use of the FluidFM as a micro-balance to grasp and precisely measure the mass of micro-sized objects was pioneered.

2.3.1. Force controlled patch-clamp in whole-cell mode

Patch-clamp is the reference technique in terms of voltage-gated ion-channel characterization. Although widely employed in the laboratories of industry and universities, it suffers from low throughput and the necessity of a highly skilled operator. It is the aim of this project to understand if the force-sensing capabilities of the FluidFM can be of advantage in this field. In particular, patch-clamp in the whole-cell configuration is addressed because of its importance in the investigation of voltage-gated ion-channels.

In collaboration with Prof. Abriel (Department of Clinical Research, University of Bern), the strategy was to first demonstrate the ability to record activity of well-known voltage-gated ion-channels through the FluidFM microchanneled cantilever. I studied the sealing behavior of the cell membrane within the aperture of FluidFM probe and compared to results obtained on conventional patch-clamp made with pulled glass-micropipettes. Two fundamental biological systems have been targeted: first, the potassium hERG channel because of its well-known and slow dynamics, then on the NA\textsubscript{v}1.5 sodium channel because of its fast activation (sub-millisecond). The latter, was used to understand the limits of the FluidFM probe in terms of accuracy of the voltage-clamp circuitry in combination with the FluidFM probe.
At this point the added value of the force sensing capabilities could be exploited. Isolated cardiomyocytes have been used to demonstrate that the FluidFM is capable of simultaneous whole-cell patch-clamp recordings and force sensing. For the first time reported, generated force and transmembrane ionic current could be directly correlated and measured through the same interrogation device. In parallel, advantages in terms of stability, cell viability and noise have been addressed in the framework of this project.

2.3.2. Force-controlled SICM

Scanning Ion Conductance Microscopy is a non-contact imaging technique of the scanning probe microscopy family. The roots of such technique are strictly related to patch-clamp. Indeed, the used electronics and probes are the same, with minor differences. The only fundamental addition needed is an XYZ piezo scanner that is used to manipulate standard glass micropipette. The technique is presented in Chapter 6. As the FluidFM naturally includes precise XYZ positioning actuators, it was my task to understand if the technique was affine with the FluidFM probes too.

Therefore, the first reached milestone was to critically evaluate the SICM technique applied to the FluidFM. Aspects such as resolution, probe geometry influence, electrical impedance and applicability of standard SICM models have been considered both computationally with the use of the software COMSOL and experimentally on defined substrates and cells.

This level of understanding being reached, the possibility to improve in terms of imaging robustness and speed compared to conventional SICM setups is envisioned, by making profit of the force sensing capabilities. It was our goal to demonstrate a novel SICM concept, in which two controllers are working on two different physical signals (ionic current and deflection), in unison. In other words, to implement a sort of backup feedback loop driven by the deflection signal of the FluidFM cantilever that would intervene when the feedback of the SICM fails to maintain the minimum substrate-probe distance.

2.3.3. FluidFM micro-balance

The third FluidFM application pioneered with the FluidFM in the framework of this thesis raised from a fruitful discussion with Dr. Vassalli (CNR Institute of Biophysics, Genoa) where he proposed to use the FluidFM to i) catch single micro-objects and ii) to weight them by following the induced change in the resonance frequency of the cantilever.
The goal within this project was to investigate resonance frequency shift detection in both air and liquid environment after adding a mass to the probe aperture. Based on this and on other limitations (e.g. immobilization hurdles, refer to Chapter 4.3.4), 5 μm diameter borosilicate spheres were selected, which were fixed by suction at the probe aperture of oscillating tipless FluidFM probes. The resonance frequency shift was measured either by common frequency spectra acquisition or with the use of Phase-Lock-Loop (PLL) controllers. The measured values were validated by precise estimation of the volume of each immobilized sphere by means of AFM imaging on a calibration grid. In air, the measurements were precise and offered high resolution to follow the frequency shift, whereas in water, the lowered Q factor diminished the sensitivity of the measurement.

2.4. Structure of the thesis

Chapter 1 provides an introduction on the electrophysiology field and its general importance, containing a brief overview of the patch-clamp technique and its challenges, including a discussion on the automated patch-clamp systems that are on the market.

Chapter 3 contains the discussion of the developmental work done on the FluidFM to enable ionic current sensing through the microchanneled cantilever and the challenges encountered. The available instrumentation is described, and the most important results about the electrical characterization are presented.

Chapter 4 is about the use of Focused-Ion-Beam technique to fabricate special aperture design for particular FluidFM applications. Characteristics and problems related to the FluidFM cantilevers are treated in this Chapter, that concludes with the detailed protocols for the fabrication of FluidFM apertures for i) patch-clamp, ii) injection and extraction, iii) bacterial adhesion applications.

Chapter 5 summarizes the force controlled patch-clamp recordings obtained with the developed setup.

Chapter 6 presents the FluidFM used as a novel SICM-AFM hybrid imaging tool.

Chapter 7 shows the results of using FluidFM tipless cantilever as a tool to accurately and precisely weight micro-objects.

Chapter 8 gives an overall conclusion and outlook.

The results presented in Chapters 5, 7 and 8 either have been submitted (force-controlled patch-clamp) or are in the process of being submitted (force-controlled SICM and FluidFM...
as a microbalance). For each topic a brief introduction and state of the art analysis is given, together with a discussion of the difficulties encountered during realization. A conclusive paragraph at the end of each Chapter summarizes the respective most important results and proposes an outlook in the future work.
3. Development of the pc-FluidFM

Aim of this Chapter is to describe the developmental work I have carried out on the FluidFM to make the system compatible with electrophysiology recordings, i.e. patch-clamp, and scanning ion conductance microscopy.

The major milestones were i) the insertion of an electrode in the Cytoclip holder, ii) electrical and mechanical noise shielding and ii) development of the necessary software for simultaneous force and electrophysiology recordings.

3.1. Available Instrumentation

FluidFM system
The FluidFM system used in this thesis comprehends a Nanosurf FlexAFM headscanner with a 10 μm ranged piezo actuator and an ES2 controller. The software Easyscan2 was used to control the AFM. When needed, the z-actuator was driven externally using the z-axis input available from the break-out box. Signals of interest (e.g. vertical deflection, piezo position, etc.) were accessed from the same break-out box via BNC cables. The scanner actuator of the FlexAFM is decoupled from the z-actuator. XY movements are achieved through an electromagnetic actuator that is externally drivable through the X- and Y- inputs of the ES2 controller breakout box.

A special headstage-probeholder has been designed from Nanosurf to accommodate the Cytoclips, on which the FluidFM probes are permanently glued. A programmable pressure controller completes the FluidFM system that allows precise and stable application of pressure with mbar precision in a range from -800 mbar to +1000 mbar.
The FluidFM was accommodated on an inverted optical microscope (Axiovert TV100, Zeiss, Jena, Germany). A microscope stage (Nanosurf, Liestal, Switzerland) allowed to move the FluidFM independently from the specimen in X and Y direction. A set of Long Distance (LD) objectives from 5x to 40x magnification was available. The microscope was equipped with a fluorescent Xenon light source (XBO 75) and fluorescent filter sets for Rhodamine and FITC fluorescent dyes.

An environment controlled chamber completed the system that allowed temperature control (The Box & The Cube, Life Imaging Services, Basel, Switzerland).

**Lock-In Amplifier and PLL**

A Lock-In amplifier (Zurich Instruments, Zurich, Switzerland), was used when dealing with AC signal. The amplifier comprehended double PLLs and PIDs. PLLs have been used to track resonance frequency changes in the micro-balance project (Chapter 7) and PID was used as z-controller in the first SICM tests (Chapter 6.3).

**Patch-Clamp amplifiers**

Two Patch-Clamp amplifiers were at our disposition: an EPC7 (HEKA Elektronik, Lambrecht, Germany) and a PICO2 (Tecella Inc., Foothill Ranch, CA, USA).

The EPC 7 is an analogue, high quality patch-clamp amplifier that features all the electronics specifically designed for patch-clamp experimenting (e.g. capacitance compensation, pipette offset compensation, etc.). The EPC 7 is all-analogue meaning it is not computer controllable, but only using the front panel analogue knobs. The digitalization of the signals of interests happened through National Instruments acquisition cards and a self-written LabView program (Chapter 3.6). This amplifier features a small pre-amplifier that is appositely designed to be mounted extremely close to the measuring site. Being all analogue, this amplifier revealed to be the best choice in term of continuous DC measurement and represented our choice for all the experiments involving SICM (Chapter 6.3).

The PICO 2 Patch-clamp amplifier is one of the smallest patch-clamp amplifier on the market. The PICO 2 is USB powered and controllable only by the computer interface. It revealed to be less suitable for DC measurements as the digitalization introduced artefacts in the recorded signal. Thus, this amplifier was not used in combination with SICM experiments (Chapter 6.3). This amplifier could be directly mounted on the AFM because of the reduced size. All the compensation steps (i.e. capacitance and offset compensation) is done automatically by a mouse-click. In addition, this amplifier offers capacitance
compensation up to 100 pF, which is good in combination with the FluidFM as its probes showed capacitance values in average higher than conventional glass micropipettes. The recorded data are analogically accessible through a BNC adapter and can be recorded with external acquisition methods (NI DAQ cards and LabView in our specific case).

**Nanonis SPM controller**

When it came to test the capabilities of the FluidFM for SICM, we relied on a Nanonis control system (SPECS Zurich GmBh, Zurich, Switzerland). Nanonis control systems are fully digital controllers and compatible with any kind of SPM technique. The Z-piezo actuator of the FlexAFM scanner was addressed via the break-out box Z-piezo input, as well as the XY AFM actuator.

The controller comes with a fully-capable SPM software, one of the most advanced on the market. In addition, different controller dynamics are pre-programmed and readily available (e.g. logarithmic controller).

A wide range of add-on modules (e.g. Lock-In amplifiers, HV piezo controllers, etc.) can be added to the base controller anytime to increase controller capabilities and performance.

**Autolab Potentiostat**

An Autolab Potentiostat (Metrohm, Herisau, Switzerland) was used for the acquisition of Electrical Impedance Spectroscopy. The Autolab is a fully computer controllable potentiostat/galvanostat especially designed for electrochemical applications. The measurements were performed in a full-metal faraday cage to reduce picked-up noise.
3.2. Electrode insertion

History of the probeholder

In a first phase, the project envisioned the developmental work done to adapt the FluidFM technology to JPK AFM standards. Only later, the FluidFM technology shifted towards a Nanosurf-based system thus requiring the redesign of the probeholder. Here I briefly report on the developmental work done during this transition, together with the modification that allowed the insertion of an electrode in the probeholder itself.

The first prototype was developed to fit a NanoWizard 1 BioScience AFM (JPK Instruments, Berlin, Germany). The FluidFM probes (at that time with double their size) were fixed onto a drilled probeholder by means of a standard JPK spring (Meister et al., 2009). A small O-ring ensured liquid tightness between the FluidFM probe and the polycarbonate\(^7\) probeholder (Figure 3.1). I developed several versions of probeholders. Firstly to solve mechanical stability problems (i.e. mechanical stability of the tubing, FluidFM probe fixation to reduce movement relative to the probeholder upon pressure application, etc.), second, to integrate an electrode to allow measuring of ionic current through the microchannel. Figure 3.1 gives an overview of the evolutionary journey of the probeholder, in the switching process from a JPK-based system to a Nanosurf based system.

\(^7\) Polycarbonate is rated as a good material for patch-clamp applications because of low dielectric loss (Wonderlin et al., 1990)
With the help of computer aided design a new AFM probeholder that solved the issues recognized in the first prototypes has been designed, i.e. i) large reservoir volume, ii) weak mechanical connection between FluidFM probe and probeholder and iii) insertion of an electrode in close proximity to the FluidFM probe. The obtained probeholder was conceived to be used on a JPK AFM. The reservoir volume was reduced to 15 µL by integrating it in the probeholder and having one side connected to the pressure controller tubing that allowed to change the reservoir’s internal pressure. The connection between the FluidFM probe and the probeholder itself was improved by replacing the metal spring with a clamp fixed with screws and a silver wire was positioned in the reservoir.

![Image](image.png)

Figure 3.2 Thin design of the FluidFM probe holder compared with the second generation, more robust design.

I designed different types of clamps and tested their mechanical stability, together with the electrical characteristics of the system (E.g. capacitance and noise). The first one had a light design and was fabricated with electrical discharge machinery (EDM). Cemented carbide was used as material because of its strength. However, this material revealed to be too brittle and easy to brake. The same geometry was therefore produced in plastic PEEK and carbon-fiber reinforced polymer (Figure 3.2). The plastic version of this clamp succeeded in holding the FluidFM probe tightly against the O-ring, even though their higher flexibility compared to the metal one did not considerably improve the mechanical connection. For this reason, we proposed a second design (Figure 3.2) which is bulkier but easier to produce with conventional CNC milling machines and was produced in both plastic and metal.

Eventually, while designing the probeholder for the Nanosurf system, Cytosurge solved the mechanical coupling of FluidFM probes and probeholder by irreversibly gluing them together, resulting in a strong and stable mechanical connection (Figure 3.3). The main advantage of this solution is the increase of manipulation simplicity for the end-user. However, the irreversible gluing makes their reutilization difficult. In addition, the

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8 Wire EDM machine was used: a biased voltage is generated between the tool (wire) and the workpiece. When the distance between the two becomes small enough, a spark is produced because of current flowing. As a consequence, material is removed.
The probeholder is fabricated using high precision CNC machining, a costly process. The cost of the consumable therefore is the main drawback of this solution. Injection molding will be the way to go to reduce costs. The material of the probeholder (polycarbonate) and the composition of the glue can be an obstacle for some surface functionalization treatment or for some cleaning protocols.

**Electrode insertion and isolation**

The foremost goal of this thesis was to measure ionic current through the hollow FluidFM cantilever, in the same way that is done with the use of glass micropipettes in patch-clamp setups. Seen their widespread application in electrophysiology and their simple and reproducible fabrication, we used a two-electrode setup with Ag/AgCl electrodes. The working electrode is inserted in the reservoir that is integrated in the probeholder, whereas the second one is immersed in the bath and connected to the ground (Figure 3.4). 150 mM KCl electrolyte has routinely been used as standard test solution.

The electrode in the reservoir is incorporated in the cap that seals the reservoir, to facilitate its renovation and substitution in case of damage. Different electrodes and designs have been proposed, starting with the use of commercially available AgCl pellets. The latter was soldered onto an MMCX connector pin that was itself soldered onto a wire (Figure 3.5a).
Even though working well, this solution revealed to be too rigid and thus easy to break. In addition, changes in the pressure in the reservoir may change the shape and position of the liquid meniscus. As a consequence, the level of the meniscus may change relative to the inserted electrode together with its active surface area (and therefore of its electrical resistance to electron transfer). To avoid this inconvenience I used 200 μm diameter silver wire coated with Teflon (Science products GmbH, Hofheim, Germany). A hole was drilled in the tubing connector of the Cytoclip and inserted an electrode that has been fixed and isolated with epoxy glue (Figure 3.5b).

From the beginning of the development, an O-ring was envisioned to seal the isolated reservoir form the bath. However, electrical impedance measurements revealed that this sealing method was ineffective to electrically isolate two aqueous based electrolyte solutions. Since only one O-ring is used, it is likely that a thin aqueous film is wetting the O-ring, building an electrical bridge for the ions.

The problem of the O-ring wetting is solved by dipping the rear part of the Cytoclip and the connector in molten Paraplast (Sigma Aldrich, Montana, US) (Figure 3.6). The Paraplast was heated on a hot plate at about 90 °C. Alternatively, a thin stripe of Parafilm can be used too.

**Approach on PDMS substrate**

To prove the tightness of the Cytoclip connector, O-ring and Paraplast coating, I envisioned an experiment where a FluidFM probe with a flat aperture (Figure 3.7) is
pressed against a PDMS substrate. The probe used for this experiment has been prepared with the FIB according with a method similar to the one described in the flattening protocol presented in the fabrication of the probes for patch-clamp (Chapter 4.3.1).

![FluidFM probe milled with FIB to obtain a flat aperture.](image)

A voltage bias of -5 mV was applied between the bath electrode and the electrode in the reservoir. With the use of the Tecella Pico2 amplifier, the ionic current was measured while approaching the PDMS substrate (Figure 3.8a). As the probe reached the substrate, the current drastically dropped close to zero, proving that the measured ionic current indeed is flowing through the microchannel and the aperture at the pyramid’s apex.

![Figure 3.8: Ionic current recorded while approaching and retracting the probe on a PDMS substrate. As the probe’s aperture is closed by the PDMS, the ionic current drops to zero indicating closing of the electrical circuit. Applied voltage: -5 mV](image)

The electrical resistance of the PDMS seal has been measured by applying a voltage squared pulse of 50 mV and measuring the resulting current. The current difference measured before and after the appearing capacitive peak (Figure 3.8b) allows to calculate the resistance via the classical Ohm’s law (2.36 GΩ).
3.3. AFM Adapter and Cytoclip connector

The cap for the Cytoclip ensures a tight mechanical and electrical connection for both electric and pneumatic access. In fact, the tubing for the pressure controller and the wire to the reservoir’s electrode are mounted through it. Another challenge is to provide an interface between the low-noise current amplifier (or pre-amplifier) and the recording site. This interface performs an important task: in fact, patch-clamp recording aims to measure electrical currents in the picoampere range for whole cell configuration, and sub-picoampere for single-channel recordings or for SICM experiments. This kind of measurements require proper metallic shielding of the measurement site and a signal amplification as close as possible to the signal source (Molleman, 2002). For these purposes an AFM connector has been designed that interfaces the amplifier to the Cytoclip connector that fulfilled different functions: i) mechanical support for the amplifier or pre-amplifier used, through a BNC connector; ii) shielding of the unprocessed signal from external noise sources and iii) both pneumatic and electronic connections.

To confer mechanical support, the AFM connector is directly fixed onto the AFM through a mask that is press-fitted on the AFM’s backside and where the AFM connector can be screwed onto it. The mask has been 3D printed because of its particular geometry that would be difficult to mill with traditional CNC machines (Figure 3.9).

Figure 3.9: 3D-printed AFM mask to interface the AFM connector to the AFM.

The AFM connector is composed by two parts: the mask, already introduced in the above paragraph and the connector itself (Figure 3.10a). The connector is made of aluminum to provide the necessary electrical shielding, because the signal at its inside is not yet amplified. The connector provides connections for both the pneumatic tubing and for the electric wire (Figure 3.10b). The tubing is connected through self-designed connectors presented afterwards. The electric wire is secured with standard MMCX connector on the interface with the Cytoclip, whereas a more robust BNC connector interfaces the current amplifier (Figure 3.10c/d).
DEVELOPMENT OF THE PC-FLUIDFM

Figure 3.10: Custom-made AFM connector. a CAD of the design connector. The connector is made of two parts: i) a plastic mask that is press-fitted on the AFM, ii) a connector cage that accommodates the tubing and the silver wire. b Backside CAD view of the AFM connector. The view has a section where the pneumatic and electric connections for the Cytoclip are positioned. c Photograph of the finished product. The amplifier is interfaced through a BNC connector. d Photograph of the opened AFM connector showing the silver wire that connects the Cytoclip at one side through an MMCX connector and the low-noise amplifier on the other side with a BNC connector.

It is difficult to find miniaturized pneumatic connectors commercially available that ensure pressure tightness for low pressure (max 2 bar) but offer a fast and simple connection mechanism. I therefore designed a method that allows to plug flexible tubing together. The male connector comprises of a stainless steel tube (0.5 mm syringe needle) with rounded apertures. One side is permanently glued with the flexible tubing (Figure 3.11a). The female connector is a plastic cone inserted in the rubber tubing that has the function to facilitate insertion. A seal forms between the stainless steel tube and the rubber tubing that ensures tightness up to several bars. To facilitate handling, a plastic sliding-bearing is press-fit onto the entrance cone of the female adapter and of the male adapter (Figure 3.11 b/c).

Figure 3.11 : a CAD model of the connector: a metallic tube (blue) is permanently glued on one side to the flexible tubing (white), building the male adapter. The female connector is a plastic cone (yellow) glued on a flexible tubing. b Photograph of the finished male and female adapters. c Photograph of the pneumatic fittings.

The Cytosurge controller designed for the FluidFM system has a maximum pressure output of 1 bar.
3.4. Electrical noise shielding

When sub-picoampere currents have to be measured, there are some rules of thumb that have to be taken into account: first, the current amplifier (or pre-amplifier) has to be positioned as close as possible to the measuring site, in order to avoid that the signal of interest is contaminated and corrupted by electromagnetic noise. As this countermeasure is usually not enough, I build a special dish-holder that would shield the measurement site.

![Figure 3.12: a CAD model of the petri dish holder. The base has the shape that fits the microscope stage of the FlexAFM, while the holding ring is designed to hold WillCo dishes. Magnets are envisioned to facilitate exchange of dish. b Photograph of the manufactured stage. Aluminum ensures shielding the measurement site from electromagnetic noise.](image)

Figure 3.12 shows the designed petri dish holder. The base is made to fit the microscope stage of a FlexAFM (Nanosurf. Liestal, Switzerland), whereas the holding ring is designed to fit WillCo dishes (WillCo Wells, Amsterdam, The Netherlands). The advantage of these dishes is a short rim that fits under the AFM. In addition, the bottom of these dishes is made of glass coverslips that, among others, has the advantages of better imaging properties and broader surface chemistry opportunities.
3.5. Electrical characterization

3.5.1. Electrical impedance

The electrical properties of the developed FluidFM system have been assessed by Electrical Impedance Spectroscopy (EIS). Detailed measurements are reported in the Appendix 9.1.

The electrical impedance is a measure of the resistance that a circuit exhibits to a current passage. Impedance knowledge increases of importance when dealing with alternate currents (AC), i.e. in frequency domain, because it is dependent on the frequency. The bandwidth of the system can therefore be determined, i.e. the frequency range in which the system shows better performance. In patch-clamp, fast currents events (especially sodium currents) are not longer than few millisecond. To be able to measure them, the used system must have enough bandwidth. The measurements were performed in 150 mM KCl solution and an Autolab potentiostat (Metrohm, Herisau, Switzerland) was used.

Figure 3.13 shows the EIS of a tipless FluidFM probe with 2 µm aperture fabricated with the shadow mask technique (refer to Appendix 9.1). Fitting the result with a simple equivalent circuit model\textsuperscript{10} revealed the presence of a capacitance of about 30 pF (Figure 3.13, upper inset). Conventional glass pipettes show between 5 to 15 pF. The FluidFM setup behaves as a low pass filter with a cut-off frequency around 600 Hz. The Bandwidth can be further increased by isolating the microchannel membrane (Appendix 9.1).

\textsuperscript{10} It is assumed that the microchannel and solution resistance are the only non-negligible resistances. The microchannel is modelled with a resistor and a capacitor in parallel, whereas the solution is modelled with a resistor only. i.e. and R[CR] circuit.
3.5.2. Streaming current

The streaming potential or streaming current is an electro-kinetic transport phenomena that occurs due to the charge displacement in the electrical double layer (EDL). The displacement shall occur by an external force that tangentially shifts the liquid phase against the confining walls (Figure 3.14). Counter ions in the diffuse layer of the EDL are carried downstream from the pressure driven flow, resulting in the so called streaming current. The streaming potential $\Phi$ generates according to the streaming current (Chun et al., 2005). As a consequence, the streaming potential moves the counter ions in an opposite direction of the flow, slowing it. The latter effect is known as the electro-viscous effect (Vainshtein and Gutfinger, 2002).

Figure 3.14: Schematics showing the electro-kinetic streaming potential effect. An electrical potential appears when a pressure driven flow is induced in a microchannel filled with an electrolyte (Chun et al., 2005).
Among others, the research of Frank et al. (van der Heyden et al., 2005) showed that the streaming current is proportional to the pressure gradient and increases with the channel height and is a function of the salt concentration. In particular, it remains constant for KCl concentrations below 10 mM, whereas it strongly decreases by at higher salt concentrations.

The same effect is present in FluidFM probes when there is flow. I measured the streaming current in FluidFM probes by sweeping the applied pressure from 0 to 300 mbar back to −300 mbar and to 0 mbar eventually. The pressure was changed in steps of 50 mbars. At the beginning of the experiment, the current has been set to zero compensating for the junction potentials. Figure 3.15 shows the result of such experiment, where every 50 mbar step corresponded to about 1.5 pA of streaming current. The peaks at the beginning of a pulse that have capacitance-similar shape are artefacts introduced by the pressure controller.

Frank et al. (van der Heyden et al. 2005, Fig3), for a similar channel both in height and material, observed a streaming current of about 25 – 30 pA/bar. With the FluidFM setup 10 pA were measured with 300 mbar pressure, i.e. ~33 pA/bar, in agreement with their work.

The streaming current has been proposed as an efficient way to transduce flow energy to electric energy (Yang et al., 2003). In addition, more relevant in this context, the streaming currents can be exploited to provide information on the electric properties at the solid-liquid interface and can be used as an integrated analytical tool.

### 3.5.3. Diffusion and flow rate

**Diffusion**

Since the microchannel is the biggest resistor in the electrical circuit, its electrical resistance can be precisely measured. The resistance of the microchannel, when filled with an electrolyte, is dependent on the ionic strength of the solution. Having two solutions
ELECTRICAL CHARACTERIZATION

with different ion concentration in the FluidFM cantilever and in the bath, allows to measure solution exchange dynamics by monitoring the ionic current.

When two solutions with different ion concentrations are in contact, a diffusion process takes place. As diffusion we understand the movement of species from regions of high concentration to regions of lower concentration, driven by a concentration gradient. The *Fick’s Second Law* gives the concentration vs. time relation:

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 J}{\partial x^2}
\]

Equation 3-1

Where \( D \) is the diffusivity and \( J \) the diffusion flux. With the assumption of an infinite source (i.e. the microchannel is much smaller than the bath and the reservoir), the following solution will describe the concentration profile:

\[
C(x, t) = C_s \text{erf} \left( \frac{x}{2\sqrt{Dt}} \right)
\]

Equation 3-2

Figure 3.16: Diffusion measurement of a FluidFM cantilever with 4 μm opening. 100 mM KCl bath concentration. 150 mM KCl reservoir concentration.

Figure 3.16 shows the ionic current measured over time when a difference of 50 mM of KCl concentration was present. The experimental setup was prepared using 100 mM KCl bath solution and 150 mM KCl in the FluidFM probe’s reservoir. No pressure has been applied, allowing the estimation of the diffusion process inside the cantilever. A fit of the data with the function presented before (Equation 3-2) confirms that the measured current arises from the diffusion phenomena, revealing a diffusivity value of about 20 cm²/s (18 cm²/s in water at 25°C according to literature (Gosting, 1950)).

**Flow rate estimation from electrolyte exchange**

Monitoring the electrical resistance of the microchannel leads information about tiny variations in salt concentration. Here I exploited this simple measurement to estimate the flow rate in the FluidFM cantilever. The experiment consisted of having two different salt concentration in the bath and in the reservoir of the FluidFM probe. 100 mM KCl...
concentration was used in the bath, whereas the reservoir was filled with a solution of 150 mM.

Figure 3.17 Solution exchange in the microchannel. 

**a** Current and pressure versus time. Two regimes are visible: the fast transients in the current correspond to the streaming current, whereas the slow transients arise from the exchange of solutions with different ionic strength. 

**b** Schema explaining the behavior of the current in a. First, the microchannel is filled with liquid B. Then, overpressure is applied to release solution B. Eventually, the pressure gradient is reversed and Solution A replaces solution B.

Figure 3.17 shows the solution exchange experiment: First, the channel is filled with solution B (150 mM KCl). Then, overpressure dispenses solution B in the bath. The increase in current is the streaming current resulting from the flow (Chapter 3.5.2). Subsequently, the overpressure is removed (streaming current goes to zero) and the solutions exchanged by suction. The current linearly drops to eventually reach a plateau when solution A entirely replaced solution B. By measuring the time of the linear transient, and knowing the volume of the microchannel, one can estimate the flow rate, which in this case was $Q = 1.8 \times 10^{-15} \text{[L/s]}$ at 100 mbar.
3.6. Data acquisition environment

As pointed out in the previous Chapters, the setup has been developed to perform low noise patch-clamp experiments. The same setup must be suitable for scanning ion conductance microscopy (SICM) too. Therefore, I was obliged to create a different software written in LabView. The underlying challenge was to write a routine to synchronize the data generated from the AFM with the electrophysiology data of the patch-clamp amplifier, for simultaneous recording of force and current signals, while controlling AFM or SICM. The difficulty arises from the high level of synchronization needed between generation and acquisition of the electrophysiological protocols, with sub-millisecond precision. Also, the different signal types of electrophysiology experimenting and of the AFM require different acquisition architecture, which complicates their synchronous acquisition.

LabView (National Instruments (NI), Austin, Texas), allows to efficiently create so called Virtual Instruments (Vis), in a graphical programming interface that is merged with the graphical user interface of the program. Almost all hardware manufacturer supports LabView, making it an efficient solution when building test-setups in a laboratory context where data usually have to be acquired and analyzed and a high degree of flexibility is needed.

The NI experimental setup comprehends two NI data acquisition PCI cards and relative BNC connector boxes. The acquisition cards both feature analog inputs and analog outputs (two) channels. The development, architecture and capabilities of the software written is described in detail in the Appendix 9.2. I wrote two distinct programs, one for patch-clamp experiments and one for SICM imaging.
3.7. Conclusions

I developed the necessary hardware to allow low-noise electrical recordings with the FluidFM system in electrophysiological conditions. For this purpose, I designed specially shielded connectors and dish holders. In addition, I created a software interface to simultaneously acquire and manipulate data of the AFM and of the electronic amplifier, especially interesting for SICM experiments. Minor experiments have been carried out to understand the electro kinetic effects that take place in the microchannel of the FluidFM probes, as the streaming current and diffusion.

LabView revealed to be an ideal data acquisition environment in these circumstances: the high flexibility offered and the relatively intuitive programming language allows continuous adapting of the software to the changing experimental needs. However, even though the software allowed us to carry on the envisioned proof of concepts, its performance and functions are far away from those of commercially available alternatives (example: Nanonis SPM controller and software).
At the beginning of the project, not only the FluidFM was at its first developmental stage, but transitioning from CSEM to SmartTip limited the availability of probes in terms of both number (because of several defects) and design (tipless or pyramidal tip). We envisioned applications in biology and material science to pioneer with the FluidFM technology and to prove its potential. However, the proposed applications needed specially shaped probe apertures. Using the focused ion beam technique (FIB) to drill application-specific geometries was a straightforward decision. Indeed, being merged with a scanning electron microscope, FIB confers a high degree of control and versatility. For this purpose, I used FluidFM probes appositely fabricated by SmartTip having a pyramidal probe without any aperture, from now on simply referred to as “closed probes”.

Figure 4.1 Scanning electron microscopy images of the FluidFM cantilevers fabricated or inspected with the focused-ion beam technique. (Guillaume-Gentil et al., 2014a).
In addition, I used FIB to observe the structure of the different FluidFM probes and to inspect the newest prototypes.

This Chapter will give a detailed description of different custom FluidFM probes, comprehending the FIB protocols optimized to drill the different apertures at the apex of the pyramidal probes for the corresponding applications.

4.1. Generals about Focused Ion Beam (FIB) technology

The first developments of FIB date back to 1974, with the attempts of controlling high-energy focused ion beams for maskless lithography purposes towards a novel microfabrication technique (Seliger and Fleming, 1974). FIB enables imaging, localized milling (etching) and deposition with nanometer precision of conductors and insulators (Reyntjens and Puers, 2001). While the imaging happens to a mechanism similar to the scanning electron microscope, milling uses high ion current beam to achieve physical sputtering of the material. In addition, even though not used in this thesis, the FIB can be used to deposit localized films with the same principle of chemical vapor deposition (CVD), without the need of masks (Thornell and Johansson, 1998).

![Concept of the CrossBeam technology](image1)

**Figure 4.2 a** Concept of the CrossBeam® technology developed by Zeiss. In the center, in vertical position, there is the electron optical column. The focused ion beam column is on the left and the gas injection system is depicted on the right. **b** Photograph of the Zeiss NVision40 FIB-SEM microscope. Source: [www.zeiss.com](http://www.zeiss.com).

