

DIPLOMARBEIT:

SETUP AND APPLICATION OF A
SCANNING ION CONDUCTANCE MICROSCOPE

Ausgeführt am Institut für
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der Technischen Universität Wien

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durch

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Zusammenfassung

Ein Rasterionenleitungsmikroskop (Scanning Ion Conductance Microscope – SICM) wurde aufgebaut und in Betrieb genommen. Das Rasterionenleitungsmikroskop wurde aus einer konventionellen Patch-clamp Meßeinrichtung in Zusammenarbeit mit einem Mikropositionierungssystem konstruiert. Die Patch-clamp Pipette wird als Sonde verwendet. Die Sonde misst Ionenströme so dass während des Meßvorganges kein physischer Kontakt mit der Probe stattfindet.

Das Mikroskop visualisiert stark unregelmäßige Oberflächen auf nicht-destruktive und verformungsfreie Art und produziert dreidimensionale Bilder der Probe. Im Gegensatz zu anderen Mikroskopen bietet das Rasterionenleitungsmikroskop den Vorteil lebende Zellen in ihrer natürlichen Umgebung abbilden zu können ohne sie beschädigen oder abtöten zu müssen.

In Pisa wurde in Kooperation mit Prof. Dr. Mario Pellegrino (*Dipartimento di Fisiologia e Biochimica, University of Pisa*), Prof. Dr. Donatella Petracchi und Prof. Dr. Cesare Ascoli, (*Institute of Biophysics, National Research Council Italy*) ein Prototyp gebaut und getestet. Eine Erweiterte Version wurde am *Institut für Verfahrenstechnik, Umwelttechnik und Techn. Biowissenschaften* an der *Technischen Universität Wien* installiert. Als Beispielapplikation wurden Aufnahmen von roten Blutkörperchen gemacht.

Abstract

A Scanning Ion Conductance Microscope (SICM) for research and educational purposes has been constructed and put in operation. The Scanning Ion Conductance Microscope is constructed from conventional patch clamp devices in conjunction with a micro-positioning system. The patch clamp pipette is used as a probe. The probe senses ion currents hence no physical contact to the specimen takes place during scan.

The microscope visualizes highly irregular surfaces in a non-destructive and deformation-free way and produces three-dimensional pictures of the specimen. As opposed to other kinds of technical microscopes the Scanning Ion Conductance Microscope provides the advantage of the ability to visualize living cells in their natural environment without the need to damage or kill them.

A prototype has been built and tested in Pisa in cooperation with Prof. Dr. Mario Pellegrino of the *Dipartimento di Fisiologia e Biochimica (University of Pisa)* and with Prof. Dr. Donatella Petracchi and Prof. Dr. Cesare Ascoli, *Institute of Biophysics (National Research Council Italy)*. An advanced version has been installed at the *Institute of Chemical Engineering (Technical University of Vienna)*. As example application pictures of red blood cells have been taken.

Acknowledgments

This work was supported by Prof. Dr. Frank Rattay, Dr. Ille Gebeshuber and Prof. Dr. Helmut Stachelberger at the Technical University of Vienna and by Dr. Donatella Petracchi, Dr. Cesare Ascoli and Prof. Dr. Mario Pellegrini at the Dipartimento di Fisiologia e Biochimica "G.Moruzzi" in Pisa.

Statement

I hereby certify that the work presented in this diploma thesis is my own and that work performed by others is cited.

Ich versichere, diese Arbeit selbstständig verfasst, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt und mich auch sonst keiner unerlaubten Hilfsmittel bedient zu haben.

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“This equipment came from diverse technological epochs and had been smuggled into this, the Outer Kingdom, from a variety of sources, but all of it contributed to the same purpose: It surveyed the microscopic world through X-ray diffraction, electron microscopy, and direct nanoscale probing, and synthesized all of the resulting information into a single three-dimensional view.

If Hackworth had been doing this at work, he would already be finished, but Dr. X’s system was a sort of polish democracy requiring full consent of all participants, elicited one subsystem at a time. Dr. X and his assistants would gather around whichever subsystem was believed to be farthest out of line and shout at each other in a mixture of Shanghainese, Mandarin and technical English for a while. Therapy included but was not limited to: turning things off, then on again; picking them up a couple of inches and then dropping them; turning off nonessential appliances in this and other rooms; removing lids and wiggling circuit boards; incense-burning; cable-wiggling; putting folded-up pieces of paper beneath table legs; drinking cappuccino and sulking; invoking unseen powers; — and a similarly diverse suite of troubleshooting techniques in the realm of software.” [Ste95]

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Chapter 1

Introduction

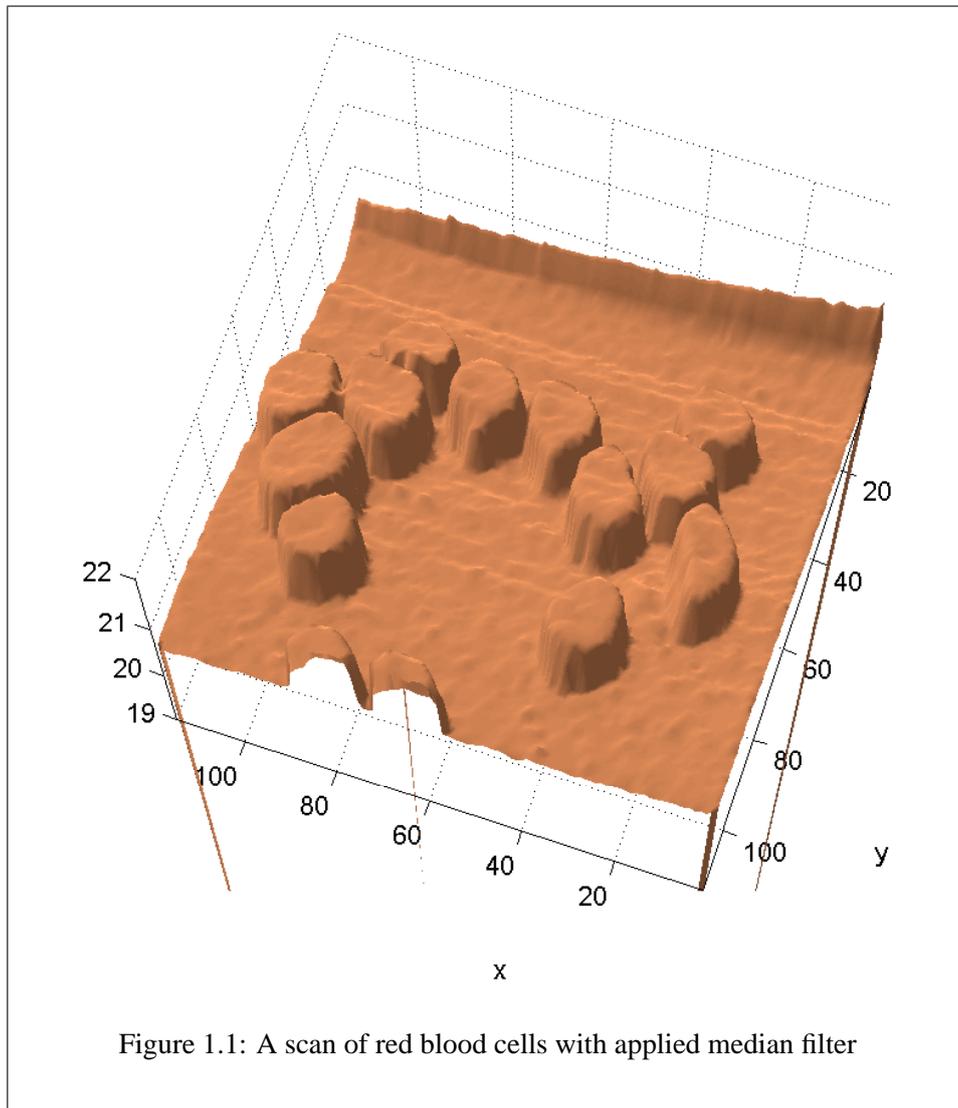
The principle of the scanning ion conductance microscope (SICM) was invented at the University of Santa Barbara by the Hansma Group AFM Lab [Hea89]. It belongs to the family of scanning probe microscopes and works with specimen in conductive solution at room temperature and atmospheric pressure.

The SICM uses conventional patch clamp devices for the measurement of ion currents. The patch-clamp pipette is used as scanning probe. Due to the functional principle of sensing ion currents no physical contact to the sample is necessary during the scan. The movement of the scanning probe is accomplished by a piezo actuator.

The microscope is able to visualize highly irregular surfaces in a non-destructive and deformation-free way and produces three-dimensional pictures of the specimen. As opposed to other kinds of technical microscopes the SICM provides the advantage of the ability to visualize living cells in their natural environment without the need to damage or kill them. The specimen needs only enough fixation to prevent them from drifting. Even dynamical processes on the cell surface can be measured.

Applications of the SICM range from corrosion research, quality control and examination of technical and biological membranes to specialized medical and pharmaceutical topics regarding cell and ion-channel behavior.

The SICM is an excellent research tool with an array of promising applications in the fields of Biology, Micro- and Nanotechnology. At the present time



no commercial SICMs are available. The motivation for this thesis was to show that a reliable working SICM can be constructed at reasonable cost and that actual research work can be done with this SICM.

Therefore the scope of this thesis was to construct a working SICM. This device should be set up with special regard to low cost-components and flexible experimentation. Test scans of various surfaces and in particular of cell membranes have been taken as proof of concept. An example scan of red blood cells is shown in Fig.1.1. The image range for this scan is $60 \times 60 \mu\text{m}$.

The SICM we constructed excels in its working area which is up to $80 \times 80 \mu\text{m}$ at resolutions down to 20 nm. The resolution depends directly on the opening diameter of the micro-pipette. The control circuit of the piezo micro-positioning system is working in closed loop mode to compensate hysteresis effects of the piezo element. Special precautions have been taken in the scanning sequence to prevent the micropipette tip from breaking. Instrumentation methods and measurement algorithms have been optimized in order to scan biological surfaces in reasonable time.

In chapter 2 the theory and modes of operation of different kinds of scanning probe microscopes are described. The basics of the patch-clamp technique are explained and the principles of the SICM is presented.

The experimental setup developed in this thesis is described in chapter 3. The numerous items constituting the SICM are individually explained.

Control of the measuring sequences is carried out by the software. The used algorithms, functions and controlling structures are described in chapter 4.

Often used methods and work steps necessary for measurement with the SICM are introduced in chapter 5. Detailed instructions for the handling of the SICM and some obtained pictures can also be found in this chapter.

To take the SICM from its present prototype status to a fully fledged research tool some enhancements have to be done. These are described in chapter 6.

Chapter 2

Theory

2.1 Scanning probe microscopy

While scanning probe microscopes (SPMs) vary widely in application and resolution the basic principle is the same: SPMs use a probe which senses a physical quantity on a very small length scale. According to the type of SPM the measured quantity changes in a significant way when the probe approaches the sample surface. This quantity is either recorded directly (in so-called constant height mode) or used to adjust the vertical position of the probe above the sample surface while scanning [Mea88]. Usually either the sample or the probe is moved by a piezo actuator.

Since SPMs do not use optics there are no lenses and the resolution of the SPM is generally dependent from the tip size and the length scale at which the measured quantity is changing.

Most SPM's can operate in two modes: *constant current* or *constant height mode*.

In *constant current mode* the probe is moved at a specified distance above the surface thus following the topology of the specimen. The height dependent signal (current) is kept constant this way. Since the height has to be controlled constant current scans are slower than constant height scans. In constant current mode the scanned surface may be somewhat irregular.

In *constant height mode* the height of the tip over the surface is fixed. The changes in the signal are recorded and an image of the surface is derived from them. This means that the irregularities of the scanned surface may not exceed the constant height. In return constant height scans are very fast as there is nothing to be controlled.

Depending on the measured quantity and the corresponding requisites to probe and sample every scanning probe microscope is suitable for more or less specialized applications. Since the Scanning Tunnelling Microscope was invented, many applications and different types of SPMs have been developed. Some of the most important types of SPMs will be discussed in the following chapter.

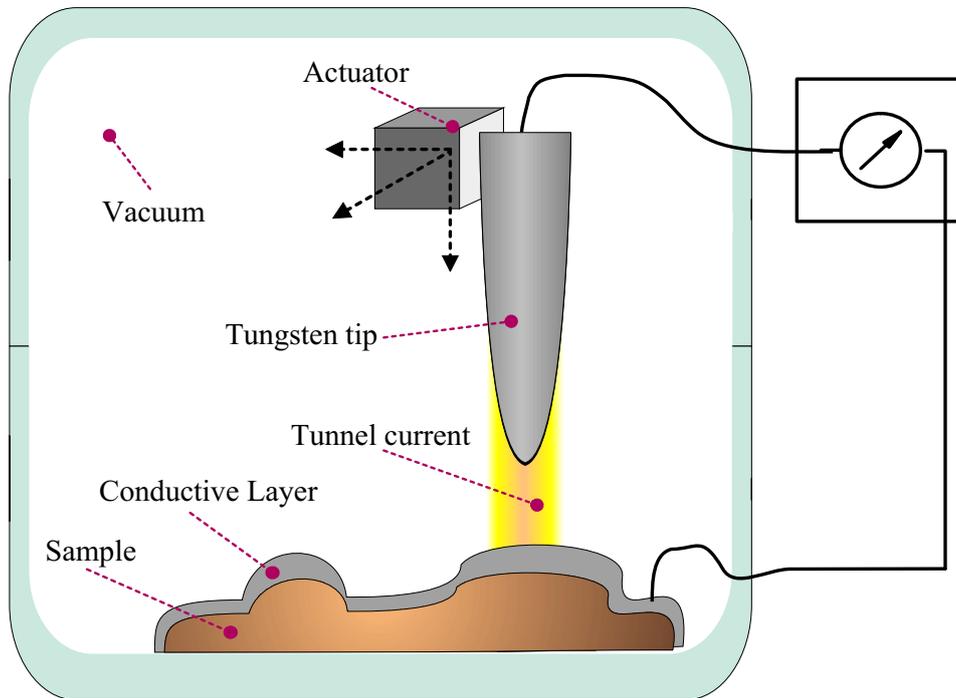


Figure 2.1: Principle of a STM

2.1.1 Scanning tunnelling microscopy

The invention of the Scanning Tunnelling Microscope (STM) in 1982 has introduced new ways of examining surfaces with resolutions down to atomic levels [Bea82]. For the first time it was possible to reach atomic resolutions without the physical barriers of diffraction. A detailed review can be found at [BR87].

The STM works according to the following principle: The scanning probe is a metallic tip (usually made from tungsten wire) biased with a voltage against a conducting sample surface. The voltage induces a small current (approximately 1 nA) between tip and the surface. This current is called Tunnelling current. It stems from the non-vanishing probability density of electrons in the immediate vicinity of the sample surface.

To detect this current the whole measurement is usually carried out in ultra high vacuum (UHV). The vacuum also prevents contamination of the surface. The current depends on the distance between tip and surface. Non-vacuum STMs exist but are only utilized for special purposes.

The probe is mounted on a three-dimensional piezo actuator and is moved in a raster scan over the surface. At each grid point the current between tip and surface is measured and used to control the vertical position of the piezo actuator. In this way, the sample surface can be followed and an image of it is obtained.

With a Scanning Tunnelling Microscope it is possible to obtain highly detailed images of conducting surfaces, for example metals. However this method is not appropriate for biological samples because of their poor conductive properties. The samples have to be either very thin, or to be covered with a conductive layer — in most cases a gold layer applied by vacuum deposition is used.

The STM can be used for micro-structuring: by reversing the polarity of the tip single atoms can be picked up from the surface and be placed at a desired point.

Crucial elements of the STM are the control electronics which have to be fast and accurate as well as the shape of the tip. The latter has to be as sharp as possible — ideally as wide as some atoms at the very end — a result that is not easily obtained nor reproducible on a regular basis. The small working distance between tip and sample and the lateral insensitivity of the probe easily lead to tip-crashes.

Scanning tunnelling microscope (STM)**Signal:** Tunnelling current**Probe:** Metallic tip**Resolution:** Down to subÅ**Requisites:** Conducting surface, usually UHV

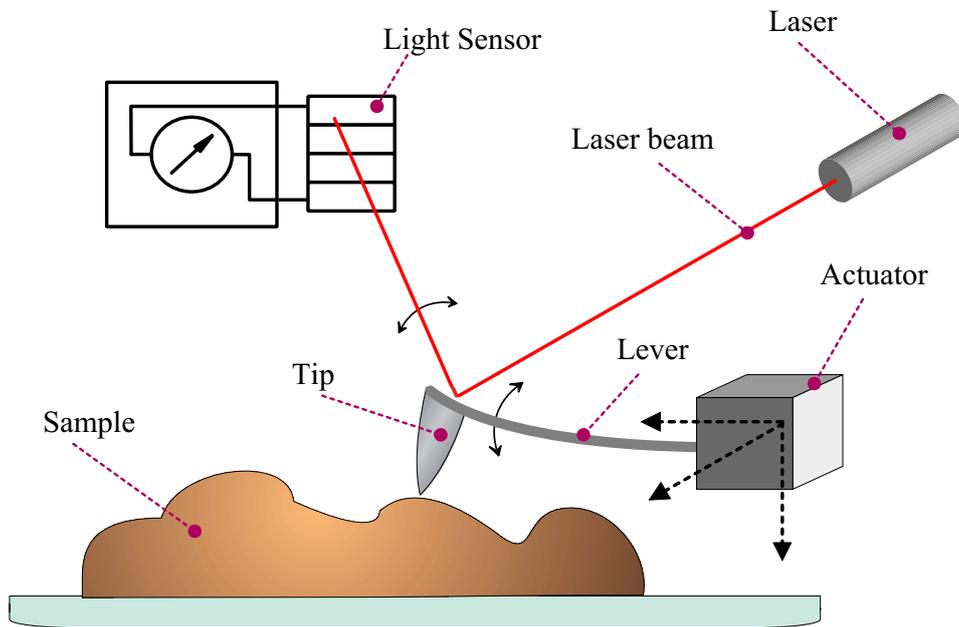


Figure 2.2: Principle of a AFM

2.1.2 Atomic force microscopy

The Atomic Force Microscope (AFM) operates by measuring attractive or repulsive forces between the tip and the sample [BQG86]. In its repulsive *contact mode* a tip at the end of a flexible leaf spring or “cantilever” touches the sample.

As a raster-scan drags the tip over the sample, a detection apparatus measures the vertical deflection of the cantilever, which indicates the local sample height. Thus, in contact mode the AFM measures hard-sphere repulsion forces between the tip and sample. In most cases, the detection apparatus consists of a laser beam which is reflected at the cantilever. This beam acts as a contact-free pointer and its deflection is detected by a light-sensor [Aea88].

In so-called *non-contact mode*, the AFM derives topographic images from measurements of attractive forces. The lever is excited with vibration at its resonance frequency. When the tip is now attracted by near atoms (without touching them), the vibration frequency changes. With this method it is possible to achieve sub-atomic resolution. This mode does not allow the imaging of samples immersed in liquid.

Yet another possibility is the *Attractive Mode Force Microscope* (AMFM). When the probe tip is brought within a distance of 2 nm to 20 nm of a sample, the tip is pulled towards the sample by van der Waals forces as well as by the surface tension created by water molecules that condense between tip and sample. Monitoring the attractive force produces a topographic map of the samples surface.

Atomic Force Microscopes can achieve a resolution of 10 pm, and unlike electron microscopes, these devices are able to image samples in air and in liquids [MDH87] [Dea89].

The cantilever of an AFM has to be very flexible in order to exert lower downward forces on the sample. On the other hand the resonance frequency of a AFM cantilever should be high to enable fast scans. Both requirements can be fulfilled by using cantilevers with a very small mass. Commercial vendors manufacture almost all AFM cantilevers and tips by microlithography processes similar to those used to make computer chips.

In principle, AFM resembles the record player as well as the stylus profilometer. However, AFM incorporates a number of refinements that enable it to achieve atomic- or even sub-atomic scale resolution. For high resolutions the AFM has to operate in UHV since dust and condensed water would interfere with the measurement. AFMs are highly developed and commercially available.

Atomic force microscope (AFM)**Signal:** Deflection of a cantilever**Probe:** sharp tip on a flexible cantilever**Resolution:** 10 pm**Requisites:** Regular surface, UHV for high resolutions

2.1.3 Other techniques

Friction force microscope (FFM): The friction between a thin tip (the probe) and the specimen surface is measured. The goal is to investigate friction on an atomic scale. Usually modified AFMs where the torque of the tip is measured are used for this method.

Magnetic force microscope (MFM): [AP03] The construction is similar to a AFM but the tip is coated by a magnetic thin film. MFMs are used to investigate the magnetic domain structure with resolutions from 25–100 nm . The MFM has been especially useful in studying magnetic recording devices such as computer hard disks.

A very important problem in MFM is the separation of the magnetic image and the topography. To solve this problem the magnetic measurements are executed by means of two-pass method. In the first pass the topography is determined in contact or semi-contact mode. In the second pass the cantilever is lifted to a specified height and scanned using the stored topography (without the feedback). As a result the tip-sample separation during second pass is kept constant. During the second pass the short-range van der Waals force vanishes and the cantilever is affected by long-range magnetic force only. Both the height-image and the magnetic image are obtained simultaneously with this method.

Electrostatic force microscope (EFM): In this microscope the metallic scanning tip is charged electrically. The electrostatic forces between the charged tip and electric charges on a surface are measured.

Scanning thermal microscope (SthM): This technique adds a highly miniaturized thermistor to the AFM tip. SthMs are used for the characterization of thermal gradients with 200 nm spatial resolution and 0.2°K temperature resolution [WW86].

Optical absorption microscope : It can be used to determine the chemical composition of a surface by using absorption spectroscopy. Shining a laser on a sample heats up some atoms more than others and the temperature differences can be detected by a thermal microscope. Varying the wavelength of the laser gives an absorption spectrum of the surface which in turn gives its

chemical composition. Resolutions down to 1 nm are possible, making it possible to record the spectrum of a single molecule.

Scanning acoustic microscope (SAM): A project to develop a scanning acoustic microscope actually predates the invention of the STM. Stanford researchers have been imaging samples with sound, using a sonar-like technique. Resolutions of 40 nm have been obtained by using sound frequencies of 8 GHz and cooling the sample to less than 0.5°K in liquid helium.

Molecular dip-stick microscope : An application of the Atomic Force Microscope, the molecular dip-stick illustrates the versatility of the new microscopes. To measure the thickness of very thin lubricant films, the tip of an AFM is lowered into the lubricant like a dip-stick. The tip feels a strong attractive force from surface tension when it touches the lubricant and then a repulsive force when it reaches the underlying surface. The molecular dip-stick can determine the depth of a lubricant to an accuracy of 5 Å.

Shear force microscope (SHFM): [Kam95] The probe is mounted on a piezoelectric actuator which drives the probe at a frequency close to one of its resonant modes. The tip is mounted in such a way that its direction of vibration is parallel to the sample surface. As the probe is moved into close proximity of the sample, the amplitude of oscillation decreases due to damping from the van der Waals interactions. Sub-Ångstrom changes in the tip to sample separation can be detected in the feedback signal. This signal is generated by focusing a laser at the tip and measuring the intensity of the diffraction image.

Scanning near-Field optical microscope (SNOM) : An optical microscope is not able to give a resolution better than about 200 nm, or about half the wavelength of visible light. This limit can be circumvented by using a near-field technique where either the light source or the detector is smaller than wavelength of the light and is brought very close to the specimen. Using this method resolutions about 40 nm can be achieved.

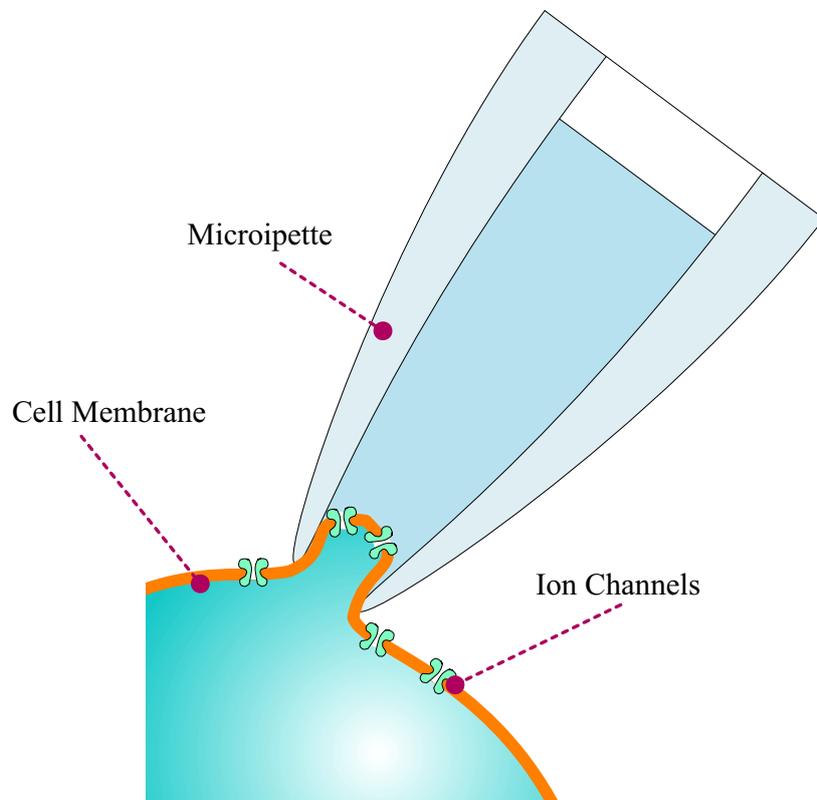


Figure 2.3: Patch-clamp

2.2 Patch-clamp technique

Patch-clamping is an electro-physiological method used to monitor the ion current of single ion-channels in the membranes of living cells [NS92]. These currents are so small that they cannot be easily detected due to the background noise in the measurement setup. The dimension of these current pulses is in the pico-ampere range. To isolate the signal from noise, a glass tube, pulled to a diameter of approximately $1 \mu\text{m}$ is brought near the cell surface. Detailed information regarding patch clamp pipettes is given in chapter 3.6.