All the FIB work presented in this thesis has been done with a ZEISS NVision40 FIB-SEM microscope (Carl Zeiss, Jena, Germany) at the ScopeM facility of ETH Zurich. The Nvision40 is a combined scanning electron and focused ion beam workstation, whose ion beam is generated from a liquid metal source of Gallium.
Challenges and risks

On the one hand, the fabrication of the FluidFM probes with special geometries (prototypes) in the cleanroom stage is long and expensive, and does not ensure ending with a high-yield wafer. On the contrary, it has been usually the case that the first wafer of probes showed defects having the roots in the microfabrication steps (e.g. bonding problems, incomplete etching of the microchannel, damaged pyramids, asymmetric or misplaced apertures etc.). On the other hand, the FIB work confers a high degree of versatility, but also an additional, elevated investment of financial as well as time resources. To give an idea, an experienced user may invest two full days of work to drill the opening of 30 FluidFM probes. This time includes an end-user quality control, probes preparation and sorting, and the effective FIB work. The latter may take only half a day if the protocols have been extensively tested and, without any particular discount, will cost 500 CHF per hour (including the operator).

The variability between the probes poses a further challenge: in fact, each design variation obliges to deeply inspect the geometry of the probe and to redefine the processing parameters. In addition, variations in the FIB microscope in terms of stability and beam intensity can alter the conditions and require a subsequent parameter optimization.

4.1.1. Preparation of the FluidFM probes for FIB

SEM-FIB holder

I fabricated a probeholder for the SEM/FIB stage that could accommodate up to twenty FluidFM probes. The geometry of the probeholder was designed to allow milling of both top-aperture and side-aperture probes (described afterwards). Indeed, the holder can be positioned with two inclinations: on its side wall, to have the cantilever perpendicular to the ion beam (Figure 4.3), or on its flat plane, to bring the pyramid wall parallel to the ion-beam. Details will be discussed in the milling protocols paragraph.

The FluidFM chips were fixed in a sandwich-like structure with a thin copper sheet that makes electrical contact with the top carbon coating. The metallic holder then ensures electrical contact to the ground of the SEM/FIB stage.

*Hint:* The best way to mount the FluidFM probes on the SEM/FIB holder is to cut them out from the wafer in a row-like fashion, so that the FluidFM probes results to be naturally aligned and equally spaced.
Figure 4.3: a: Custom probeholder with 20 FluidFM probes mounted in the inclined mode, with a 54° angle, in order to fabricate top-aperture probes. b: Lateral section/schema of the probeholder. The metallic sheet is pressed on top of the FluidFM probes to electrically connect the carbon coating on top of them.

Preliminary quality control of closed pyramidal probes

As the FIB milling process is demanding in terms of time and costs, it is therefore good practice to recognize defected cantilever at an early stage. The quality control performed in our lab included three steps: i) to identify major defects (e.g. missing cantilever or bonding problems, see Figure 4.4 a) with the help of a low magnification desktop stereomicroscope (Zeiss, Jena, Germany). ii) to determine the presence of extraneous particles inside the microchannel (Figure 4.4 b) and iii) to confirm complete etching of the sacrificial material (Figure 4.4 c). The last two points were done by imaging with an inverted optical microscope at 20x magnification with a LD objective (Zeiss, Jena, Germany).

Figure 4.4: a) Micrograph of a major defect: part of the structure is missing. b) Micrograph of an aperture-less cantilever showing particles inside the microchannel (small black dots in proximity of the apex, highlighted by the red circle. c) Incomplete etching of the microchannel visible with the microscope. The red arrow shows the beginning of the remaining sacrificial material. Scale-bars: 30 μm.
Carbon coating

One drawback of SEM and FIB systems is that the specimen has to be conductive, otherwise electrical charging may occur which will deviate the beam in unpredictable way. Not only this prevents to achieve high imaging resolutions, but also the ion beam may shift from the wanted position considerably reducing the milling precision. The FluidFM probes are made of quartz, silicon and silicon nitride: all are insulating materials. The probes have therefore been coated with a conductive material. The choice fell on amorphous carbon coating, especially because it is a non-destructive process, simple to be removed afterwards with oxygen plasma etching. It is important to entirely remove the carbon after the milling process as the surface of the FluidFM probes is often modified by further chemistry, depending on the needed characteristics (e.g.: hydrophobic, anti-fouling, etc.). Carbon was sputtered over the sample by means of evaporation with a conventional rod fed source (Cressington Scientific Instruments, Watford, England). The thickness was not precisely measured, but theoretically couple of tens of nanometers were deposited by evaporating carbon for 10 s three times according to the instrument specifications.

4.2. FluidFM Probes and their characterization

This chapter will present the standard FluidFM probes that are fabricated by SmartTip (Enschede, The Netherlands), including the probes that are already fabricated with an aperture. The following paragraph will present the properties that all the probes have in common, whereas the next chapters will treat the single probes separately.

Inspection of the microchannel as well as the pyramidal probe revealed to be indispensable in order to understand the behavior of the probes during field-testing and to tackle the diverse problems that we were facing with the early prototypes (i.e. clogging, leaking, and non-reproducibility). In addition, to optimize the fabrication protocols for different aperture designs, characterization work was indeed needed. Especially, parameters like the thickness of the pyramid walls have to be known as the milling time depends on it.

The FluidFM probe

FluidFM probes are fabricated with a rectangular cantilever made of silicon nitride. Standard FluidFM probes are entirely fabricated by standard microfabrication techniques, ideally ensuring a high yield. The channel is fabricated by etching a silicon sacrificial layer sandwiched between 350 nm silicon nitride layers. The microchannel spans from the center of the FluidFM chip to the very end of the cantilever (Figure 4.5a), for about 1500
μm and is bonded to a glass wafer that stabilizes it and increase handling. The channel is contacted through a hole in the glass wafer which is made by sand blasting.

Figure 4.5 a Micrograph of the FluidFM microchannel filled with fluorescein dissolved in water to highlight its length. The upper and lower walls of the channel are stabilized with the incorporation of pillars. b Section of the microchannel showing the hollow nature of the FluidFM cantilever.

A section of the microchannel is shown in Figure 4.5 b. Its height can be changed by tailoring the thickness of the sacrificial layer. We worked with three different heights: 1.5 μm, 1 μm (which became a standard) and 200 nm. The height of the microchannel is a critical parameter as it determines two central properties of FluidFM cantilevers, i) the hydrodynamic resistance (Dörig et al., 2013) and ii) the spring constant (Chapter 7.1.2).

<table>
<thead>
<tr>
<th>Microchannel Length</th>
<th>Microchannel Width</th>
<th>Microchannel height</th>
<th>Cantilever Width</th>
<th>Cantilever height</th>
<th>Cantilever length</th>
<th>Resonance frequency</th>
<th>Spring constant¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>~1500 μm</td>
<td>30 μm</td>
<td>1 μm</td>
<td>36 μm</td>
<td>1.9 μm</td>
<td>200 μm</td>
<td>~80 kHz</td>
<td>~2 N/m</td>
</tr>
</tbody>
</table>

Table 4-1 Summary of the most important physical characteristics standard FluidFM cantilever

¹ Measured with the Sader method (Sader et al., 1999).
4.2.1. Inspection of standard cantilever

The FIB has repeatedly been used to inspect the microchannel of the different FluidFM probes. Here I shortly present the structure of the standard FluidFM cantilever, i.e. Tipless and Apex300\(^2\). Both have a microchannel height of 1μm, 30μm wide. The walls of the microchannel nominally measures 350 nm.

![Image of FluidFM probes](image)

Figure 4.6: a section of a Tipless probe. In the upper panel, the section of the microchannel integrated in the cantilever is visible. In the lower panel the section is made through the opening, highlighting the cylindrical geometry of the Tipless aperture and the ending of the microchannel. b Section view of a part of the microchannel. Circled in red are debris bigger than a micrometer. c Section of a Apex300 probe. The continuation of the microchannel towards the top of the pyramid is visible. Red arrows indicate residuals of the microfabrication in the hollow pyramid.

FIB section of the standard FluidFM probes, i.e. tipless and apex. Figure 4.6a shows a section view of the tipless probes highlighting the aperture and the thick walls. Figure 4.6b, instead, shows a section of the standard apex probes. The aperture measures 300 nm.

The inspections revealed two kind of problems: on the one side, debris are present in the microchannel that have bigger dimension than the aperture itself. This may lead to an unwanted valve-like behavior of the probes that allows suction but not overpressure application. On the other hand, the pyramid of the walls are very thin: 80 nm. Consequently, the pyramid itself might bend under application of force\(^3\). On the other side, such a thin membrane is also difficult to mill with the FIB, which is visible from the not entirely etched walls at the section level.

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\(^2\) Apex300 refers to a pyramidal tip with an aperture at its apex, with a nominal diameter of 300 nm.

\(^3\) The very thin walls might bend under application of force, but they will not break. We tested several tips with force-spectroscopy routines up to 1000 nN. Subsequent SEM imaging did not show any damage (data not shown).
4.2.2. Inspection of FluidFM probes without aperture

Most of the pioneering applications with the FluidFM requested particular geometric properties of the probe aperture. Since the production on a wafer scale is expensive and time consuming, we agreed on some universal properties regarding the pyramid and the cantilever properties of closed probes, i.e. without aperture. The aperture was then meant to be individually drilled by means of FIB. This approach gave the flexibility to produce application-specific geometry and to adapt the design depending on the results obtained from the FluidFM experiments.

Even though different designs have been produced, the two batches hereafter presented are the most relevant ones. The basic properties of the FluidFM probes without aperture concerned the two components of the FluidFM probe, i.e. i) the cantilever, and ii) the pyramidal probe.

4.2.2.1. Cantilever

The parameter that represents the properties of the cantilever is the spring constant $k$. Depending on the application one would like to have a rather softer or a rather stiffer cantilever. E.g. gentle static contact on a soft cell requires a soft cantilever as the measurement of sub-piconewton does. A stiffer cantilever is desired when working in dynamic mode (higher resonance frequency) or when it comes to measure big forces (e.g. mammalian cell adhesion). The standard FluidFM cantilever exhibit a spring constant of about 1.8-2.8 N/m, which is considered a stiff cantilever. We therefore agreed in fabricating cantilevers with lower spring constants for the applications that needed higher force sensitivity (e.g. bacterial adhesion).

Cantilever spring constant: 

$$k[N/m] = \frac{3EI}{L^3},$$

$E$ is the elastic modulus [Pa], $L$ the length of the cantilever and $I$ [m] the moment of inertia of the cantilever cross section. Without changing the material, the two geometric options to change the stiffness of the cantilever are the length or the thickness. While the first option would be the most straight-forward and influent, it implies the change of the (expensive) masks used for the microfabrication of the probes. Therefore, we choose the option of reducing the thickness by lowering the channel height from 1 μm to 200 nm which demanded only minor changes on the fabrication protocols. By doing so, the spring constant is reduced to values around 0.02 N/m.
Figure 4.7: a SEM view of a sectioned soft, no aperture, pyramidal cantilever. The microchannel is 200 nm height. b SEM view of a section of a standard, no aperture pyramidal cantilever. The microchannel is 1 μm in height.

In Figure 4.7 a comparison between the same cantilever design but with different microchannel heights is shown. The drawback of having such a small microchannel is its increased hydrodynamic and electric resistance. While increased hydrodynamic resistance is not a problem for the filling of the cantilever, the increased electric resistance is indeed a problem as it is prohibitive for electrophysiology experiments.

4.2.2.2. Pyramidal probe

The other feature that is possible to modify is the pyramid itself. Although the basis of 10 μm and slope of 54.7° of the pyramid are fixed, i) the thickness of the walls and ii) the sharpness of the edges can be changed with reasonable efforts.

i) Pyramid wall thickness

The wall thickness of the pyramid is relevant for two different reasons: first, thick walls allow to create an aperture at the apex which does not only guarantee a connection to the microchannel but that can be carved for particular function too. In other words, thicker walls allows to fabricate 3D apertures instead of 2D. This is indeed the case for probes employed for patch-clamp and will be deepened later (Chapter 4.3.1). However, thicker walls are unwanted in the case of syringe-like probes, as it becomes more difficult to bring the aperture very close to the apex. Figure 4.8 shows FIB sections of pyramidal probes two probes with the same design but different wall thickness (80 nm vs. 450 nm). Sectioning has been achieved with the use of 150 pA milling current.
Figure 4.8: a SEM view of the section of a pyramidal probe without aperture, with 80 nm thick walls. b SEM view of a pyramidal probe without aperture with a wall thickness of 450 nm.

ii) Oxidation sharpening

Figure 4.9: a SEM picture of a pyramidal probe that underwent the oxidation sharpening treatment. Note the sharper corners. Inset: FIB angle view of the sharpened apex. b Non-oxygen sharpened probe.

Especially to improve the penetration efficiency of the plasma membrane, it was a fabrication option to perform additional oxidation-sharpening step. To keep it short, it consists in an oxidation/etching treatment which enhances the sharpness of corners (Ravi, 1991). Figure 4.9 shows a comparison between a treated pyramid to a non-sharpened one.

iii) Apex sharpness

Many AFM applications of the FluidFM require a sharp probe, especially the ones involving imaging or membrane penetration. During their micro fabrication, the use of lithography masks that are not precise enough or because of mask misalignment, lead to blade-like pyramidal tops (Figure 4.10a). Indeed, a blade-like apex results when the two orthogonal basis of the pyramid are not perfectly same in length. This problem was affecting the majority of the FluidFM probes and lowered the availability of sharp probes as 80% had no pointy apex. The problem has been solved introducing E-Beam defined lithography masks that guarantee a perfect squared-shape to the basis of the pyramid (Figure 4.10b).
4.3. FIB protocols for closed FluidFM probes

Aim of this paragraph is to give a comprehensive overview on the fabrication of the apertures that have been used for i) patch-clamp, ii) SICM, iii) adhesion measurement bacteria, iv) injection or extraction experiments.

*Hint 1*: although the Zeiss NVision40 allows for simultaneous SEM and FIB, it is not advised to make use of this feature, because the electron beam enhances re-deposition of the ablated material and contributes to beam drifting, as the sample is charging while imaging.

*Hint 2*: The FIB gun objectives (i.e. lenses for different current intensities) are supposed to be perfectly aligned. However, it is often the case that an offset exists. For this reason, when possible, it is suggested to image with the same current that is used for milling.
4.3.1. Patch-clamp probes

Goal
This application aims to demonstrate compatibility of the FluidFM system with the patch-clamp technique. As pointed out in the dedicated Chapter 1.3.1, the most important achievement is a tight seal between the membrane of the patched cell and the aperture of the probe. Ideally, the contact between the probes’ aperture and the cell must show gigaohmic electrical resistance, in order to allow for high quality recording. The aperture has to be designed so that the seal resistance is maximized for the given aperture-less, pyramidal probes.

Standard Apex300 probes are not considered an adequate choice because of the square section of the pyramid. In fact, the limited ability of the cell membrane to bend (minimal bending radius around 100 nm) will prevent a tight seal to happen in the concave corners of the pyramid. For this reason, we decided to design and fabricate by our own the probes for patch-clamp. A cylindrical aperture was envisioned, thus providing a circular surface where the seal could happen. Additional details on the proposed design are explained in the following paragraphs.

![Diagram](image)

Figure 4.11: **a** With the geometry of the standard Apex300 FluidFM probes, high quality seal formation is prevented from the sharp and concave corners. **b** The customized aperture takes advantage of the increased wall thickness of the FluidFM closed probes to fabricate a cylindrical aperture at the apex that has a seal-friendly geometry.

Two different sealing opportunities have been identified: one mimicking the conventional sealing mechanism, while the other consists of enhancing the seal by introducing an oil-based coating. The two approaches require different geometry, as the seal location is different; both are hereafter presented.

**AFM inclination compensation**
On the AFM, the cantilevers are mounted with an inclination. In the case of the FlexAFM this inclination is from 8° to 10°, whereas the JPK AFM has an inclination of 11°. In order to have a levelled contact with the cell, or with the substrate, this angle is compensated during the FIB milling. Figure 4.12 shows an example on how the inclination of the AFM
head is compensated by tilting the FIB milling plane 10° with respect to the plane defined by the cantilever.

Figure 4.12: a Example on how the compensation for the AFM inclination is executed. The milling plane is tilted by 11° with respect to the cantilever plane. b Drawing of the 10° tilt introduced to compensate for the AFM inclination.

4.3.1.1. Conventional sealing method

For conventional sealing method, we understand here the mechanism for which the sealing is achieved by adhesion of the cellular membrane to the aperture of the probe, triggered by the application of negative pressure (i.e. suction). In conventional glass micropipettes the seal happens in its first micrometers (Figure 4.13a). The building up of the high ohmic resistance (i.e. the gigaseal) happens in this region. It is accepted (Suchyna et al., 2009), that a conductive, aqueous layer is present between the cellular membrane and the glass of a micropipette, which thickness is in the range of 1 nm. Making use of this value and knowing where the location of the sealing process, one can estimate the resistance in an optimal case.

Figure 4.13b shows a SEM view of the sealing region of a planar patch-clamp chip (Curtis and Baldwin, 2008). Importantly to note are the dimensions of this sealing region in both the case of a glass micropipette and for planar patch-clamp chips. In these cases, the patch\(^4\) can extend for several micrometers.

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\(^4\) The patch is understood as the portion of the membrane of the cell that is sucked into the probe. The area being patched.
To create the space for the sealing to happen, together with SmartTip, we agreed to produce closed pyramidal probes with increased wall thickness. Figure 4.13c shows the section of such probes with 450 nm thick walls. With this design, we are constrained in the red-circled region as a seal is not likely to happen in the square-sectioned portion of the pyramid.

We aim to reproduce the geometry of planar patch-clamp chips at the apex of the FluidFM probe. Since the thickness of the pyramid walls is limited, the height of the cylindrical aperture is dependent on the aperture diameter (Figure 4.14a). In Figure 4.14b we calculated the expected seal resistance in function of aperture diameter and aperture height. The calculations have been performed assuming a conductive aqueous gap between the cellular membrane and the probe of 1 nm (Suchyna et al., 2009). This result is published in the supplementary information in the patch-clamp dedicated Chapter (See 5.4.6). The highest seal resistance obtainable with this approach is between 70 MΩ to 250 MΩ.

Lowering the aperture diameter would improve the seal resistance. Diameters down to 100 nm are feasible with the FIB (lower is difficult to be achieved because of the high aspect ratio of the aperture), however, the immediate drawback of small apertures is the difficulty to enter whole-cell configuration. In fact, a smaller aperture area limits the maximum force applicable through suction, which may not be sufficient to disrupt the patch.
I therefore aimed to produce cylindrical apertures in the apex of the FluidFM probes that mimics the ones used in planar patch-clamp.

4.3.1.1.1. Protocol

1. Mount the samples on the holder after having coated them with carbon.

1st step: Apex flattening

1. Position the SEM/FIB holder on to the 36° inclined side (cantilever axis parallel to the FIB emission gun as shown in the following figure:)

2. Compensate for the AFM angle: Set the SEM tilt angle to +10° (adapt this value depending on the AFM head inclination – Nanosurf with corrected AFM-nose: 8°).

3. Bring the first probe at the ideal working distance from the SEM gun (Zeiss NVision40 of EMEZ: 4.8 mm). Follow the SEM procedure to have a sharp image of the pyramid.

4. Ramp up the FIB gun
5. Before imaging with the FIB gun, set the imaging current at 10 pA. This current is also used for the milling process. *Observation:* This current is adapt only for small milling volumes (here ~500 nm$^3$). If the process takes too much time, higher currents are a viable option too.

6. Switch to the FIB mode and center the view on the apex. With the fine rectangle milling tool draw a rectangle of the desired dimensions over the apex. 

**Parameters:** *Width:* about 200 nm bigger than the desired cylinder diameter (e.g. ~600 nm for a 400 nm aperture). The reason is that an inclined plan is being milled (trapezoidal shape). *Height:* depending on the width, set the height in a way that the whole apex is being milled away. *Depth:* 1 μm is safe.

Following figures show a probe before and after the beheading step:

![Probe before and after beheading](image)

**2nd step: hole drilling**

1. Retract the SEM/FIB holder to a safe distance and position the stage so, that the FIB gun will be perpendicular to the previously milled plane: rotate the stage 180° around the z-axis. Set stage tilt angle $T = 18^\circ$ to bring the cantilever perpendicular to the FIB gun.

![Stage tilt angle](image)

2. Subtract from the tilt angle the compensation angle introduced in step 2nd point of the flattening step: the FIB gun is now perpendicular to the plane milled in the 1st milling step (flattening).

3. Upper the stage and bring it again at the working height.

4. Center and focus the SEM on the probe.

5. Switch to FIB, center and focus on the flat plane on top of the pyramid at an adequate magnification.

6. Launch the *feature mill* tool and load the actual image.

7. Draw the circle (filled) of the desired diameter in the center of the plane at the apex.
8. To avoid drift, image again the probe in FIB mode at moderate speed. The scope is to slightly charge the substrate, to the point where the image is not drifting anymore. Do this by toggling between imaging and load image in feature mill until the image position is stable.

9. Once the image is stable, start the milling process.

10. Milling parameters: **milling current: 10 pA** are a good compromise between speed and precision. Lower current will take too much time to mill deep holes, and this makes also small drifts compromise the result. The milling time depends on the diameter of the aperture (longer for larger holes). **Milling time:** for 400 nm diameter a milling time of 100s is enough.

Observation: The milling time can be critical because of over-milling. It is advisable to sacrifice one probe after the first milling to ensure that the milling process happens as wanted. A section through the hole does the job.

11. Be sure that the milling step is complete. This is very important for good seal formation:

4.3.1.1.2. **Summary:**

Results: When milling high-aspect ratio features, beam drifts becomes more and more crucial: deeper structures require longer etching times, thus the drift results in larger displacement, eventually impeding the mill of deep structures. However, if the quality of the carbon coating is good, one can drill very small apertures. The smallest feature that can be milled is defined by the bottom part of the aperture: in fact, the result of this kind of processing brings to a conical-like geometry (Figure 4.15b). Another limiting factor is the FIB current: lower currents make a more defined beam width. This would be in favour of using 1 pA currents instead of 10 pA, however, 1 pA currents have not enough energy to etch high-aspect ratio apertures.
4.3.1.2. Enhanced sealing method

Since the space available for conventional sealing mechanism to happen is too restricted to obtain gigaohmic resistances (see previous Chapter), I investigated the option of an enhanced seal that is triggered by a coating deposited on the pyramidal probe. This concept is inspired in the pioneering work of Collins et al. that used a number of hydrocarbon mixtures (mainly oils, waxes and Parafilm) to improve stability of gigaseals in range of the giant patch method\(^5\) (Collins et al., 1992). The details about this mechanism are presented in the patch-clamp dedicated Chapter 5.3. Following this reasoning, the sealing is no longer happening inside the aperture via suction application, but is supposed to happen on the outer surface of the probe (Figure 4.16).

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\(^5\) Giant patch method: excised membrane patches with diameters of 12 μm to 40 μm.
The square geometry of the pyramid is difficult to be uniformly coated because of the sharp corners. I therefore envisioned localizing the sealing region around the aperture. The flattening step introduced in the first step the protocol for the fabrication of the probes designed for the conventional sealing method (4.3.1.1.1) is accentuated for this purpose (Figure 4.16). In Figure 4.17 an example of the obtained result is shown.

Figure 4.17: SEM of a FluidFM probe designed for the oil-enhanced sealing method.
4.3.2. SICM or Simultaneous SICM and AFM

4.3.2.1. SICM

Based on simulation work presented in the Chapter 6.3.2, I concluded that an increased area surrounding the aperture might improve the ionic-current squeezing effect and therefore improve the performance of the SICM.

In addition, this design allows getting apertures down to 10 nm without reaching prohibitive electrical impedance values. Indeed, the nanometer-sized section area of the aperture has a limited thickness and becomes rapidly bigger because of the pyramidal geometry of the FluidFM probe. In the case of nanometer-sized pulled nanopipette the probe has a conical geometry, which is less steeper compared to the FluidFM pyramid, i.e. shows an elongated shaft. For this reason, the narrow section area is longer and responsible for an important increase in electrical impedance. Indeed, it is interesting to note that even though glass micropipettes can reach comparable sub-micrometer dimensions too, their impedance increases about two orders of magnitudes (up to 500 MΩ), considerably complicating their usage in terms of electronics requirements. In the case of the pyramidal probes of the FluidFM, the aperture can be designed to be only few nanometers thin. Behind the aperture, then, the section rapidly increases to the size of the microchannel.

Figure 4.18: a Aperture < 50 nm after an apex flattening step. b 50 nm aperture after a rounding step.

The fabrication of these probes is indeed very simple: a single flattening step is needed to flatten the apex of the pyramid under constant SEM control. In this way, apertures down to few tens of nanometers are readily obtained. However, the result is a triangular or trapezoidal shape because of the AFM tilt angle compensation. If desired, one can obtain a round aperture by adding a milling step. However, by doing so, the minimum aperture size that can be obtained is of 50-80 nm, determined by the beam size at the smallest current intensity (1 pA).
4.3.2.1.1. Protocol

**Apex flattening** – for details, refer to the flattening step proposed for patch-clamp probes (Chapter 4.3.1.1)

1. Position the SEM/FIB holder on to the 36° inclined side (cantilever axis parallel to the FIB emission gun

2. Compensate for the AFM angle: Set the SEM tilt angle to +10° (adapt this value depending on the AFM head inclination – Nanosurf with corrected AFM-nose: 8°).

3. Bring the first probe at the ideal working distance from the SEM gun (Zeiss NVision40 of EMEZ: 4.8 mm). Follow the SEM procedure to have a sharp image of the pyramid.

4. Switch to the FIB imaging and center the pyramid with adequate magnification. Remember to image with the same current objective used for the milling step. 10 to 40 pA are appropriate. The process can be accelerated with higher currents.

5. With the FIB tools, draw a fine rectangle on the ending of the pyramid with is big enough to reach the hollow part of the pyramid.

6. Make sure that the option “switch to SEM+FIB mode when milling” is activated in the FIB settings: this ensures live SEM view during the milling process.

7. Start the milling and center the SEM view over the apex using the shift beam

8. As soon as the aperture is visible and the desired size reached, be fast in interrupting the milling

   The result, up to this step, is a triangular or trapezoidal shaped aperture (see Figure 4.18a). One can choose to add another milling step to round off the aperture as shown in Figure 4.18b.

**Aperture rounding:**

1. Follow the second step of the protocol of the patch-clamp probes (hole drilling). The only substantial difference is the intensity of the current used. The lowest milling current is desired, because it has the narrowest beam.

   **Use 1 pA current.** Minimum aperture diameter approximately 50 nm.

   **HINT:** The fabrication can be done without live SEM view during the milling process: simply define the correct milling height by correctly setting the height of the fine rectangle tool.

4.3.2.2. Simultaneous SICM and AFM

By avoiding the flattening step introduced for both the previous probes and by directly mill a sub-micrometer aperture beneath the apex, it is possible to keep the sharp apex intact.
Figure 4.19 shows the result of such fabrication. Although not exploited in range of this thesis, these probes are optimal for simultaneous AFM and ion conductivity measurements. Indeed, the probe can be scanned over a surface in AFM mode and the current monitored to reconstruct conductivity map. A similar work is presented in Chapter 6.2 where PDMS containing silver nanoparticles is characterized with the use of standard Apex300 FluidFM probes.

Figure 4.19: Aperture appositely drilled sideway to leave the sharp apex intact. The functionality of AFM imaging is therefore preserved.
4.3.3. Injection and extraction

Injection:

*Force-Controlled Fluidic Injection into Single Cell Nuclei*

Orane Guillaume-Gentil, Eva Potthoff, Dario Ossola, Pablo Dörig, Tomaso Zambelli, Julia A. Vorholt.

*Small, 2013*

*Injection into hard-wall cells*

*Tentative authors list:*

Orane Guillaume-Gentil, Vincent Martinez, Dario Ossola, Tomaso Zambelli, Julia A. Vorholt.

In preparation.

Injection into mammalian cells was one of the first demonstration of the potential of the FluidFM for single-cell manipulation (Meister et al., 2009). However, the injection was not yet reliable as it was the supply of working FluidFM probes. Later, as the probes availability and quality became acceptable, the injection application was deepened further. In particular, Injection into mammalian cell nuclei (Guillaume-Gentil et al., 2013). The requirement for this application are two: i) a sub-micrometer aperture is needed to inject small amount of liquid into the nucleus, ii) the sharp apex of the pyramid has to be preserved during the FIB processing to ensure an efficient cellular membrane penetration. The latter, has been a challenge over a long period of time. In fact, because of not precise enough lithography masks used during the micro fabrication process that were creating blade-like apexes instead of pointy apexes (See 4.2.2). On the other hand, the imaging through the FIB beam is a destructive process. Especially sharp edges becomes smooth already after short period of imaging. Unfortunately the imaging with the FIB is an obligatory step, therefore the user has to carefully avoid to image the apex of the probes that are used for injection or extraction (refer to the protocol).

![Figure 4.20](image)

*Figure 4.20: a* The first design was a circular aperture drilled orthogonally to the wall of the pyramid. *b* Later, the aperture was drilled orthogonally to the cantilever, i.e. milling the pyramid from the top. *c* Triangular aperture milled orthogonally to the front wall of the pyramid. The triangular shape maximize localization of the aperture towards the apex.
For injection purposes, we tried different fabrication approaches that were dictated both form changes in the design of the probe design and from the functionality during the experiments. Firstly, I was drilling circular apertures perpendicular to the wall of the pyramid (Figure 4.20a). These probes had the problem that the aperture too far away from the apex. In this case, very few injected liquid entered the cell and the majority ended in the bath (see Figure 4.21). To avoid this kind of problem the circular aperture was then milled along the axis of the pyramid. In this way, the aperture could be positioned much closer to the apex (Figure 4.20b). However, this approach did not solve the problem of the aperture protruding from the cell, as the resulting elliptical opening is still naturally extending along the wall of the pyramid. In addition, with this fabrication approach it is more difficult to avoid damages at the probe rising form the milling process and is very sensitive to beam drifts.

![Critical positioning of the aperture.](image)

Figure 4.21: Critical positioning of the aperture. When the aperture is not fully inserted into the cell, leak is observed. It is plausible that the internal pressure of the cell will prevent the liquid to enter.

I made a step back and kept milling with the FIB perpendicular to the pyramid wall (this also maximizes milling efficiency) and switched from a circular aperture to a triangular one. This geometry allows to fit the aperture between the two adjacent pyramidal walls, closer to the apex (Figure 4.20c). Milling from this direction is problem-free as long as the pyramidal walls are thin. When dealing with thicker walls, it is better to slightly change the FIB incidence angle and to mill parallel to the pyramid’ rear wall (Figure 4.22). By doing so, the aperture can be positioned higher towards the apex, because it is milled in the rear wall itself, whilst the bottom of the triangle has to contact the hollow compartment of the pyramid. In addition, it confers the advantage of making the process not vulnerable to overtime milling. This is especially important when processing pyramids with very thin walls (e.g. 80 nm) too.
Challenges

Typically, the size of the triangular aperture is in the order of 200 nm. Therefore, the position and size of the triangular aperture must consider the thickness of the pyramid walls. In fact, as it is the case with the 450 nm thick walls, the aperture can be placed very close to the apex but without being connected to the hollow part of the probe (Figure 4.22). The lack of sharp probes was major problem that compromised the efficient production of these probes in the early stage. The majority of the probes had indeed an apex similar to a blade and not pointy (4.2.2). The solution for this problem was the introduction of E-beam fabricated lithography masks.

Working with sub-micrometer apertures always poses clogging risks. Indeed, around 50 % of the probes are still affected by this limitation. Most likely, clogging problems arises from residual particles in the microchannel, as pointed out in 4.2.1. These micro-object, when reaching the aperture, can act as a valve and compromise the functionality of the probe. The real problem is the difficult identification of debris in the microchannel without actually testing them. This means, all the probes are being milled with FIB but only a little part of them is working. Of course, time and money is lost here.
4.3.3.1.1. Protocol

The protocol presented hereafter is valid if using closed pyramidal probes with 450nm wall thickness. Some parameters have to be changed if thinner walls are being processed.

**Milling of the aperture. Milling direction: parallel to the rear wall of the pyramid**

1. Position the SEM/FIB holder on the flat side (0°)
2. Set the stage tilt angle to 18° to bring the FIB beam parallel to the rear wall of the pyramid.
3. Bring the SEM stage up to the SEM/FIB coincidence point (Zeiss NVision 40 of EMEZ: Working distance = 4.8 mm)
4. Centre and adjust SEM frame on the pyramid probe with adequate magnification

*REMEMBER:* for this application, the sharpness of the apex plays a critical role. Be sure not to damage it by over-imaging it with the FIB. Ideally, only two images of the apex are needed to be taken in the FIB mode.