By applying some negative pressure on the pipette it is likely that the cell is sucked to the pipette opening. Thereby the concealed area (the so-called *patch*) of the cell membrane is electrically isolated from the surrounding solution. In this way the influence of electrical background noise is blocked by the pipette (see

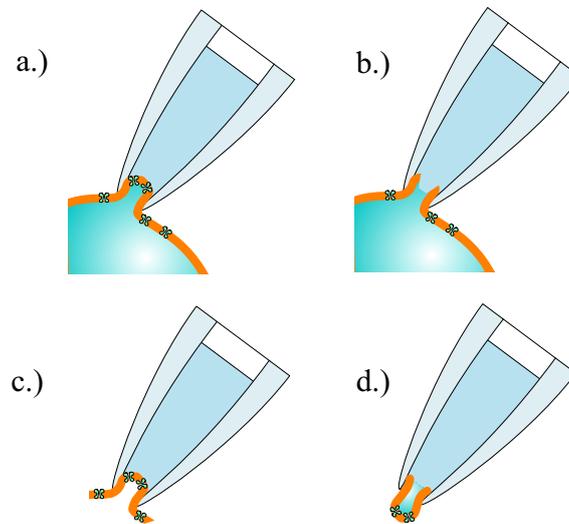


Figure 2.4: Cell configurations:

- a.) Cell-attached
- b.) Inside-out
- c.) Whole-Cell
- d.) Outside-out

Fig.2.3).

This phenomenon is called the forming of a gigaseal (the resistance is usually greater than $1 \text{ G}\Omega$). The electrical properties of the ion channels in the patch can be investigated. If ion-channel density and pipette opening diameter are appropriate, it is possible to record the ion current from single channels.

When forming a gigaseal, the cell membrane sticks tightly to the glass pipette thus providing electrical isolation to the patch. Through various kinds of manipulation different cell-configurations can be achieved without de-attaching the patch. In this way not only single channels can be measured in the so called *cell-attached configuration*, but it is also possible to break the patch to get access to the cytoplasm of the cell. This *whole-cell configuration* corresponds to a conventional intra-cellular recording.

Furthermore it is possible to measure the properties of the patches with the cytoplasmic side directed to the bath or to the pipette (*inside-out* or *outside-in configuration*). See Fig.2.4 for illustrations.

A variant is the loose-patch method: hereby there is no gigaseal formed between the pipette and the cell. In this case a lot of noise is introduced to the measurement and it gets hard to identify the signal.

The patch-clamp method is widely used in laboratories to examine the behavior of Ion channels and transmitter-receptors. Today patch-clamping is probably the most widely used electro-physiological method for the investigation of ion channels. In 1991 the Nobel price in physiology was granted to Erwin Neher and Bert Sakman for developing this method.

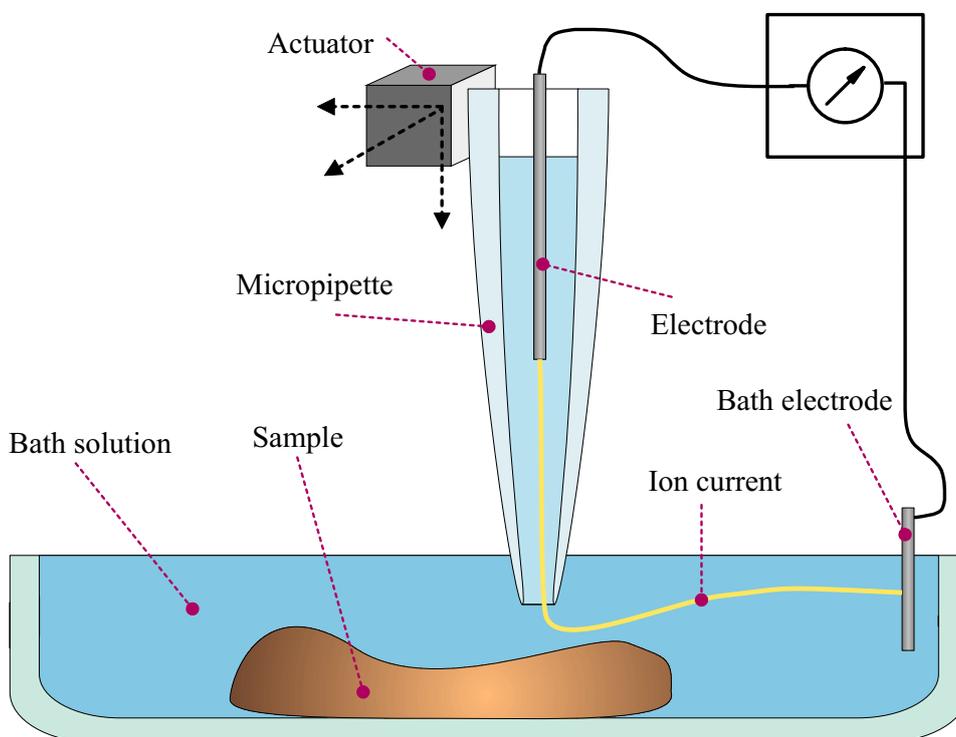


Figure 2.5: Principle of a SICM

2.3 Scanning ion conductance microscopy

A Scanning Ion Conductance Microscope (SICM) is a Scanning Probe Microscope which has been developed to image the topography of non-conducting surfaces that are covered with electrolytes at atmospheric pressure [Hea89].

The scanning tip of a SICM consists of a pulled out glass tube (called micropipette) filled with electrolyte and containing an electrode. Similar pipettes are used in the patch-clamp technique. A difference to patch clamp pipettes is the opening diameter of the tip which should be smaller than for patch-clamp applications. As will be shown below the longitudinal resolution of the SICM corresponds directly to the opening diameter of the pipette.

The imaging technique of the SICM is based on the measurement of the ion current between two electrodes. One electrode is placed inside of the pipette, the other electrode is located in the bath solution (See Fig.2.5). To avoid electrical drift the bath solution and the solution inside of the pipette should be the same.

The insulating sample is also placed in the bath solution. If there is a current flow between the electrode inside the pipette and the bath electrode it has to pass through the opening of the pipette. The current is at saturation value when the opening is relatively far from the surface.

When the tip of the pipette approaches the insulating sample surface, the current drops due to the decreasing volume of conductive solution between tip and sample (with decreasing volume the electrical resistance increases). This current decrease is detected and used to keep the pipette tip at a defined distance to the surface.

To obtain pictures the tip is moved by a raster scan over the surface. In every point of the raster the tip is moved towards the surface and the flowing ion-current is measured.

The current decrease indicating the near surface is measured differentially: If a defined difference to the precedent measurement is exceeded, the tip stops and the position is read by the software. The differential approach makes the procedure independent of voltage drift.

A common source of voltage drift is the evaporation of bath solution — changing the chemical potentials between bath and the solution inside the pipette.

When the surface is detected, the tip retreats a programmable height and moves to the next raster point. In this way breaking the tip at irregularities of the sample surface can be avoided.

It is also possible to use the SICM to record local ion currents stemming from the cell. This is resembling the loose-patch method of patch-clamp experiments.

Applications of biological nature are of primary interest. Dr. Donatella Pettrachi suggested the investigation of growing neurons. By repeatedly scanning the neurons it is possible to obtain detailed topographic and chronological information about their growth patterns.

Dr. Yuri Korchev (*MRC Clinical Sciences Centre, Imperial College London*) recently managed to localize single active ion channels on the surface of living cells with a combined whole-cell voltage clamp and SICM imaging system [[Kea00](#)].

The combined use of the apparatus as scanning probe microscope and as patch-clamp device will also bear new insights into cell function. In this case the SICM

is used to localize specific ion channels. The pipette is then moved to these ion channels and is used to measure their properties with the patch-clamp technique.

In medical terms research on matters like cell signalling and ion transport regulation will help to understand diseases such as cystic fibrosis, and how cardiomyocytes are preserved during metabolic stresses such as hypoxia and ischaemia.

Other possible application possibilities are in the field of micro-structuring. It should be possible to use the SICM for galvanic or chemical deposition. This could be of special interest when trying to interface to cells or as additional method to established micro-structuring procedures.

Since the occurring currents are in the nano-ampere range we use a patch-clamp amplifier to detect them. The electrodes have to be of second order to measure ion currents. To archive that, we put silver wire in chlorine-bleach overnight. On the next day the silver wire is coated with a layer of AgCl.

The assumption that the lateral resolution of the SICM depends directly on the opening diameter leads to the question how to calculate the height dependence of the current as function of the geometrical properties of the pipette. The model from the diploma thesis of E. Schwab [Sch90] was used to calculate the current when approaching the sample surface.

In this model, the total resistance of the pipette is composed of a height-dependent part R_H between pipette tip and sample surface and an constant part given by the ion-conductible liquid inside the frustum of the tip. The remaining resistance of the bath solution is neglected in this model.

The resistance R is in general proportional to length L and inversely proportional to cross-section A and conductivity κ :

$$R = \frac{L}{A\kappa} \quad (2.1)$$

Thus, applied to the resistances of the frustum R_F (with the pipette diameter r_p and the inner diameter r_i) and the hollow cylinder R_H (with the outer diameter r_o and the height h):

$$R_F = \frac{L_k}{r_p r_i \pi \kappa} \quad (2.2)$$

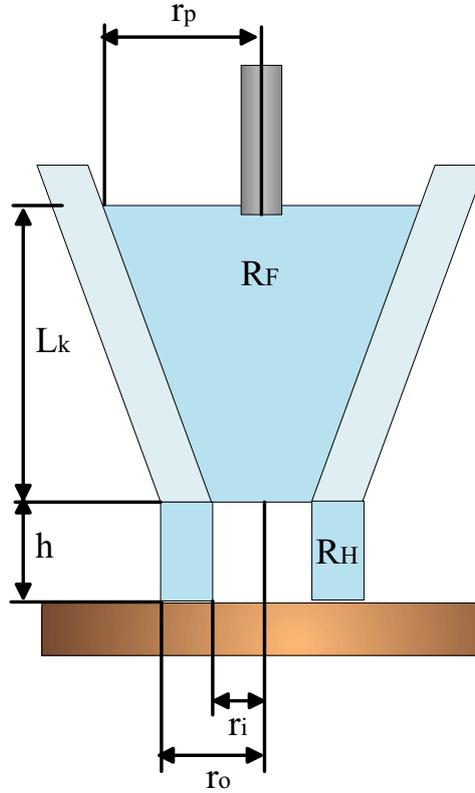


Figure 2.6: Model of a micro-pipette tip

$$R_H = \frac{\ln\left(\frac{r_o}{r_i}\right)}{2\pi h \kappa} \quad (2.3)$$

Giving a total resistance R_T :

$$R_T = R_K + R_H = \frac{U}{I} \quad (2.4)$$

And resolved for the current I :

$$I = \frac{U \kappa \pi}{\frac{L_k}{r_p r_i} + \frac{\ln\left(\frac{r_o}{r_i}\right)}{2h}} \quad (2.5)$$

When the tip is far away from the surface ($h \rightarrow \infty$) the so-called saturation current (I_{sat}) is flowing:

$$I_{sat} = \lim_{h \rightarrow \infty} \frac{U \kappa \pi}{\frac{L_k}{r_p r_i} + \frac{\ln\left(\frac{r_o}{r_i}\right)}{2h}} = \frac{U \kappa \pi}{\frac{L_k}{r_p r_i}} \quad (2.6)$$

To get a usable quantity the current is normalized to the saturation current I_{sat} :

$$\frac{I}{I_{sat}} = \frac{1}{1 + \frac{\ln(\frac{r_o}{r_i})r_p r_i}{2L_k h}} \quad (2.7)$$

The normalized ion current is only dependent on the geometrical properties of the pipette and the height h above the surface.

From this theoretical considerations some information about the ideal geometry of the pipette can be derived. When measuring ion currents it is advantageous when the current decrease begins at a greater distance above the specimen surface. This should prevent the tip from crashing into the sample. This means for pipettes used in the SICM that the electrode has to be near the opening — the frustum has to be as short as possible. Furthermore the wall thickness of the pipette should be large. With these premises the influence of the height-dependent resistance R_h is dominant. See chapter 3.6 for details on micro-pipettes.

Equation 2.6 also gives us a possibility to estimate the opening diameter from the measured resistance. With $\frac{U}{I_{sat}} = R_{pip} = \frac{L_k}{r_p r_i \kappa \pi}$ we obtain to:

$$r_i = \frac{L_k}{R_{pip} r_p \kappa \pi} \quad (2.8)$$

With the knowledge of R_{pip} , r_p and κ we can estimate the frustum length L_k and are able to calculate the opening diameter. The step-wideness for scans can only be set to a good value if we have an estimation for the opening diameter.

A result of this computation is shown in Fig.2.7. A calculated and a measured approaching curve are displayed. The geometric properties of the pipette have been estimated and applied to the model calculation. The stated simple model seems to reproduce the behavior of the approaching tip very well.