The next steps do not have to be done in all cases. The procedure described hereafter is optimal in order to minimize drift. They are not needed if the beam is stable and if the probes are not charging.

5. Set the imaging current of the FIB gun to **10 pA**. This current intensity is ideal for the probes with 450 nm wall thickness. Lower current will excessively increase the milling time causing even a small drift to become critical. For 80 nm wall thickness, **1 pA** current is advised.
6. Switch to the FIB mode in reduced mode. Center the pyramid by avoiding to include the apex in the live window (i.e. try not to image the apex.). In this way, only part of the pyramid body is damaged by the imaging but not the sharp probe.
7. Exit reduced mode and take an image of the complete pyramid.
8. Lunch **feature mill** and import the image just taken.

*HINT:* during definition of the milling pattern, it may be advisable to keep imaging the pyramid in reduced mode. This will prevent the complete
discharging of the probe and will avoid drift due to initial charging when starting the milling process.

9. Draw the desired geometry in the feature mill tool. The drawing below refers to a 200 nm wide triangular aperture milled in a closed pyramid with 450 nm thick walls.

![Feature mill tool drawing]

The red, discontinuous circles are used in the feature mill tool to guide the positioning of the pattern. The basis of the triangular aperture is placed at least at 800 nm from the apex. Thick black lines denotes the view of the pyramid from the FIB angle. Discontinuous, black lines denotes the presence of the 450 nm thick walls. The aperture must cross these lines to obtain a functional probe.

10. Mill for **15s, with 10 pA**. These parameters are optimized for a 200 nm triangle. Adapt the time in function of the size (more time for bigger size) and the thickness of the pyramidal walls.

**Summary** – In conclusion, we obtained best results milling a triangular aperture parallel to the rear wall of the pyramid. In this way, milling overtime became non-critical. Many parameters affect the efficacy of these probes for mammalian cells or yeast injection. One of these, as said, is the sharpness of the probes. Depending on the rigidity of the membrane, high stiffness of the cantilever is also wanted. For example, the probes fabricated with the E-Beam masks that were introduced to solve the blade apex problem, revealed to be softer than the previous batch (~1 N/m instead of 3 N/m). The consequence is an increase in sensitivity but a decrease of detectable range, i.e. the maximum measurable force is lower and not enough to measure the forces needed to penetrate cells or yeasts with rigid membranes. However, the limiting factor is still the high clogging probability when working with sub-nanometer apertures and overpressure, indicating that the debris detected in the microchannel are affecting the performance of these probes.
4.3.3.2. Extraction

*Publication in preparation:*

*Extraction and analysis from single mammalian cells*

*Tentative authors list:*

Orane Guillaume-Gentil, Rashel Grindberg, Dario Ossola, Tomaso Zambelli, Julia A. Vorholt.

The probes used for extraction are similar to the ones fabricated for Injection in both design and fabrication. The only difference is the size of the aperture, i.e. bigger. Bigger sizes facilitates lysis of the cell of interest and minimizes the risk of clogging. For this reason, we fabricated apertures between 400 nm to 600 nm. In this case 10 pA beam current, for 50 s was used for pyramid with 450 nm walls.

4.3.4. Bacterial adhesion

*Publication submitted*

*Bacterial Adhesion Force Quantification by Fluidic Force Microscopy*

Eva Potthoff, Dario Ossola, Tomaso Zambelli, Julia A. Vorholt.

The ability of the FluidFM system to efficiently and reliably measure adhesion forces of mammalian cells on different substrates with increased throughput has already been demonstrated with the use of tipless probes (Potthoff et al., 2012). The next challenge was to exploit the AFM force sensitivity by measuring adhesion of bacteria. For this purpose, different apertures designs have been fabricated with the FIB.

In conventional adhesion measurements, the cell or bacteria are irreversibly attached onto a tipless cantilever (Peters et al., 2012) to give so called bacterial probes. The cantilever is then brought in contact to the desired substrate and retracted again after a defined contact time. As each bacteria has to be singularly attached to the AFM probe, this technique is labor intensive and limits the throughput drastically (most studies analyzed 3 cells only (Beaussart et al., 2013). The closed fluidic system embedded in FluidFM probes offers the unique advantage of reversible immobilization of bacteria and cell, allowing the use of the same probe for serial measurements. Direct comparison between cells is therefore only feasible with this technique.

Forces involved in bacterial adhesion are several orders of magnitude lower than those involved in mammalian cells adhesion. Whereas for the latter case a stiff cantilever was
ideal (high maximum measurable force), it is of impairment for the measurement of much smaller forces. To increase the force sensitivity we agreed in reducing the height of the embedded microchannel (refer to Chapter 4.2.1). However, it was an open question whereas the application of suction through such a thin microchannel was still possible or not.

The bacteria that we envisioned to reversibly immobilize with the pyramidal FluidFM probes in physiological conditions are *Escherichia coli* and the *Streptococcus pyogenes*. E. coli are rod-like shaped (Figure 4.23a), whereas the streptococcus pyogenes is spherically shaped, naturally occurring in chains of two or more cells (Figure 4.23b). We designed special apertures geometry to increase immobilization efficiency.

![Figure 4.23: a SEM picture of a colony of E- Coli bacteria. Source: www.wikipedia.org. b Pair of streptococcus pyogenes bacteria. Source: www.waterscan.rs.](image)

### 4.3.4.1. Apertures for E. Coli immobilization

To efficiently and reversibly immobilize E-coli bacteria we design hemi-cylindrical apertures. This bacteria-complementary shape ensures robust trapping mechanism, that ensures directional immobilization. Figure 4.24a shows the result of such a probe drilled in closed pyramidal probes with 80 nm walls. Subsequently, we switched to FluidFM probes with smaller spring constant, featuring a 200 nm high microchannel instead of 1 μm (Figure 4.24b/c). These probes have thicker pyramidal walls (450 nm) that forced the introduction of a second flattening milling step (see protocol).
Figure 4.24: Custom FIB probes for bacterial adhesion. a Hemi-cylindrical aperture for E. coli immobilization. Drilled in a closed FluidFM probe with 80 nm thick walls. b E-coli immobilization aperture milled on a soft, closed FluidFM probe with 450 nm thick walls. A second milling step is required to position the hemi-cylindrical aperture closer onto the substrate.

Fabrication protocol:

i) Protocol for 80nm thick walls – monostep

Milling of the hemi-cylindrical aperture:

1. Position the SEM/FIB holder on to the 36° inclined side (cantilever axis parallel to the FIB emission gun)

2. It is good practice to compensate for the AFM angle: Set the SEM tilt angle to +10° (adapt this value depending on the AFM head inclination – Nanosurf with corrected AFM-nose: 8°).

3. Bring the first probe at the ideal working distance from the SEM gun (Zeiss NVision40 of EMEZ: 4.8 mm). Follow the SEM procedure to have a sharp image of the pyramid.

4. Switch to the FIB imaging and center the pyramid with adequate magnification. Remember to image with the same current objective used for the milling step. 10 pA currents are ideal, but 40 pA are appropriate too if one wants to accelerate the process.

5. Open the feature mill tool and draw a circle with the desired dimensions in the appropriate location (see following drawing)
6. The milling process can be followed in real time and ended manually when finished. However, as an indication, the following parameters are valid: ~200 s @ 10 pA for hole diameter of 700 nm or ~40 s for a hole of the same size @ 40 pA.

ii) **Protocol for 450nm thick walls: with flattening**

*Hint:* the flattening step can be done before or after milling the cylindrical aperture. Here we prefer the second option because it gives better control on the depth of the hemi-cylindrical aperture.

**Milling of the cylindrical aperture:**

*Follow the steps 1-4 of the previously described protocol*

5. Open *Feature Mill* tool and draw the circle to be milled with position and dimensions according to the illustration below:

Light, discontinuous lines indicate the 450 nm thick walls

6. Keeping the same holder position (i.e. same FIB incidence angle), take an image in FIB mode of the result.

7. With the *fine rectangle* tool, draw a rectangle with the *fine rectangle* tool.

8. The best way to control the process is to switch to the SEM + MILL mode and follow the process live. When the desired degree of flatness is achieved, interrupt the milling process.

10 to 40 pA are appropriate for this processing step.
4.3.4.2. Apertures for S. Pyogenes immobilization

S. Pyogenes bacteria present a spherical-like shape. To immobilize this kind of bacteria we envisioned a cylindrical, apex aperture similar to that proposed for patch-clamp experiments (Paragraph 4.3.1.1.1).

Probe positioning and alignment is the very same as described in the protocol fabrication of patch-clamp probes (Paragraph 4.3.1.1.1). The difference relies in the use of higher milling current. Indeed, in this case bigger apertures are needed (up to 900 nm). Using 10 pA milling currents, 250 s milling time are required. Better parameters are 50 s milling time with 40 pA milling current. Figure 4.25a shows typical result with these parameters. It is important mentioning that over-milling is critical in this case. In the section view of Figure 4.25b, one recognizes that over milling would cause the cylindrical aperture to reach the backside of the pyramid, which is for obvious reasons unwanted.

Figure 4.25: Custom FIB probe for S. Pyogenes adhesion measurements. a SEM picture of the pyramid. Aperture diameters ranged from 300 nm to 900 nm. b SEM picture of a sectioned custom FIB probe. The nan-channel is visible in the pyramid. Bacteria are trapped in the cylindrical aperture.

4.4. Conclusion

The FIB allowed us to solve two distinct problems: on the one hand, it gives answers on the probes structure by imaging cross-sections of the cantilevers. On the other hand, it allowed us to bypass the shortage of top-aperture FluidFM probes and to design special apertures for the scope of pioneering promising applications. Even though expensive and time consuming, the FIB revealed to be an essential tool for the development of the FluidFM.

I milled more than 960 FluidFM Probes that have been used in the following projects: Force-controlled electrophysiology, mammalian cells and bacterial adhesion measurements, mammalian and bacterial cell injection, mammalian cells extraction, FluidFM for scanning ion conductance microscopy and for the FluidFM as a nanolithography tool.
5. Force controlled patch-clamp in whole-cell configuration

Patch-clamp is the application that stimulated the concept of using microchanneled cantilevers as force controlled micropipettes, and thus fostering the invention of the FluidFM. In this Chapter, we demonstrate the ability of the FluidFM to perform whole-cell patch-clamp recordings, even on the most “hostile cells” for this technique: Isolated adult mice cardiomyocytes. In addition, we deeply discuss the opportunities and weaknesses of the FluidFM system for single cell electrophysiology.

An overview the electrophysiology topic and on its importance has already been given in the introduction of this thesis (Chapter 1.1). Direct competitors with our technology are automated patch-clamp devices. However, no other technology is able to offer high quality electrophysiology recordings together with the force sensitivity of an AFM. Planar patch-clamp is probably the technology that show at most the potential to become a valid alternative to conventional patch-clamping. Especially, high quality recordings have been obtained with a special planar-chip design proposed in the Cytopatch2 (Chevalier et al., 2014), an automated patch-clamp instrument presented in the introduction of this thesis (Chapter 1.3.4.1). The nano-inspired innovation efforts presented in Chapter 1.3.4.2 are treated as a different category because of their inability to precisely control the transmembrane potential.

Here we will detail the whole-cell configuration, the mostly used patch-clamp configuration to investigate the dynamics of ion channels, and, in particular, voltage-gated ion-channels. The patch-clamp technique still offer the highest quality electrophysiological recordings when performed with conventional glass micropipettes and sets the standard benchmark.
The results presented in this Chapter are the output of the fruitful collaboration with the Ion-Channel Group of the Department of Clinical Research of the University Bern, directed by Prof. H. Abriel in the framework of an interdisciplinary project granted by the Swiss National Foundation. In Abriel’s group, a PostDoc, Mohammed Yassine Amarouch was appointed having a solid experience with standard patch-clamp. The collaboration revealed to be crucial for the success of this project in all its phases, from the project definition to its experimental execution. Together, we identified the ideal model systems, which were first characterized by means of conventional patch-clamp in Bern. In a second phase, their expertise provided us with the biological system that could exploit at best the FluidFM capabilities: freshly isolated mice cardiomyocytes. The proximity with the collaborators revealed to be of fundamental importance. Indeed, isolation was carried out in Bern early in the morning of an experimental day and I was able to travel to Bern in time to grab the freshly isolated cells, in order to immediately travel back to Zurich to perform the experiments on the FluidFM setup.

5.1. Introduction

5.1.1. Whole-cell patch-clamp

The whole-cell patch-clamp configuration was briefly described in the introductory Chapter of this thesis. Aim of the whole cell configuration is to achieve contact to the intracellular environment of the selected cell and thus control the transmembrane potential, which is mandatory for the investigation of voltage-gated ion channels. Figure 5.1 proposes a cartoon of a micropipette in contact with the cell membrane in whole-cell configuration.

![Whole-cell configuration](http://sites.sinauer.com/neuroscience5e/animations04.01.html)

The whole-cell technique allows to bring the electrode in the micropipette in direct electrical contact with the cytoplasm. In this configuration, i) the transmembrane potential can be controlled with the use of special amplifiers. Thus, we can simultaneously record the ionic current mediated via transmembrane transporters including ion channels. ii) In the ideal
case (spherical cells), the activity of whole cell population of ion-channel present in the plasma membrane is recorded, because they all sense the same transmembrane potential. In practice, the membrane potential is not constant on the entirety of the cell (especially when dealing with large cells as neurons or cardiomyocytes), meaning that rather local currents are recorded. An effect known as space-clamp problem (Bar-Yehuda and Komggreen, 2008). iii) The micro-pipette usually have few micrometer aperture diameter and is typically filled with a solution mimicking the intracellular composition in terms of ions (species and concentration) and molecules (ATP, GTP etc.), or with compositions that allow to study ion channel dynamics under particular conditions (e.g. in absence of a particular ion specie).

Two different recording modes are commonly available when working in whole-cell configuration. The voltage-clamp and the current-clamp mode. In the first one, the voltage is kept constant with the help of an operational amplifier, while the fluctuation in current are recorded. The current-clamp mode does the opposite: the current is kept constant, while the voltage variations are recorded. The voltage-clamp technique is used when the movement of ions across the plasma membrane is of interest, whereas the current-technique is used when changes in the transmembrane potential are of interest (e.g. to record action potential of excitable cells).

**Whole-cell configuration access** – Figure 5.2 schematically summarizes the protocol to achieve whole-cell configuration. The first steps are shared between all the patch-clamp modes, i.e. one has to approach the pipette to the cell and achieve the gigaseal. It is common practice to follow the procedure by monitoring the currents elicited by a voltage pulse (~10 mV, ~10 ms). Two important values are derived from such a measurement: i) the resistance of the seal, by measuring the ionic current difference between the minima and maxima plateau of the pulses; ii) the RC value of the capacitance transients. If the seal resistance gives information about the seal quality, the capacitance contains information about the cell being patched, e.g. the cell volume (Satoh et al., 1996).
Figure 5.2: Whole-cell access procedure. As with every patch-clamp configuration the pipette is approached on the cell and the gigaseal is formed (cell-attached). Subsequently, suction pulses are applied to disrupt the membrane in the patch and to obtain access to the cytosol.

Before electrode approach, the capacitance transients are appropriately compensated with the dedicated circuitry provided by patch-clamp amplifier. As contact is reached, a moderate increase of the resistance is noticed together with the appearances of small capacitance peaks. While gigaseal formation occurs, the resistance drastically increases, leaving in the current signal only fast capacitance transients (Figure 5.2). Subsequently, suction pulses are commonly applied in order to favor the whole-cell configuration by rupturing the membrane. Successful break-in is confirmed by a decrease in capacitance transient decay speed, as the capacitance of the cell membrane is added to the system, which is about 1 μF/cm² (Miller, 1992).

Cell dialysis – An often unwanted effect is the so called cell dialyzing or wash-out effect. Since the volume of a cell is smaller than the volume of the electrode, the cell’s intracellular solution is exchanged with the artificial solution of the pipette in little time. This process is faster for the ions than for large intracellular molecules, but every molecule having a size smaller than the aperture of the pipette is eventually washed out. This effect has both positive and negative consequences. The unwanted effect is the loss of important cellular components, including molecules and proteins, affecting the long-term current recordings. In fact, it is common to observe a spontaneous decrease in currents amplitude with time. An effect known as rundown of the recorded currents. Interestingly, it has been shown that cytosolic molecules indeed have a direct influence on the rundown effect as their presence may stabilize particular conformations of ion-channel proteins (Loussouarn et al., 2003) The advantage, on the opposite, is the possibility to control the intracellular ionic composition and to deliver a multitude of agents of interest (e.g. dyes, etc.) in the cytosol.
5.2. Encountered difficulties

As the patch-clamp project started the FluidFM technology just emerged and the upmost concern was the limited availability of defect free probes slowing down systematic studies. We decided to switch from tipless FluidFM probes with circular aperture to FIB-customized pyramidal FluidFM probes because the first ones did not show promising preliminary results. With the use of tipless probes, a seal of a few MΩ only was observed, the reason being the geometry of the microchannel. Indeed, the circular aperture is too thin to allow appropriate membrane adhesion and the rectangular section of the microchannel impedes tight adhesion of the patched portion of membrane.

The majority of the probes had leakage problems, due to the microfabrication process. In addition, the first half my thesis we were constrained from the absence of AFM laser signal. The reasons were different: i) capacitance contribution of the reflective coating, ii) defects in the microfabrication and incompatibility with the at that time new AFM hardware. However, patch-clamp experiments could be equally performed by carrying out manually the approach in the very same way done in conventional patch-clamping, i.e. monitoring the increase of electrical impedance of the probe when in close proximity with the cell (refer to Chapter 6). Therefore, even without force control we could at least recognize issues related to the seal formation.

Seal formation is crucial in patch-clamping. We tested different FluidFM probe design (from tipless to different apex geometries), and it was clear from the beginning that the square section of both the microchannel and the pyramid would have represented an obstacle for the attainment of high quality seals. The new strategy was therefore to design a pyramidal probe with thicker walls so that a circular aperture could be milled at the very apex (see FIB Chapter, 6). However, the drawback of FIB milling is that it is a time consuming and expensive technique. Summed with the low yield of defect-free FluidFM probes, turned systematic experimenting into a challenge.

Contamination and clogging problems also affected the efficiency of the experiments. Contamination presented as thick traces of undefined substance delivered from the microchannel under some not totally understood circumstances. Clogging is related to the images shown in the Focused Ion Beam Chapter (see Chapter 4.2) where particles bigger than the aperture are present in the pyramid and in the apex. Under these conditions, the probes were delivering liquid for short time before clogging or they were showing a valve-like behavior, i.e. where only suction was applicable.
Gentle contact on cell – When the objective is to gently position the probe on top of a cell, the applied force is of fundamental importance. Indeed, the cell is a very soft material and the applied force must be minimal to avoid excessive deformation and damage. Under these circumstances, the stiffness of the probe determines the minimum detectable and thus applicable force. The FluidFM probes used for the proposed experiments present a spring constant of about 2 N/m which translates in a minimal force setpoint of a few nN. Even though this force does not compromise the integrity of the cell, mechanical deformation is visible. Probes with spring constant of an order of magnitude lower would fit better this application, but are not available. Development of such probes would involve either longer or thinner cantilevers. Both options are challenging: i) thinner cantilever oblige to fabricate a thinner microchannel, which would in turn increase the electrical impedance to values that would not allow electrophysiological recordings. ii) longer cantilever involve the redesign of the masks used in the microfabrication steps, which is a demanding process both in terms of costs and time, where time is measured in months.

The second observation related to gentle probe positioning is that the cell is a deformable and adaptive body. The consequence is that application of a constant force results in a constant squeezing of the cell by the probe because the cell cannot react with the same force. The effect is similar to the indentation of viscous material. When using the relatively stiff FluidFM pyramidal probe to approach a cell, it might be important to readily reduce the setpoint of the force controller as soon as contact is detected, to avoid excessive squeezing. After approach, the force setpoint was set to zero. The controller therefore ensured that in average no force is applied on the cell and excessive cell deformation was prevented. This procedure was needed due to the relatively stiff probes (~2 N/m), but the only ones at our disposal.
5.3. Oil-enhanced sealing / Oil manipulation – a pilot study

Improvement of the seal between plasma-membrane and the glass of the micropipette has long been a topic when high-resistance seals are needed on large diameter pipettes (12 to 40 μm). The technique is known as the Giant Membrane Patch (Hilgemann, 1989). It has been reported the use of different hydrocarbon mixtures consisting of oils, waxes and Parafilm (Collins et al., 1992). The correct mixture has to be defined as a compromise between seal stability and resistance.

In range of a parallel strategy to conventional sealing and along with the literature research presented above, we aimed to obtain high seal resistances by getting rid of the conductive, aqueous layer between pipette and plasma membrane. (Figure 5.3)

![Figure 5.3: Oil-based coating. The coating is represented in Orange. The role of the oil-based coating would be to dissolve the outer layer of the plasma membrane, and fuse with the hydrophobic tails of the lipids belonging to the cytosolic membrane layer.]

By coating the probe with a lipophilic layer, the outer membrane would likely fuse with it and there and no physical space will be left for the aqueous layer usually present between the bilayer and the uncoated glass. For this purposes we designed specially shaped aperture (refer to the Chapter 4.3.1.2, related to the fabrication of these probes) which present a flat plane around the aperture. Our strategy was to coat this surface with a lipophilic layer that, if needed, can be regenerated after each cell contact. Indeed, regeneration of the coating in-situ would be an interesting aspect that would simplify and allow for serial patch-clamp recordings. Regeneration of the coating is envisioned by introducing a cleaning step (solvents or soaps) and a subsequent immersion in clean oil. This type of regeneration would allow to reuse the same probe on a multitude of cells allowing serial patch-clamp and thus enhancing the throughput of the system.

The coating must i) be limited in thickness to avoid encapsulation of the whole pyramid, must ii) cover the surroundings of the aperture and must iii) must be stable in time, i.e. must not flow spontaneously into the aperture. We limit the thickness of the oil coating by ensuring a hydrophobic surface of the cantilever. With a hydrophobic coating, the layer spontaneously spread on the surface of the cantilever under the action of surface-tension.
To perform the experiments, we relied on squalene oil (Sigma-Aldrich, Montana, USA), a natural hydrocarbon organic compound. This oil has been chosen because it is naturally occurring in nature and has a moderate viscosity, which favors formation of films. All the experiments, except for the patch-clamp where the corresponding intracellular and extracellular solutions are used, were performed in 150 mM KCl liquid environment. The substrate is the glass bottom of the WillCo Wells, used as received.

5.3.1. Results

Oil deposition in liquid environment

Oil coating of glass micropipettes happens by dipping the pipette directly into the oil reservoir or by passing it through a film of the hydrocarbon mixtures (Hilgemann, 1989). This strategy cannot be adopted for FluidFM probes because dipping would result in a macroscopic droplet encapsulating the entire cantilever, compromising its functionality as an AFM sensor. We solved this problem by depositing an oil droplet (with contact-diameter ranging from 50 μm to 200 μm, m) acting as a reservoir on the substrate itself by using FluidFM tipless cantilever with 8 μm aperture diameter. The squalene was filtered through a 0.22 μm filter and pipetted into the reservoir of the tipless FluidFM probe. FluidFM probes were used as received, i.e. without any surface treatment. Figure 5.4 shows a droplet of squalene deposited with an 8 μm tipless probe. Overpressure was applied manually, via a syringe, until a droplet of oil was visible at the aperture. The probe was then approached to the surface and retracted as the force setpoint was reached.

![Figure 5.4: Deposition of a squalene droplet through an 8 μm tipless FluidFM probe.](image)

In a second moment, the free end of the probe to be coated, filled with the desired solution (intracellular solution, for whole-cell patch-clamp) is inserted under optical control into the previously deposited oil droplet. In order to achieve a uniform and reproducible behavior, we tested different surface modifications of the FluidFM probe that are hereafter summarized.
Oil based coating

It is common practice to treat the surface of FluidFM probes 2 minutes oxygen plasma (PDC-32G; Harrick Plasma, Ithaca, New York) to ensure smooth filling of the microchannel when working with aqueous solutions. Indeed, oxygen plasma treatment ensures removal of organic contaminants and, turns the surfaces more hydrophilic. However, hydrophilic surfaces are usually oil repellent. Indeed, as shown in the image sequence of Figure 5.5a-d, in the attempt of inserting a 2 μm tipless FluidFM probe into the squalene oil droplet, the latter is pushed away. During the insertion procedure, the ionic current is continuously monitored (data not shown). With a hydrophilic cantilever, electrical contact is never lost, meaning that a layer of water surrounds the cantilever ensuring a pathway for the ions.

Figure 5.5: Comparison between hydrophilic and hydrophobic cantilever. A 2 μm Tipless FluidFM cantilever is inserted in a droplet of squalene. a-d Sequence of images showing the insertion of the hydrophilic cantilever into the squalene droplet. The oil is repelled. e-f Same insertion sequence of the hydrophobic cantilever into the oil droplet. The oil is attracted and spontaneously sucked into the microchannel. The fluorescent marker added in the microchannel solution highlights its replacement by the oil.

Silanization – We used Sigmacote® (Sigma Aldrich, Montana, USA) to deposit a silicone monolayer on the cantilever to make it hydrophobic. The silanization was performed overnight in atmospheric pressure, in a sealed petri dish were the probes were placed besides a droplet of Sigmacote solution. By spontaneous evaporation, the silanes will form a self-assembled hydrophobic monolayer on all the exposed surfaces. The probes were treated in oxygen plasma for 2 minutes immediately before silanization. After filling the microchannel with 150 mM KCl with the addition of 1 μg/mL of fluorescein dye, the next step was to insert the cantilever in a droplet of squalene to obtain the desired oil-based coating. Figure 5.5e-f show fluorescence microscopy images sequence of the insertion of the hydrophobic cantilever into the squalene droplet. In contrast to the behavior observed with the hydrophilic cantilever, the oil adheres to the surface and spontaneously replaces the aqueous solution in the microchannel even though positive 100 mbar were applied. In this case, electrical contact is lost at the very moment the cantilever enters in contact with the oil droplet. Because of the surface energy, the oil pulled into the cantilever
could not be expelled by the application of overpressure. This behavior compromised the functionality of the probe as the electrical contact was unstable, i.e. only present when forcing high flow rates with the use of a syringe as a piston.

To avoid the above-mentioned behavior, we eventually developed a sealed silanization chamber for FluifFM probes that allows a constant application of overpressure in the microchannel, that shall avoid the silane mixture to enter the microchannel. Figure 5.6 shows the micrographs of the oil coating procedure synchronized with the electrical signal that was used to calculate the electrical resistance of the probe. A customized FIB cantilever (Chapter 4.3.1.2) with hydrophobic outside and hydrophilic microchannel is inserted into the droplet of squalene. As predicted, the squalene is pulled onto the cantilever but does indeed not enter the microchannel, confirming the validity of the above described silanization procedure.

![Micrograph sequence of a custom hydrophobic/hydrophilic FIB probe inserted into the squalene droplet. Upon contact with the droplet, the aperture is immediately sealed. When the probe is retracted, the seal is maintained, indicating successful coating of the probe. Eventually, overpressure pulse of 500 mbar restores the connection, which remains stable when going back to 0 mbar.](image)

During insertion of the probe into the oil droplet, a positive pressure of 100 mbar is maintained. As the probe touches the surface of the oil droplet, the latter is spontaneously pulled onto the cantilever and the electrical signal drops, revealing a resistance of about 4
GΩ. The seal is maintained also when the cantilever is extracted from the oil droplet. By application of 500 mbar of positive pressure, the electrical connection is restored and, importantly, is stable upon release of the positive pressure.

5.3.2. Conclusions & Outlook

We presented a simple method to obtain a thin, uniform oil-coating of the FIB-modified FluidFM probe that to not compromise the electrical connection through the aperture. Further investigation is needed to confirm the supposed mechanism explained in Figure 5.7. The amount of oil present on the aperture surroundings has to be characterized to assess the validity of this method. Only few experimental approaches have been performed on cells to test the formation of the gigaseal, but did not give any conclusive result about the effectiveness of the technique yet. Preliminary experiments (not shown here) showed immediate sealing between the squalene layer deposited on a tipless FluidFM probe and a HEK 293 cell. However, the use of tipless probes involve a big interaction area between probe and cell (the upper half of the cell being entirely covered with oil) that compromised cell integrity, thus no electrophysiological recording was possible. Consequently, the sealing properties have to be confirmed with the use of pyramidal probes. We assume that the use of pyramidal probes will not compromise cell viability because of the localized interaction.

![Figure 5.7: Expected coating mechanism.](image)

However, the strategy has the potential to become a valid protocol for serial patch-clamping with the FluidFM. In addition, the concept of In-situ coating regeneration has been demonstrated by depositing oil reservoirs close to the measurement site using a second FluidFM probe.

Addition – in range of these investigations, the deposition of oil droplet in a liquid environment suggested the use of liquid droplet as a regenerative probe, i.e. the oil droplet can be used as a probe itself. Different oil composition could give interesting insights in hydrophobicity/hydrophilicity properties of the substrate or the interaction of biological
systems with different oil/lipid mixtures can be studied. In addition, the use of sub-micrometer apertures of the pyramidal FluidFM probes could increase the lateral resolution in the order of the smallest droplet diameter that can be achieved. Furthermore, by filling the cantilever reservoir with oils, lipid, or other thinkable mixtures, insight into their interaction forces can be gathered. The AFM has already been used for this kind of studies, but with extremely low throughput because of the complexity level. The FluidFM can give a substantial technological advantage in these fields. For example, force between droplets in aqueous environment was measured to elucidate electrostatic double layer interactions (Dagastine et al., 2004). Other studied the membrane fusion dynamics and protein complexes involvement (Abdulreda et al., 2008), or forces involved in droplet systems (Tabor et al., 2012). Interestingly, a liquid droplet can be used as a transporting vehicle too (Bhushan and Ling, 2009).
5.4. Publication – Force-controlled patch-clamp using AFM

The work presented hereafter is part of the following article, and is in the submission process:

*Force-controlled patch-clamp using atomic force microscopy*

Dario Ossola, Mohammed-Yassine Amarouch, Pascal Behr, Janos Vörös, Hugues Abriel, Tomaso Zambelli
Submitted.

All the results presented in this Chapter, where not explicitly stated, will be part of the above mentioned article.

5.4.1. Introduction

Ion channels are pore-forming proteins that provide pathways for the controlled movement of ions across the plasma membrane as well as the membrane of intracellular organelles. The channel open-close mechanism depends on different stimuli, which are of mechanical, chemical or electrical nature. In particular, voltage-gated channels are sensitive to the transmembrane electric potential and are involved in generation and transmission of action potentials in excitable cells such as neurons and cardiomyocytes.

Dysfunction of ion channels, also referred to as channelopathies, has been linked to many human disorders such as cardiac arrhythmias and epilepsies (Abriel and Zaklyazminskaya, 2013). Channelopathies are a result of either genetic mutations or acquired malfunctions of ion channels. Acquired channelopathies may result from drug exposures that alter ion channel function such as the voltage gated potassium channels (hERG). It is especially crucial for pharmaceutical industries to recognize drug interaction with ion channels as early as possible in the screening process, as a late failure translates in severe loss of investment. For example, several drugs have been withdrawn from the market as they were shown to inhibit the hERG channel with potentially life-threatening consequences (Fermini and Fossa, 2003).

Cellular electrophysiology techniques, in particular the patch-clamp method (Neher and Sakmann, 1976) are employed to address questions such as the role of ionic current in normal physiology and disease, and to investigate the effects of newly developed drugs on ion channels (Xu et al., 2001). Even though the technique is well implemented in many laboratories, the patch-clamp remains a low efficiency procedure even for skilled personnel. In conventional patch-clamp, a glass micropipette containing an electrode is optically approached onto a selected cell in-vitro by monitoring the impedance increase
FORCE CONTROLLED PATCH-CLAMP IN WHOLE-CELL CONFIGURATION

(Korchev et al., 1997). Finally, the cell membrane is pulled into the pipette by negative pressure application until a seal of high electric resistance (gigaseal) is formed with the probe of the pipette (Hamill et al., 1981). Further suction will rupture the cell membrane in the pipette providing electrical access to the cytoplasm allowing the so-called whole-cell configuration of the patch-clamp technique. The latter is the most widespread mode used to study voltage-gated ion channels, as it remains the only established protocol to accurately control the transmembrane potential. In automated patch-clamp, pipettes are replaced by glass chips with etched holes while the process of sealing and local membrane rupture has been replaced with automated suction and software control system (Dunlop et al., 2008). However, these instruments are only suitable for electrical recording of stable cell lines expressing the ion channel of interest.

Nanotechnology has inspired the miniaturization and parallelization of electrophysiology tools (Kwiat et al., 2013) focused mostly on intracellular measurements. Several groups were able to monitor action potentials from neurons and cardiomyocytes (Fertig et al., 2002). Yet, for some of these techniques, like the vertical silicon electrodes array technique, the amplitude of the recorded action potentials was dampened 10-fold compared to the patch pipette (Robinson et al., 2012). Furthermore, no recording of ionic currents in the voltage-clamp mode reflecting the voltage-gated ion channel activity has been shown with these nano-devices.

To improve the efficiency and the application range of patch-clamp we attempted an alternative approach aiming to control the contact of the pipette apex with the cell membrane. By rendering it as gentle as possible, the cell should not be perturbed thus enhancing the quality of the measurement. Starting point is the atomic force microscopy (Binnig et al., 1986) (AFM) with its force feedback sensitive in the picoNewton regime thanks to the optical beam deflection (OBD) method (Meyer and Amer, 1988). Saenz et al. used an AFM with conventional OBD cantilevers to measure cell elasticity variation of cardiomyocytes during contraction, while extracellularly stimulating with micro electrodes (Saenz Cogollo et al., 2011). Yet, the use of extracellular microelectrodes does not allow for the control of the transmembrane potential, impeding simultaneous electrophysiology studies. Others measured the transverse displacement and force generated by contracting cardiomyocytes with an AFM (Chang et al., 2013; Domke et al., 1999). Further, the field of force-controlled glass pipettes was pioneered by Shalom et al. (Shalom et al., 1992) introducing the concept of bent glass micropipettes provided with a mirror for the OBD AFM laser deflection, whereas Ito et al. mounted a glass pipette onto a tuning fork (Ito and Iwata, 2011) and Drake et al. ingeniously affixed it to a dual flexure optical lever (Drake et
al., 2014). However, to-date no electrophysiological recordings have been obtained with such force-controlled glass pipettes.

Figure 5.8: Force controlled whole-cell patch-clamp with the FluidFM. a Schematic cartoon of the FluidFM setup in whole-cell configuration. The membrane patch in the probe aperture is ruptured to connect the electrode inside of the pipette with the cytosol. The patch-clamp electronics (schematized with an inverting operational amplifier) clamps the transmembrane potential. Changing the transmembrane potential modifies the activity of voltage-gated ion-channel as reflected in ionic current. The OBD system monitors and regulates the deflection of the cantilever via actuation of the z-piezo. b Scanning electron micrograph of a FluidFM cantilever coated with a thin gold layer to enhance its reflectivity. At the far end, the pyramidal probe is visible. Inset reveals the hollow nature of the cantilever with a section through the pyramid made with FIB c Zoom-in of the pyramidal probe used for patch-clamping. The circular aperture at the pyramid’s top is milled with FIB. d FIB section of a typical probe highlighting region where the seal is formed.

As an AFM-based tool, we relied on the FluidFM for our development: a force-controlled nanopipette which takes advantage of microchanneled OBD AFM cantilevers directly connected to a pressure controller (Guillaume-Gentil et al., 2014a; Meister et al., 2009) (Figure 5.8a). The technical challenge was threefold: i) the ionic tight insertion of two Ag/AgCl electrodes connected to a patch-clamp amplifier in the fluidic pipeline, ii) the milling of the probe aperture with focused ion beam (FIB) with the most appropriate geometry for patch-clamp, and iii) the custom software simultaneously recording the output data from the AFM control electronics as well as the patch-clamp amplifier.
We selected Na\textsubscript{v}1.5 voltage-gated ion channels expressed in human embryonic kidney (HEK 293) cell lines as benchmark biological system to demonstrate the FluidFM ability to carry out experiments relevant for electrophysiology, and isolated adult mouse cardiomyocytes to exploit the force control capabilities. Na\textsubscript{v}1.5 voltage-gated ion channels are demanding because of their quick kinetic response while cardiomyocytes are one of the most difficult cells to “patch” as they usually react with seal-damaging contractions once approached with the pipette.
5.4.2. Methods

FluidFM setup

Probes: Pyramidal, FluidFM probes without aperture were supplied by Cytosurge (Zürich, Switzerland). The cantilevers have nominal spring constant of 3 N/m. However, calibration with Sader method (Sader et al., 1999) revealed lower effective spring constant of 1.9 N/m. Cantilever have a rectangular shape with 200 μm length and 36 μm width. The channel is 1 μm high, 30 μm wide and about 1.5 mm long. Pyramid wall thickness is 450 nm. Cantilevers are made in silicon nitride. FluidFM probes were received as loose chips for FIB modification. Modified chips were then sent back to Cytosurge, which mounted them on a Cytoclip (supplementary Fig. 1) that fits the AFM probeholder (Nanosurf, Liestal, Switzerland) modified for the FluidFM.

FIB milling: The FluidFM probes were coated with a thin layer of carbon (Cressington, Dortmund, Germany) before being imaged and milled with a Zeiss NVision40 FIB-SEM (Carl-Zeiss, Jena, Germany). Probes were first flattened starting from the apex with a 10° angle to compensate for the tilt angle of the AFM probeholder (FIB probe: 30 kV @ 10 pA). The cylindrical aperture were milled perpendicular to the resulting surface at beam current of 10 pA for a duration dependent on the depth and diameter of the aperture (e.g. 50 s for 450 nm wall thickness and 300nm diameter).

FluidFM probe filling: before filling the FluidFM probe, the solution was passed through 100 nm filter and degassed in a 10ml syringe where vacuum/vent cycles were manually applied until no more air bubbles formed. Negative pressures have to be applied during seal formation; degassing prevents creation of air bubbles during said phase. Before immersion, the backside of the Cytoclip and its connector were carefully immersed in wax (Paraplast; Sigma-Aldrich, St. Louis, Missouri) (supplementary Figure 5.13).

FluidFM probe cleaning: Prior to the experiment the cantilever was cleaned for 2 min in air plasma (PDC-32G; Harrick Plasma, Ithaca, New York). After each interaction with cells the cantilever was cleaned with the subsequent protocol: (i) immersion in 10X Terg-a-Zyme® (Sigma-Aldrich, St. Louis, Missouri) for approximately 10 s in which series of positive and negative pressure pulses are applied. (ii) Immersion in ultrapure water (Millipore Corporation, Billerica, Massachusetts) with applied overpressure for approx. 5 s, (iii) Immersion in 5 % Sodium Hypochloride (Hänseler, Herisau, Switzerland) for approx. 10 s with series of positive and negative pressure pulses applied. (iv) Immersion in ultrapure water twice for 10s. After the cleaning procedure the electrical resistance of the probe is compared with the initial value. If the resistance remains higher, the probe was not used further.

Cellular electrophysiology

Hardware: Pico 2 USB-powered Patch-clamp Amplifier (Tecella, Foothill Ranch, California) interfaced to a personal computer and driven by the WinWCP software (University of Strathclyde, Glasgow, Scotland) was used to record ionic currents in whole-cell voltage-clamp mode. Two Ag/AgCl electrodes of 0.5 mm diameter were used. One inserted in the Cytoclip and one in the bath solution connected to the ground. The electrodes were prepared by electroplating in 3 M KCl.

Na+1.5 current recording: HEK293 cells stably expressing Na1.5 were used and the sodium current was recorded at room temperature (23-25 °C) using the same setting as described for hERG current recording. For
whole-cell recordings, the leak and the residual capacitive currents were subtracted off line. The cells were bathed with an extracellular solution containing (in mmol/L): NaCl 130, CsCl 5, CaCl$_2$ 2, MgCl$_2$ 1.2, HEPES 10, Glucose 5. pH 7.4 was adjusted with NaOH. FluidFM cantilevers were filled with an intracellular medium containing (in mmol/L) CsCl 60, aspartic acid 50, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, EGTA 11, Na$_2$ATP 5. pH was adjusted to 7.2 with CsOH.

**Current recording on mouse ventricular cardiomyocytes:** ionic currents were recorded at room temperature (23-25 °C) from isolated adult mouse ventricular cardiomyocytes. For whole-cell recordings, the leak and the residual capacitive currents were subtracted off line. The cells were bathed with an extracellular solution containing (in mmol/L): NaCl 140, KCl 5.4, CaCl$_2$ 2, MgCl$_2$ 1.2, HEPES 10, Glucose 5. pH 7.4 was adjusted with NaOH. FluidFM cantilevers were filled with an intracellular medium containing (in mmol/L): KCl 120, CaCl$_2$ 1.5, MgCl$_2$ 5.5, HEPES 10, K$_2$EGTA 5, and Na$_2$ATP 5. pH was adjusted to 7.2 with KOH.

**Isolation of mouse ventricular cardiomyocytes:** A mixed genetic background male mice were euthanized by cervical dislocation. Hearts were rapidly excised, cannulated and mounted on a Langendorff column for retrograde perfusion at 37°C. Hearts were rinsed free of blood with nominally Ca$^{2+}$-free solution containing (in mmol/L): 135 NaCl, 4 KCl, 1.2 MgCl$_2$ , 1.2 NaH$_2$PO$_4$ , 10 HEPES, 11 glucose, pH 7.4 adjusted with NaOH and subsequently digested by a solution supplemented with 50 μM Ca$^{2+}$ and collagenase type II (1 mg/mL; Worthington, Lakewood, New Jersey) for 15 minutes. Following digestion, the atria were removed and the ventricles transferred to nominally Ca$^{2+}$-free solution, where they were minced into small pieces. Single cardiac myocytes were liberated by gentle trituration of the digested ventricular tissue and filtered through a 100 μm nylon mesh. Cells used for electrophysiology were washed 3 times and the Ca$^{2+}$ concentration was progressively raised to 1 mmol/L within ~30 minutes. Then, the cell suspension was stored at room temperature (23-25°C) until use, within 6 hours after isolation.

**Hardware control and Data acquisition**
Ion-channel activation protocols were generated and acquired within the WinWCP software (University of Strathclyde, Glasgow, Scotland) at 10 kHz. The AFM was controlled with the EasyScan 2 software (Nanosurf, Liestal, Switzerland. AFM and patch-clamp amplifier were connected to a data acquisition card (NI-PCI 6221; National Instruments, Austin, Texas) through coax cables. A self-written LabView virtual instrument (National Instruments, Austin, Texas) constantly synchronizes and stores data of the AFM (deflection, piezo position and approach DC-motor) and of the patch-clamp amplifier (ionic current). Signals are low-pass filtered analogically by the Pico 2 amplifier and acquired at a sampling rate of 10 kHz. AFM data and DC-ionic current were averaged every 1 ms prior to storage.

**Seal formation**
Electrical resistance of the probe was measured each time before approach, after seal formation, when withdrawn and after cleaning. The resistance was calculated with Ohm’s law based on the current elicited by 10 or 15 mV voltage steps. Approach was done both by monitoring the probes’ resistance with the above described method (not presented) or by following the DC-ionic current. In the latter case, a voltage bias of -10 mV or -15 mV during the approach procedure. As the current and force control were stable suction was applied with the help of a syringe. When the seal formed we set the holding potential to -90 mV. Cells that showed difficult or no access to whole cell configuration were discarded.
5.4.3. Results

**Electrical properties of microchanneled AFM cantilevers**

The formation of a seal between pipette probe and cell membrane is the initial and crucial step of patch-clamp experiments. The properties of the aperture of a glass pipette in the region where the seal formation occurs during patch-clamp experiments was subject to extended studies both for conventional patch-clamp as for planar patch-clamp chips because of its role in seal quality (Sordel et al., 2010; Suchyna et al., 2009). Especially, the aspect ratio of the aperture determines the seal resistance value since it reflects the surface area where the high resistance is formed between glass internal wall and cell membrane. For our study, we considered Si$_3$N$_4$ FluidFM probes with closed pyramid (i.e. without any lithographically obtained aperture) and having a wall thickness of 450 nm (Guillaume-Gentil et al., 2013). We produced an aperture at the pyramid apex with FIB in order to maximize the expected seal resistance. The optimal geometry is a compromise between aspect ratio and aperture diameter (Supplementary Figure 5.12), refer to the end of the Chapter). We milled cylindrical apertures with a diameter of about 350 nm that resulted in a height of 500 nm (Fig. 1c,d). This geometrical configuration theoretically leads to seal resistances in the order of 150 MΩ, enough to record in whole-cell configuration (Fertig et al., 2002).

Having defined the probes, the second step was to make the FluidFM setup compatible with electrophysiological measurement after inserting an Ag/AgCl electrode into the reservoir of the cantilever holder (Cytoclip). A macroscopic coating with wax around the insertion point ensured an ion-tight connection. In addition, a custom metallic holder for the Petri dishes onto the x-y stage of the microscope was conceived acting as Faraday cage shielding from electromagnetic noise together with a connector that interfaces the Cytoclip and the patch-clamp amplifier (Supplementary Figure 5.13), refer to the end of the Chapter). When filled with 150 mM KCl (mimicking physiological conditions) and immersed in the same solution, a FluidFM probe (Figure 5.8b) with a cylindrical aperture of 350 nm (Figure 5.8c/d) has a typical electrical impedance of the order of 30 MΩ. We report in detail in Supplementary Figure 5.14 that the major impedance source is the microchannel (~90 %) (Figure 5.8b, inset) having a length of 1500 μm, width of 30 μm and a height of only 1 μm. Therefore, the hollow pyramid and cylindrical nano-aperture only partially affect the overall resistance. Compared to glass microelectrodes, where the narrow probe prolongs for several micrometers (thus generating high impedance), we can
reduce the aperture to few hundreds of nanometers without facing prohibitive impedances in the gigaohmic range.

Assessing the FluidFM performances with NaV1.5 ion channel

To evaluate the performances of FluidFM for force controlled electrophysiology, we used HEK-293 cells stably expressing NaV1.5 ion channels. NaV1.5, a cardiac voltage-gated Na+ channel involved in generation and propagation of electrical impulse in the heart, was chosen for its fast activation kinetics (less than 1 ms) representative of the group of fast activated voltage-gated ion channels. We consider the challenging properties of this channel a reference to evaluate the limits and the potential of the developed setup.

After selecting a cell in the Petri dish, we positioned the FluidFM probe on top of it under optical control with an estimated separation of ~100 µm. The final approach was performed using the AFM force-control in contact mode with the smallest setpoint just above the background noise (~1 nN). For the first time reported, we could simultaneously monitor ionic current and the force of a nanopipette as function of the probe-membrane separation (Figure 5.9a) while patch-clamping a HEK-293 cell, gently and automatically. The force control engages the piezo actuator at the very moment the probe makes contact with the cell and keeps the force constant. At the same time, a decrease in the ionic current is observed, i.e. an increase in nanopipette resistance (Figure 5.9a, arrow 1). While the contact between the probe and the membrane is often lost in conventional patch-clamping, vibrations or cell-volume changes did not affect our measurement because they are promptly compensated by the automated force feedback of the AFM. The result is a stable and robust patch-clamp recording setup.

To induce the formation of a seal, we applied suction with a 5 mL syringe (Figure 5.9a, arrow 2) analogous to that of standard patch-clamp protocols(Hamill et al., 1981), which caused an abrupt decrease of the ionic current indicating seal formation. When the ionic current stabilized, we switched to a voltage-pulse protocol (Figure 5.9b) that besides facilitating the seal resistance measurement (presented approach: R_{seal} = 99.6 MΩ) gives information about the capacitance (e.g. 102 pF in whole-cell on adult cardiomyocyte). Since we initially compensated the pipette capacitance (17 pF, conventional pipettes typically from 5 pF to 15 pF) prior to approach with the dedicated electronics, the sudden appearance of capacitance transients demonstrated rupture of the membrane patch(Hamill et al., 1981), i.e. attainment of the whole-cell configuration.
The seal resistances we routinely achieved were in the order of 100 to 150 MΩ permitting electrophysiological recordings of large ionic currents with sufficient signal to noise ratio. Occasionally, we observed the formation of a gigaseal both with HEK-293 cells and adult cardiomyocytes (see below and Supplementary Figure 5.15), yet this is a puzzling phenomenon that is not expected because of the geometry of the probe aperture (Supplementary Figure 5.12), an event also known in planar patch-clamp chips (Curtis and Baldwin, 2008).

Figure 5.9: Force-controlled whole-cell Na\textsubscript{V}1.5 currents on HEK-293 cells. a Approach and access to whole-cell configuration. Cantilever spring constant: \( k = 1.8 \text{ N/m} \). Pipette resistance \( R_p \) measured 26.2 MΩ and seal resistance \( R_{\text{seal}} = 99.6 \text{ MΩ} \). The FluidFM probe is lowered to the point of contact (recognized by a change in deflection together with a reduction in ionic current, i.e. increase in resistance, marked by the arrow 1). The force control engages automatically and maintains constant the force signal. Little negative pressure manually exerted with a 5 mL syringe is applied after ~40 s (arrow 2) and a seal is obtained. In this particular experiment, access into the whole-cell configuration happened spontaneously, visible as a perturbation in the ionic current signal. Stable current after perturbation indicate cytosol access too. Conventional positioning guided by pipette resistance increase can be performed too, as the force-control can be engaged or disengaged at any time. b Whole-cell access is confirmed by the appearance of capacitive transient current induced by application of a 10 mV square pulse. The applied voltage, the measured current before whole-cell access and current after whole-cell access are displayed from top to bottom. The compensating capacitance of 17 pF was determined before the approach whereas we measured a whole-cell capacitance of 102 pF. c Sodium currents recording elicited from a series of voltage pulses of 5 mV increments. Typical fast inward voltage gated sodium currents are recorded. d Current-voltage relationship of the recorded sodium current.
We recorded currents in response to conventional voltage pulse protocols (Figure 5.9c). Superimposed whole-cell Na+ currents of the NaV1.5 channel recordings are shown in Figure 5.9c. The recorded currents are elicited by a conventional voltage pulse protocol exemplified in the inset: we depolarized the membrane by 5 mV steps, from a holding potential of -100 mV to a maximum of +20 mV repeated with 2 s interval. Thus, we plotted the current-voltage relationship curves of the inward sodium peak current. The current recordings have been compensated off-line for leak and capacitance as described in the methods. These recordings confirm the specific electrophysiological characteristics of the NaV1.5 ion channel.

As described in the literature (Gellens et al., 1992), we recorded a typical voltage gated sodium current characterized by a fast activated inward current (time to peak ~1.0 ms) followed by a slower inactivation. The relatively high leak currents caused by the obtained sub-optimal seal resistance together with a high access resistance, causes the I-V curve to deviate from the expected (Hodgkin-Huxley) description of Na+ currents (Hodgkin and Huxley A F, 1952): On the one hand, the low resistance ratio between $R_{\text{seal}}$ and $R_{\text{access}}$ stretches the I-V curve to higher values as the true voltage sensed from the membrane is smaller than the command potential (Perkins, 2006). On the other hand, the uncompensated series resistance limits the bandwidth of the voltage follower, causing the loss of voltage control. The consequence is a shift to more negative values of the I-V curve and a discontinuity in correspondence of its peak (-65 mV), as described by Sherman and others (Sherman et al., 1999). Concluding so-far, these recordings unequivocally demonstrate the feasibility of force-controlled whole-cell measurements.

Recording simultaneously whole-cell currents and contraction force on freshly isolated mouse adult cardiomyocytes

Adult isolated cardiomyocytes are challenging cells for conventional patch-clamp because of their spontaneous contractions that are known to compromise the seal between the probe of the pipette and the cell membrane. We thus tested these cells to demonstrate the benefit of the force-feedback feature using the protocol described above for HEK-293 cells. In contrast to conventional patch-clamp, we observed that recordings with the FluidFM from contracting cardiomyocytes were readily possible. Relying on the force control, we were able to obtain seals and record ionic currents even from cardiomyocytes that were already contracting before starting the approach procedure whereas such cells are usually discarded in conventional patch-clamp experiments. Figure 5.10a shows the process of the force-controlled probe positioning and seal formation, where force and ionic current
are simultaneously displayed. For the shown approach we used a FluidFM probe with an aperture diameter of 450 nm and a resistance $R_p$ of 18.4 MΩ. With this type of apertures we expected seal-resistances not higher than 50-60 MΩ (Supplementary Figure 5.12). Indeed, the seal of Figure 5.10a had a final value of $R_{\text{seal}} = 47$ MΩ. Increase in ionic current amplitude shortly after last increase in negative pressure ($t = 35$ s) suggests rupture of the patch, i.e. access in whole-cell configuration. Cardiomyocyte contractions were visible as prominent modulation in the force signal, where the involved forces peak at 31.5 nN with the force control turned on. An approach and seal formation on a non-contracting cardiomyocytes is presented in Supplementary Figure 5.16.

Figure 5.10: Simultaneous whole-cell current recordings and contraction force on primary mouse cardiomyocytes. a Force and ionic current recording during approach on a contracting cardiomyocyte with enabled force control. The force increases simultaneously with a drop in ionic current as the FluidFM probe makes contact with the cell (arrow 1). The force-control automatically engages as the force setpoint is reached. Not labelled arrows indicate slight increase of suction force through a 1 mL syringe. Increase in ionic current amplitude, at about 35 s consequently to further suction, indicates rupture of the patch (i.e. attainment of whole-cell configuration). Resistance of the probe before contact: 18.4 MΩ, final resistance of the seal (in whole-cell): 47 MΩ. Peaks with a maximum value of 31.5 nN appear in the force curve due to the spontaneous contractions of the cardiomyocytes. b Zoom-in of the contraction marked with an asterisk in Figure 3a. In addition, the relative position of the piezo actuator is shown. As contraction starts, the force control drives the piezo actuator in order to bring the force signal back to the set-point. This allows to measure the contraction amplitude (~800 nm) and the uncompensated force amplitude (~33 nN). After a short perturbation in the ionic current, no difference in seal resistance is measurable before and after the contraction. c With the force control disengaged, the effective contraction amplitude (and thus the force) is measured simultaneously to evoked transmembrane currents. Contraction is induced by depolarization of the membrane


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potential in whole-cell configuration. Na⁺, Ca²⁺ and K⁺ ion currents are recognizable in the recorded trace: the first two in the fast inward peak and K⁺ as the slow and outward current that follows the depolarization step. Maximum force amplitude: 166 nN. The contraction movement starts with ~2 ms delay after the inward ionic current peaks.

The peculiar piezo movement commanded by the force feedback during a contraction event is displayed in Figure 5.10b. Every time that a change in the deflection (i.e. force) is detected following a contraction event, the piezo is actuated and retracted of about 800 nm (comparable to the values in the works by Saenz (Saenz Cogollo et al., 2011) and Domke (Domke et al., 1999)) in order to maintain the measured contact force equal the set-point value (AFM “constant contact force” mode). After the contraction, the leak current stabilized again at the initial level, indicating that the patch was not disturbed by the mechanical stress due to the contraction.

To measure ionic currents from cardiomyocyte together with the actual exerted contraction force in z-direction, after getting into whole-cell configuration we disengaged the force feedback. We used extracellular and intracellular solutions (i.e. bath and probe-filling solutions) containing sodium, calcium and potassium ions which are implicated in the excitation contraction coupling of cardiac cells, to follow their combined ionic current development during contraction. We applied membrane depolarizing voltage steps to trigger an action potential while simultaneously measuring ionic currents and force amplitude (Figure 5.10c). Depolarization induced Na⁺, Ca²⁺ and K⁺ voltage-gated ion channel activation. Fast, inward Na⁺ and Ca²⁺ currents ($I_{Na-Ca} = -1.5$ nA with pulse step from -110 mV holding potential to -45 mV for 20 ms) and slow, outward K⁺ current are depicted in Figure 3c. Contraction started ~2 ms after detection of the Na⁺ and Ca²⁺ current peak, and generated a transverse force of 166 nN on the FluidFM cantilever.

These experiments illustrate the ability to locally and quantitatively record both ionic currents and exerted force through the very same probe which is a unique feature of the FluidFM technology. Notably, even with disengaged force-control we noticed that neither the cell nor the seal are compromised by cell contraction. We assume that the ability of the FluidFM cantilever to react by bending, compared to the stiff nature of glass micropipettes, has a crucial role in preserving the probe-cell contact integrity. In whole-cell configuration contraction can be induced by electrical stimulation while monitoring the amplitude of the contraction (Supplementary Figure 5.17 and Supplementary video).

**Serial patching and serial injection in cardiomyocytes.**

In terms of costs and automation, it is desirable to perform patch-clamp experiments in a serial fashion. Such a procedure implies the use of one probe to address different cells, an unpopular concept in conventional patch-clamp given that used glass micropipettes are
difficult to clean with the tiniest probability to achieve satisfactory seals on their second use. Kao et al. first suggested a protocol to clean used planar patch-clamp chips with 6.15% sodium hypochlorite solution (Kao et al., 2012), motivated by the high costs of the chips. For similar motivation, we adjusted that protocol to clean the FluidFM probes after every contact with a cell allowing us to achieve multiple times whole cell configuration on different cells, with the same probe. After cleaning, we checked the electrical resistance of the probe and reused it only if it did not show any deterioration. Results shown in Figure 5.9 are from the fifth cell patched with one single probe, obtaining similar recordings on 3 out of 5 patched cells. Serial patching is an additional step towards automatized patch-clamp and increased throughput.

Besides serial patching, Figure 5.11 shows how the patch-clamp technique combined with atomic force microscopy can be used as an injection tool. Once that whole-cell configuration is attained, exchange of solution readily happens by diffusion or can be driven by overpressure application. Especially, other technique (e.g. micro-injection (Shubeita et al., 1992)) succeeded to inject into myocardial cell with very low viability, but never in freshly isolated adult mouse cardiomyocytes (Ruiz-Meana et al., 1999). The challenge is not to induce hypercontraction in the cardiomyocyte because this steers to cell death in less than 30 s. The breakthrough accomplished with the FluidFM is twofold: i) the injection protocol does not lead to hypercontraction, and ii) the procedure can be repeated multiple times as the cell is left undamaged. We patched the cardiomyocyte of interest in whole-cell configuration as described above, with the intracellular solution loaded with Lucifer Yellow dye. In whole-cell configuration, we let the dye spontaneously diffuse into the intracellular environment. No morphological change was observed upon retracting the probe at the end of the injection experiment. We established the whole-cell configuration twice on the very same cell, which survived further 25 minutes after probe retraction for a total of 45 min from approach to cell death. The measured relative intensity after the second injection was about three times higher than after the first injection. In addition, when "showered" with calcium containing intracellular solution, the cardiomyocytes showed spontaneous activity.
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Figure 5.11: Serial injection protocol of Lucifer Yellow dye in a cardiomyocyte. The cell is approached twice with the same probe and both times whole-cell configuration is achieved. When in whole-cell configuration, we let the dye diffuse naturally into the cardiomyocytes for approx. 5 min. The insets show the intensity profile of the drawn line. The cardiomyocyte did not show any visible damage or morphological alteration and survived 25 min after the second injection. a First approach on the chosen cardiomyocytes. b Withdrawal of the probe after first injection. c Micrograph in the fluorescence channel after the first injection. Inset shows the intensity profile at the location marked with a white line. d The procedure is repeated again (i.e. approach, injection and probe withdrawal). e Micrograph of the cardiomyocyte after the second injection. No morphological change is visible. f Micrograph in the fluorescence channel after the second injection. The profile shows about three times higher relative intensity than in c. The signal over background (S/B) ratio after the first injection was 1.30, while after the second injection it was 1.42, the relative intensity increases almost three folds after the second injection by subtracting the background intensity.

An additional advantage of not inducing hypercontraction is the possibility to acquire fluorescence images with longer exposure times. In the Supplementary Figure 5.18 we propose another version of the micrograph of Figure 5.11 where different intensity thresholds of the Lucifer Yellow dye are plotted, highlighting intracellular boundaries.

To our knowledge, serial single-cell injection into freshly isolated cardiomyocytes has never been achieved because cells do not survive the removal of the patching micropipette. The serial injection protocol and repeated whole-cell procedures on single cardiomyocytes offer to the biology field a new tool to address open questions. In addition, the potential effects of bioactive molecules could be tested using the serial injection procedure.
5.4.4. Discussion

In this study, by merging patch-clamp and AFM via the FluidFM, we have developed a new tool for electrophysiology that enables investigation of voltage-gated ion channel activity in whole-cell configuration, while simultaneously controlling the force exerted on the studied cell. This unique feature is beneficial from two perspectives: on the one hand, the piezo actuator compensates for unwanted mechanical perturbations making the probe-membrane contact gentle and consequently, the electrophysiological recording which is stable for longer periods. On the other hand, the electrophysiological data are enriched with the information on generated or exerted forces helping to answer open questions for complex biological systems where forces and ion-currents are intimately co-acting. In particular, the mechanobiology field can benefit from the AFM-assisted patch-clamp approach introduced here, e.g. to understand sensing and transduction of mechanical stimuli on cardiomyocytes (Hersch et al., 2013), or to study the relatively new discovery of so called mechano-gated piezo ion channel (Martinac, 2012).

We showed that it is possible to establish a serial patch-clamping protocol (e.g. recordings presented in Figure 5.9d resulted from the 5th approach on a cell). Serial patch-clamping is not an option for conventional glass microelectrodes as efficient cleaning of the glass surface after establishment of a gigaseal can be hardly accomplished because membrane proteins are present in the patched membrane region which strongly adsorb in a denatured form on the glass surface during seal formation (Suchyna et al., 2009) and are difficult to be removed. However, little effort has been made to reuse glass micropipettes because of their low cost. Besides increasing mechanical and temporal stability of the seal, a reduced aperture size lowers the amount of proteins that bind in the dome. We cleaned the FluidFM probes after each cell contact by modifying the protocol proposed to clean planar patch-clamp chips (Kao et al., 2012) (that uses sodium hypochlorite solution) by adding a cleaning step with a protease enzyme-containing detergent. In addition, defects introduced in the membrane after retraction of the probe become less critical with sub-micrometer probe apertures allowing a single cell to be patched several times. Indeed we succeeded to inject fluorescent dye twice in the same cardiac cell. We therefore show how the whole-cell injection method becomes a valid alternative to conventional micro-injection techniques that are still either rather large glass-micropipettes (Mueller et al., 1980) or technically demanding (Stephens and Pepperkok, 2001).

The scope of this study was to explore the potential of the FluidFM for patch-clamp. The major challenge that novel micro- and nano-devices have to face in order to compete with
glass micropipettes is the achievement of high ohmic seals (gigaseal). Low resistance seals introduce problem of recording quality as the they likely are i) temporally unstable and ii) cause of imprecise transmembrane potential control. Whereas the stability of the obtained seal is in our case not an issue because of the small patch area (as discussed above), the electrophysiology recordings presented in Figure 5.9c,d show that the FluidFM indeed is affected from the low obtained seal resistances. As pointed out in the result section, both resistance and capacitance of the FluidFM probe contribute to decreasing the bandwidth of the system. The consequence of it is less control over the transmembrane potential which results in deformed I-V relationships (Sherman et al., 1999). However, the cause for low-resistance seals is in this case well understood: it is the square section of the pyramidal probe that impedes the building of giga-ohmic resistances.

We are convinced that the obtained results prove the potential of the FluidFM for electrophysiology field and indicate the way to follow in the future. Three aspects must be addressed: i) the microchannel resistance, ii) the cantilever stiffness and iii) the aperture geometry. A strategy to bypass the microchannel resistance will encompass the integration of an electrode in the cantilever. A lower resistance down to few MΩ (probe and aperture resistance, see Supplementary Figure 5.14) can be foreseen easing the recording of sub-picoampere currents related to single channel activity. Moreover, cantilevers can be designed to be longer and thus softer because the cantilever resistance is no more affected by the microchannel size with an integrated electrode. In this way, gentler approach and still higher force sensitivity could be achieved. Pyramidal probes will be substituted with cylindrical one of high aspect ratio (Dörig et al., 2010) mimicking the geometry of conventional glass micropipettes. The circular cross section and the height of the cylinder will let the patched membrane to span enough to build giga-ohmic resistances.

5.4.5. Conclusion

The FluidFM combined with the patch-clamp technique is shown here as a novel tool that allows interrogation of mechanophysiology systems otherwise not possible. In addition, the whole process has the potential to be fully automated eliminating the need of highly skilled operators, as the AFM and the pressure controller are fully programmable. Furthermore, the scanning nature of the FluidFM makes the developed tool ready for Scanning Ion Conductance Microscopy (SICM) (Hansma et al., 1989; Korchev et al., 1997) too. This technique has proved to be an impressive imaging tool (Novak et al., 2009) especially for living cells, being a non-contact technique. SICM allows the experimenter to obtain high resolution topography images of the cell before the patch-clamp procedure
aiding the precise targeting of the desired patch location on the cell (Julia Gorelik et al., 2002).