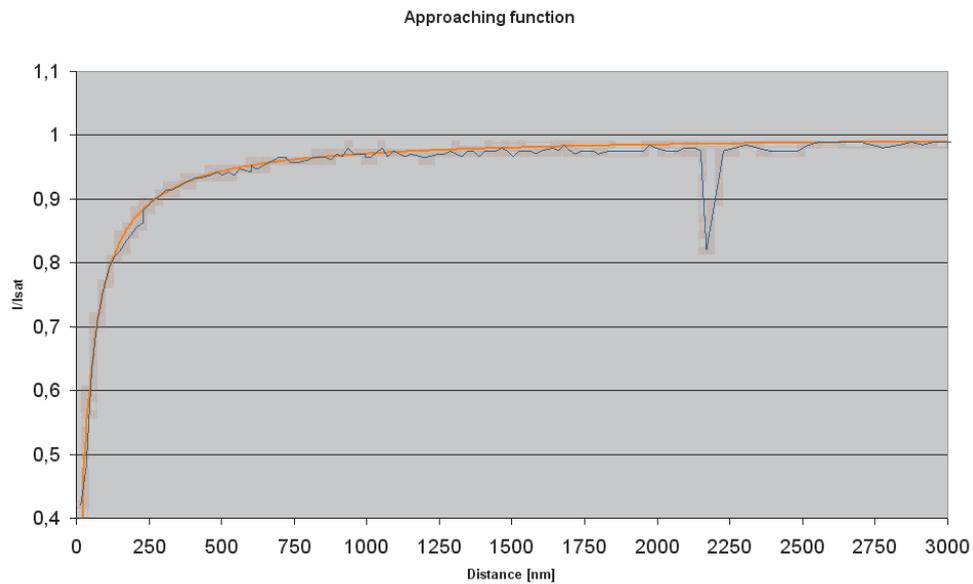


Figure 2.7: Calculated and measured approaching curves
(the spike at 2200 nm is a measuring artifact)

Scanning Ion Conductance Microscope (SICM)

Signal: Ion current

Probe: Micro-pipette

Resolution: About 20 nm

Requisites: Non-conducting sample in conductive liquid

Typical Applications: Visualization of living cells

Chapter 3

Experimental setup

The most important consideration in the design process was to keep the setup flexible. The SICM therefore consists of two parts.

The SICM *scanning head* (Fig.3.1) is designed to be easily detachable from any optical microscope and also works without any optical microscope at all. The scanning head consists of the micro-manipulator holding the piezo actuator. Electrically, the pre-amplifier is the most sensitive part of the setup. To keep signal paths short the pre-amplifier is directly holding the pipette and is mounted on the piezo actuator.

The SICM pre-amplifier could also be exchanged for the probe head of another type of SPM. By utilizing the head as moving part (as opposed most other working SICMs) we have plenty of room left on the stage of the optical microscope. This free space can be used for a perfusion bath or another micro-manipulator – devices that are necessary when doing complex experiments with cells. The compact build of the micro-manipulator is important to reduce mechanical vibration.

The *instrument rack* (Fig.3.2) holds the controlling devices. It is mechanically separated from the optical microscope and the scanning head to prevent vibration. The only connection consists of various cables, these are fixed to the vibration damping steel plate. The instrument rack is placed on a trolley for more mobility.

A good working place should be free of mechanical vibration and acoustic noise (e.g. slamming doors) as well as of electrical noise (especially on the ground–line).

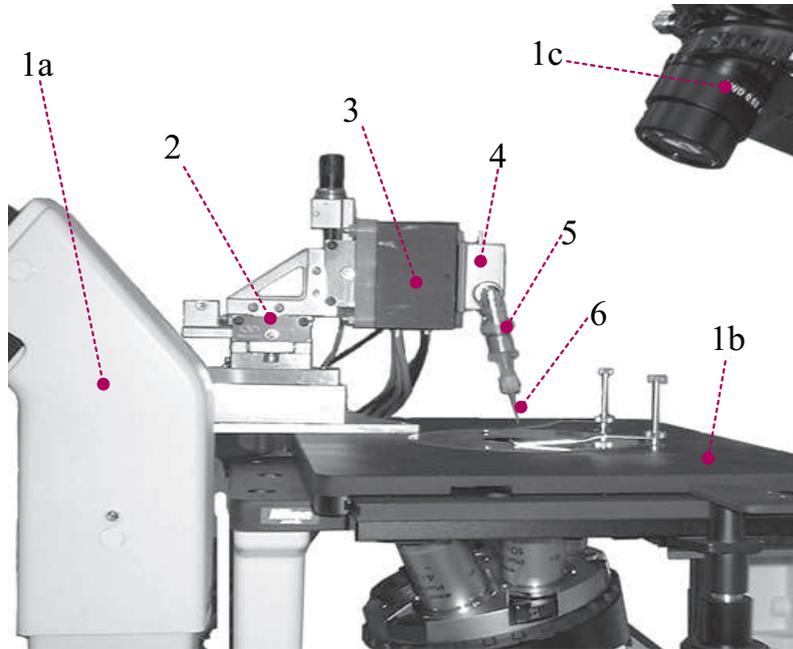


Figure 3.1: The Scanning Head of the SICM

Hardware Elements

1. (a) Optical microscope
(b) Object table
(c) Condenser
2. Micro-manipulator
3. Piezo-actuator
4. Headstage
5. Pipette holder
6. Micropipette

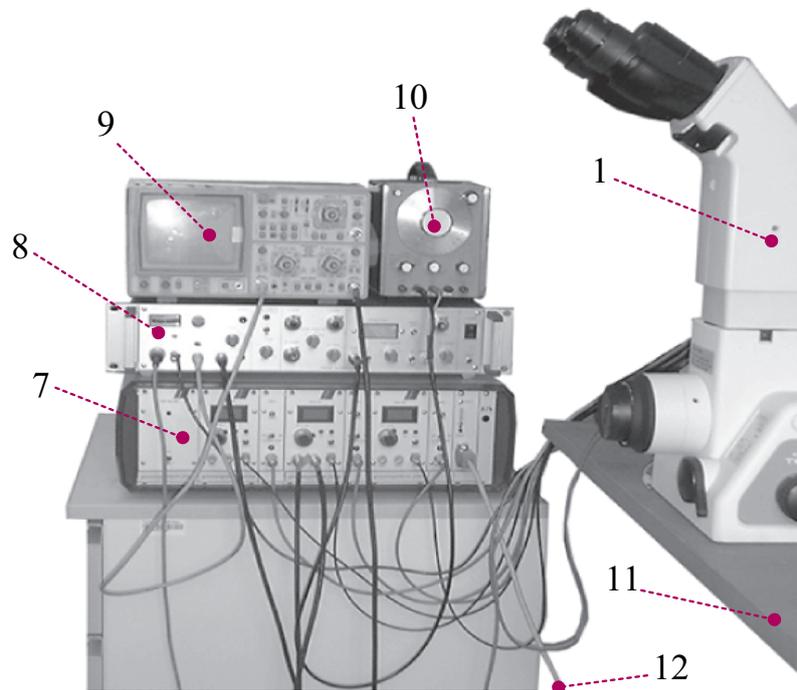


Figure 3.2: The Instrument Rack

Hardware Elements

- 7. Piezo controller
- 8. Patch-clamp amplifier
- 9. Oscilloscope
- 10. Function generator
- 11. Vibration damping
- 12. Connection to PC – data acquisition

Faraday cage (not shown)

Pipette puller (not shown)

3.1 Optical microscope

The purpose of the optical microscope is to aim the pipette tip to the target area. To scan the specimen access from above is required. The optical part of normal microscopes is therefore in the way so the most important criterium is that the microscope is an inverted microscope. For the same reason the condenser of the microscope should have a long working distance. Problems arise when trying to get good picture quality: It is necessary to use objectives with a high numeric aperture. This in turn requires a short working distance of the condenser. For these reasons it is not possible to acquire optimal pictures when the SICM head is mounted on the microscope.

A Nikon Eclipse TE300 inverted microscope is used for this thesis. This microscope offers professional optical properties and can be equipped with phase-contrast or Nomarsky interference contrast devices, which will come handy when working with cells.

Phase-contrast microscopy is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells. Transparent structures may be visualized in phase microscopy by producing contrast from refractive index inhomogeneities in the sample rather than from light absorption inhomogeneities. Contrast is obtained by converting phase changes into amplitude changes. Phase microscopy is well suited to studies of living tissue as samples do not have to be fixed and stained in order for internal structure to become visible.

Nomarsky microscopy, also called differential interference contrast (DIC) microscopy is a modification of phase microscopy. The technique essentially acts as a high-pass filter that emphasizes edges and lines. Out-of-focus refractive index changes will be blurred and have a shallow spatial gradient in the focal plane, they will therefore not contribute much to the contrast of the image.

The condenser stand of the microscope can be tilted backwards which gives additional room at the cost of good lightning.

3.2 Micro-manipulator

The micro-manipulator is mounted on the stage of the microscope. The manipulator is used for rough positioning of the pipette and to place it near the sample surface. The models usually used for patch clamping purposes are not useable for our application since they do not offer the required mechanical stability to mount the heavy piezo-actuator.

The used model Newport M-461-XYZ is made of steel and shows no drift. Its travel is 13 mm in every direction and it provides mounting possibilities for the piezo-actuator. All three axes can be equipped with stepper-motors.

When lowering the scanning head turn the knob anti-clockwise. A turn of $\frac{1}{4}$ corresponds to a movement of 60 μm .

3.3 Piezo-actuator

The whole raster scan is done by a *Tritor 3D 100SG* piezo element from *Piezosystem Jena*. This actuator is compact and provides excellent mechanical stability. It offers up to 100 μm motion in every direction (80 μm in closed loop mode) and it is equipped with an integrated strain gauge measurement system for overcoming the effect of hysteresis. Resolution for controlling each axis is 12 bit which corresponds to 20 nm (resolution can be enhanced by the use of a pull down resistor).

Inside the piezo element the actual position is measured by strain gauges which provide data for a feedback loop that prevents the piezo element from drifting and nullifies the hysteresis normally observed in piezo movement. This is automatically done by the piezos controller when switched to closed loop.

3.4 Headstage

The headstage is mounted directly on the piezo actuator. Note that in our setup the moving part is the micropipette and not the sample. This is unusual but has the advantage that one can look at the sample through the optical microscope any time even during scan. The headstage contains the sensitive amplifier that constitutes the current-to-voltage converter, as well as components for injecting test signals into that amplifier. The headstage is capable of changing its range according to the performed experiment. For further information see the manual [\[HEK\]](#).

On one side of the pre-amplifier is a BNC connector which holds an ankle on which the pipette-holder is mounted. Thus the pipette holder can be slightly tilted to let light from the optical microscopes condenser pass and to have the shadow of the tip in the view-field of the optical microscope for observation.

On top of the headstage a pin jack carries a high quality ground signal which is useful for grounding the bath electrode or nearby shields. As bath electrode also a AgCl covered silver wire is used. For this reason the bath electrode and the pipette electrode should be produced in the same process to prevent them from having a potential difference.

Note that the metal casing of the probe is also connected to the signal, and therefore **must be insulated from the ground** and thus, also from the piezo actuator.

The MC-7 is an accessory to the headstage. it is used as a model circuit for testing purposes. This circuit simulates an open pipette, a gigaseal or a whole-cell configuration. When in use it gets plugged to the headstage instead of the pipette holder.

3.5 Pipette holder



Figure 3.3: The pipette holder and a pipette

The holder (shown in Fig.3.3) is provided with the patch clamp amplifier. It consists of Teflon and polycarbonate and has a small pipe leading out sideways to apply pressure to the pipette. This pipe is needed when doing patch clamp experiments to apply negative pressure to the tip in order to establish the gigaseal. In the SICM Setup it is sometimes used to apply positive pressure to the tip when entering the bath solution to prevent the tip from getting clogged by debris.

The pipette electrode has to be welded to the contact centerpiece. Mounting to the headstage occurs over a BNC connector. For cleaning the pipette holder methanol is used.

3.6 Micropipette

The pipettes are produced by pulling glass tubes in a device called pipette puller. The pipette is characterized by its wall thickness, opening diameter and tip length. See chapter 2.3 for some calculations.

Pipettes can be pulled of soft glass, borosilicate or aluminiumsilicate glass or quartz glass. In section 5.1.1 a description of the actual pulling process will be given.

Soft glass tubes tend to have huge opening diameters and are sometimes toxic to cells. Quartz glass has a very high melting point only reachable with expensive laser pullers but offers superior electrical properties. Laser pullers also allow for the best process control and can produce the smallest opening diameters (about 30 nm) reproducible. Aluminiumsilicate glass also produces useable tips which are slightly longer and a bit more fragile but offer a smaller opening diameter. With the available puller the best results have been obtained by using borosilicate glass tubes.

For patch clamp experiments it is usual to cover the pipettes with hydrophobic substances like Sylgard to improve the electrical properties of the tip. It is also not uncommon to heat-polish the tip in a so called micro-forge. This method leads to more rounded tips. None of these procedures are necessary when using the tips in the SICM.

Usually it is best to pull the tip fresh when it is used but it is possible to store them dust free with a small amount of silica for several months. Still, the danger of clogged tips leading to tip-crashes is increasing with storage time.

Most experiments for this project have been done with borosilicate tubes (diameter: 1 mm, wall thickness: 0.3 mm) resulting in tips with opening diameters between 150 nm and 400 nm. The used puller did not produce reproducible results. With the aid of a good puller it should be possible to obtain opening diameters down to 15 nm [CGL92].

An interesting approach is the use of micro-fabricated tips as described in [PH91]. These are etched from silicon and offer an opening diameter of about 250 nm. The advantage is the high mechanical robustness allowing high-speed scans.

3.7 Piezo controller

The amplifier system corresponding to the piezo actuator was also chosen from Piezosystem Jenas product range. A modular system containing power amplifiers and position control modules for all three channels (named ENV) as well as an interface board (EDA2) is used. The interface board allows control of the piezo by

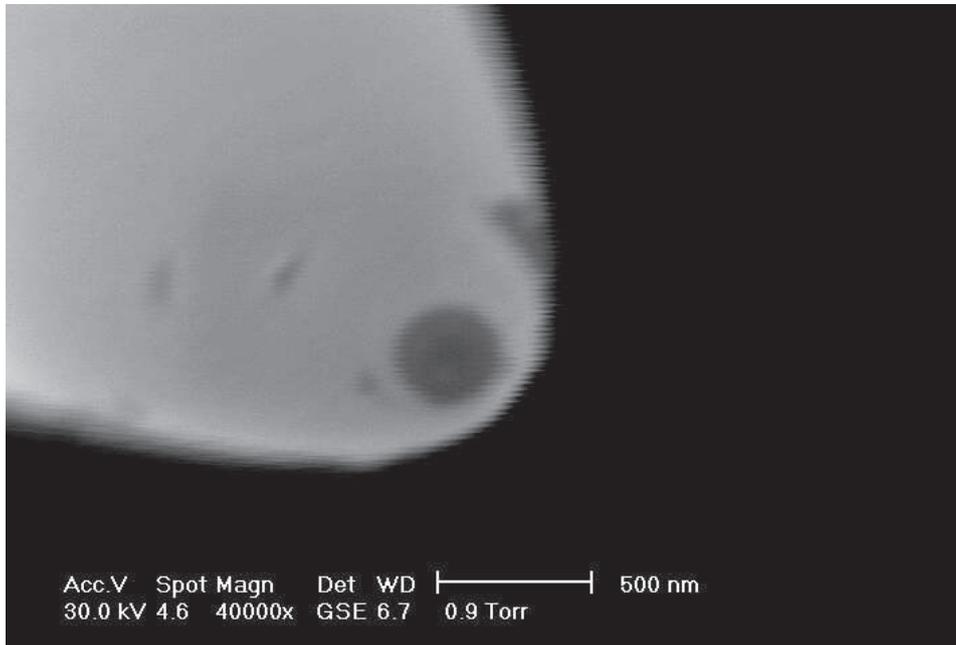


Figure 3.4: Scanning electron microscope image of a micropipette

a PC via a RS232 serial connection. A programmable microprocessor is also available in this unit and can be utilized for future enhancements. Detailed information can be found in the manual [Pie]

It is also possible to apply a modulating control voltage signal directly to each module. This feature is used to control the vertical movement when scanning a surface.

3.8 Patch-clamp amplifier

The model used is a Heka EPC 7 [HEK]. This is a classic patch clamp amplifier with a good signal to noise ratio. It can be used for a variety of experiments. The device consists of the main amplifier and a pre-amplifier also called the *headstage* or *probe*.

The main unit contains the power supply, the signal processing electronics and all of the controls. The EPC 7 offers an extremely wide bandwidth and the inte-

grated transient cancellation and series-compensation functions add to the versatility of the amplifier. Another very useful feature is the SEARCH-mode which keeps the current signal from drifting off-scale.

The EPC 7 also offers a manually adjustable constant current. When the unit is switched to VOLTAGE CLAMP mode this feature can be used to generate a dc signal as stimulus. A constant stimulus has the advantage of reduced artifacts when the ion current is sampled. This drastically improves the accuracy of the measurements and thus, the stability of the scanning process.

3.9 Oscilloscope

A two-channel digital oscilloscope is used for various measurements and for signal visualization. This device is essential when tracking errors.

3.10 Function generator

This instrument is used mainly for testing purposes. It produces a square signal. For most experiments a direct-voltage offset can be generated by the patch-clamp amplifier. With adapted software signal-generation can also be done by the Computer via the NI-DAQ data acquisition card (NI-DAQ).

3.11 Vibration damping

Since the tip-sample distance of the SICM is quite small vibration of the apparatus has to be prevented. All parts of the construction are selected to be robust and compact leading to high resonance frequencies.

The vibration damping system consists of a steel plate of 45 kg resting on four tennis balls. The optical microscope holding the scanning head rests on this plate. This setup is simple yet effective — operation of the microscope runs smoothly despite the four track heavy traffic street running alongside our institute building. A similar construction with squash balls has been described in [OR95].

Vibration sensibility test were also made. For these test the tip was held at $1\ \mu\text{m}$ over a glass surface then typical vibration sources (heavy steps, knocking on the table, slamming doors etc.) were applied. The only thing that caused the tip to move noticeably was slamming the rooms door.

3.12 Data acquisition

The measured ion current is sampled to a computer running Matlab via a National Instruments data acquisition card. The computer controls the piezo, detects the drop in ion current and records the data for successional imaging and analysis.

The analog output of the data acquisition card also provides the voltage slope that drives the piezo in vertical direction during scan. The piezo controller is connected to the computer via a RS232 cable to the COM2 port of the PC. The lateral movement of the piezo is controlled using this signal path.

The NI-DAQ data acquisition card provides 8 AD channels (inputs) and 2 DA converters (analog outputs) as well as 24 Digital I/O lines. The resolution of the converters 12 bit. Since currently one input for the measured current and one output channel to drive the piezo is used there are enough resources for future enhancements. In particular timed output of stimulation signal pulses should be done with this card.

Process control is done with Matlab running on a P133 PC (Matlab uses this computer to full load). The Systems has 128MB RAM and the installed OS is Windows 98 SE. Windows 98 offers better timing and stability for real-time purposes than its successors.

3.13 Faraday Cage

Since the measured signal is very small it can easily be distorted or overlayed by electrical background noise. To optimize the signal-to-noise ratio the working area is shielded by a removable faraday cage.

The most common noise sources — the 50 Hz hum radiated by the power lines and high-frequency radiation from computers and monitors are eliminated



Figure 3.5: Narishige Pipette puller

by this shielding. The Faraday cage must be mechanically de-coupled from the microscope to prevent vibration pick-up.

3.14 Pipette Puller

We used an puller from Narishige, model PD-5. It is a Brown-Flaming type horizontal puller. Pipettes are heated by a platinum filament without current control. The force is applied in two phases. The first pull is selectable in a range from 0–10 g. When a micro-switch is triggered by the shaft the second pull is initiated with a adjustable force from 0–100g.

To adapt the PD-5 for the pulling of SICM-pipettes the filament had to be changed. Still the pulled pipettes were not very reproducible.

The puller used in this thesis was donated by Prof. Dr. Graf (AKH Vienna). Since it was needed by another research group it has not been available for the latest months. Information on pipette-pullers in general can be found at section 5.1.1.

Chapter 4

Software and mode of operation

In the following chapter the software operating the SICM will be discussed. The functional principles of the SICM will also be outlined. The controlling software is written in Matlab (V6R12). Matlab is well suited for testing and visualization purposes.

As starting situation of a scan it is assumed that the pipette tip is placed in the solution and in a range of max. 40 μm above the specimen. When using the SICM the piezo has to be initialized and then the tip has to be moved manually into an appropriate position (see 5.2). A constant voltage is generated by the EPC 7 as signal. This voltage should be adjusted to a little bit below 5 V.

Initialization of the SICM is done by evoking the routines `initeda` and `initdaq`. For reset purposes the `kill` function can be used. After invoking `kill` re-initialization is possible.

`initeda`: Initializes the piezo-controller by switching it to computer control and moving the piezo to the starting position.

The program creates a global variable `s` which contains the handle for the serial port. Explanation of commands sent to this port can be found in [Pie].

`initdaq`: Initializes the data acquisition card. The analog input is now ready to sample the pipette current. The analog output is controlling also the vertical axis of the piezo and is also set to its starting position.

Here the global variables `ai` and `ao` are defined. Those carry the handles for the analog in- and output. The sample-rate is set to 1KHz for both channels and a value according to a height of $40\ \mu\text{m}$ (the maximum height for the vertical axis when) is set for the output channel.

This is done by the subfunction `movez(var)`. This function utilizes the movement of the piezo by sending the according voltage to the analog output. The parameter `var` is to be given in μm .

kill is a cleanup-routine. If something goes wrong and it is necessary to manually interrupt a scan this program should be called afterwards to clean the buffers and close all open instruments.

approach(startheight, Istopratio) The recording of an *approaching curve* is done by invoking the program `curve=approach(startheight, Istopratio)`. The measured approaching curve is saved in `curve`. The parameter `startheight` is to be given in μm and specifies the starting height of the approach.

The piezo is moved down step by step and in every point an average of 20 samples is taken. For this movement the (slower) method of controlling the piezo via the EDA-controller is used. An advantage is that the full range of $80\ \mu\text{m}$ on the vertical axis can be used with this method.

At the beginning of the measurement the saturation current is sampled (as an average of 5×20 samples). The approach stops at an defined ratio between actual current and saturation current (the parameter `Istopratio`). When stopped the tip is retreated from the surface and the obtained curve is displayed. This method delivers accurate absolute current values.

raster This routine is used for the actual raster scan of the surface. The parameters of the scan like x- and y-range, step-width etc. have to be changed in the code accordingly to the experiments needs. The code for this function is shown below. To start a scan set the parameters in the code and evoke the function with `map=raster`. The obtained data is saved in the variable `map`.

```

function map=raster;
% handle of the analog input
global ai

% parameters of the scan
Istopratio=0.999;
startheight=30;

xstep=0.25;
ystep=0.25;
xmin=0;
xmax=10;
ymin=0;
ymax=10;
tapheight=3;

% initialization
line=[];
map=[];
height=startheight;
x=xmin;
y=ymin;
movez(height)
movea(1,x)
movea(3,y);

% raster loop
while y<ymax
    while x<xmax and x>xmin
        % find the surface
        height=probe(Istopratio);
        line=[line height];
        % retreat from surface
        height=height+tapheight;
        movez(height);
        % move to the next step
        x=x+xstep;
        movea(1,x);
    end
    % next line
    % switch direction
    xstep=xstep*(-1)
    x=x+xstep;
    y=y+ystep;
    to the next line
    movea(1,x);
    movea(3,y);
    % append line to map
    map=[map;line];
    line=[];
end
% return to startpos
movez(startheight)
movea(1,xmin);
movea(3,ymin);

%calculate number of elements
x=xmin:xstep:(xmax-xstep);
y=ymin:ystep:(ymax-ystep);
% show surface map
surf1(x,y,map,[80 50])
colormap(copper)
shading interp
xlabel('x (m)')
ylabel('y (m)')
zlabel('z (m)')
view(120,30)

```

When scanning, the vertical piezo position is controlled by a voltage delivered by the analog output of the NI-DAQ card. This method is much faster than the step-by-step method used in the approach function. The controlling voltage is dropped in a slope, thus the pipette is moved towards the surface. While pipette

moves the output of the patch-clamp amplifier (the actual ion-current) is sampled at 1 KHz and analyzed in realtime.

An average of 20 samples is taken and compared with the last measurement. If the difference exceeds a defined ratio, the voltage slope is stopped and the position of the tip is determined by the function `readheight`.

This method is very fast and sensitive. Since the result is only dependent of the actual and the previous measurement, slow voltage drifts do no interfere with the measurement as long as the current signal stays in the input range of the analog-digital converters of the NI-DAQ data acquisition card.

Further speed enhancements are possible when using a higher sample frequency. With a higher sample frequency it is possible to move the piezo faster and still detect the current drop in time.

The measured height value is saved in a two-dimensional map. To prevent the tip from breaking it is now lifted some μm and moved to the next raster point. Lateral movement is carried out with the EDA-unit since it is not time-critical.

The schematic on the following page (Fig.4.1) illustrates the signal paths in the SICM.