5.4.6. Supplementary Figures

Figure 5.12: (Supplementary) Size optimization of the cylindrical aperture to be drilled with FIB. Seal formation region is limited in the walls of the cylindrical aperture drilled by FIB at the apex of the pyramidal probe. On the contrary, it is unlikely that the cellular membrane adheres tightly in the concave corners of the inner facets of the pyramid, thus preventing the formation of high-ohmic seals. The resistance of the seal was estimated by assuming the presence of an aqueous gap of 1 nm between the probe and the cell’s membrane with a buffer conductivity of 1.79 S/m for of 150 mM KCl. This assumption results from observations of seal size and resistance in conventional glass micropipettes (Suchyna et al., 2009). In our geometry, the pyramid wall thickness determines the depth of the drilled aperture where the seal may happen, i.e. the aspect ratio. We calculated the seal resistances for cylindrical apertures having a diameter $d$ from 40 nm to 1 μm. The region marked with a star corresponds to the seal resistances (between 100 and 300 MΩ) reached with the geometry of FluidFM probes (approx. $d = 200 - 500$ nm and $h = 200 - 600$ nm). The top curve ($h = 3500$ nm) mirrors the dimensions of a standard glass micropipette. The region marked with a double star marks the expected seal resistances with conventional micropipettes. Higher seal resistances are achieved with high aspect ratios. However, small aperture diameters have consequent drawbacks too: on the one hand, they push the FIB performances to the limits (700-nm deep apertures are challenging to be obtained with a diameter smaller than 100 nm). On the other hand, a minimal amount of force is needed to tear off the lipid bilayer from the cell’s cortex (lipid bleb model(Merkel et al., 2000; Milton and Caldwell, 1990)). The force exerted by suction is proportional to the aperture area. A small aperture might not exert enough force to pull the membrane deep enough and to eventually tear off the dome of the patch. Indeed, we could not attain whole-cell configuration with apertures lower than 200 nm. Measurements with planar patch-clamp demonstrate that seal quality of few hundreds of megaohms are enough to record currents in whole-cell mode (Fertig et al., 2002). We therefore chose apertures of approx. 350 nm diameter, that routinely gave seal resistances in the range of 120 MΩ.
Figure 5.13: (Supplementary). Modification of the FluidFM setup for patch-clamp experiments. a Bottom view of the FlexAFM (Nanosurf, Liestal, Switzerland) with customized Cytoclip (Cytosurge, Zurich, Switzerland) and AFM/Patch-Clamp amplifier interface. A full-metal AFM connector that fits the FlexAFM has been designed and manufactured. The connector fulfills two roles: i) it serves as interface for the pneumatic tubing and for the electrode in the Cytoclip reservoir; ii) it shields the electrode connection wire from electromagnetic noise ensuring at the same time that the patch-clamp amplifier is positioned as close as possible to the measurement site. b Cytoclip with custom tubing and electrode connector. The backside of the Cytoclip and connector was immersed in molten wax (Paraplast; Sigma-Aldrich, St. Louis, Missouri) to avoid any ionic current leak from the integrated O-ring. c Petri dish holder designed and manufactured to accommodate WillcoDishes (WillCo Wells B.V., Amsterdam, Netherlands). Besides conferring the mechanical stability requested for AFM recordings, the holder acts as a faraday cage shielding the recording site from electromagnetic noise.

Figure 5.14: (Supplementary) Electrical properties of the FluidFM probes. a Electrochemical impedance spectroscopy (EIS) of a pyramidal FluidFM cantilever with a 400-nm aperture measured with an Autolab Potentiostat (MetroOhm, Switzerland) from 0.01 Hz to 100 kHz in a two Ag/AgCl electrode configuration. The 150 µm long cantilever with an embedded microchannel 1 µm thick and 30 µm wide pyramidal probe was filled with a solution of...
150 mM KCl identical to that of the bath. It showed a resistance of 29.3 MΩ at the lowest frequency of 0.01 Hz. After breaking the cantilever, the approximately 1500 µm long microchannel connecting the pyramidal probe to the reservoir showed a resistance of 20.1 MΩ. The data were fitted with an equivalent circuit neglecting the electrodes resistances and capacitances, which are assumed to be lower than the impedance of the microchannel. Thus, the fitted equivalent circuit only features the capacitance and resistance of the microchannel in series with the resistance of the Cytoclip’s connection channel. The fit reveals a system capacitance of about 15 pF, in accordance to the empirical capacitance compensation used at the beginning of the patch-clamp procedure. Conventional glass micropipettes have a capacitance generally ranging from 5 to 15 pF.

The DC electrical resistance of the different parts constituting the FluidFM cantilever have been simulated with COMSOL Multiphysics (Comsol Multiphysics GmbH, Germany). We modelled a probe with the same size of the actual ones and then calculated the resistance of i) the microchannel (l = 1500 µm, h = 1 µm, w = 30 µm), ii) the pyramidal hollow probe and (iii) the cylindrical aperture (d = 400 nm, h = 600 nm). The total simulated series resistance is in accordance with the EIS and measures about 30 MΩ. The microchannel has a calculated resistance of 30.4 MΩ, the pyramid of 1.0 MΩ and the cylindrical aperture of 2.6 MΩ in good accordance with the measured value of 29.3 MΩ.

The simulation confirms that the microchannel mostly contributes to the overall impedance. Therefore, the next probe generation is thought to have an embedded electrode in the cantilever bypassing the microchannel resistance. It will be then possible to fabricate probes with sub-micrometer apertures having a smaller impedance than pulled micro glass pipettes (because of their elongated probe shape (Schanne et al., 1968)) becoming interesting probes for scanning ion conductance microscopy too.

COMSOL simulation methods: meshing was accomplished with the physics controlled mode and with the extremely-fine setting. The stationary case has been simulated with a potential drop of 100 mV applied at the beginning of the microchannel and the grounded probe aperture. The electric potential distribution was computed with the electric currents (ec) physics module. As buffer, we chose water with a conductivity of 1.79 S/m. The simulation does not take into account any electrokinetic effect (e.g. streaming potential) or charges separation induced from surface charges (Chun et al., 2005).
FORCE CONTROLLED PATCH-CLAMP IN WHOLE-CELL CONFIGURATION

Figure 5.15: (Supplementary). Gigaseals occasionally formed with HEK-293 cells and with adult mouse cardiomyocytes. For reasons exposed previously about the geometry of the FluidFM probe (see Supplementary Fig. 1), we do not fully rationalize the nature of these gigaseals. **a** Seal resistance and force during approach and seal formation on HEK-293 cell. Arrow 1 marks the moment where contact is achieved, which also corresponds to the increase in the resistance. We reduced the setpoint to zero (arrow 2) and subsequently switched to constant height.

**b** Seal resistance and current at an absolute time of 450 s. The resistance was $R_p = 24.7 \, \text{M} \Omega$.

**c** Seal resistance and current at an absolute time of 147 s. The resistance was $R_p = 36.7 \, \text{M} \Omega$, and the seal resistance was $R_{\text{seal}} > 10 \, \text{G} \Omega$. 

Figure 5.15: (Supplementary).
Suction was initially applied with the help of a pressure controller (Cytosurge, Zurich, Switzerland) (arrow 3). Arrow 4 points the force peaks generated when we switched from the pressure controller to a 5 mL syringe. Eventually (arrow 5) a seal resistance > 2 GΩ has stably formed. $R_p = 24.2$ MΩ. Applied voltage during approach: -10 mV DC. Arrow 4 points the force peaks generated when we switched from the pressure controller to a 5 mL syringe. Eventually (arrow 5) a seal resistance > 2 GΩ has stably formed. $R_p = 24.2$ MΩ. Applied voltage during approach: -10 mV DC.

**b** Ionic current and deflection during seal formation on a cardiomyocyte. Measured pipette resistance $R_p = 24.7$ MΩ. Seal resistance was monitored with the WinWCP software (University of Strathclyde, Glasgow, Scotland) by application of 5 mV square voltage pulses. We applied little suction with the use of a 5 mL syringe (arrow 1). Contraction of the cardiomyocyte (arrow 2) did not impede the formation of the gigaseal. Indeed, after about 8 s the amplitude of the current pulses drastically dropped and the calculated seal resistance was 14 GΩ (arrow 3). We disabled the force control for this particular experiment (constant-height mode). The graph shows the current pulses raised by a voltage stimulus of 15 mV. Measured pipette resistance $R_p = 36.7$ MΩ. Arrow 1 marks the moment of contact with the cell. Short after suction application (arrow 2) we recorded the formation of the gigaseal (arrow 3). During the overall procedure, i.e. approach and seal formation, the chosen cardiomyocytes was contracting, visible in the data from the spikes in the deflection signal. Inset shows the operation of the force control. During contraction, the piezo actuator (green curve) moves in the z-direction to keep constant the deflection signal. Interestingly, the measured current is minimally influenced from the contraction confirming the benefit of the force control. Indeed, a seal resistance bigger than 10 GΩ is measured and is not lost upon cardiomyocytes contraction.

**Figure 5.16:** (Supplementary). Approach on a non-contracting adult mouse cardiomyocyte. We aim to demonstrate the tangible advantage conferred by the force control of the FluidFM technique. Contrary to what observed in conventional patch-clamp, the approach and seal formation procedure does not become more difficult when patching contracting cardiomyocytes. We therefore show here an approach on a non-contracting cardiomyocytes followed by seal formation that can be compared with Figure 3a of the manuscript. Seal formation occurs in a similar way on both systems. After an initial decrease in current amplitude due to contact with the cell, the seal is obtained at approximately $t = 65$ s upon suction application. The pipette resistance measured $R_p = 18.4$ MΩ, seal resistance stabilized at 52 MΩ.
FORCE CONTROLLED PATCH-CLAMP IN WHOLE-CELL CONFIGURATION

Figure 5.17: (Supplementary). Efficiency of the force control evaluated by inducing contraction of the cardiomyocytes with electrical stimulation and active force control in cell-attached mode. The stimulation signal is a composition of two square voltage waves: one is used to monitor the seal resistance (10 mV, 250 ms), while the stimulation signal (100 mV, 350 ms) superposed to it was triggered by the user. A With active force control, the position of the FluidFM probe is constantly adjusted in order to keep the force constant. Indeed, the piezo movement shows a steep retraction phase in concomitance with the beginning of the contraction, followed by a slower relaxation. The transversal contraction amplitude measures approximately 400 nm. B We measured cantilever’s deflection simultaneously with the ionic current. A relatively low seal resistance $R_{\text{seal}} = 61 \, \text{M} \Omega$ is responsible for the higher current amplitudes but did not impede the electrical stimulation. By active force control, the deflection signal represents the error that drives the controller. Perturbation in the deflection signal is rapidly brought back to the setpoint by the compensatory movement of the piezo actuator.

Figure 5.18. (Supplementary). Boundary in the cardiomyocytes. We demonstrated serial injection of Lucifer Yellow dye in a single cardiomyocyte without inducing hypercontraction. It is thus possible to image the cell minutes after the injection (here, approximately 10 minutes) instead of a couple of seconds only. Indeed, conventional microinjection techniques consistently induce hypercontracture of the injected cardiomyocyte leading to cell death in about 30 s (Ruiz-Meana et al., 1999), thus not allowing for an accurate imaging of the dye intensity distribution in the cell. Here we present the same image showed in Fig. 4f highlighting intracellular compartmentalization. We plotted five different intensity thresholds, to emphasize the non-uniform distribution inside the cardiomyocyte. Similar Lucifer Yellow distribution is observed in hypercontracture propagation experiments, i.e. in cells that are connected with an intercalated disc to the one that has been injected (Ruiz-Meana et al., 1999).
5.5. Whole cell hERG recordings

After careful consideration, these whole cell-recordings have not been included in the above-presented publication. Motivation is expressed at the end of this Paragraph.

Acquired channelopathies may result from drug exposures that alter ion channel function such as the voltage gated potassium channels (hERG). Indeed, increased risk of cardiac arrhythmias engendered market withdrawal for several drugs (Fermini and Fossa, 2003) that were shown to inhibit the hERG channel. The ability of structurally different compounds to block this channel and induce cardiac arrhythmias points out the importance of screening for hERG inhibition during drug development process. For this reason the hERG channel draws a lot of attention from the electrophysiology point of view and is deeply studied. In addition to the NaV1.5 voltage gated ion channel, we selected human embryonic kidney (HEK-293) cell lines expressing hERG, a cardiac voltage-gated K+ channel. We used hERG as a benchmark first for its high importance in pharmaceutical drug screening, as the vast majority of drugs affect its function (Vandenberg et al., 2012). Second, for its slow kinetics representative of the slowly activated voltage-gated channels.

5.5.1. Methods

The exact same setup used for the recording of Na\textsubscript{v}1.5 ion-channel has been used for the hERG recording (refer to Chapter 5.4.2), with the following details:

HEK293 cells were transfected with DNA complexed to JetPEI (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. DNA concentrations were 1 \(\mu\)g of pCDNA3.1-hERG, and 1 \(\mu\)g of pIRES-CD8. Eight hours after transfection, the cells were isolated and seeded in glass dishes (WillCo-Dish; WillCo Wells B.V., Amsterdam, Netherlands) at low density. Twenty-four hours after transfection, the resulting potassium current was recorded at room temperature (23-25 °C) under whole-cell voltage clamp conditions. For whole-cell recordings, the leak current was subtracted off line. The cells were bathed with an extracellular solution containing (in mmol/L): NaCl 140, KCl 5.4, CaCl\textsubscript{2} 2, MgCl\textsubscript{2} 1.2, HEPES 10, Glucose 5. pH 7.4 adjusted with NaOH. FluidFM cantilevers were filled with an intracellular medium containing (in mmol/L KCl 145, EGTA 10, MgCl\textsubscript{2} 1, MgATP 5 and HEPES 5. pH 7.3 adjusted with KOH.
5.5.2. Results

To evaluate the performances of FluidFM for force controlled electrophysiology, in addition to the fast Na_v1.5 channel, we used transiently transfected HEK-293 cells with DNA encoding the hERG channel.

![Figure 5.19: Force-controlled whole-cell hERG currents on HEK-293 cells. a Representative hERG currents expressed in HEK-293 cells measured in whole-cell configuration with active force control subsequent to series of voltage pulses applied in 20 mV increments. The inset illustrates the voltage pulse protocol. Lower figure: I-V relationship of the tail current. b Upper figure: sodium currents recording elicited from a series of voltage pulses of 5 mV increments. Typical fast inward voltage gated sodium currents are recorded. Lower figure: I-V current of sodium peak current.](image)

We recorded currents in response to conventional voltage pulse protocols (Figure 5.19a/b). Superimposed whole-cell K⁺ currents of the hERG channel recordings are shown in Figure 5.19a. Currents were elicited from a holding potential of -80mV with 3 s pulses to test potentials up to +180 mV in 20 mV increments followed by a potential of -110 mV for 2 s (step protocol see inset) and repeated at 1 s intervals. Amplitude of the peak tail current versus voltage has been constructed from the obtained traces to obtain the voltage dependence of current activation. Typical slow outward potassium current was recorded during the prepulse phase of the protocol (form -80mV to +180 mV). This first current component plausibly represent a mixed K⁺ currents related to the activation and inactivation of hERG channel as well as the activation of endogenously expressed potassium channels in HEK-293 cells (Zhou et al., 1998). At +80 mV the current amplitude was 238 pA. On the other hand, to isolate the hERG component from the prepulse related currents, a test pulse to – 110 mV was applied allowing the recording of tail potassium current related only to the deactivation of hERG channels. At -110 mV the maximal tail amplitude was -184 pA.
Summary – Even though the above presented whole cell recordings are undoubtedly elicited by voltage gated ion channels, it cannot be stated with security if the recordings contain hERG currents or only endogenous channels. Reason of this skepticism is the low amplitude of the tail currents and the shift of the I-V curve towards higher membrane voltage values compared with those reported in the literature (Zhou et al., 1998). The current recordings should be confirmed with additional experiments. Better seal resistances would help to obtain higher quality recordings. In addition, whole-cell patch-clamp recording from a control experiment with the use of non-transfected HEK cells would help in discerning the activity of endogenous channels from transfected hERG channels.

5.6. Long-term stability

It was difficult to compare the stability of patch-clamp recordings obtained with the FluidFM, which does not present a gigaseal, with those obtained on conventional patch-clamping systems that do feature gigaseals. For this reason, the result here presented was excluded from the publication.

We were able to measure whole-cell Na⁺ and K⁺ currents with the active force-control for more than 45 min on a single cell (Figure 5.20). Compared to conventional patch-clamp, where a typical recording is hardly maintained longer than 20 min, this result potentially confirms the added stability conferred from the AFM force control that dampens the mechanical stress of the cell membrane in correspondence of the probe aperture reducing the cell damage. However, a direct comparison with conventional patch-clamp is difficult as no gigaseal is obtained here.

Figure 5.20 shows whole-cell recording of fast Na⁺1.5 currents on HEK-293 cells evoked from a holding potential of -100 mV with pulses of 50 ms at -20 mV repeated with a frequency of 1 Hz. The graph shows the superposition of the current traces recorded by stimulating with the above described depolarization step for 27 minutes without interruption except for stimulation with the ladder protocol presented in Figure 5.9 at t =147 s and t = 759s. The total duration of the experiment in whole-cell configuration was 46 minutes. The recording has been voluntarily ended, and not because of cell lost. Inset Figure 5.20 shows a zoom-in of the fast inward sodium current with its typical fast raise and slower decay.
5.7. Conclusions & Outlook

Scope of this study was to investigate the potential of the FluidFM for patch-clamp, although a posteriori we realize that the development of the machine was maybe too premature for such an ambitious challenge. Indeed, we invested a lot of time understanding the challenges of the system as the supply of FluidFM probes was very limited and did not allow systematic experimenting, which is especially important in the development stage of novel techniques. Nevertheless, optimizing the geometry of the probes’ aperture and in combination with freshly isolated cardiomyocytes, we obtained nice and promising results that are unprecedented. For the first time, we demonstrated the simultaneous current recordings in whole-cell configuration and localized force detection through the very same probe.

The major challenge that novel micro- and nano-devices have to face in order to compete with glass micropipettes is the achievement of high ohmic seals (gigaseal). Low resistance seals introduce problem of recording quality as the they likely are i) unstable and ii) cause of imprecise transmembrane potential control. Whereas the stability of the obtained seal is in our case not an issue because of the small patch area, the electrophysiology recordings presented above, show that the FluidFM indeed is affected from the low obtained seal resistances. As pointed out in the result section, both resistance and capacitance of the FluidFM probe contribute to decrease the bandwidth of the system. The consequence of it is less control over the transmembrane potential which results in altered I-V relationships (Sherman et al., 1999). However, the cause for low-resistance seals is in this case expected and well understood, as it is the square section of the pyramidal probe that impedes the building of giga-ohmic resistances. In line with these arguments we
envision to use a recently developed FluidFM prototype that feature a tube-like aperture instead of the conventional pyramid. Figure 5.21 shows some SEM images of the probe. These probes solves two problems: i) the circular cross section, together with the high aspect ratio shall allow for the formation of gigahmic seals and ii) no further FIB modification is needed, reducing thus the cost of the consumable.

![Figure 5.21: SEM images of FluidFM tube prototype probes.](image)

In addition, the use of softer cantilevers could give unique insights in gigaseal formation or membrane mechanics. In fact, softer cantilevers allow for the detection of forces with sub-piconewton resolution, allowing to detected membrane rupturing events. The use of softer cantilever will allow for a force-controlled contact onto cells in a much delicate manner, which on the one side is good for cell viability and for long-term experimenting. On the other side the exerted force during seal formation could be better controlled, allowing optimization of seal formation procedure by systematic study of the involved forces.
6. Force-controlled Scanning Ion Conductance Microscopy (SICM)

Back in the late 80s, glass micropipettes were already used for single cell manipulation and with the invention of the patch-clamp technique showed their great potential as electrodes too. Scanning Probe Microscopy made his way through the literature: STM and AFM in particular. Hansma et al. was the first to make an SPM with glass micropipettes and called it the Scanning Ion Conductance Microscope (SICM) (Hansma et al., 1989).

The aim of this subproject was to develop a novel setup for scanning ion conductance microscopy, no longer with conventional glass micropipettes, but with the FluidFM pyramidal probes. The setup is similar to the one used for patch-clamp experiments as the electronics requirements are the very same.

Part of the work presented here was carried out with Livie Dorwling-Carter in the framework of her Master thesis, under my supervision.

6.1. SICM: state of the art

High spatial resolution observation of living cells is of great value in many branches of cell biology and medicine. Since its invention, Scanning Probe Microscopy (SPM) has become increasingly more popular in these fields. The most successful technology of the SPM family is the Atomic Force Microscope (AFM). In the last decade in particular, the AFM proved to be a powerful tool for cell biology research because it offers ultrahigh resolution in real time under near-physiological conditions. However, imaging of soft samples like cells has always been a challenging task for the AFM: it is difficult to prevent the sharp scanning-probe to damage the sample being imaged. Because of the applied force, in
addition, the image contrast depends on the rigidity of sub-membrane structure instead of membrane surface topography (Le Grimellec et al., 1998).

Hansma et al. specifically designed the SICM for biology and electrophysiology. It is particularly useful to image soft structures in an electrolyte without actual mechanical contact (Hansma et al., 1989). In general, the resolution of the AFM is higher than that of SICM because of the sharper apex of AFM probes. However, on living cells, the lateral resolution of AFM is considered to be in the range of 50-500 nm (Häberle et al., 1992) whereas the resolution of SICM is set by the inner opening diameter of the pipette probe and can be low as 10 – 20 nm (Rheinlaender et al., 2011).

The SICM, as it was invented in the 1989, uses the same micro pipettes used for the measurement of intracellular potential and patch-clamping. These pipettes are usually pulled from glass capillaries with an initial outer diameter of 1-2 mm. Using a controlled pipette puller one can reproducibly create sharp micropipettes with apertures down to some tens of nanometers (Figure 6.1a). Even though a small aperture diameter translates in higher imaging resolution, a compromise has to be found between it and the electrical resistance, which can easily increase up to several hundreds of megaohms in high-resolution micropipettes⁶. A high pipette resistance is a problem if the leakage resistance ($R_L$), that is of interest for measuring the pipette-surface distance (Figure 6.1b), becomes smaller than the pipette resistance ($R_P$). In this case, $R_P$ would dominate the overall potential drop, masking the signal of interest.

6 E.g. SICM nanopipette with aperture diameter of 10 nm reported to have 1.2 GΩ resistance. See supplementary information of the referenced article (Shevchuk et al., 2006).
Both the micropipette and the bath are filled with electrolyte solutions mimicking salt concentrations found in physiological conditions (i.e. ~150 mM KCl or NaCl). Two Ag/AgCl electrodes complete the setup, with one electrode located in the micropipette and the other in the bath, to measure the ionic current flow through the pipette aperture. The same is done in conventional patch-clamp systems.

The imaging procedure of the traditional SCIM is similar to that of the AFM: the micropipette is mounted on an X, Y and Z piezoelectric actuator and is lowered towards the substrate while monitoring the current flow between the two electrodes. When the aperture of the micropipette is in close proximity with the specimen (typically at distances of about a pipette radius), a decrease in the current amplitude is observed (Figure 6.2). This is caused by the presence of substrate hindering the flow of ions and therefore an increase in the leak resistance ($R_l$).

Figure 6.2 shows a typical approach on a glass surface. The micropipette has an inner aperture radius of 150 nm, comparable to those used for patch-clamping. The current decreases from a constant value to almost zero in a range comparable to the radius of the micropipette.

![Figure 6.2: Current versus distance curve during approach on glass in 150 mM KCl. Inner probe radius: 150 nm. The feedback controller modifies the height of the probe to maintain constant the user-defined setpoint (Nitz et al., 1998).](image)

For imaging purposes a feedback controller (PID) is implemented in the system (Figure 6.1b), that actuates the Z-piezo to keep constant the drop in ionic current explained above. Usually, a drop of ionic current in the order of 0.2 % to 3 % is used as feedback signal.
6.1.1. Imaging modes of SICM

Since its invention, the SICM technique has been constantly innovated. In the following paragraph we will give a brief overview of the most common SICM imaging modes.

6.1.1.1. DC or static mode

The DC Mode is the original SICM mode and the simplest. Essentially, DC potential is applied between the two electrodes and the current is monitored. The amplified current is used as the input of the feedback loop, which maintains constant the probe-sample distance (Figure 6.3a) according to the ionic current amplitude versus sample distance relationship in Figure 6.2. The main advantage of this mode is its simplicity. However, it has two major drawbacks: on the one hand, its performance strongly depends on the long-time stability of the overall conditions. Temperature, evaporation, electrodes contamination and corrosion, for example, can cause significant DC offsets on the current baseline, which in turn have a direct effect on the current-based feedback control. On the other hand, the distance range in which the probe senses the sample is small. This means that during scan, the probe is blind to near structures that are higher than the separation between probe and surface. Collision with neighboring surfaces may bend the probe as a consequence of increased shear forces. Such a bending induces a tilt of the probe aperture with respect to the surface, which in turn results in a higher ionic current. The feedback-loop of the SICM will therefore push the probe even further towards the sample, eventually ending in a probe crash causing damages to both the probe and the sample. For this reason this mode is seldom used for imaging, or is used at higher distances (Korchev et al., 2000a), reducing however the sensitivity, and therefore the resolution of this technique (Chen and Baker, 2011; Rheinlaender and Schäffer, 2009).
Figure 6.3: Schematics of the different modes of SICM. a non-modulated mode (DC mode). As shown in d, the approach curve becomes smaller as the probe is approached to the substrate. The signal is fed to the feedback controller to maintain the distance constant. b Distance-modulated feedback (AC mode). c Hopping feedback mode, d DC approach curve used to control the position of the pipette in DC mode; e AC approach curve when modulating the pipette with a constant distance (Δd) which generates a modulated current $I_{\text{MOD}}$. $I_{\text{DC}}$ and $I_{\text{AC}}$ respectively represent the DC and AC (peak-to-peak amplitude) components of $I_{\text{MOD}}$. f In the adaptive resolution scanning, the overall surface roughness is first estimated by a prescan of small number of imaging points to estimate surface roughness. A rescanning is then achieved with adjusted resolution (rougher surfaces are scanned with a higher resolution) (Chen et al., 2012).

6.1.1.2. AC Mode

The AC Mode introduces a distance modulation (dynamic mode) in the order of tens of nanometers depending on the experimental condition, which originates an AC component in the monitored ionic current when getting close to the substrate. As it is shown in Figure 6.3e, the current signal does not show any AC component when away from the surface, but as the probe is lowered, the AC component increases due to squeezing of the conductive pathway between probe and sample. A lock-in amplifier is used to detect the changes in the modulated current amplitude at the frequency that is applied to the probe oscillation, which is then used to control the feedback controller. The control mechanism works in the opposite way in respect to the DC-Mode. The AC signal is in fact increasing with decreasing probe-substrate distance (Figure 6.3e).

This technique has two main advantages: on the one hand, the AC component of the ionic current is less sensitive to drifts in the DC current, which constitute one of the limiting factors of DC-SICM. On the other hand, measurements shown in Figure 6.4 demonstrate
that one can obtain both much higher sensitivity and better signal to noise ratio monitoring the lock-in response instead of the current signal alone (Donnermeyer, 2007). The better sensitivity allows to work closer to the surface compared to DC-SICM. Moreover, simultaneous recording of the DC ionic current can provide additional information about sample surface by comparing the images obtained in AC- and DC-Mode (Pastré et al., 2001). Whereas SICM is usually blind to surface features which are higher than the probe-sample distance, the AC-SICM mode increases the sensing range. Like the advantage of tapping mode over static mode in AFM, the AC-SICM allows to scan surface features with higher aspect ratio.

![Figure 6.4: ionic current and lock-in response monitored while probe is approached on an oil/water interface. Both S/N ratio and sensitivity in lock-in detection is larger than in the ionic current signal alone (Donnermeyer, 2007).](image)

### 6.1.1.3. Hopping, or spectroscopy Mode

The spectroscopy mode, also commonly known as “hopping mode” (Novak et al., 2009) repeats the approach of the micropipette for every desired pixel of the image: the height information of every datapoint is extracted by retracting the electrode by a certain distance and approaching it again to the surface while monitoring the current. As the drop in the current reaches a previously defined threshold the approach stops and the height value is stored (Figure 6.3c) The main drawback of this technique is the lower imaging speed compared with the AC/DC SICM techniques. However, spectroscopy mode SICM allow to image structures with high aspect ratio.

### 6.1.1.4. Fast Hopping Mode

The drastic increase in imaging time caused by the hopping mode led to innovative imaging technique. One of them is presented in Figure 6.3f, where an estimation of the sample topography is obtained with a pre-scan. Zhukov et al developed a technique called FSICM (fast SICM) that combines the advantages of the DC mode and the hopping mode
(Zhukov et al., 2012). First, an estimation of the sample is obtained by hopping mode. The pipette is then scanned along this estimation line while the current is being measured. A more detailed image is reconstructed with the current measured in the second passage and repeated over the whole image. At the moment, this is the fastest SICM technique that has been developed with 40 μm lines scanned in 102.4 ms only. However, abrupt changes in topography perpendicular to the fast scanning direction are still a challenge to be imaged and this speed is achieved only once the estimation procedure is finished.

### 6.1.2. State of the art of hybrid SICM systems

A comprehensive review of the functionalities of SICM for cellular physiology is referenced (Lab et al., 2013). In this paragraph, we focus on most important and innovative detection methods that have been combined with SICM.

#### 6.1.2.1. Photonic information with SICM

To simultaneously acquire photonic information with the high resolution topography, scanning near-field optical microscopy (SNOM) and confocal fluorescence microscopy have been combined with SICM.

The SCIM/SNOM combination has been achieved by using the glass nanopipette as a light source. An optical fiber was back-inserted in the micropipette and a thin layer of metal was used to coat its walls to improve light confinement (Figure 6.5a). Morphological features can be resolved with the SNOM technique and correlated with the SICM topography information.

![Figure 6.5: a Schematic diagram of a hybrid SICM/SNOM system. (Korchev et al., 2000c) b Spatial correlation of cell topography obtained with SICM and fluorescence information obtained by confocal microscopy (J Gorelik et al., 2002).](image-url)
The combination with confocal microscopy is somewhat more straightforward, as an SICM system is usually already mounted on an inverted microscope. Known as scanning surface confocal microscopy (SSCM), this technique is powerful especially to investigate molecular dynamics in living cells (J. Gorelik et al., 2002), see Figure 6.5b.

### 6.1.2.2. Scanning electrochemical microscopy and SICM

Scanning electrochemical microscopy (SECM) provides spatial mapping of electrochemistry on a wide range of materials. Different methods of SECM/SICM combination have been reported in the literature. The most common one consisted in coating the glass micropipette with a metal layer which is subsequently isolated, except for the very probe ending (Takahashi et al., 2010). This hybrid systems allow to control the distance of the pipette with the SICM method while simultaneously gathering electrochemical information thanks to the redox reactions happening at the electrode.

![Illustration of the SICM/SECM hybrid system.](image)

With the use of double barrel θ pipettes, the functionality of SICM is further enhanced and allows probing of electrochemical dynamics even in air. Indeed, with a double barrel pipette, the ionic current flows from one pipette to the other, through a liquid meniscus. This setup is known with the name of scanning electrochemical cell microscopy (SECCM) (Ebejer et al., 2013).

### 6.1.2.3. Patch-clamp and SICM

Patch-clamp and SICM setups are extremely similar in nature. It is therefore a natural consequence that the two system has been merged. SICM can improve the patch-clamp setup by providing precise spatial localization of the micropipette. The basic idea is to scan the surface of a cell with the SICM technique to find the interested region. The same pipette is then used to retrieve physiological information by patch-clamp means. The most
prominent work in this direction is from Novak et al. that solved the prohibitive high electrical resistance of nanopipettes for patch-clamp experiments, by developing a protocol of controlled breaking of the pipette. With this methodology, they managed to achieve high resolution images as well as high quality electrophysiological recordings (Novak et al., 2013). This method is also known as \textit{smart patch-clamp}.