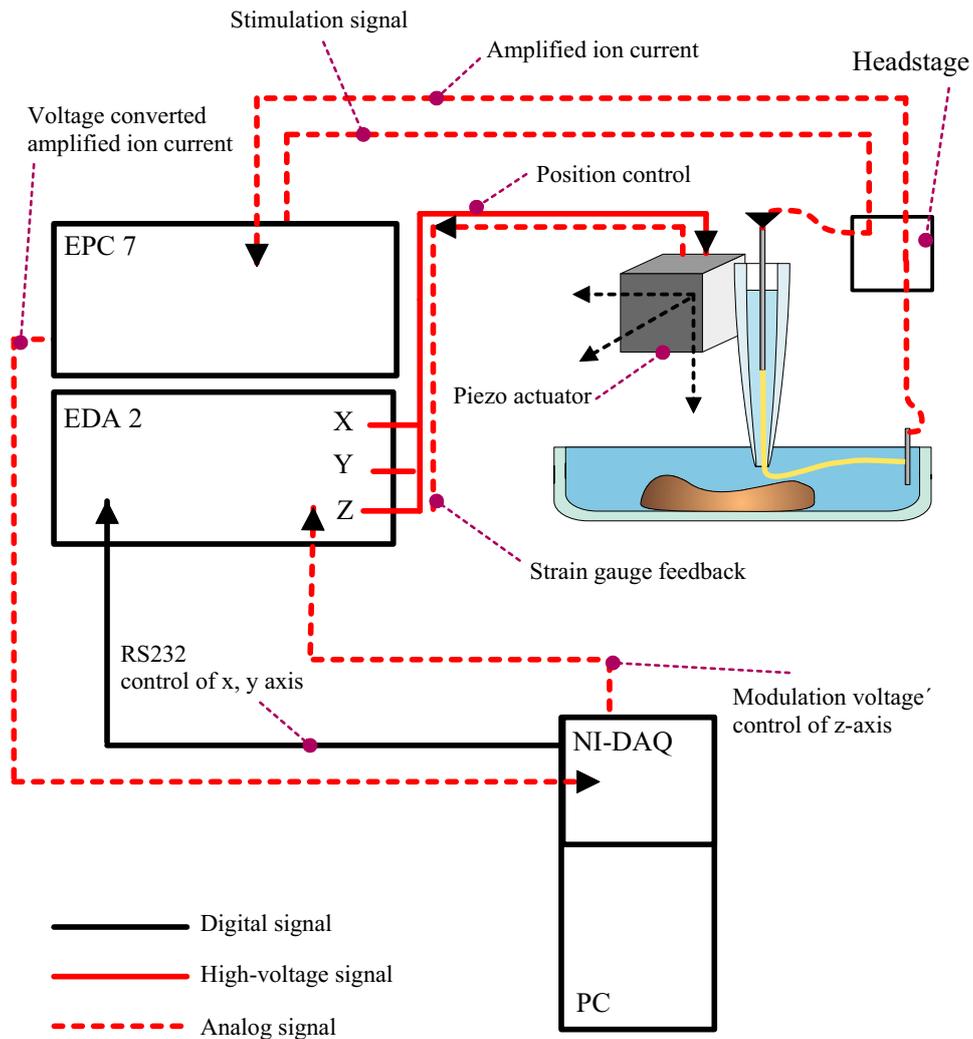


Figure 4.1: Diagram of the signal paths of the SICM

Chapter 5

Using the SICM

This chapter has two aims. It should serve as a manual for the laboratory including step by step tutorials for the various tasks needed to perform microscopy on the SICM-Setup. For the reader it is used to present the used techniques and routines and to show how scans are accomplished.

5.1 Preparations

Not all of the preparations below have to be done every time the SICM is used. Testing the electronic components – the EPC 7 patch-clamp amplifier and the piezo controller EDA will only be necessary when something seems wrong or after longer periods of inactivity.

Preparing electrodes and maybe samples will occur more often while pulling pipettes is daily work. The section 5.1.6 explains the routine operations that have to be done before starting to use the SICM.

5.1.1 Pulling micropipettes

The micropipette constitutes the actual probe of the SICM. It senses the ion current and determines the lateral resolution of the scan. Since it is made from glass it is also a very fragile part and tends to break. For this reason and for better reproducibility always batches of about 10 pipettes should be pulled.

In principle the required small opening diameters are obtained by heating up a glass tube until it begins to melt. Then a longitudinal force is applied, pulling the tube apart until it is tearing. To get reproducible tips so called pullers are used.

In the puller the clamped glass tube is heated up by a platinum filament or by a laser beam. The force is applied by electromagnets or by gravity. Often the tubes are pulled with varying forces or in several pulling cycles.

The following types of pullers are commonly used:

Vertical pullers use weights to exert pulling force to the pipette. Usually pipettes are pulled in two turns. Vertical pullers are very popular for electrophysiological applications like patch-clamping but are not suited well for the use in the SICM: reachable tip opening diameters are about 300 nm and the produced tips tend to be long.

Brown-Flaming Type Pullers work with electromagnets. They allow a lot of control over temperature and applied forces. It is possible to pull pipettes with diameters about 100nm reliably.

Exact temperature control is always a problem when using filaments. They tend to change their behavior with air pressure, humidity and operating time.

Laser pullers also allow to pull quartz pipettes. The glass is heated with a laser. This allows for very high melting points and accurate temperature control. These units are very expensive but excellent. Diameters down to 30nm are no problem.

Since the resolution of the SICM is directly dependent of the tip diameter a laser puller is recommended for serious research.

The puller described in section 3.14 was used for this thesis. The produced tips were not very reproducible. The pulled pipettes should be as short and thin as possible. The pulled pipette should have a length of about 4.5 cm to match our setup.

5.1.2 Electrodes

The electrodes are the metallic conductors that connect the pre-amplifier with the pipette filling and the ground-line with the bath solution. To prevent voltage drifts the electrodes have to be of second order thus the used silver wire has to be covered by a layer of silver-chlorate AgCl. We use a silver wire with a diameter of 0.1 mm — small enough to match into the pipette.

There are three possibilities to chlorinate the wire. In preparation they should be cleaned with alcohol and sanded with fine sandpaper.

- The easiest way is to put them in bleach overnight (for 12 h).
- You can put the wire in chloride-solution (20 mM to 100 mM) and connect it to the anode of a DC source. Another wire is connected to the cathode and also entered in the chloride-solution. With a current 1mA it takes approximately four minutes. The current should be small to prevent bubbles from developing. The required time gets longer with small currents but the AgCl layer gets more durable [ND96][p.63].
- The fastest method is to melt AgCl in a ceramic bowl over a bunsen burner (melting point is about 600°C). Then dip the electrode in the melted AgCl to chlorinate the wire.

Always prepare the bath electrode and the pipette electrode in the same procedure to be sure they have the same properties. The chlorinated electrodes have a dark gray coating and should be replaced or re-chlorinated once a month because the AgCl coating gets scratched off when the electrode is inserted into the pipette. Store chlorinated electrodes sheltered from light since the AgCl layer is light-sensitive.

To match to our pipettes, the pipette electrode should have a length of 4 cm. The pipette electrode is then soldered into the pipette holder. The bath electrode is soldered to a small alligator crimp.

Some cells are damaged by silver ions. When working with such cells, the bath electrode has to be connected to the bath solution over a so-called agar bridge. A instruction how to built an agar-bridge can be found in [ND96][p. 64].

5.1.3 Preparing a sample

A good testing sample consists of a blood smear on an object holder, fixated with methylene alcohol. In the optical microscope the single red blood cells are recognizable.

When fixated with methylene alcohol these cells are flat disks about $2\ \mu\text{m}$ high and $7\ \mu\text{m}$ in diameter. These dimensions are ideal for testing the SICM. To prepare the sample the proceeding is the following:

- A small drop of blood is placed on the surface of a clean glass slide near the end. If blood is taken from the finger, care must be taken to avoid touching the slide to the skin.
- The slide is held between two fingers and the thumb of the left hand with the drip of blood on the upper surface towards the right. (Reverse for the left handed individual). An edge of the spreader slide is placed on the first slide to the left of the drop of blood and is pulled to the edge of the drop. The angle between the two slides will vary according to the size of the drop and the viscosity of the blood. The approximate angle for normal blood is 30 to 40 degrees.
- The drop of blood should be allowed to bank evenly behind the spreader which is then pushed to the left in a smooth, quick motion. The more rapid the motion, the shorter and thicker the smear. The smear should cover approximately half the slide with a gradual transition from thick to thin. No ridges should be present and the end (called the "feather edge") should be smooth and even. In the feather edge the red blood cells should not be routinely overlapped.
- Allow slide to dry completely.
- Fixate the slide with Methanol for max. 30s.

We did not stain our samples because the red blood cells are clearly visible but it is possible to do so. A sample prepared with this method can be used up to two weeks.

5.1.4 Testing the EPC 7

The EPC 7 – the patch-clamp amplifier – measures the small ion current with a highly sensitive operational amplifier circuit. The current is also converted to a voltage and filtered.

Initial checkout

As a first step we check the basic current-measuring circuitry of the EPC 7. This test used to check the operativeness of the EPC 7 main unit.

- Connect the function generator to **STIM. INPUT**.
- Connect the **CURRENT MONITOR** output to the oscilloscope.
- Set the **MODE** switch to *TEST*.
- Set the **GAIN** selector to 10 mV/pA .

Any Signal applied to the **STIM. INPUT** connector should be reproduced with the same amplitude (but inverted) at the **CURRENT MONITOR** output. A voltage of about 1 V is appropriate [HEK].

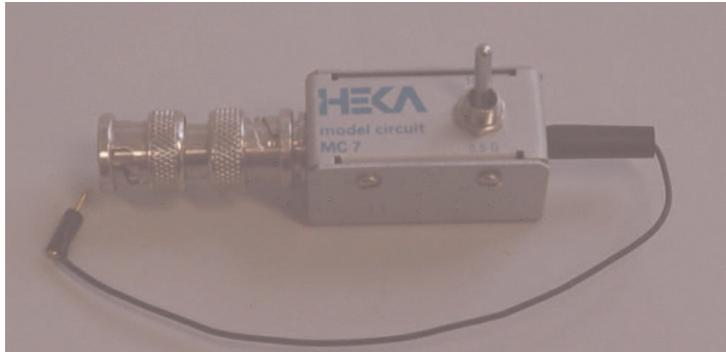


Figure 5.1: The MC-7 model circuit

Testing the headstage

For further Tests the *MC-7 Model Circuit* is used. With this circuit the headstage is checked. The model circuit has three settings to offer:

1. In the *10 M Ω setting* the *MC-7* simulates a pipette that is open to the bath solution .
2. In the *center position* a gigaseal is simulated, leaving only a capacitance of about 5 pF. Now the **C-FAST** control on the EPC 7 can be used to cancel the capacitive spikes resulting from stimulus pulses.
3. In the *0.5 G Ω position* a “model cell” in the whole-cell configuration (see Fig.2.4) is switched in. In the *VOLTAGE CLAMP* mode, this can be used to verify the **C-SLOW** controls and the action of series resistance compensation on the EPC 7. Note that because of poor electric properties of the internal switch, the model circuit introduces excess random noise above the level that can be obtained with a gigaseal [HEK].

The input circuitry of the probe can be damaged by static electricity. For this reason the input terminal should not be touched unnecessarily. Also, it is a good idea to be grounded when handling the scanning head.

We use the model circuit to check the functionality of the headstage.

- Mount the *MC-7* to the BNC-connector of the headstage.
- Connect the plug of the *MC-7* to the **GND** connector on top of the headstage.
- Connect the function generator to **STIM. INPUT** on the EPC 7.
- Connect the **CURRENT MONITOR** output of the EPC 7 to the oscilloscope.
- Set the *MC-7* to $10\text{ M}\Omega$.
- Set **MODE** switch to *SEARCH*.
- Set **GAIN** selector to 10 mV/pA .
- Set **STIM. SCALINGEPC** to $.01$.

When applying a square signal from the function generator to **STIM. INPUT** the signal should be reproduced on the oscilloscope. By switching the *MC-7* to the other modes you can watch the behavior of a gigaseal or a whole-cell configuration.

If only drifting noise is measured the isolation between the headstage and the piezo actuator should be checked before sending the EPC 7 to maintenance. This has been the most common error in the course of the project.

5.1.5 Testing the EDA unit

To check the functionality of the piezo actuator and its control unit (EDA) we use the *DEMOEDA* software provided by the manufacturer.

When switching the EDA unit on or off always take care that the **OPEN/CLOSED LOOP** selector is set to *open* for all channels. Be sure that the EDA-unit is turned on and set all channels to closed loop mode.

- Invoke the *DEMOEDA* program by clicking its icon.
- When the connection is established. The program should show "RS232 aktiv" in the upper left corner.
- Click the Tab `Standardfunktion`.
- Enter an amplitude (`Amplitude`) of 70 μm for each channel.
- Enter a frequency (`Frequenz`) of 50 Hz for each channel.
- Write the data to the EDA by clicking `alles schreiben`.
- Test each channel by clicking `start`. Now you should hear a humming noise – this is the sound of the vibrating piezo. Stop the channel (`stop`) and proceed with the next one.

The *DEMOEDA* program is also used to configure the EDA-device. See manual for details [[Pie](#)].

5.1.6 Preparing the microscope

The following procedures are necessary to begin any measurement with the SICM.

The thin plastic tube needed to fill the pipette is produced by heating a plastic needle protection cap over a very small flame. If the flame is too big it will burn the plastic instead of softening it. When the plastic seems uniformly soft pull it slowly apart. Cut the produced hollow plastic fiber at the thinnest part.

Filling the pipette

- To fill the pipette with solution dip the pipette tip in filtered solution for some seconds (tip filling).
- Insert the thin plastic tube of the filling syringe in the back of the pipette and fill it to a length of 1 cm. There should be no loose drops of solution in the rest of the pipette (back filling).
- Knock on the side of the pipette with your finger repeatedly to get rid of small air bubbles.

Mounting and positioning of the pipette

- Put the pipette in the pipette holder in a way that the electrode runs up to the pipette tip.
- Now place the petri dish containing the sample on the stage of the optical microscope and plug the pipette holder to the headstage.
- Twist the pipette down until it nearly touches the surface of the solution but is still slightly tilted. The tilt should be enough to let light pass to the very tip of the pipette. The headstage EPCHS should be in a high position when you do this.
- The last step is to mount the bath electrode. Connect it to the small jack on top of the headstage EPCHS and let the chlorinated silver wire dip into the bath solution.

After these steps everything is prepared to start measuring: All components are tested and the pipette is filled and positioned over the sample, ready to enter the bath solution. By now the desired sample should be centered in the view-field of the optical microscope.

5.2 Obtaining data

As a start, *Matlab* should be invoked on the PC. To move the piezo actuator to a defined upper position, initialize it by executing the function `initeda` in Matlab. Now the EDA-controller is activated and the position of the pipette is set.

For initialization of the data-acquisition system call the function `initdaq`. The next step is to insert the pipette into the bath solution.

- Connect the function generator to **STIM. INPUT** on the EPC 7.
- Connect the EPC's **CURRENT MONITOR** output to the oscilloscope.
- Set **MODE** switch to *SEARCH*.
- Set **GAIN** selector to *10 mV/pA*.
- Set **STIM. SCALING** selector to *0.01*.
- Apply a signal from the signal generator to **STIM: INPUT**.
- Now, with the pipette not touching the solution, there should be no signal visible at the oscilloscope. Now is a good time to calibrate the oscilloscope by switching its input to GND and adjusting the line to zero.
- Move the pipette down with the micromanipulator until it enters the bath. At this point some pressure can be applied to the pipette if a tube is attached to the pipette holder. When the bath solution is dirt-free this is not necessary.

In the moment the pipette enters the bath the signal can be seen on the oscilloscope. This indicates that the electrical circuit is closed and the pipette is not clogged.
- Move the pipette tip further down manually. It should now be immersed in the solution but still some millimeters away from the sample surface. The signal on the oscilloscope should be stable and not too noisy. If there is much noise the Faraday cage should be mounted now.

5.2.1 Measuring the pipette resistance

From knowing the applied voltage and the resulting current the pipette resistance can be calculated and the opening diameter of the tip can be roughly estimated.

- Calculate the applied voltage by multiplying the voltage from the function generator (the oscilloscope can be used to measure the voltage) with the value of **STIM. SCALING**.
- Then measure the current answer (reconnect the oscilloscope to **CURRENT MONITOR** if necessary). Multiply the voltage displayed on the oscilloscope with the value of **GAIN** on the EPC 7. The obtained value is the current flowing through the pipette.
- To calculate the resistance simply use Ohm's law $I = \frac{U}{R}$. The resistance should be some $M\Omega$.

The correlation between resistance and pipette diameter was developed in chapter 2.3.

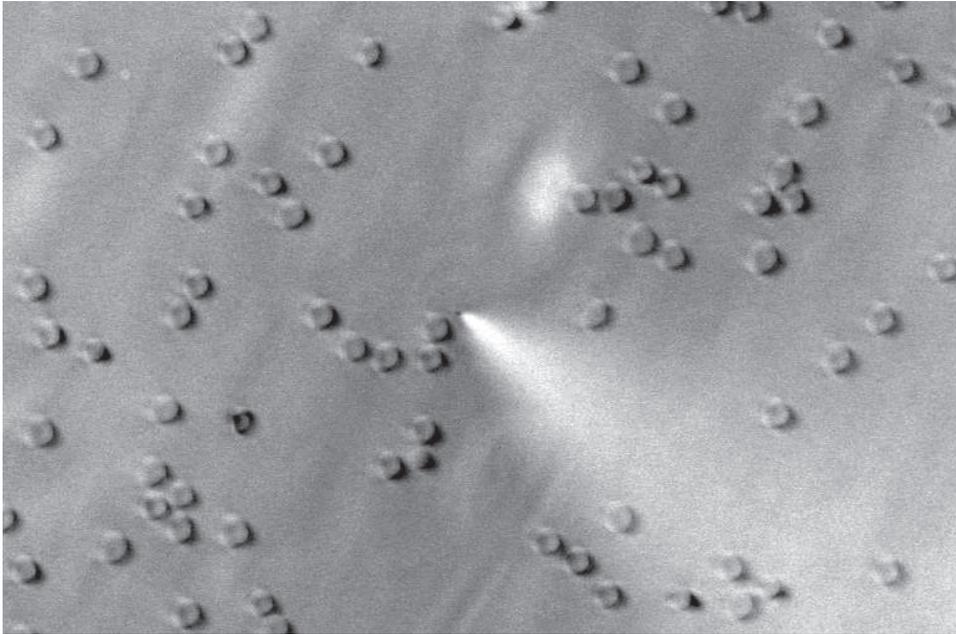


Figure 5.2: The tip near the sample surface viewed under the microscope

5.2.2 Approaching the sample

- Get the pipette tip manually near the target position with the micromanipulator
- Try to bring the tip into the focus of the optical microscope.
- Now move the focus down to your sample and then a little bit up again.
- Move the pipette down with the micromanipulator until the tip is in focus again.
- Repeat the last steps until the tip is near the sample surface. This may take some practice — the result should look similar to Fig.5.2. In the shown case the tip is approximately $20\ \mu\text{m}$ above the cells.
- To test if the surface is in reach of the tip call the program `probe(0.998)`. This will give you the distance to the surface. If the resulting height is zero, lower the tip ($\frac{1}{4}$ turn anti-clockwise) and try again.

5.2.3 Recording an approaching curve

Before we start, the pulse generator should be turned off and detached from **STIM.INPUT**. Instead of the pulse signal applied before we will now apply a constant signal. The advantage of a constant signal is the achievement of a smoother curve and fewer measuring artifacts.

To apply a constant signal we use the Patch-clamp amplifier itself. Turning **VHOLD** on the EPC 7 until the answer on the oscilloscope is slightly less than 5V provides a good signal since the analog-to-digital converters of the NI-DAQ data acquisition card have a range of 0–5 V.

To record an approaching curve, be sure that the tip is in proximity of the surface. Then enter `approach(val)`. Good values are about 0.98 — too low values will destroy the tip by crashing it into the surface.

Now the tip is moved towards the surface step by step. In every point the current is measured, recorded and compared to the initial saturation current. This takes much longer than the method that will be used to raster the surface but is more accurate in absolute terms.

Since this program relies on absolute current values, it is prone to drift and measuring errors.

It is inevitable to experience some broken tips. When the tip breaks the current jumps to a significantly higher value. After a tip-crash some shards of the destroyed tip can usually be identified in the optical microscope.

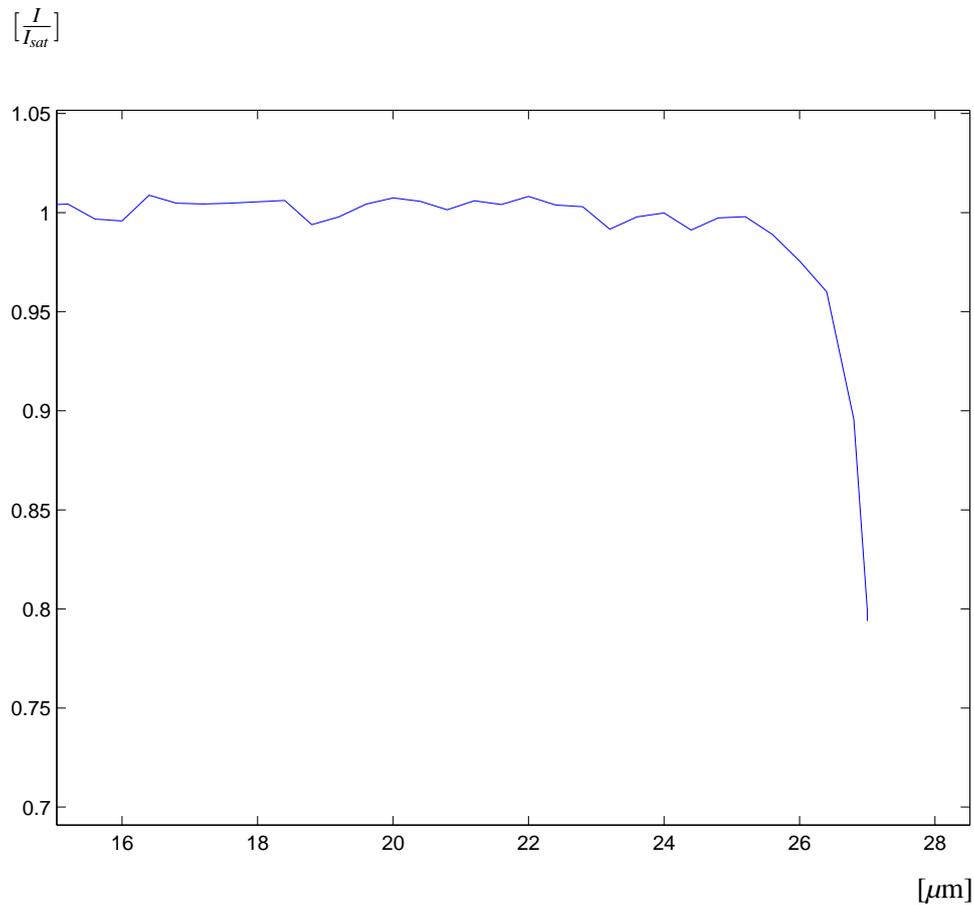


Figure 5.3: Approaching curve

5.2.4 Starting a scan

When the tip and the specimen are in position, type `map=scan` to start the raster scan. The obtained data is saved in the matrix `map` which you can use for further visualization and analysis. You will have to change the values of the program according to your needs before you start. The Faraday cage should be attached and any vibration should be prevented during scan.

When viewing through the microscope the scan begins in the upper left corner of the scanning area – keep this in mind when positioning the tip. Also, the tip should start its movement over glass and not directly on the specimen.

5.2.5 Processing and visualizing the data

After a scan the obtained data is stored in Matlab's workspace.

The recorded data will contain unwanted peaks and other failed single measurement points. To remove them and to generally enhance the quality of the picture you should apply a median filter. A median filter not only removes unwanted peaks but also sharpens the edges.

With Matlab's visualization routines a broad range of views can be computed without any programming.

The entire workspace or selected matrices can be stored and recalled using the `save` and `load` commands in Matlab.

Some Matlab commands:

`map2=medfilt2(map)` generates a median filtered version of `map` stored in `map2`.

`map=dlmread('filename')` reads numeric data from the ASCII delimited file `filename` into matrix `map`

`dlmwrite('filename',map,delimiter)` writes matrix `map` into an ASCII-format file, using `delimiter` to separate matrix elements. A comma (,) is the default delimiter. Use '^' to produce tab-delimited files.

`plot(map(xcut,:),y)` produces a profile of `map` at the position `xcut` along the y-axis. To get profiles along the x-axis use the form `plot(map(:,ycut),x)`. The lists `x` and `y` must match the dimensions of `map`.

`showmap(map,x,y)` computes a lighted surface view of `map`. The lists `x` and `y` must match the dimensions of `map`. An example: if the dimension of `map` is $10 \times 10 \mu\text{m}$ with a step-size of $0.5 \mu\text{m}$ the matching lists are produced by: `x=[0.5,0.5,10]`; and `y=[0.5,0.5,10]`.

5.3 Performed tests

In the beginning we recorded many approaching curves to get the process under control. A lot of experimentation went into the preparation of the pipettes and debugging of the first software versions. The first experiments were performed blind since there was no inverted microscope available. The setup used is shown in Fig.5.4. In September 2000 the first successful approaches were presented at the “WE-Heraeus Course – Nanophysics” at the Technical University in Dresden [Sch00].

In late summer 2001 I received the invitation to Pisa and continued the project there. Some problems soon got obvious: the initial approach to scan the surface by step-controlling the tip via the EDA-controller was much too slow.

Therefore we came up with the idea to use an analog output to modulate the controlling voltage, simultaneously sampling the current to stop the movement when detecting the surface. This worked out very well. In addition to the higher scanning speed tip breaks got significantly infrequent due to the dynamic nature of the process and the increased sensitivity.

With the working prototype we experimented mainly with red blood cells prepared like described in chapter 5.1.6.

The microscope was set up in Vienna again, this time in the described configuration. Fine tuning of the software was done and the scanning head was installed on an easily detachable plate for more versatility.