SICM can be used in parallel with conventional patch-clamp too. In this case, a cell is patched in whole cell mode by a patch-clamp micropipette that monitors the transmembrane potential. While scanning the SICM nanopipette onto the cell’s surface, opening events of single channels caused by the SICM are detected in the transmembrane potential. This configuration allowed the functional mapping of ion-channel (Korchev et al., 2000b).

6.1.2.4. \textbf{Simultaneous force detection and SICM}

The most crucial category of hybrid SICM systems in relation to the FluidFM are those with force sensing capabilities. So far, we distinguish a category where tuning forks are used in combination of glass pipettes and one where optical read out methods are used.

\textbf{Glass micropipettes and tuning fork}

Tuning forks be used to measure interaction forces between substrate and probe, even though not in a direct way and with the need of particular calibration method (Castellanos-Gomez et al., 2009). A group in Japan (Ito and Iwata, 2011) and one in South-Korea (An et al., 2014, 2012). However, only the group in Japan demonstrated the application of their setup in a liquid environment.

\textbf{Fountain pen micropipette}

One of the first attempt of combining glass nanopipettes with AFM dates back to 1992 (Lieberman et al., 1994; Shalom et al., 1992). These probes are specially designed glass pipettes with a bent ending (Figure 6.7). The deformation of these pipettes has been sensed both with OBD and quartz tuning force methods. The combination with OBD envisioned the gluing of a small mirror close to the pipette ending, allowing to use these probes in combination with an AFM. However, their high stiffness (> 10 N/m) makes them less suitable for static imaging of soft materials like cells.
Shear force microscope

The SICM combined with a shear-force distance control is another successful combination. In this system, air filled periscopes are submerged with the probe and carry the laser beam that is used to detect lateral movements of the pipette (Figure 6.8). A piezo provides the lateral vibrational movement typically in the range between 10 to 70 kHz. The amplitude of the lateral motion is dependent on the amount of shear forces and is used as a feedback signal for the Z-controller (Böcker et al., 2007).

Deep atomic force microscope

Maybe one of the most creative innovation in the context of hybrid SICM systems, Drake et al. (Drake et al., 2014) fixed a glass micropipette on a soft suspension system which movement is monitored with a conventional OBD method (Figure 6.9).
Figure 6.9: Schematics of the SICM/AFM hybrid of Drake et al. The pipette is suspended on two triangular arms, which deflection is monitored with OBD system. (Drake et al., 2014).

This system proved to be capable for contact AFM imaging with simultaneous ion conductance mapping of different substrates, e.g. deep trench or flat polycarbonate Isopore™ filter.

6.1.3. Foreseen advantages of the FluidFM and SICM hybrid system: Project goal

The final objective aims to combine the AFM and the SICM techniques together exploiting both of their advantages. On the one side the imaging gentleness of the SICM and the single cell interventions made possible from the pipette-like probe (patch-clamp, injection, etc.) while keeping the high force sensitivity of the AFM. One can envision different imaging modes of an AFM-SICM hybrid system:

- **SICM only**: The advantages of SICM imaging can be directly adopted by the FluidFM system. In addition, i) the use of AFM cantilevers makes the imaging in DC-Mode less prone to damage the sample. Indeed, the stiffness of a cantilever compared to a rigid, vertical glass micropipette is orders of magnitude lower. As a consequence, eventual force interaction of the probe with the specimen will cause the cantilever to bend, avoiding irreversible damage to both the probe and the sample. ii) FluidFM cantilever can be designed with aperture sizes comparable to pulled nano-pipettes, but show lower resistance (40-60 MΩ compared to 100-800 MΩ, depending on the fabrication method (refer to Chapter 4.3.2.1).

- **Static AFM and IC**: The AFM can be used in static mode to follow the topography of a substrate, while the ion conductance is simultaneously measured. This can deliver unique insight in sample conductivity and topography.
- **Dynamic AFM and IC.** Used in dynamic mode, the AFM can be used to follow the topography of softer samples. With the added phase information, insight in sample stiffness are gained too. Simultaneously, the DC ionic current\(^7\) can be measured and correlated to the topography information.

- **SICM & AFM hybrid controller:** While using the FluidFM in SICM mode, the deflection of the cantilever continuously provides information on the contact forces. Driving a second controller which aim is to limit the contact forces, the SICM imaging process can be improved. Especially, the scan speed in DC mode can considerably increase.

- Switching from SICM to AFM mode with the FluidFM can be made easy. In addition, other modes are imaginable, especially by combining new pieces of equipment not mentioned in this context.

In the next paragraph, the SICM & AFM hybrid controller concept is deepened, together with some preliminary experiments of AFM with simultaneous ion conductance.

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\(^7\) Depending on the impedance of the FluidFM cantilever, the limited bandwidth (usually below 1 kHz) limits the possibility to access the AC signal while working at the resonance frequencies of the cantilever (30-50 kHz).
6.1.4. FluidFM and SICM at a glance

The information flow between FluidFM components for ion-conductance distance control is shown in Figure 6.10. DC-mode is achieved using a traditional patch-clamp amplifier (Heka EPC7 or Tecella Pico2), marked in green. AC-mode requires the use of a Lock-In amplifier (HF2-LI, Zürich Instruments), highlighted in red, instead of the patch-clamp amplifier. At the beginning, the PID and the signal acquisition is taken over from a self-written LabView program (National Instruments, Austin, Texas).
6.2. AFM imaging and simultaneous ion-conductance mapping

Before deepening the SICM capabilities of the FluidFM, its operation as an AFM with simultaneous mapping of local ion-conductance is a straightforward application. In this imaging mode, the FluidFM probe is scanned over a specimen with the distance controlled by the AFM. During the scan, one can simultaneously record a map of the ionic conductance by measuring the ionic current through the microchannel of the FluidFM cantilever (Figure 6.11).

![Diagram of proposed experiment](image)

- Figure 6.11: Schematics of the proposed experiment. The conductive Ag/Ag PDMS is connected to the ground, acting as an electrode. A voltage bias is applied between the two electrodes and the current measured. Simultaneously, the setup is operated in AFM mode. Experiment carried out in 150 mM KCl.

This imaging mode has been investigated together with the LBB PhD student Pascal Behr. The details on these experiments and their discussion will be reported in his PhD Thesis and are in the process to be published in the following article:

*Multi-parameter imaging using microchanneled AFM probes with integrated Ag/AgCl electrode,*

Pascal Behr, Dario Ossola, Alexandre Larmagnac, Janos Vörös, Tomaso Zambelli

In preparation.

In this paragraph, I will limit to give a brief summary on the work done in this direction.

**Experiment design:**

Two experiments were envisioned: i) to measure topography and potential map of grounded and floating ITO electrodes on glass substrates in static AFM mode and ii) to measure topography and local conductivity map of Ag-PDMS electrodes imaged in AFM dynamic force mode. Apex 300 probes supplied by Cytosurge were used without further treatment. The DC ionic current was measured with a PicoAmp 2 amplifier (Tecella Inc, Foothill Ranch, USA). The current signal was forwarded to the user input of the FlexAFM controller. Imagining of both signals was achieved within the EasyScan2 software (Nanosurf, Liestal, Switzerland).
6.2.1. Electric potential mapping of ITO substrate

Indium Tin Oxide (ITO) is a transparent, conductive oxide and has been patterned onto a glass coverslip (Microvacuum, Hungary). The patterned ITO coating had a thickness of 10 nm and was deposited in such a way to leave an isolated island in the middle of the sample. This part was not connected to the ground, i.e., was a floating electrode, bridged from the electrolyte in the solution only. On the contrary, the rest of the ITO was grounded. A bias voltage of 100 mV was applied and the ionic current between the Ag/AgCl electrode in the FluidFM probe and the grounded ITO portion was measured while scanning the surface in static AFM mode.

The experiment showed that a difference in current amplitude was detectable from the isolating glass substrate to the ITO, but also between the grounded ITO electrode and the floating one. The result confirmed feasibility of local ionic conductance measurement simultaneous to AFM imaging. Differences in current amplitude are plausibly correlated to the interaction of the probe with the electrical Double layer or with the Diffuse Double Layer of the specimen.

6.2.2. Dynamic AFM and simultaneous IC mapping on Ag-PDMS electrode

The previously described experiment showed the simultaneous acquisition of IC information while imaging in contact mode. The contact mode is less suitable for soft or adhesive substrates, therefore we demonstrated that the dynamic mode is suitable as well.

Electrodes made of conductive PDMS\(^8\) were analyzed. These electrodes were fabricated incorporating silver particles in PDMS. The Ag-PDMS electrode was used as ground electrode and the ionic current was monitored while scanning its topography in both static and dynamic force AFM (Figure 6.11). The bath and the cantilever were filled with 150 mM KCl. The electrodes were biased with -100 mV. Moving the probe on an exposed and connected silver particle should trigger electro-deposition of Cl\(^-\) ions, thus generate an ionic current. The result was a high resolution image of the topography and the conductivity map together in the same scan.

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\(^8\) PDMS: Polydimethylsiloxan. A silicon based organic polymer.
Figure 6.12 gives an example of the data gathered in this configuration, where both insulated silver particles and exposed particles are recognizable by superimposing the conductivity map.

Figure 6.12: Superposition of topography (3D information) and conductance map (color code: darker translates in higher ionic current) of a Ag-PDMS electrode. Silver particles embedded in PDMS are insulated (grey arrows) and no change in conductance is visible. Whereas exposed silver particles show an increase in ionic current (red arrows).

6.2.3. Summary

The FluidFM indeed helped to characterize composite materials that have heterogeneous conductivity properties. Exposed silver particles can be distinguished with high contrast from those embedded in PDMS and thus isolated. However, it is still difficult to draw conclusions about their connection pathway.

No other technology can exploit the maximum capabilities of AFM and SICM without major compromises. In fact, the FluidFM is a fully capable AFM with added opportunities thanks to the integrated microchannel. In this particular case, filled with electrolyte and with the integration of a low-noise current recording setup, ionic-currents in the sub-picoampere range are measurable. In conclusion, the setup allows to retrieve the topography based on the AFM and to simultaneously gather information on electrical surface properties. In addition, by carefully choosing the salt-species in the electrolyte one can alter the conduction pathway and thus trigger particular localized electrochemical reactions.
6.3. SICM with the FluidFM

In this Chapter, I will deepen the application of the SICM technique to the FluidFM. Firstly, the affinity of the two systems is demonstrated by utilizing the FluidFM as a scanning ion-conductance microscope only. Then, the properties of FluidFM probes for SICM will be deepened by COMSOL simulations. Finally a novel, true combination of AFM and SICM will be presented, where two controllers will work in unison for high speed DC-SICM.

The result presented hereafter are part of the following publication:

Simultaneous SICM and AFM with microchanneled cantilevers
Dario Ossola, Livie Dorwling-Carter, Pascal Behr, Harald Dermutz, Janos Vörös, Tomaso Zambelli
In preparation

6.3.1. Force and Ionic current spectroscopy

The first proof of the simultaneous recording of SICM and AFM operation was done by force spectroscopy means in 150 mM KCl solution. The probe was approached on the substrate by conventional AFM spectroscopy protocols and stopped by trespassing a force threshold. The $Z$ dependency of deflection and ionic current was acquired and plotted versus probe position. Figure 6.13a shows the curves during an approach on a glass substrate obtained with standard Apex300 FluidFM probes. Thanks to the deflection signal, the contact point can be detected with high precision. The inset shows the drop in the current prior to contact, which constitutes the portion of signal of interest for the operation of the SICM. It is interesting to compare approach curves on different substrates.
Figure 6.13 Simultaneous force and ionic current spectroscopy with a standard Apex300 FluidFM probe in 150 mM KCl solution. 

**a** Approach on a glass substrate. Inset shows the current drop before contact. Contact point marked by the orange vertical line. 

**b** Deflection and current vs. z position on two different substrate: glass and PDMS. The PDMS deflection curve shows different contact times of the FluidFM apex features, reflected in the sealing dynamics observed in the current signal. 

**c** Schematics of the proposed closing mechanism of the Apex300 on PDMS.

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Approach curves on glass and on PDMS are shown in Figure 6.13b. Before contact, the current curves had the same shape. After contact, the two signals differ substantially. In the case of glass, which being a hard surface does not deform, the current dropped only slightly. As expected, the deflection steeply increased with a nearly linear slope. On PDMS, which is softer, both the current and the deflection exhibited step-wise behavior. This can be explained with the help of the schematics of Figure 6.13c. When contact is reached, the lower part of the probe touched and indents the PDMS (first less steep slope of the deflection). Simultaneously, the current abruptly decreases followed by a transition moment, where the probe is further pushed into the PDMS to eventually reach the backside of the aperture and close it completely. At this point, the slope of the deflection becomes steeper, because more volume is indenting the PDMS, and the current almost drops to zero, because the aperture is sealed.
6.3.2. Validation via COMSOL simulations

In this project, a new technique to carry out scanning ion conductance microscopy was developed, which no longer relied on the usual glass micro- or nano-pipettes but on the FluidFM pyramidal probe as a scanning probe. Figure 6.14 shows the FluidFM probes that have been used in this paragraph compared with a standard SICM nanopipette.

Several models based on both numerical simulation and analytical models (Adenle and Fitzgerald, 2005; Del Linz et al., 2014; Edwards et al., 2009; Rheinlaender and Schäffer, 2009; Thatenhorst et al., 2014) have been developed to better understand and characterize the working principles of the SICM. They especially allowed to study the resolution of the SICM and to assess the changes in the ionic current depending on the geometry of the probe, the topography of the surface, the probe-sample distance, the maximum slope of approach, and to predict image artefacts in SICM. In this paragraph the above mentioned models for our probe geometry is validated. For this purpose, the approach current signal obtained with standard Apex300 FluidFM probe and custom, FIB-milled FluidFM probe apertures down to 50 nm (Figure 6.14) is compared. Typical probe apertures had a diameter in the range of 80 nm – 550 nm while the corresponding resistance ranged around 22-55 MΩ in 150 mM KCl. The filling of the probe was made easier by O₂ plasma treatment for 2 min or by pre-filling the microchannel with CO₂.

The aim of the Finite Element Modelling is to create an evaluation tool that allows to assess the influence of different parameters of the SICM-FluidFM setup. Particularly, changes in the geometry of the FluidFM probe (probe diameter, inner over outer radius ratio, cone angle) can be easily assessed through the proposed model. The results can be used to better design pyramid apertures and to understand the experimental result, especially important during imaging (not shown here). Figure 6.15 shows the 3D model of the FluidFM probes apertures used in this instance.
Figure 6.15: 3D models of the used FluidFM probes. a The aperture of the standard apex 300 probe. b Aperture model of the apertures milled per FIB. c Snapshot of the simulation of the ionic current through an Apex300 probe. d Simulation snapshot of the ionic current through a customized aperture geometry. The sensitive area is where the streamlines are denser.

Figure 6.15c/d are snapshots of the FEM simulation for the two probe designs. The streamlines density indicate ionic current amplitude. Indeed, they get denser under the probe’s aperture indicating the sensing location.

The simulation considers all the geometrical parameters (lateral wall thickness, aperture diameter and shape, aspect ratio, etc.) as well as the substrate characteristics (topography, inclination, etc.).

6.3.2.1. Experimental validation of the simulation results

An ionic-current approach curve onto a glass substrate is presented in Figure 6.16. Even though this measurement revealed to be noisy because of sub-optimal gain settings of the current amplifier, the overlay with the simulated data confirms the correctness of the FEM model. The saturation $I_{sat}$ current in agreement too, being in the same order of magnitude ($I_{sat}$ simulation: 229 pA, $I_{sat}$ experimental 111pA). The z-distance dependency is also fitting well. The reader should note that no fitting parameters are used for the overlay. The simulation precisely reproduce the reality and predicts with confidence the experimental results.

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9 Details in Dorwling-Carter's master thesis.
Figure 6.16: Overlay of simulated (red cross) and experimental (blue line) data. Approach of a standard Apex300 FluidFM probe on a glass substrate in 150 mM KCl solution. Saturation current normalized ($I_{sat}$ simulation: 229 pA, $I_{sat}$ experimental: 111 pA).

In Figure 6.17 a comparison of the simulated current approach curves between the Apex300 and the custom FIB geometry is shown. The latter shows a steeper dependence from the distance to the substrate, thus enhanced sensitivity.

Figure 6.17: Comparison of simulation result between the apex300 geometry and the custom FIB geometry in 150 mM KCl solution.
The simulated approach curves shows good agreement with the models proposed for glass nanopipettes. Figure 6.18 shows the model described in Equation 6-1. However, it results difficult to obtain a precise fitting in all the regimes of the curves. Indeed, a good fit in the region close to the substrate comes at the expenses of an imprecise fitting at higher distances; already mentioned in literature (Del Linz et al., 2014):

\[
I(z) = I_0 \left( +1 \frac{z_a}{z} \right)^{-1} \quad \text{with} \quad I_0 = \frac{V_0}{R_m + R_{tip}}
\]

Equation 6-1
6.3.3. SICM imaging

The results presented in this paragraph have been obtained using the FluidFM in SICM mode, i.e. the probes are used as SICM pipettes and not as AFM cantilevers, without force control. In this case, the goal was to obtain images in non contact mode, with the feedback controller working on the ionic current and not on the deflection signal. Nonetheless, the deflection signal was also recorded to observe where the probe “touched” the sample during SICM scanning. Two different setups have been used for this purpose. The first part was obtained with a setup comprehending a lock-in amplifier (Zurich Instruments, Zurich, Switzerland), in order to allow the testing of the AC mode. In a second phase, we had the opportunity to test the FluidFM setup with a Nanonis full control system (Specs, Zurich, Switzerland) which came with a number of advantages described later. The EPC 7 patch-clamp amplifier (HEKA, Ludwigshafen, Germany) was used to measure the ionic current. To assess the capabilities of the system we used patterned PDMS as a substrate, with known dimensions. The characteristics of the substrates used are specified in each of the following paragraphs.

6.3.3.1. DC and AC imaging comparison

In this experimental environment, the scanning of the probe, and thus the imaging display, was controlled by the Nanosurf program. The AFM controller was deactivated as the ES2 controller cannot be driven by external signals, which in our case would have been the signal from the current amplifier. I relied on the PID controller of the Lock-in amplifier to control the Z-piezo of the AFM scanhead. The PID output was connected to an input of the FlexAFM controller and displayed as an image to obtain the topography. Following the output of the PID therefore allowed to graphically represent the topography of the sample by the proper electrical connections. Bath and cantilever were filled with 150 mM KCl solution.

Figure 6.19: a SEM picture of the Silicon TGX calibration grid of Micromasch. Step height: 1 μm. Grid repeat period: 3 μm. b After using the calibration grid as a mold for PDMS, the latter features a complementary shape, which is depicted here.
For the subsequent comparison of DC and AC SICM, standard Apex300 FluidFM probes were used. The same 25 μm² square area was scanned in the two modes to better compare them. In both modes, the scanning speed was 2 s per line with 256 points/line corresponding to a sampling rate of 128 points/s.

**DC scanning mode**
The current signal was demodulated at 0 Hz with the Lock-In amplifier and was used as input for the in-built PID controller. The coefficients of the PID were estimated first via the Auto-tune function embedded in the Lock-in amplifier and subsequently manually adjusted by toggling between two different setpoints, to optimize the response of the controller. The output of the PID was sent to the Z-piezo input of the FlexAFM controller.

![Figure 6.20: SICM in DC mode in 150 mM KCl.](image)

- **a** The deflection signal contains information on eventual contact between the specimen and the scanning probe.
- **b** Friction force signal confirms that the probe is in non-contact regime.
- **c** Output of the SICM controller, i.e. topography of the specimen.
- **d** Ionic current signal, i.e. error of the controller. Regions where the controller fails to maintain the setpoints corresponds with the probe touching the substrate (compare with deflection signal in a).

Figure 6.20 shows the result of the PDMS grid imaged in DC mode and 150 mM KCl solution. The deflection and lateral force signals are almost constant, revealing that few contacts occurred during the scanning. The PID designed for feedback (integrated in the Lock-In amplifier) was fast and efficient enough to lead to few errors and maintain the
current constant. The piezo movements, actuated by the PID controller, are consequently representative of the topography of the sample. The output voltages converted in real size (500 nm/V) revealed a height of about 952 nm and a pitch of 3.05 μm, well in agreement with the specifications of the specimen.

**AC scanning mode**

The FluidFM cantilever was excited with the Lock-In amplifier with an amplitude between 100 mV and 500 mV, corresponding to about 20 nm to 60 nm oscillation amplitude at the free end of the cantilever. The deflection signal was then demodulated at the same frequency to get the oscillation amplitude. The excitation frequency that showed the highest substrate-probe distance sensitivity was chosen. This frequency was found by frequency sweep means in close contact with the substrate. Usually, a value close to 450 Hz was chosen. The excitation frequency was limited by the impedance of the FluidFM probes, that behave as a low-pass filter with cut-off frequency around 800 Hz, impeding the readout of faster signals (Chapter 3.5.1).

Figure 6.21 shows the results of scanning the same PDMS area in AC mode, in 150 mM KCl solution. The deflection signal reveals mechanical interaction between the probe and the substrate when transitioning from the high feature to the lower base. It is plausible that the pyramid probe impedes a fast lowering of the probe, commanded by the SICM controller. This explanation is supported by the good correlation between deflection and controller error (i.e. current) signals. Indeed, the controller fails to maintain the setpoint because of the probe touching the substrate, which has a delay effect on the physical lowering of the probe.
Figure 6.21: Imaging in AC SICM mode in 150 mM KCl solution. Oscillation frequency: 466.18 Hz. 

- **a** Deflection signal during scanning.
- **b** Friction signal during scanning.
- **c** Topography. The features appear sharper compared to the DC mode.
- **d** Current signal, i.e. error of the Z-controller.

Figure 6.21: Imaging in AC SICM mode in 150 mM KCl solution. Oscillation frequency: 466.18 Hz. 

- **a** Deflection signal during scanning.
- **b** Friction signal during scanning.
- **c** Topography. The features appear sharper compared to the DC mode.
- **d** Current signal, i.e. error of the Z-controller.
6.3.3.2. DC-SICM with Nanonis™ SPM controller

In range of a collaboration with SPECS Zurich GmbH (Zurich, Switzerland), the SICM with the FluidFM system was tested using a commercially available SPM controller. The advantage of this controller over the previously presented setup (Lock-In amplifier) is foremost the fully functional and complete software. Additionally, pre-programmed, in-built controller with logarithmic behavior enhances the performance of SICM. To finally prove the image quality achievable with the FluidFM system in SICM mode in combination with the Nanonis SPM controller, two additional measurements are proposed here. The first on a PDMS calibration grid featuring 500 nm high steps. The second one on primary rat hippocampal neurons to finally demonstrate the ability of SICM to image soft and delicate biological samples.

**PDMS calibration grid:**

The substrate was PDMS featuring stripes of 500 nm high. 20 mV voltage bias was applied between the electrodes in 150 mM KCl. Imaging speed was 5 μm/s. The setpoint was initially set at -354 pA and was continuously adjusted to stay as close as possible to the substrate, while ensuring no contact with the help of the deflection signal. Off-sets of normal force and lateral deflection have been compensated a posteriori.

Figure 6.21 summarizes the result of the experiment showing an excerpt of a 15 μm × 15 μm DC-SICM scan with 256 × 256 lines. While the topography data precisely reveals the dimensions of the specimen, the error signal (i.e. the current) confirms the good performance of the controller. In fact, the error deviation remained in the noise. Analysis of the deflection data revealed contact of the probe while transitioning from the top of the feature into the valley, as already discussed previously. Interestingly, the lateral deflection correlates with the shape of the aperture. Ascending the obstacle gives a constant friction value, corresponding to the pyramid side touching the PDMS. On the contrary, descending the valley, the whole aperture itself is probably interacting with the substrate because the SICM controller anticipates the valley and lowers the probe too early.
Figure 6.22: DC-SICM imaging with the help of Nanonis™ controller. Voltage bias: 20 mV. Scan speed was 5 μm/s. Fast scan direction: right to left. Performed in 150 mM KCl. 

- **a** Topography of the specimen. The height is in good agreement with the specs of the calibration grid ($h_{\text{meas}}=564$ nm).
- **b** Current signal, i.e. controller error. The almost complete absence of recognizable pattern indicate an efficient behavior of the Z-controller.
- **c** Deflection signal. Contact is revealed when transitioning from a high point to a valley.
- **d** Torsion signal reveals an interesting pattern that can be correlated with the aperture shape of the FluidFM probe.

**Hippocampal neuron:**

The SICM technique was initially invented to image living cells in physiological conditions, in non-contact mode. Therefore used the FluidFM to image primary rat hippocampal neurons from E17 embryos with conventional Apex300 FluidFM probes. I was limited to the imaging of the neurites, as the Z-piezo range of the FlexAFM is limited to 10 μm. for this reason it was not possible to image the body of an entire cell.

The bath and the cantilever were filled with filtered (0.22 μm) serum-free medium (High Glucose Dulbecco’s Modified Medium (DMEM, Life Technologies Ltd) mixed with 1 % GlutaMAX (Catalog # 61965-026, Life Technologies Ltd.) and 1 % antibiotic antimycotic (Catalog # 15240-062, Life Technologies Ltd.). The details about culture treatment of the primary neuron, medium used and substrate preparation are described in the referenced article (Dermutz et al., 2014).
Figure 6.23: DC-SICM imaging of hippocampal neuron. a Micrograph of the FluidFM cantilever in proximity of the neurons. b 3D representation of the obtained topography. c 2D representation of the topography. The gentle nature of the technique allows to discern neurites on different height levels. d Deflection signal. Low current setpoint has been chosen, therefore contact is detectable. The contact forces do not exceed 30 nN, so that the neurites are not being displaced during scan. e Error signal. Reaching the maximum extension of the piezo actuator lead to the higher error area on the lower part of the image.

Figure 6.23 shows a 50 μm × 50 μm scan of a network of neurites. Even though in the deflection signal the features are visible, the resulting force is always lower than 30 nN, preventing any damage and displacement of the neurites. The presence of the mechanical interaction is due to the choice of a low setpoint, meaning little scanning distances to obtain maximum resolution. The deflection signal constitutes an additional information that allows an informed choice of the setpoint, so that the scan always happen at the ideal conditions. The image allows distinguishing the different neurites and understanding the complicated 3D network that they form.

6.3.3.3. Summary

These measurements confirm the aptitude of the FluidFM system to be operated as a scanning ion-conductance microscope, with the unique advantage to provide simultaneous force information with AFM sensitivity. By fabricating probes with smaller aperture dimension, the FluidFM has the potential to surpass the quality of conventional SICM systems because of the reduced probes’ impedance. In fact, nano pipettes with aperture diameter in the range of 10 -100 nm diameter show resistances that exceed 1
FORCE-CONTROLLED SCANNING ION CONDUCTANCE MICROSCOPY (SICM)

GΩ (Shevchuk et al., 2006), whereas FIB customized FluidFM probes with 50 nm apertures stays in the range of 30 MΩ.

6.3.4. Double controller

In the previous paragraph, the cantilever deflection has been treated as an additional information source only. The purpose of the experiments presented in this context is to demonstrate that this additional information can be used to improve the DC-SCIM performance. A novel controller is designed, by fusing the SCIM and AFM signals. The dual SICM-AFM controller would be a distinguishing feature of our system in comparison with the glass pipettes setups by implementing an additional force feedback from the AFM in the control of the probe movement. This would especially allow to avoid excessive force interaction with the sample. Indeed, a common problem in SICM scanning is that the presence of an obstacle on the sample can lead to a tilt of the probe due friction force. A tilt of the scanning probe induces an increase of the ionic current sensed, as the aperture “opens”. Consequently, the SICM controller move the probe closer to the sample to keep the setpoint constant, worsen the situation and may cause damage the sample and the probe. I aimed to take advantage of the sensitive force detection of the AFM. By judging the amount of force detected, one can improve the decision about the optimal scan distance.

In this context, a controller that runs in parallel to the SICM Z-controller was added, which task was to keep the deflection at zero. As an advantage, the risk to damage a soft sample when scanning in DC SICM mode is reduced and the scan speed can therefore be increased. The proof of concept is proposed here, which aims is to demonstrate the potential advantages of such a setup.

The dual controller behavior was firstly simulated in Simulink (MathWorks, Massachusetts, USA) with simplified assumptions, then the setup was tested on a Nanonis™ controller.

6.3.4.1. Dual controller model

The simplified model considered in this instance was a forced simplification of AFM and SICM dynamics. Indeed, no dynamic effects are considered in this model. At this stage of an estimation of the potential of this technique was needed. Nevertheless, comprehensive simulation of the fused controller is necessary as a next step to understand the limits and the dynamics in the frequency domain.
Figure 6.24 shows the block diagram of the Simulink model where two controllers are running in parallel. The SICM controller shall keep a constant set point on the ionic current, while the force controller keeps the set point of the deflection to zero. The fusion of the controllers' outputs was here simplified to an arithmetic weighted addition. The final position of the piezo is then converted in ionic current and deflection according to the implemented physical model. The model simulates the scanning of a pyramidal probe (Figure 6.25).

**SICM Physics** – The ionic current value was calculated using the simulated current approach curves presented in 6.3.2. The difference of the piezo position with the real substrate shape (solid lines in Figure 6.25b) gives a current amplitude. The result is eventually compared with the setpoint and used as a feedback signal for the SICM controller.

**AFM physics** – Contact force arises from the interaction of the 3D geometry of the probe with the substrate, which ends with a convoluted trajectory. This trajectory has been computed in Matlab and is represented with dashed line in Figure 6.25b. The generated force is computed by comparing the probe position with respect to the convoluted substrate. The force is then calculated via *Hooke’s Law*: \[ F = k \Delta x. \] To further simplify the dynamics, attractive forces were excluded by forcing the output of the deflection physics to zero in the case that the probe-sample distance is greater than zero.

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10 Hooke’s Law: the force needed to extend or compress a spring is proportional to the displacement: \[ F = k \Delta x. \]
With the above described assumption, an SCIM scan was simulated. Figure 6.26 shows the result. The model simulated the scan of a 6 μm line with a probe velocity of 1 μm/s. Scan direction was set from left to right. Figure 6.26a shows the PID output signal. The controller did indeed retract the probe in proximity of the obstacle, to keep the current constant. The PID output therefore refurred the sample topography. The error signal confirmed good controller performance, because the transient error signal occurring by topography change was compensated in less than 50 ms and the PID output followed the topography profile with fidelity. (Figure 6.26a/b). However, a closer look at the virtual distance between the probe position during scan and sample convoluted profile (Figure 6.26c), revealed contact with the substrate. By correct functioning of the SICM scan, the probe should be higher than the surface, thus resulting in a virtual overlapping (i.e. probe height was subtracted to sample height) negative in sign. Close to the step edges, the lateral walls of the pyramid collided with the sample. Therefore, the value of the virtual overlapping became positive. It is in this region that I aimed to design a parallel running controller, exploiting the AFM deflection signal to force further retraction of the probe in case of collision.
Figure 6.26: Simulation of the simplified SICM controller. The pyramidal probe is scanned over a 700 nm step feature. Simulated scan size: 6 μm. Scan speed: 1 μm/s. Scan direction from left to right. 

a) To maintain a setpoint constant, the PID controller of the SICM retracts the piezo and the output is a representation of the topography.

b) The error signal show good performance of the controller.

c) Plot showing the distance between the probe and the convoluted substrate. Where the SICM is working in non-contact, the distance is negative, whereas in proximity of the feature, the pyramid touches the substrate with the side walls.

In range of the Simulink simulation, the properties of the block responsible of fusing the PID outputs can be varied to understand the response of the control system. An optimal compromise was found by adding a weight gain of 1000 to the AFM PID output signal. The result is presented in Figure 6.27. The resulting topography is a combination of the SICM and AFM controllers (Figure 6.27a), being the sum of the SICM output signal and of the AFM output signal amplified by a factor of 1000 (Figure 6.27c/d). By analyzing the virtual overlap between substrate and probe, the two regimes are recognizable (Figure 6.27b): the first, where the separation is negative, is characterized by a domination of the SICM controller. Indeed, negative separation indicates non-contact scan. The second regime shows a separation close to zero. Here, the AFM controller gains the upper hand and forces probe retraction avoiding collision (compare with SICM only case of Figure 6.26c).
The physics that drives the two controllers imposes them to work one against the other (Figure 6.27c/d): when the probe is in contact with a feature of the substrate, the SICM controller is prone to lower the probe, as the $I_{DC}$ setpoint cannot be reached. On the contrary, the AFM controller detects the deflection and attempts to retract the piezo actuator. Thus, the two controller work in opposing directions. It is by correctly setting the addition weighting that a profitable behavior is achieved. Figure 6.27e/f show the error signal of the two controllers. The error of the SICM in the situation where the AFM controller “wins”, increases. This leads the SICM to lower the probe in order to reduce the error. For this reason, a measurable error is present in the AFM controller too. In conclusion, the AFM controller output must induce a strengthened retraction: to react to the deflection signal and to counteract.

Even though the model proposed is oversimplified, it gives insights on how the two controllers may coexist and drive a common actuator. A more elegant and complete approach would be to model the components of the system in the frequency domain which will be needed. A fusion using Kalman or $H_{\infty}$ filter can be a good choice for the purpose here exposed. This kind of sensor fusion has been often a topic in the nanopositioning field, where sensors with different dynamic range and sensitivity are fused to improve position control performance (Sebastian et al., 2012). However, such a deep study will go
beyond the purpose in this context. Here I aimed to address the potential benefits of a dual SICM/AFM controller in the range of a proof-of-concept.

6.3.4.2. Dual controller imaging

In practice, as the deflection signal can drift from the ideal zero, the force controller was made by a proportional component. Indeed, removing or increasing the integrator time to high values allows for the presence of a steady state error together with a fast response time. To test the performance of the system, a specimen with known dimensions has been chosen. The sample featured 500 nm height stripes made of PDMS repeated every 3 μm. A silicon AFM calibration chip was used as a mold.

Figure 6.28 shows the effect of the additional force controller during imaging. The PDMS sample was scanned first in SICM mode. The force controller was activated only at about half of the image. In this particular experiment, the proportional gain of the force controller has appositely been exaggerated with the purpose to highlight its contribution. The profiles extrapolated from the topography (Figure 6.28a) compare a line scan performed with and without simultaneous deflection controller. The contribution is especially prominent when scanning into the valley. Figure 6.28b shows the contribution of the force controller. It is inactive in the upper part of the image and active in the lower part. Compared with the force trace (Figure 6.28d), it demonstrates the added value of the dual controller. Indeed, in the upper part of the image, i.e. without force controller, the involved force is higher. With active force control, the attractive force was compensated. Repulsive force was partially compensated, except for one instant, where the force stayed high. By comparing with the current signal (Figure 6.28c), one notices that in this region the measured ionic current is low, which is in agreement with the detected high force. It is plausible that the probe lowering command of the SICM, at this position, was too high to be overcome by the force controller, delaying the effect of the latter. In fact, when this point is surpassed, the SICM signal is supposed to become smaller and the force controller overshoots. It is interesting to note that, as wanted, the force controller does not interfere with the scan as long as the probe does not mechanically touch with the substrate, ensuring contact-free imaging in ideal conditions.
FORCE-CONTROLLED SCANNING ION CONDUCTANCE MICROSCOPY (SICM)

Figure 6.28: Comparison of imaging with and without force controller in 150 mM KCl. Upper part of the image is SICM only. The force controller is activated in the lower part. Scanning direction: right to left. Speed 0.5 s/μm. Gain of force controller appositely oversized to highlight contribution. a Topography. The upper half is imaged in SICM mode only, whereas the lower half had both controller active. The underneath graph shows profiles comparison of SICM only (black line) and active dual controller (red line). Contribution of the force controller is especially noticed when moving into a valley. b Force controller output. The controller is deactivated in the upper part of the image. The controller retracts the probe when it touches the substrate. c Ionic current channel. d Force signal. The lower half therefore is the error signal of the AFM controller.

Figure 6.29 show a scan example where the reactivity of the force controller has been reduced. Activating or deactivating the force controller has little effect on the topography (Figure 6.29a), but analyzing the force signal, a significant reduction of the involved force is visible. Figure 6.29b shows that with active force control the forces are buried in the noise, whereas when deactivated, they sensibly increase. Interestingly, the measured force bends the cantilever towards the sample. The “stickiness” of the PDMS can be a reason for this, but also an unpredicted interaction with the 3D geometry of the FluidFM
probe aperture with the substrate. With active force control, the force involved is about 8 times lower (Figure 6.29d, inset).

**Figure 6.29:** SICM/AFM scan with moderate force control gain. Scan direction: left to right. Scan speed 5 μm/s. a Topography. b Ionic current: minimum in the current is recognizable in concurrence with a maximum in the response of the AFM controller (c). c Output of the force controller. The controller was transiently deactivated in the middle part of the image (flat signal). d Force signal.

The systematic force recording available with the FluidFM is particularly useful to understand the interaction of the probe with the sample when scanning via SICM. The resolution of SICM is higher when the probe-sample distance is small. With a conventional, blind DC-SICM setup, the only way to find the best working distance is by trial and error, which eventually leads to a contact with the substrate. In the FluidFM case, the deflection signal gives continuous information about the applied forces and ensures that the sample is scanned at the minimal distance allowed by the experimental conditions. Importantly, the spring constant of the FluidFM cantilever is much lower (< 2 N/m) compared to the one of glass micropipettes (> 100 N/m). A mechanical interaction between probe and substrate is therefore generates much less forces in the case of the FluidFM. For this reason, the presented technique allows to operate the FluidFM close to the surface, as the interaction forces can be monitored and limited with the intervention of a second controller working on the force.
6.3.4.2.1. Imaging speed

The aim here is to demonstrate the added value of the dual controller setup to image in DC-SICM at high speed. Figure 6.30 shows the acquired image of 10 μm × 10 μm with a speed of 30 μm / s (3 lines/s). To achieve an image with this resolution in conventional hopping-mode SCIM (256 × 256 lines), a typical time of 15-20 minutes is needed. The image shown here was acquired in less than 2 minutes, thus much faster compared to other SIC microscopes because the force control allows to image in DC-SICM, with conventional AFM scan rates.

Figure 6.30: High speed SICM/AFM scan. Scan size: 10 μm × 10 μm. Scan speed: 30 μm/s. Acquisition time: 85.5s. a Topography. b Ionic current signal. c Force controller output.

6.4. Conclusions

It has been demonstrated that the proposed novel setup has the potential to overcome the performance of conventional SICM systems under many aspects: i) in terms of resolution, ii) in terms of speed and iii) in terms of versatility. The resolution is mainly dependent on the aperture sizes, which are comparable between the two systems, with a light advantage of the FluidFM that offer probes with lower electrical impedance when reaching nanometer-sized apertures. In terms of speed, the technique proposed has the advantage to allow true scanning of a substrate. For reason exposed in the previous paragraphs, the DC mode is not really a practicable option because of the limitation related to the use of rigid glass pipettes. The FluidFM permits fast, DC-SICM with an added sensing ability that controls the forces involved during scan (the AFM).

However, a lot has still to be characterized in order to fully understand the potential of the system. The imaging dynamics of the dual controller have been here superficially addressed: an in depth study of the control theory applied to this system is mandatory. Especially, because it is still challenging to find the right balance between the gains of the
two controllers. In addition there are more imaging modes (e.g. AC SICM & AFM) to be
tested and limitations to be assessed.

The functioning of the force controller in static mode requires that the probe interacts with
the substrate with a force, to build the deflection signal. As anticipated before, cantilevers
are softer than glass micropipettes. However, the ones used in this occasion have are
relatively stiff (k = ~2 N/m) and impede the detection of sub-pico newton forces. The use
of softer cantilever may help the application of the SICM/AFM dual controller on soft
materials like cells. If the electrical impedance of the FluidFM soft probes (Chapter 4.2.2)
is not prohibitive for this kind of application, they might considerably improve the
performance of the presented technique.
7. Weighting micro objects with the FluidFM

Since the advent of Micro Electro-Mechanical Systems, resonators have found a wide range of applications, from their use as frequency references for timing purposes (van Beek and Puers, 2012) to their implementation as highly sensitive mass sensors (Davis et al., 2007). AFM probes are cantilevered resonators and have already been proposed as mass sensors (Boisen et al., 2011). Here the FluidFM is exploited as a mass sensing resonator, together with its ability to precisely lock micro objects at the cantilever aperture with the help of microfluidics (Dörig et al., 2013).

Weighting of micro objects with AFM has found applications in many disparate ranges. Hassenkam and others, working in the oceanography field, were interested in the dissolution of micrometer-sized shields known as coccoliths in different pH conditions, to assess the impact of ocean acidification on its buffer capabilities. What they did was to attach the micro crystals at the end of an AFM cantilever and, based on frequency shift measurement, they assessed the dissolution rate of such fascinating crystals (Hassenkam et al., 2011). Dr. Massimo Vassalli, who was informed about the work on the coccoliths and who knew the hurdles behind the work of Hassenkam, during a visit to the FluidFM laboratory of our group came up with the idea to use the FluidFM as a microbalance and convinced us that the FluidFM showed indeed interesting objects immobilization advantages. In an intensive, two-weeks long visit in Zurich, we brought that idea onto the workbench and performed preliminary measurements towards the use of the FluidFM as a micro-balance. Additionally, the monitoring of the frequency shift would be helpful to monitor the microchannel filling process and to monitor in real time the volume of liquid that can be spilled, either in air or in liquid (e.g. oil droplets in water).
WEIGHTING MICRO OBJECTS WITH THE FLUIDFM

The results are reported hereafter and will be presented in the following article:

*Exploiting FluidFM technology for rapid and precise micro-object weighting.*

D. Ossola, J. Vörös, T. Zambelli, M. Vassalli

In preparation

7.1. Introduction

A micro- or nano-mechanical mass sensor relies on the monitoring of changes in the resonance frequency of a resonator when its mass changes. A multitude of different designs can be found in the literature. By miniaturization of the devices, the sensitivity to mass changes can be drastically improved pushing researchers to race towards the theoretical limit of detection of such systems (Ekinci, 2004). Recently, the use of a single carbon nanotube that resonates at 2 GHz allowed the detection of adsorbed mass changes with a resolution of $1.7 \, \text{yg}^{11}$, which corresponds to the mass of a proton (Chaste et al., 2012). However, these kind of devices need to be operated in extremely controlled environment such as ultra-high vacuum and are usually limited in dynamic range. This means that they allow measurements at extremely high resolutions, but for limited amount of analyte only.

AFM cantilevers are resonators too, and have been extensively used to measure the mass of the adsorbed or attached mass (Boisen et al., 2011; Waggoner and Craighead, 2007). FluidFM cantilevers are still resonators and possess the additional ability to lock micrometer sized objects on their aperture by applying suction. Figure 7.1 illustrates the proposed procedure to weight micro objects with the FluidFM. Beads of different dimensions are deposited on the substrate and picked up with a tipless FluidFM probe. The resonance frequency is determined with and without the bead attached to the cantilever. From the arising shift in resonance frequency, it is possible to estimate the value of the added mass.

The procedure can be used both in air and in liquid environment. However, the sensitivity of the system is considerably reduced in a liquid environment because of the reduced resonator quality factor.

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$11 \, \text{yg} = 10^{-24} \, \text{g}$
Compared to similar techniques, the FluidFM present several advantages that are exploited in the experiments presented in this Chapter. First, the position of the mass along the resonator has an impact on the resonance frequency shift and complicates the interpretation of the measurement (Dohn et al., 2007). This problem is not present with the FluidFM as the micro objects are trapped each time at the same place. In addition, throughput is considerably increased as the cantilever has not to be exchanged between measurements, simplifying comparison of the weighted objects.

7.1.1. State-of-the-art of cantilevered mass sensors

Many devices have reached extremely high sensitivity exploiting micro- or nano-resonators to detect changes in absorbed mass. Usually, the highest sensitivity have been achieved with the use of nano structures such a carbon nanotubes (Chaste et al., 2012). Here the focus is on existing mass-sensing technologies based on micro cantilevered devices.

7.1.1.1. Detection methods & measurement methods

Detection methods:
Different detection methods have been developed to measure cantilever deflection down to sub-angstrom resolution. A comprehensive summary of read-out methods with the analysis of their advantages and disadvantages can be found in the referenced review (Boisen et al., 2011).

The detection methods commonly used for micro cantilevered devices can be reduced in two categories: i) optical beam detection (OBD) and ii) electronic detection. While the first one is well known as it is the most widespread detection method used in AFM, electronic detection is growing interest because of its parallelizing potential.
Optical read-out – The read out used is the OBD method, commonly adopted in AFM. This method gives the highest deflection sensitivity, however it is relatively bulky and is of difficult parallelization.

Capacitive read-out – In this method the cantilever itself consists in one plate of a capacitor. Changes in plate separation upon deflection lead to variation in capacitance, which is measurable. The read-out does not affect cantilever properties, but the fabrication is more complex because the capacitance-detection has to be integrated on the device with CMOS technologies.

Piezo read-out – Comprehend piezo-electric and piezo-resistive read-out methods. The advantage of the piezo-electric approach is the ability to use the same principle for both sensing and actuation. On the other side, piezo-electric material are difficult to implement in standard microfabrication processes and are mostly suitable for dynamic measurements only. The piezo-resistive approach offer great opportunity of parallelization in both air and liquid environment. However, it must be deposited onto the cantilever, thus altering the mechanical characteristics of the sensor.

Measurement methods:
Cantilevered sensors basically offer two measurement methods: static and dynamic. Figure 7.2 summarized the two working principles: in static mode, the cantilever is not excited and the deflection is measured. In dynamic mode, the sensor oscillates at the resonance frequency, whose change is related to different physical interaction, e.g. mass increase.

Most of cantilever-like biosensors are operated in the static mode because operated in liquid environment. Indeed, static deflection is not dampened from viscous effects, thus the liquid does not affect the performance of the sensors. The topic of static, cantilevered biosensors has been extensively covered by the excellent referenced reviews (Carrascosa et al., 2006; Lavrik et al., 2004; Ziegler, 2004) Here I will limit to briefly mention their main characteristics.
Figure 7.2 Two different operation modes of cantilevered mass sensors. **a** Static deflection measurement. Bimorph cantilevers are used to obtain asymmetrical behavior. **b** Dynamic mode. The cantilever is driven at its resonance frequency. Added mass is related to the detected resonance shift. (Tamayo et al., 2013).

In static mode, the cantilever deflects as a result of stress difference on the two sides of the cantilever. The surface stress can be mainly generated in two ways: i) by fabricating bimorph cantilever, i.e. made of two layers of different material. Bulk changes in the material cause them to expand differently, thus bending the cantilever. Extremely sensitive thermometers have been fabricated with this method. ii) by functionalizing one side only of the cantilever. Substances interacting with the surface will change the surface stress and the cantilever will bend.

Driven in the dynamic mode, cantilevered resonators are commonly used as mass sensors by detection of resonance frequency shift. This mode shows its maximum potential in air or in vacuum, where damping effects are minimized, capable of measuring masses on the order of attograms. (Ilic et al., 2001). The biggest challenge of this kind of resonator is their use in liquid, as the $Q$ factor is affected from viscous damping. Recent developments suggested the use of higher resonant modes in liquid to reduce hydrodynamic loading of the cantilever (Basak et al., 2006). Their use has been quite popular in the range of viscosity measurements (Agoston et al., 2005).

Further details on resonant cantilevers are presented in the Theory Chapter 7.1.2.

### 7.1.1.2. Competing technology

The technologies in close competition with the proposed mass measurement method are reported here, while sensors with static detection have been excluded. The scenario can be classified in two categories: i) a big one, comprehending all the modification of the one same technique based on placement or gluing of objects on AFM cantilever, and ii) still a niche, of micro-channeled cantilevers.
AFM cantilevers

The most commonly used method to weight objects is to position them at the free end of an AFM cantilever. Two examples are shown in Figure 7.3. The objects are either immobilized by functionalizing the surface of the cantilever (Figure 7.3a) or with the use of glues (Figure 7.3b).

Figure 7.3: Examples of mass sensing using AFM cantilever. a SEM image of an E. coli bound at the free end of the cantilever. Modified (Ilic et al., 2001). b Coccolith shell glued on a pyramidal probe of an AFM cantilever. (Hassenkam et al., 2011).

In the first case, the cantilever is loaded in liquid and subsequently dried to make the measurement. Even though much less technically demanding, this approach offers no control over number and position of the immobilized particles. On the other side, gluing the selected particles offer the advantage of choosing the particle of interest from a population, and to control the position onto the cantilever.

However, both methods are characterized by a high level of preparation difficulty, which massively limits the throughput of data points.

Fluid-filled cantilever

It is not common to find mass sensing resonator operated in liquid, as the viscosity lowers the quality factor by two orders of magnitudes compared to vacuum. Indeed, in liquid environment, a thin film of fluid is moved with the cantilever, effectively increasing the device mass and this affecting negatively its sensitivity (Waggoner and Craighead, 2007). Burg and others elegantly solved this problem by bringing the liquid inside the cantilevered sensor, similarly to FluidFM cantilevers with the difference that the microchannel is in a closed circuit (Burg et al., 2007). With this solution, the cantilever can be operated in vacuum exploiting high quality factors, but still detecting analytes in liquid (Figure 7.4). This technology combines the advantage of measurement in air with the measurement of biomolecules in a physiological environment by confining the liquid in the cantilever itself.
The authors reported detection of avidin binding on the functionalized walls of the microchannel, as well as detection of single E. coli (Figure 7.4b) with a calculated mass of 110 +/- 30 fg.

Drawback of this technique is its blindness. Indeed, the object of interest cannot be selected from a population.

7.1.2. Theory

7.1.2.1. Resonance frequency of clamped beams

Under the mechanical aspect, an AFM cantilever is a beam that is clamped from one side and is free to move on the other (Figure 7.5).

Spring constant – The spring constant $k$ of a beam like the one presented in the figure above, and by assuming a constant cross section area, is given by the following equation:

$$k = \frac{3EI}{L^3}$$

Equation 7-1

Where $E$ is the Young elastic modulus, $I$ the cross sectional moment of inertia and $L$ the length of the cantilever. For a simple cantilever with square rectangular cross section, $I$ is:
With $W$ being the width of the beam and $t$ the thickness. Inserting it in Equation 1-1 gives the spring constant for a uniform cantilever with rectangular cross section:

$$k = \frac{E W t^3}{12 L^3}$$  \hspace{1cm} \text{Equation 7-3}$$

The FluidFM cantilever differs from a conventional one by the presence of the embedded microchannel, resulting in a cantilever with a hollow rectangular cross section having the following sectional moment of inertia:

$$I_{\text{hollow}} = \frac{W t^3}{12} - \frac{W_c t_c^3}{12}$$  \hspace{1cm} \text{Equation 7-4}$$

where $W_c$ and $t_c$ are the channel width and thickness, respectively. Thus, the spring constant of a hollow cantilever is given by:

$$k_{\text{hollow}} = \frac{E}{4 L^3} \left( W t^3 - W_c t_c^3 \right) = k \left( 1 - \frac{W_c t_c^3}{W t^3} \right)$$  \hspace{1cm} \text{Equation 7-5}$$

**Resonance frequency of clamped beams**—Assuming linear elastic material properties and small deformations together with homogeneous values of $E$ and $I$, the transversal vibration of the beam of Figure 7.5 are described by the following partial differential equation (Butt and Jaschke, 1995):

$$\frac{\partial^2 U(z, t)}{\partial t^2} + \mu \frac{\partial^4 U(z, t)}{\partial z^4} E I = 0$$  \hspace{1cm} \text{Equation 7-6}$$

$\mu$ being the mass per unity length. Equation 7-6 neglects damping effects. The solution of Equation 7-6 is a harmonic function with a position-dependent and a time dependent term:

$$U(z, t) = U_n(z) e^{-i \omega_n t}$$  \hspace{1cm} \text{Equation 7-7}$$

Where $\omega$ is the frequency of the motion and the $n$ subscript denotes the vibration mode. $U_n$ is the amplitude of the vibrational mode. Insertion of Equation 7-7 in Equation 7-6 we obtain:

$$E I \frac{\partial^4 U_n(z, t)}{\partial z^4} - \mu \omega^2 U(z, t) = 0$$  \hspace{1cm} \text{Equation 7-8}$$
Using the boundary conditions arising from the fixed end\textsuperscript{12}, the modal solutions of Equation 7-8 can be written in the following form:

\[ U_n(z) = A_n \cos(\kappa_n z) - \cosh(\kappa_n z)) + B_n \sin(\kappa_n z) - \sinh(\kappa_n z) \quad \text{Equation 7-9} \]

\[ \text{with } \kappa_n = \left(\frac{\mu \omega_n^2}{EI}\right)^{1/2} \quad \text{Equation 7-10} \]

The remaining boundary conditions at the free end reveal the following relationship:

\[ 1 + \cos(\kappa_n L) \cosh(\kappa_n L) = 0 \quad \text{Equation 7-11} \]

The equation above allow only certain discrete values for \( \kappa_n L \) that can be computed numerically. Each solution relates to one particular vibration mode with the following wavelength:

\[ \lambda_n := \kappa_n L = 1.8751, 4.6941, 7.8548, (2n - 1) \frac{\pi}{2} \text{ for } n = 1, 2, 3, n > 3 \quad \text{Equation 7-12} \]

Summarizing the above-described passages, and making use of Equation 7-10, the eigenfrequency of a bending beam is finally given by:

\[ \omega_n = \frac{\lambda_n^2}{L^2} \sqrt{\frac{EI}{\mu}} \quad \text{Equation 7-13} \]

*Effective mass* – For different purposes, e.g. calibration methods like the *Sader* method (Sader et al., 1999), it is relevant to express characteristics of a resonator in terms of *effective mass* and the *effective spring constant*, to allow comparison of individual resonance modes with those of an harmonic oscillator:

\[ \omega_0 = \frac{\sqrt{k_{\text{eff}}}}{m_{\text{eff}}} \quad \text{Equation 7-14} \]

For a cantilever driven in its first mode of any square section (as long as it is uniform), the following equations are inferred from Equation 7-13:

\textsuperscript{12} For a beam fixed at one end and free at the other, the following boundary conditions are valid: At \( z = 0 \), fixed amplitude and inclination \( U(0) := 0; \frac{dU(0)}{dz} := 0 \) at \( z = L \), no torque and no force: \( \frac{d^2U(L)}{dz^2} := 0; \frac{d^3U(L)}{dz^3} := 0 \) (Butt and Jaschke, 1995). Inserted in the following general solution of Equation 7-8: \( U(z) = A \cosh(kz) + B \sinh(kz) + C \cos(kz) + D \sin(kz) \) with \( \kappa = \left(\frac{\mu \omega_n^2}{EI}\right)^{1/2} \) the Equation 7-9 is obtained.
These results assume a homogeneous cross section of the cantilever. A real FluidFM cantilever features pillars in the microchannel to confer structural stability (Chapter 4.2). The effective mass of such a geometry can only be calculated numerically. Such calculations have been performed in COMSOL and gave the same result within the simulation error (Dörig, 2013).

7.1.2.2. Frequency shift from added mass

In general, if a small mass Δm is added at the end probe of the cantilever, a lowering of the resonance frequency is expected, according to the following equation:

$$\omega_0 = \sqrt{\frac{k_{\text{eff}}}{m_{\text{eff}} + \Delta m}}$$  or  $$f_0 = \frac{1}{2\pi} \sqrt{\frac{k_{\text{eff}}}{m_{\text{eff}} + \Delta m}}$$  \hspace{1cm} \text{Equation 7-16}

Therefore, Δm can be deduced measuring the corresponding shift in the resonance frequency. In air environment, in the case of an added point source mass at the very end of the cantilever, and form Equation 7-16, the value of the added mass can be determined based on the measured resonance frequency difference (Berger et al., 1997):

$$\Delta m = \frac{k}{4\pi^2} \left( \frac{1}{r_1^2} - \frac{1}{r_2^2} \right)$$  \hspace{1cm} \text{Equation 7-17}

Where $f_0$ and $f_1$ are the resonance frequencies of the unloaded and the loaded cantilever, respectively.

**Influence of added mass position**

The position of the attached mass onto a cantilever influences the detected shift in resonance. Indeed, both longitudinal and transversal misalignments have an effect on the resonance frequency because it directly affects the effective mass of the oscillating. This effect is easily understood thinking at the first oscillation mode. The vertical movement amplitude, and thus the surface velocity of the cantilever surface, varies with position along the longitudinal axis. Therefore, a mass added at the end of the cantilever experiences higher accelerations, thus more energy is needed to theoretically maintain the amplitude constant, compared to the same mass positioned close to the clamped side of the cantilever. The closer the added mass is to an oscillation node, the less its influence on the resonance frequency is. The same is applied to higher oscillation modes, included the
torsional modes whose eigenfrequencies are influenced by the transversal position of the added mass. As higher modes are used to increase the sensitivity of the device (especially in non-vacuum environment), the distribution of oscillation nodes cannot be neglected.

Unless the mass is fabricated on a precise location on the cantilever, it is difficult and time consuming to position an object with precision and repeatability at the micro- or nano-scales. Dohn and others (Dohn et al., 2010, 2007; Xie et al., 2008) comprehensively studied and described the effect of sample position to resonance frequency, offering the framework to correct for position errors. However, sample positioning remains a hurdle because correction for misplacement implies the precise knowledge of its position, which is not always a readily available information.

7.1.2.3. Cleveland cantilever stiffness calibration

The Cleveland cantilever calibration method adds small, known masses at the free end of the cantilever to precisely deduce the spring constant (Cleveland et al., 1993).

Starting from Equation 7-16, the following relationship is found:

$$\Delta m = M = k \frac{1}{(2\pi f_0)^2} - m_{eff}$$

Equation 7-18

With $M$ defined as the end-added mass. The above equation shows that the added mass in function of $(2\pi f_0)^2$ has a linear plot whose slope is the spring constant $k$ with the ordinate intercept being $-m_{eff}$.

Assuming the model being correct, only one added mass $M$ is enough to determine the spring constant and the effective mass (Cleveland et al., 1993):

$$k = (2\pi)^2 \frac{M}{\left(\frac{1}{f_1^2} - \frac{1}{f_0^2}\right)}, \quad m_{eff} = M \left(\frac{f_1^2}{f_0^2} - \frac{f_1^2}{f_0^2}\right)$$

Equation 7-19

7.1.2.4. Phase-Lock-Loop (PLL)

When working with resonators, it is commonly of interest to drive the resonator always at its resonance frequency. However, external factors can influence the resonance frequency of a sensor (e.g. probe-sample interaction forces (Rabe et al., 1996)). In order to lock a resonator at its resonance frequency, a PLL can be used.
A PLL is a control system that controls the phase between the excitation and the output signal of a resonator, i.e. the phase is actively adjusted at a user-defined value. Figure 7.6 shows how the amplitude and the phase of a driven harmonic oscillator are dependent on the frequency. Interestingly, at resonance frequency, the phase shows an inflection point and the slope is at its maximum. This means that the smallest change in phase correspond in a big variation of the amplitude, constituting a very sensitive signal to deviations from the resonance frequency.

The PLL produces a reference signal whose phase is related to that of the system. If the resonance frequency of the system changes, the PLL detects a change in the phase and is designated to accordingly adjust the drive signal frequency in a way that the required phase is restored. By doing so, the system is constantly driven at its resonance frequency.

A basic PLL consists of a phase detector, a variable frequency oscillator and a feedback path. In our case, a PI controller controls the feedback path (Figure 7.7). The phase detector compares the phase of the signal output with the phase of a reference signal, generating an error signal that is fed to the PI controller. The latter reacts to minimize the previously calculated error by changing the frequency of the oscillator.

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13 The PLL featured in the Lock-In amplifier of Zurich Instruments was used.
With this method, the phase is maintained at the value where resonance occurs. If the PI is correctly tuned so that the error signal is stably minimized, the resonance frequency can be tracked by monitoring the output of the oscillator.

### 7.2. Objectives and Challenges

It is our objective to demonstrate the added value in terms of measurement repeatability and simplicity conferred by the use of tipless FluidFM cantilever to measure the mass of micro objects.

The strategy envisioned was to select optically micro objects with an inverted microscope and to target them with the aperture of the FluidFM probe. Once in position, suction was applied and the probe withdrawn when immobilization occurred. To overcome electrostatic interaction, which is not screened in air, objects having spherical shape have been used so that the aperture of the probe would be sealed and effectiveness of suction force maximized. The spheres had to be big enough to be seen optically, so we chose spheres of 5 μm diameter, that would fit the 2 μm aperture of the FluidFM tipless probe too.

From the theory, one finds that 1 Hz in frequency change shall correspond to an added mass at the end of the cantilever of $10^{-12}$ g (1 pg). The mass of borosilicate spheres with radius around 1 μm is the range of hundreds of pg. Thus, we needed to be able to measure a resonance frequency shift in the order of 100 Hz.

Two detection methods of frequency shift of the added mass have been tested: i) by means of frequency spectrum acquisition and ii) by tracking the resonance frequency with PLL electronics. Both cases are equivalent, but the Phase-Lock-Loop (PLL) has the ability to track changes in frequency in real time. The performance of the two methods is affected in both cases by the physical characteristics of the sensor, i.e. the quality factor and the resonance frequency. In addition, the performance of the PLL is a compromise between speed (i.e. bandwidth) and noise. Fast phase tracking has an increased noise floor because of the widened bandwidth, whereas slower tracking increases the signal to noise ratio. To understand the order of magnitude of the measurable mass, I report here a preliminary measurement done to estimate the PLL sensitivity of our system, summarized in Table 7-1.
Table 7-1: Summary of the PLL performance in air and in water. The standard deviation of the measured value denotes the noise-floor of the detection. Higher bandwidth allow for fast tracking but increases noise, whereas a small bandwidth allow to maximize the sensitivity.

From the numbers above presented, one understands that it is possible to reliably detect changes in frequency of 10 Hz also in liquid. However, in liquid, long-term fluctuations may be bigger than the standard deviation presented in Table 7-1. Fluctuations can arise from changes in temperature, fluid motion etc. As a consequence, the frequency change can be detected transiently only. In addition, mass sensing in liquid poses a range of questions that need an answer in order to correctly estimate the mass of the added value, as the effect on the hydrodynamic resistance of the added mass (Brumley et al., 2010).

7.3. Methods

Measurement setup
Tipless, FluidFM cantilevers with 2 μm circular aperture were used as received. When filled, ultrapure water (Millipore, Darmstadt, Germany) or Squalene oil (Sigma-Aldrich, Montana, USA) has been used depending on the experiment. The cantilevers were received glued on a Cytoclip and were used in combination with a FlexAFM system (Nanosurf, Liestal, Switzerland) and a programmable pressure controller (Cytosurge, Zurich, Switzerland). Where necessary, a Lock-In amplifier (Zurich Instruments, Zurich, Switzerland) has been used in Phase-Lock-Loop (PLL) mode when necessary. The cantilever was acoustically excited through a high-frequency piezo positioned in the AFM’s probeholder. The excitation signal was generated within the Lock-in amplifier and connected to the piezo excitation input of the FlexAFM controller. Excitation amplitude was set to obtain an oscillation signal amplitude of about 800 mV corresponding to about 90 nm oscillation amplitude.
Beads trapping and frequency shift measurement in air environment

5 μm, borosilicate glass beads (SPI supplies, West Chester, USA) have been deposited as received on the glass bottom of a WillCo dish (WillCo Wells, Amsterdam, The Netherlands) with the help of a needle. The beads have a reported density of 2.5 g/cm³ and a mean diameter of 5.1 ± 0.5 μm. The trapping protocol consisted in approaching an isolated bead with the FluidFM probe with a setpoint of ~20 nN. When in contact, - 800 mbar are applied with the pressure controller. Under optical control, the FluidFM aperture is carefully aligned with the bead. When the bead is trapped (gentle tapping with a finger on the AFM’s table can facilitate immobilization), the cantilever is lifted and retracted by a distance enough to avoid long-range interaction forces. To release the bead, the cantilever is brought in proximity of the substrate (~10 μm) and maximum overpressure (> 1000 mbar) is applied with the pressure controller or with the help of a syringe. Due to electrostatic force, the bead is attracted onto the glass surface again. Proximity to the glass substrate is needed to avoid reattachment of the bead to the cantilever body. In the case that overpressure was not enough to release the trapped bead, or if the bead stucked onto the cantilever, strong vibrational excitation of the cantilever (5 V excitation amplitude, resulting in >1000 nm oscillation amplitude) was always successful to clean the cantilever.

Frequency spectrums were acquired before and after catching a bead, at a distance from the substrate where long-range forces are not affecting the measurement. Frequency spectrums were fitted with a Lorentzian to extrapolate the resonance frequency shift.

For calibration purposes, the exact radius of the cached beads is measured by imaging the beads locked at the aperture of the FluidFM tipless probe onto a TGT1 calibration grid (MDT Co, Zelonograd, Russia) featuring an array of sharp probes with 10 nm nominal probe radius (see Figure 7.13b). A section in correspondence of the sphere diameter is extrapolated and fitted with the equation of a circle using the software Gwyddion (Nečas and Klapetek, 2011).