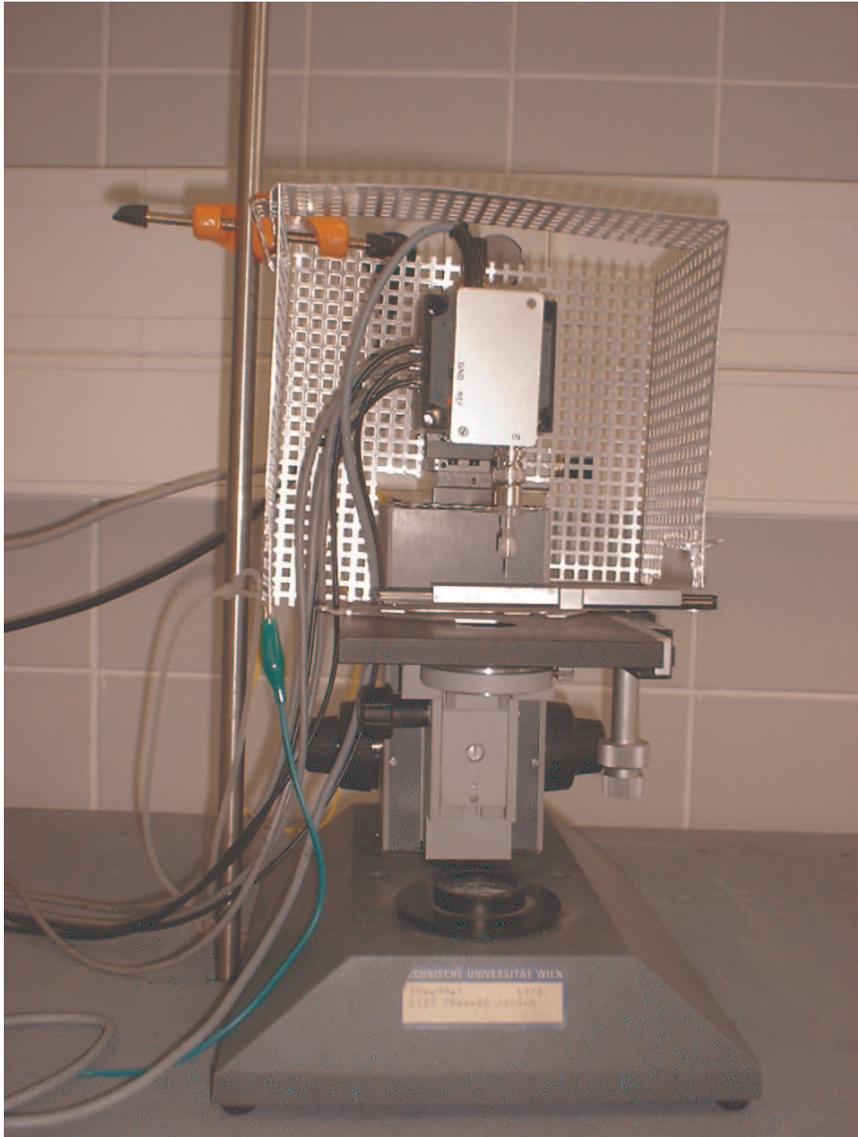


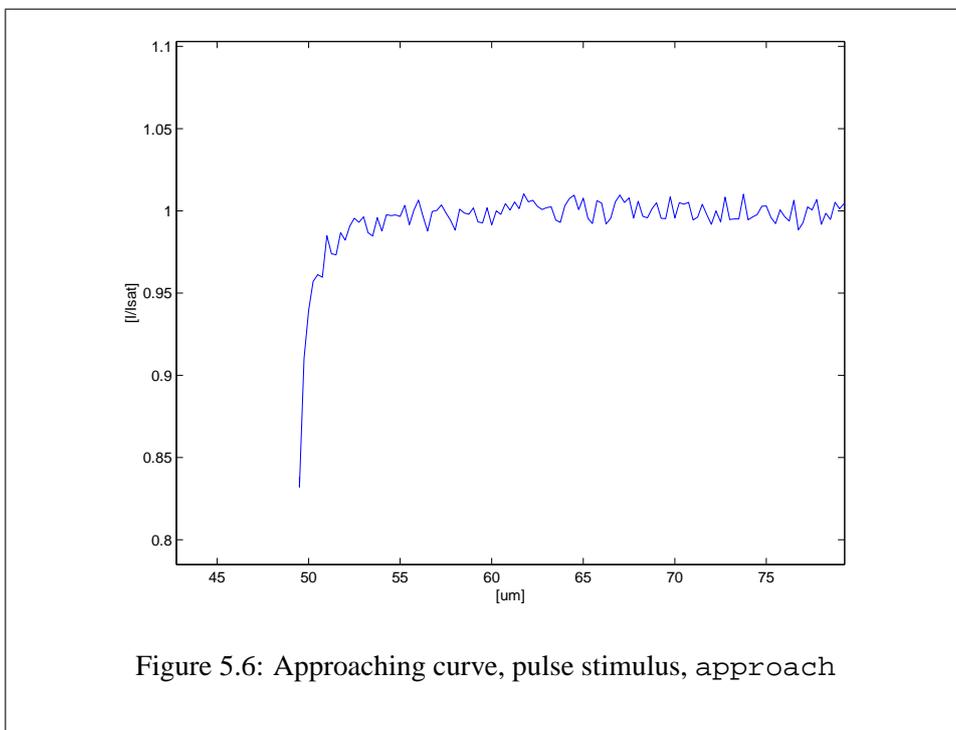
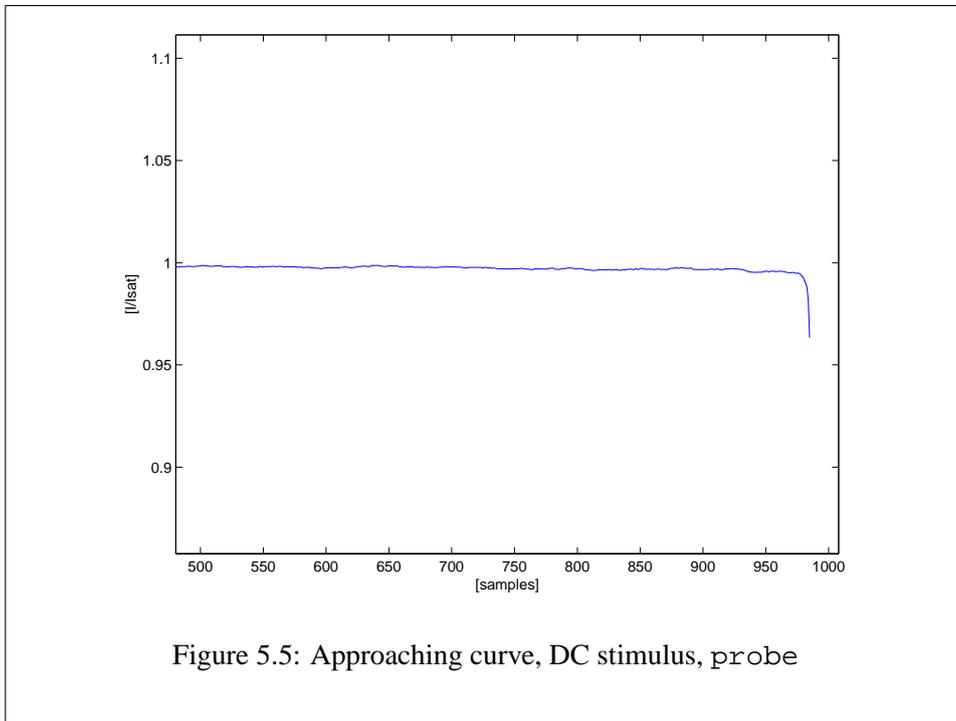
Figure 5.4: An early version of the SICM

5.3.1 Approaching curves

A SICM scan consists of a sequence of approaching curves carried out at every raster point. To gain quick evidence of the operativeness of the SICM approaching the surface is the easiest way. With the shape of the approaching curve at hand estimation of the tip diameter is also possible.

A typical approach curve is presented in Fig.5.5. This curve was recorded using DC stimulus by the EPC 7 and the function `probe` utilizing differential measurement. For comparison Fig.5.6 shows a curve that was recorded with a pulse signal and the use of the function `approach`. The DC stimulated curve is considerably smoother.

The tilting of the pipette had no noticeable influence on the curves (this would still be worth investigating again with smaller and in particular more reproducible pipettes).



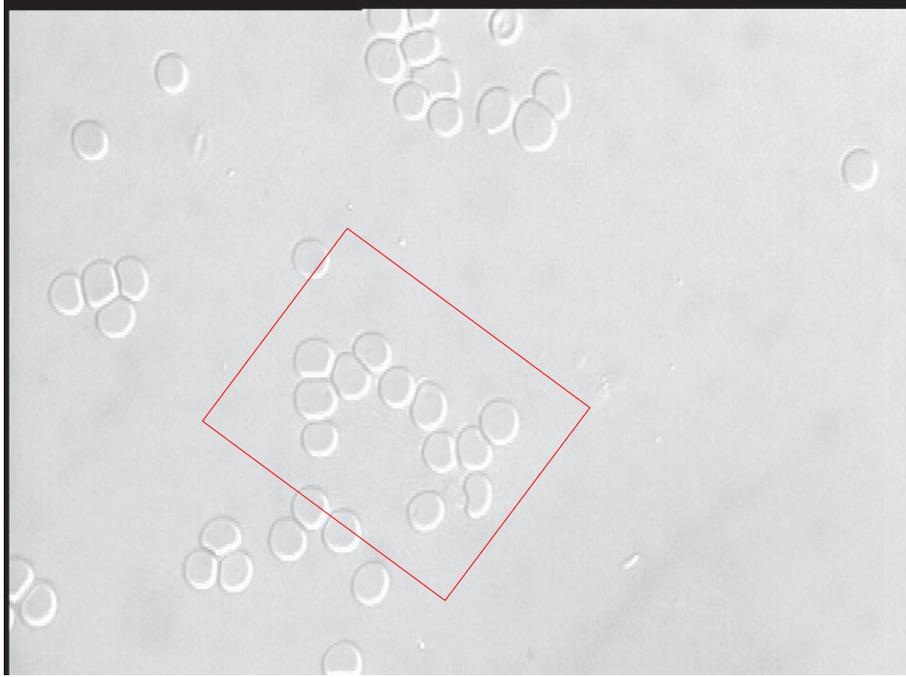
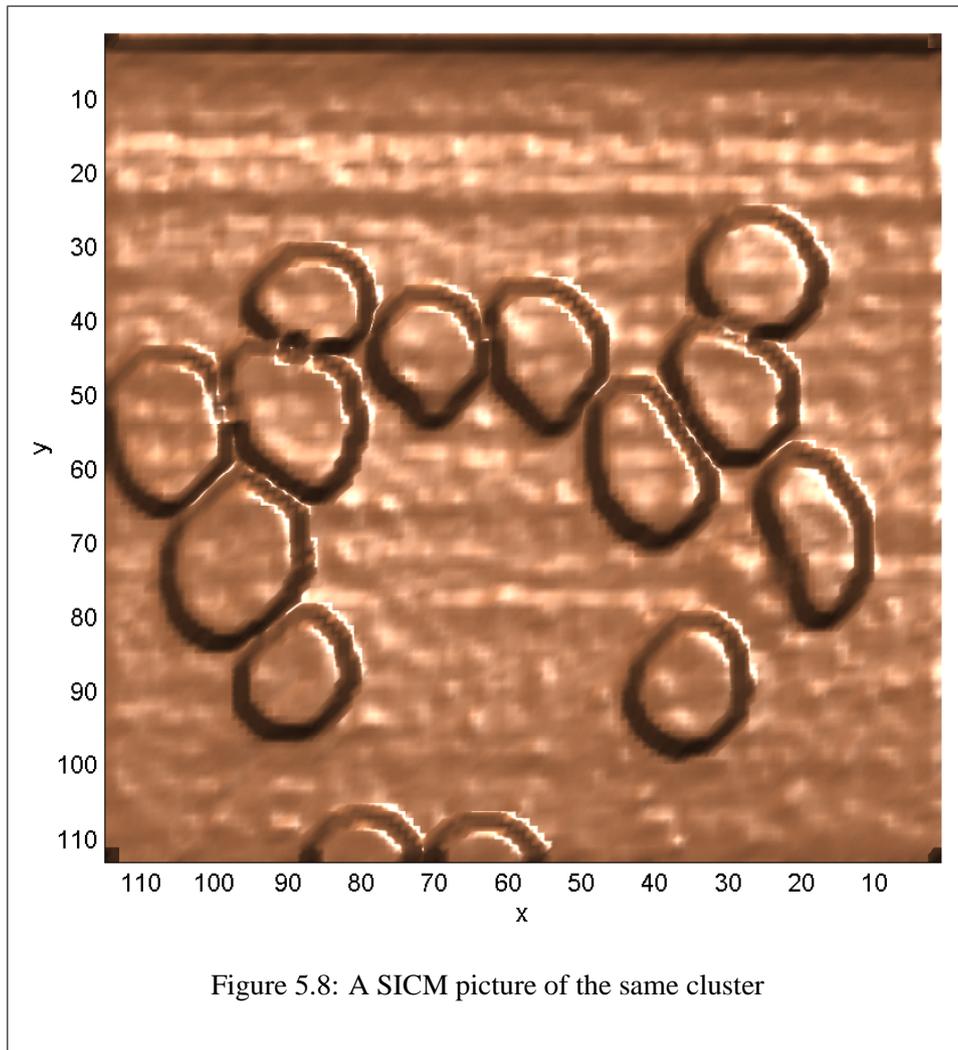


Figure 5.7: A Nomarsky phase-contrast picture of red blood cells

5.3.2 Scans