PLL frequency shift tracking

For continuous tracking of the resonance frequency, I implemented a PLL driven by a PID controller. In addition, the PLL tracking system is more sensitive than the acquisition of the frequency spectrum. This method was applied to follow the resonance frequency of the cantilever, while filling the cantilever in air, and while dispensing oil droplets in water.
7.4. Results

7.4.1. Effect of internal pressure on the resonance frequency

The resonance frequency of the FluidFM cantilever can be influenced by the application of a pressure and this effect should be evaluated to ensure a picogram-sensitive measurement protocol able to deliver 10-20% reliability. In particular, the presence of an internal pressure induces an additional stress to the microstructure that alters its elastic constant. The precise calculation of the cantilever shrinking (or dilation) due to the presence of an internal pressure, can only be performed by numerical methods. However, a rule-of-thumb estimation of the expected behavior can be obtained describing the FluidFM cantilever as a thin cylinder of external radius $R$ and thickness $\delta$. For this simplified geometry, an analytical expression exists that links the change in width to the internal pressure $P$ (Hearn, 1997):

$$\delta R = \frac{PR}{4\delta E}(2 - \nu)R$$  
Equation 7-20

where $E$ and $\nu$ are the Young modulus and Poisson ratio, respectively. If the radial dimension is changing linearly with $P$, the area moment of inertia $I$ of Equation 1-1 does change as $P^4$. The elastic constant $k$ is thus expected to behave as $P^4$ too and, in turn, the resonance frequency should be $\propto P^2$ (see Equation 7-14). To verify the expected behavior and to quantify it, the frequency change was measured as a function of the applied pressure. FluidFM cantilevers were induced to oscillate at their resonance frequency in air environment. The frequency shift was followed with the PLL. The Cytosurge pump driver was programmed to perform a cycle between -800 mbar and + 800 mbar.

Figure 7.8 shows the result of such experiment for a cantilever with open ending (Figure 7.8a) and with the aperture closed with a micro bead (Figure 7.8b). The above described prediction of a resonance frequency dependency proportional to $P^2$ is confirmed in both measurements. Indeed, the error with a second order polynomial fit is negligible. Interestingly, the variation of frequency is much less for the open cantilever compared to the closed one. Indeed, an open ending in the cantilever impedes complete building of the pressure in the microchannel. In the case of the open cantilever, a difference of about 4.5 Hz in resonance frequency was measured with + 800 mbar applied. With the closed microchannel, the increase in resonance frequency was about 15 Hz for the same pressure difference. It is important to mention that a significant source of error is given by the pressure value. Two uncertainties have to be considered: i) the pressure is measured
at the output of the pressure controller and ii) the bead positioned at the aperture of the FluidFM cantilever might not constitute a tight seal, lowering the real pressure values.

Figure 7.8: Dependence of the resonance frequency on the internal pressure of an hollow cantilever. Dashed line represents the fit with a second order polynomial. Error bars not indicated because smaller than the symbols. a Measurement performed with open cantilever aperture. b Measurement performed with closed aperture, i.e. with attached bead.

Considering the above presented results, an effective spring constant $k_{\text{eff}}$ variation in the order of $10^{-4}$ N/m is inferred, which is practically negligible$^{14}$

COMSOL based FEM simulation was used to confirm the behavior observed experimentally. The static case was only evaluated, because the study of the frequency spectrum is too demanding in terms of computational time. The cantilever was modelled with the geometry as similar as possible to the reality, with a microchannel internal pressure of $-1000$ mbar. Figure 7.9 shows the deformation of cantilever’s cross section in consequence of underpressure in the microchannel, which shall be the source of the frequency dependency on the pressure. However, when calculating the spring constant based on simulated load, we obtained $k = 2.954$ [N/m] for the cantilever without pressure and $k = 2.861$ [N/m] for $-1000$ mbar internal pressure. Even though a softening of the spring constant under negative pressure is confirmed, the absolute value reveals significant discrepancy compared to the experimental results ($10^{-1}$ versus $10^{-4}$). At this stage of the investigation it is not possible yet to completely explain this incongruence,

$^{14}$ E.g: Assuming $k_0 = 2 \left(\frac{N}{m}\right)$, $f_0 = 75000$ [Hz] gives $m_0 = 9.05 \times 10^{-12}$ Assuming no change in effective mass upon pressure application, and using Equation 7-16 for 15 Hz difference in resonance frequency gives: $k_1 = 2.0008 \left(\frac{N}{m}\right)$. 

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however, a plausible explanation resides in difference in real and simulated pressure in the microchannel as we have no means to measure the pressure in the microchannel.

To conclude, the dependency of $k$ on the applied pressure is negligible in the framework of this project.

### 7.4.2. $\Delta f$ during cantilever filling

The frequency change upon mass change can be used to detect filling and filling dynamics of the cantilever. Figure 7.10 shows a reference experiment: the cantilever was filled with water in air environment. The frequency change was tracked with the use of the PLL circuitry of the Lock-In amplifier.

As the liquid reaches the free standing cantilever, a drop in the resonance frequency is observed. Interestingly, the frequency drops with a square root behavior accordingly to Equation 7-14.
The feature demonstrated with this experiment can help when working in situations for which it is needed to know the exact moment of cantilever filling (e.g. to stop pressure before exit of the liquid). Or, to know that the liquid is indeed advancing inside the channel: this is especially useful when working with small apertures or in filling-unfavorable conditions (e.g. hydrophobic coatings, viscous mediums, etc.). Furthermore, it can be used in future as a signal for automated filling.

7.4.3. PLL in liquid, dispensing of oil droplets

After assessing the operation of the PLL in air, the performance of the PLL was tested in liquid environment, by releasing squalene oil droplets form the FluidFM cantilever. The cantilever was used as received. 100 mbar pressure were applied during the measurement presented in Figure 7.11. The phenomenon revealed to be repeatable, as it was the volume of the spilled droplets. By fitting Equation 7-14 on the frequency reduction (Figure 7.11b), the square-root decay is visible. Indeed, the volume is supposed to increase linearly because of the constant flow, thus the mass. Correspondingly, the frequency decreases with a square root slope.

It is however difficult to assess the real mass of the droplet and to discern it from the apparent mass arising from the liquid amount that is dragged together with the droplet.
7.4.4. Mass position dependence of resonant frequency shift

The shift in frequency experienced by the cantilever under vibrations depends on the exact position of the bead and there is strong dependence on both lateral and axial displacement of the attached mass, with respect to the free end (Dohn et al., 2010, 2007; Xie et al., 2008). Figure 7.12 proposes a representative result in which three beads are sequentially cached. Frequency spectrum has been measured at the beginning of the experiment, and after each attached bead. When releasing the beads far away from the substrate, the beads often get re-attached onto the cantilever by electrostatic interaction. The procedure has been repeated twice and eventually the cantilever was cleaned by strong vibrational excitation.

Interestingly, the second bead is producing a smaller reduction in frequency, as the third one that has an even smaller effect even though it is closer to the end of the cantilever. The result confirms what already reported in literature about the effect of the position of multiple particles on the resonance frequency of a cantilever (Dohn et al., 2010). The effect could be observed better by measuring the frequency spectrum one bead at a time.

Figure 7.11: Oil spilling in liquid environment with online PLL frequency tracking. a Frequency behavior during spilling events of several oil droplets, highlighting the repeatability of the phenomenon. b Zoom into (a) of the growth of a single droplet. Equation 7-14 is fitted on the data, to highlight the square root decay of the frequency upon linear increase of the volume, thus mass. c-k Optical images of the oil droplet increasing in volume at the aperture of the cantilever and eventually detaching.
7.4.5. Calibration an bead mass determination

Once the properties of the FluidFM have been addressed with the above-proposed preliminary experiment, it is presented here the measure of the mass of borosilicate beads of 5 μm in air, grasped by suction force upon selecting them on a glass substrate. The cantilever used was a tipless FluidFM cantilever with a circular aperture of 2 μm diameter which was calibrated with the Sader method (Sader et al., 1999) to give \( k = 2.15 \text{ N/m} \). The frequency spectrum was acquired before and after immobilization of the bead (Figure 7.13a). The resonance frequency was determined by fitting a Gaussian function on the top part of the resonance peak. With Equation 7-17 the mass of the bead was determined.

To validate the technique, the obtained result has been compared with a more classical approach: mass calculation via material density and volume. Since one can consider the beads having perfect sphere geometry, their radius was measured by means of atomic force microscopy imaging. Before releasing, the bead has been imaged with a TGT1 calibration grid featuring an array of sharp probes. The result is shown in Figure 7.13b, where the spherical geometry of the trapped bead is easily recognizable. The beads were imaged in AFM dynamic mode to reduce the interaction forces and avoid displacement of the bead during imaging.
Figure 7.13: Methods to determine the mass of the caught bead. a Representative frequency spectrum shift upon bead fixation. Solid line is the spectrum acquired before catching the bead and the dashed line is the frequency spectrum of the cantilever with trapped bead at the aperture. b Convoluted image of the bead scanned in dynamic mode over an array of sharp probes.

The correlation of the two mass determination methods is presented in Figure 7.14, where the mass extrapolated from the geometry and with the help of AFM imaging are plotted against the mass values calculated from the resonance frequency shift.

Figure 7.14: Correlation of the a priori mass expectation calculated from density and volume of the sphere, measured with an AFM calibration grid and the mass calculated based on the frequency shift. Dashed line represents $y=x$ function. Inset shows an example of bead diameter extrapolation from AFM image.

The mass values obtained with the two methods lies on a straight line indicating good correlation and validity of the measurement setup. In addition, to further confirm the accuracy of the measurements, we used the Cleveland method (refer to Chapter 7.1.2.3) to extrapolate the cantilever spring constant. The plot of the added mass versus $\omega^{-2}$ is
shown in Figure 7.15. The slope of the obtained linear regression is 2.30 N/m, in agreement with the value of 2.15 N/m obtained with the Sader method.

![Graph showing the obtained linear regression with a slope of 2.3 N/m](image)

Figure 7.15: Following the Cleveland method and applying the relationship of Equation 7-18 a linear plot is obtained, whose slope is the spring constant. Extrapolated \( k = 2.3 \text{ N/m} \)

With this experiment, I measured the performance of the FluidFM both in terms of mass sensitivity and in terms of facilitated procedure. In air, the frequency shift can be tracked with sub-hertz resolution, meaning that sub-picogram masses are also detectable.

### 7.5. Conclusion & Outlook

The added value of the FluidFM system for high resolution and accurate mass sensing of micro-objects is demonstrated twice: i) in terms of added mass positioning precision and repeatability and ii) easiness of operation, i.e. enhanced throughput of datapoints and iii) selection of the object of interest by optical microscopy means.

As pointed out before in this Chapter, the resonance frequency shift is sensitive of the position of the added mass. With the FluidFM this problem does not subsist, as the micro object is always trapped at the same position, i.e. at the circular aperture. In addition, the immobilization procedure is much simpler and requires only a couple of minutes per bead, comprehending the force controlled approach, suction application and withdraw of the probe from the substrate. To give an idea on the throughput of the technique, the 8 beads presented in Figure 7.14 have been weighted and imaged in a frame of about 3 h. Compared to the laborious procedure of fixing the objects of interest on conventional cantilevers, this represents a major breakthrough. Indeed, published studies on mass measurement on AFM cantilevers usually show a number of 5 to 20 measurements
(Hassenkam et al., 2011; Ilic et al., 2001) because of the extremely time consuming protocols.

Here, it has been presented the weighting of micro-object with well-defined geometry in order to maximize the immobilization force, but other model systems may have to deal with objects with irregular geometry. The problem that arises with uneven geometries is that the immobilization force is lowered because the aperture of the FluidFM cantilever is not perfectly sealed. Therefore, it can become difficult to overcome adhesion forces, especially in a dry environment. Surface modification to lower electrostatic forces is an option, or operation in liquid.

The second challenge is the sensitivity of the setup. The liquid environment offers the most interesting scenario in terms of biosensing. However, one would have to cope with the reduction of the $Q$ factor which challenges the high sensitivity of micro resonators. Theoretically, it should be possible to immobilize the object of interest in a liquid environment and transport it through the liquid-air interface in order to measure the resonance frequency in air where the $Q$ factor is less prohibitive.

In addition, along the line of the microchanneled resonator presented by Burg and others (Burg et al., 2007), refer also to Chapter 7.1.1.2, The FluidFM could be used to draw a solution form a reservoir containing some analyte, withdraw the probe to the gas phase and subsequent acquisition of the resonance spectrum. The procedure can further be exploited by using pyramidal FluidFM probes with sub-micrometer apertures that are ideal to immobilize nanometer-sized objects like bacteria or nano-crystals.
8. Conclusions and Outlook

In the framework of this thesis, I demonstrated the ability to measure ionic currents in the picoampere range through microchanneled AFM cantilevers. The properties of the FluidFM such as electrical impedance and presence of streaming potential were assessed in the first phase of the project and related to conventional glass micro pipettes. In general, the FluidFM system would benefit from an increased probes portfolio. Parameters like aperture size and cantilever spring constant strictly define where the FluidFM can be applied.

The novel experiments presented in this thesis have the form of a proof-of-concept and beside showing the potential of the FluidFM to answer open questions, they highlighted limits and problems, which are hereafter summarized, divided per application.

**Prototyping with FIB**

The FIB has proven to be a powerful technique on two sides: i) as an inspection tool to understand and identify microfabrication defects of the microchannel and ii) to fabricate application-driven apertures enabling to explore novel applications for the FluidFM. Following this strategy, we used FIB-customized FluidFM probes i) to show how the reversible immobilization of microbial and mammalian cells offered by FluidFM probe is valuable to measure adhesion forces, ii) to perform intra-nuclear injection with syringe-like apertures and iii) to perform patch-clamp recordings in whole-cell configuration using high aspect-ratio cylindrical apertures milled in the apex of thick-walled pyramidal cantilever. For all the proposed aperture designs, we provided detailed fabrication protocols for efficient utilization of FIB time.
In general, FIB-customized probes are still showing sporadic clogging problem during their use in experiments. The roots of the clogging has been individuated with FIB sectioning, highlighting presence in the microchannel of extraneous micrometer-sized particles. Syringe-like probes instead, still exhibit a high percentage of clogging (some batch reached 90% of clogged probes). It is plausible that deposits of yet unknown nature sediments in the corners of the hollow pyramid.

FIB fabrication has been chosen to define aperture geometries apt to particular tasks. Once that the shape has been defined and the application field has proven to be of broad interest, the fabrication is supposed to shift towards conventional lithography and microfabrication techniques, on wafer scale. FluidFM apex apertures with defined specifications already underwent this process, while syringe-like FluidFM probes still are under development (Berenschot et al., 2012).

**Force-controlled patch-clamp**

Using the patch-clamp FIB-modified FluidFM probes, we established a novel protocol to perform whole-cell patch-clamp. In a first phase, we demonstrated the possibility to control the membrane potential and thus to gate hERG and Na\(_v\)1.5 ion channels with the FluidFM. In particular, recordings of Na\(_v\)1.5 currents proved the ability to record fast ionic currents (sub-milliseconds), even though highlighting the limits of the setup in terms of voltage clamp, which lies in strict relationship with the high-impedance of FluidFM probes. Indeed, high access resistance together with low seal resistance impedes efficient following of the transmembrane potential, leading to misreading of rapidly changing currents. We envisioned to solve this problem by the integration of the recording electrode in the microchannel itself, to bypass the high electrical impedance of the microchannel. The electrode can be integrated following to different strategies: i) during manufacturing of the FluidFM probes, through standard microfabrication techniques (SmartTip is working on this solution) and ii) a posteriori, with different deposition techniques, e.g. conductive gold film from highly packed gold-nanoparticles deposition\(^\text{15}\).

Successively, I extended the patch-clamping protocols to isolated mice cardiomyocytes, with the aim to exploit the highly sensitive force detection of the AFM. Indeed, we demonstrated for the first time reported simultaneous mechanical and electrophysiological correlation through the same interrogation probe. In this context, electrical stimulation of

\(^\text{15}\) A pilot study has been carried out in the framework of a semester project by Kristian Cujia. Details: [https://www1.ethz.ch/lbb/Publications/Diploma_semester_works/GNP_Conductive_FIlms_based_on_Gold_Autometallography](https://www1.ethz.ch/lbb/Publications/Diploma_semester_works/GNP_Conductive_FIlms_based_on_Gold_Autometallography)
cardiomyocyte was shown with the FluidFM and the resulting transmembrane ionic currents together with the generated transversal force were recorded.

Temporal stability of the recording was assessed during whole-cell patch-clamp experiments of \( \text{NA}_v1.5 \) ion-channels without being conclusive yet. However, the gentleness of the procedure was confirmed by the viability of the cardiomyocytes upon probe withdrawal. While these kind of cells do not survive the conventional patch-clamp procedure, no visible damage was observed when retracting the FluidFM probe after having recorded in whole-cell configuration. This result allowed to demonstrate the feasibility to inject into isolated cardiomyocyte without compromising their integrity. To our knowledge, this result has not been achieved in published works yet.

In absence of gigaohmic seals, the technology is not ready yet for its employment to answer open questions in electrophysiology. However, we identified the source of the problem that relies in the geometry of the FluidFM pyramidal probe, which do not offer enough surface area to build gigaohmic resistances. Work is in progress to solve this issue by mimicking the seal friendly geometry of glass micropipettes, i.e. featuring a hollow circular aperture of high aspect-ratio. Prototypes have already been fabricated by SmartTip. However, they have not been tested for gigaseal formation yet.

Even though hindered from the above mentioned problematic, the FluidFM in this configuration is a novel tool that allows interrogation of mechanophysiology systems. Indeed, the transmembrane potential can be controlled as well as the exerted force, simultaneously. In addition, we highlighted the automation potential of the system, that could lead to a force-controlled patch-clamp platform which does not need highly skilled operators.

**Force-controlled SICM**

The SCIM and patch-clamp share the same sensing probe. in Chapter 6 was shown how the FluidFM is capable to perform SICM too. Advantages and drawbacks of the different SICM modes have been addressed and correlated to the FluidFM characteristics. The force sensing capabilities of the FluidFM proved to improve the performance of DC SICM in both gentleness and imaging speed. Imaging up to 30 μm/s have been shown not to deteriorate the image quality, thus meaning that the limits have not been pushed yet. The performance of the SICM and AFM hybrid has to be proven on living cells, as it’s the environment for which the SICM has been developed for. Two important aspects can be tackled to bring substantial improvement in imaging capabilities: i) softer cantilevers would
increase force sensitivity and thus guaranteeing lower forces during imaging. ii) increase in Z-piezo range. In fact, at present, the FlexAFM features 10 μm Z-range, which is not enough to image the whole body of the majority of mammalian cells.

I proved that the cooperation of SICM and AFM controllers may lead to a profitable imaging mode. However, I only analyzed an over-simplified model in the framework of a proof-of-principle strategy and only superficially characterized the novel imaging mode in the experimental setup. The topic has to be deepened in order to understand the dynamics of the two imaging modes when run in a parallel wise. An accurate model has to be studied first from a theoretical point of view, especially in the frequency domain. Exploiting of the complete potential is otherwise not achievable. Other imaging hybrids are worth to be investigated too, e.g. AC SICM with AC AFM or DC SICM with AC AFM to cite some.

The ability of the AFM to perform both SICM and patch-clamp allows to perform patch-clamp in a precise way, i.e. by positioning the probe with nanometer precision on interesting features, that can be scanned prior to the patching, in SCIM mode. This strategy is also knows as smart patch-clamp and its implementation with the FluidFM should be possible without encountering major obstacles.

**FluidFM as Microbalance**

A new protocol to measure the mass of micro-sized objects was established. With the use of tipless probes, microspheres of borosilicate were successfully immobilized and subsequently separated them from the glass substrate, in air environment. The measure of the mass was then validated based on the resonance frequency shift by precise determination of the volume of the spheres by means of AFM imaging with a calibration grid. The result proved that the measurement is both accurate and precise. Weighting in water proved to be feasible only with PLL tracking of the resonance shift. However, the interpretation of the data is not straightforward and need further investigation.

The simplicity of the immobilization mechanism conferred by the suction possibility of the FluidFM probes showed once again its usefulness to measure the mass of trapped micro-objects. In this thesis I applied mass sensing protocols to spherically shaped particles only. However, it is of major interest to measure mass of arbitrarily shaped objects. The ability to immobilize these kind of objects still has to be investigated, both in air and in water. Additionally, the possibility to trap objects in liquid environment and detect resonance frequency shift in air would be interesting to be considered.
9. Appendix

9.1. Electrical Impedance

Reflective coating

Any AFM cantilever, whose deflection is sensed via an OBD system needs to have its backside coated with a reflective material. Otherwise, the reflected laser may be too weak for correct AFM performance. The first prototypes of the FluidFM had the whole backside coated, the inlet too (Figure 9.1a).

![Figure 9.1: a Schematics of a FluidFM probe’s backside entirely sputtered with a reflective Cr/Au layer (shown in yellow). b Deposition of the reflective coating by using a shadow mask: the gold is locally deposited onto the cantilever only.](image)

This kind of coating is simple because it does not involve the use of masks, but caused a drastic increase of capacitance that is reflected in the EIS by lowering the cut-off frequency in sub-hertz range (Figure 9.2a). Indeed, by fitting the EIS result with a simple equivalent circuit model\(^\text{16}\) indicated a capacitance of about 76 nF (conventional glass pipettes show between 5 to 15 pF). Having charging transients lasting several seconds (Figure 9.2, inset), made the use of these probes for electrophysiological purposes impossible.

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\(^{16}\) It is assumed that the microchannel and solution resistance are the only non-negligible resistances. The microchannel is modelled with a resistor and a capacitor in parallel, whereas the solution is modelled with a resistor only. I.e. an R[CR] circuit.
The evolution of FluidFM probes envisioned the coating with a reflective layer of the cantilever only, with the use of a so called shadow mask (Figure 9.1b). The EIS confirmed our assumptions and the bandwidth increased by several orders of magnitudes, close to 1 kHz (Figure 9.3).

As the shadow mask process was only available late in the project, in the first two years of experiments we etched the gold and worked without reflective layer. This obliged us to work without force control but I could however start to investigate the behavior of FluidFM pyramidal probes in conventional patch-clamp approach.
In conclusion, the FluidFM setup behaves as a low pass filter with a cut-off frequency around 600 Hz. The Bandwidth can be further increased by isolating the microchannel membrane. For this purpose, we added a droplet of epoxy glue on top of the FluidFM probe. The resulting EIS is shown in Figure 9.4. In this case, the capacitance is further reduced to about 5 pF, well in range with values showed by conventional glass micropipettes.

![EIS of FluidFM probe with the top membrane of the microchannel isolated with an epoxy-glue film. The capacitance of the system is further reduced to 4.4 pF only.](image)

The bandwidth of the system can be further reduced by decreasing the R*C product\(^{17}\), that defines the time response constant. Figure 9.5 shows the EIS in the case where the ionic strength of the solution has been increased.

![Overlap of three EIS measurements of a FluidFM probe. Blue data refers to the normal conditions, i.e. using 150 mM KCl solution. Full circles is the amplitude, empty circles the phase. Solid line corresponds to the model circuit fit presented before. Red curves have been measured in 1 M KCl concentration. Full circles are amplitude](image)

\(^{17}\)\(\tau = R \times C\) is the time constant of an RC circuit and is the time required by the capacitor to charge to 63.2 percent.
measurements, empty circles are the phase. Solid line is the model fit. Green curves have been measured with 1 M KCl and with the top membrane of the microchannel isolated with glue.

Using KCl at 150 mM concentration, a plateau at $Z \sim 25$ $M\Omega$ and a slope of -20 dB/dec characteristic of a capacitive element is visible. We changed the 150 mM KCl solution by a 1 M KCl solution, expecting a lowering of the impedance at low-frequency, and no change in the capacitive curve at higher frequencies should occur. The pink curve in Figure 9.5 indeed show this behavior. As stated before, the bandwidth can be further increased by isolating the microchannel's top membrane. The green curve in the EIS of Figure 9.5 confirms this assumption where the -20 dB/dec slope shifts to higher frequencies.

9.2. Data acquisition environment

As pointed out in the previous Chapters, the setup has been developed to perform low noise patch-clamp experiments. The same setup must be suitable for scanning ion conductance microscopy (SICM) too. Therefore, we were obliged to create a different software written in LabView. The underlying challenge is to write a routine to synchronize the data generated from the AFM with the electrophysiology data of the patch-clamp amplifier, for simultaneous recording of force and current signals, while controlling AFM or SICM. The difficulty arises from the high level of synchronization needed between generation and acquisition of the electrophysiological protocols, with sub-millisecond precision. Also, the different signal types of electrophysiology experimenting and of the AFM require different acquisition architecture, which complicates their synchronous acquisition.

LabView (National Instruments (NI), Austin, Texas), allows to efficiently create so called Virtual Instruments (Vis), in a graphical programming interface that is merged with the end graphical user interface of the program. Almost all hardware manufacturer supports LabView, making it an efficient solution when building test-setups in a laboratory context where data usually have to be acquired and analyzed and a high degree of flexibility is needed.

The NI experimental setup comprehends two NI data acquisition PCI cards and relative BNC connector boxes. The acquisition cards both feature analog inputs and analog outputs (two) channels.

The development, architecture and capabilities of the software written is described in detail in the Appendix
Patch-Clamp LabView program

Two patch-clamp amplifier were at disposition: the PICO 2, a completely digital, computer controllable and supported from different simple free patch-clamp software and the EPC 7; an analog amplifier that needs independent data acquisition hardware. The software for this kind of amplifier is expensive and does not offer the flexibility needed to include the AFM in the measurement environment. We therefore wrote our own LabView-based software that allowed us to perform basic Patch-Clamp experiments. Hereafter we give a short overview of the program capabilities and functions.

The nature of electrophysiology experiments require a generation of series of voltage pulses. In the meantime, the acquisition of the measured current must happen, simultaneously and synchronously. The synchronization is triggered by hardware clocks on the NI board so that each generation and acquisition data packages are generated/requested at the same time (1 ms precision). This kind of data acquisition is not-continuous, it happens in a discrete way, a package of data per time. On the contrary, the acquisition of the AFM data have to happen continuously, as the one wants to monitor signals as deflection and piezo position without interruption. The different functioning of the two acquisition tasks, obliges to run them on different acquisition cards. The NI acquisition cards clocks are shared via a Link cable.

Architecture – Figure 9.6 shows the diagram of the virtual instrument for patch-clamp. The software starts with an initialization step, where the data acquisition cards are initialized, and the buffer memory is allocated. After the initialization, the data acquisition can be started anytime upon user input. Two different acquisition process happen simultaneously: the first, acquires and generates data of the electrophysiology setup, the other acquires the data of the AFM.
In the data processing step the newest data is extracted from the buffers and analyzed or averaged for visualization purposes. Eventually, the data is saved in a text file using ASCII standard.
Figure 9.7 shows the front panel of the written program. The user interface is divided in the input area and the visualization area. Hereafter, the basic functions of the program are shortly described.

- **Design of voltage protocols**: voltage transmembrane currents are conventionally studied by a sequence of voltage steps. The user can design such protocols with the in-build tool (Figure 9.8).

![In-built tool for voltage-steps protocol design.](image)

- **Electrical resistance calculation**: The resistance of the probe can be monitored in both DC mode and using square voltage pulses. In the first case, the junction potentials have firstly to be compensated. The software simply calculates the resistance based on Ohm’s law by dividing the applied voltage with the current. However, the most common way to record the resistance of the probe during a patch-clamp experiment is by applying voltage square pulses and by monitoring the current. The advantage of this method is two-fold: first, the calculated electrical resistance based on the difference between the pre-pulse and the end-pulse currents is not dependent on junction potential drifts. Second, the resulting transient capacitance peaks communicate information on the sealing process and on the access in whole-cell configuration (Chapter 5.1.1).

- **Zapping function**: to get access to the whole-cell configuration after gigaseal stabilization, electrophysiologists commonly apply a short, but high amplitude voltage pulse, known as zap. The electric field across the patch should increase causing membrane breakdown.

- **Simultaneous AFM and Electrophysiology data acquisition and correlation**: The program continuously collects AFM data (deflection and piezo position). The data is synchronized with the electrophysiology data and can be saved together.
SICM LabView program:

The above presented program had the function to allow a proof of concept of force-controlled patch-clamp. In the framework of the SICM project (Chapter 6), we were interested in showing the simultaneous operation of AFM and SICM. As the patch-clamp LabView program lacked in flexibility, we wrote a dedicated software for this purpose. Main goal of this program is to synchronize data acquisition between the AFM controller and the patch-clamp amplifier for simultaneous recording of force and current signals, while controlling the AFM for SICM. The latter means that the main AFM functions are taken over by the LabView program. The main reason is to achieve controlled approach both on force and ion conductance signals. In a first version, the distance control algorithm (PID) was also implemented in the LabView program. Afterwards, this task has been replaced with hardware-based PID controllers with better performance. In fact, high speed data acquisition of 4 different channels (z-piezo position, cantilever deflection and ionic current), their quasi real-time analysis, data display and z-position control during scanning pushes LabView without real time modules to its limits.

Figure 9.9: Front panel of the virtual instrument programmed for SICM in LabView. The left part monitors the signal of the AFM (upper display) and the current from the patch-clamp amplifier (middle display). The electrical resistance is calculated in real time and displayed in the lowest display. The top part of the right side allows the user to control the AFM approach through LabView and to set the PID parameters. In the lower part the approach curves are displayed (Here, as an example, the blue curve is the ionic current and the red curve is the deflection signal during and approach on a glass substrate).

Architecture – Figure 9.10 gives a graphical overview of the flow of data in the program. Firstly, all the variables and the acquisition cards are initialized. When ready, the acquisition is started in a continuous fashion. In a first moment, all the data is stored in
memory buffers that we refer to as first level buffers. The program draws the data to be analyzed or manipulated from the 1st level buffer. Subsequently, the elaborated data is displayed and stored in 2nd level buffers, where the data is made ready for storage.

**Hereafter, the main functions of the software are shortly described:**

- **Data acquisition and display:** The backbone of the program is the high-speed data acquisition from the AFM and from the patch-clamp amplifier and its display. In fact, it is based on these signals that the user takes all the decisions. The data is stored in a buffer for both display and storage function, allowing saving at any time. The data can be preconditioned, including offset compensating tools, filtering and calibration.

- **Electrical resistance recording:** the electrical resistance of the probe can be continuously monitored during the experiment.

![Diagram summarizing the data flow of the written program. Three layers are recognizable in the program hierarchy: The top level (orange) is the user interface. The calculations and data manipulation tasks are in the second layer (green). The physical data allocation happens in a deeper layer (blue). First, the initialization of the program takes place. After this step, the data starts to be continuously acquired and stored in a memory buffer. The data is then read from the memory buffers, manipulated and displayed, and stored in a second buffer, ready to be stored.](image)

- **SICM and AFM approach:** This function enables the approach of the probe in both SICM and AFM mode. In the first case, the motor approach is automatically stopped when a desired set-point in the ionic current is reached. The second is the conventional AFM...
approach where a set-point in the deflection constitutes the stop approach command. This approach routine allows to store and analyze the obtained approach curves. However, the curves are displayed versus time. A simple voltage to meter calibration of the piezo driving signal would allow to perform true force or current z-spectroscopy measurements.

- **SICM distance controller (optional):** A PID controller is implemented in the SICM program to allow the control of the probe’s distance based on the ionic current signal. The drawback of such a software-based controller is its 1 kHz bandwidth limitation. The program allows for toggling between the two control signals: deflection or current.


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BIBLIOGRAPHY


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Publications

In preparation:

- D. Ossola, J. Vörös, T. Zambelli, M. Vassalli; Exploiting FluidFM technology for rapid and precise micro-object weighting.

- D. Ossola, L. Dorwling-Carter, P. Behr, H. Dermutz, J. Vörös, T. Zambelli; Simultaneous SICM and AFM with microchanneled cantilevers.

- P. Behr, D. Ossola, A. Larmagnac, J. Vörös, T. Zambelli; Multiparameter imaging using microchanneled AFM probes with integrated Ag/AgCl electrode.


Submitted:


- E. Potthoff, Dario Ossola, T. Zambelli, J. A. Vorholt; Bacterial adhesion force quantification by fluidic force microscopy.

Accepted:


Oral Presentations


Poster Presentations


Supervised Student Projects:
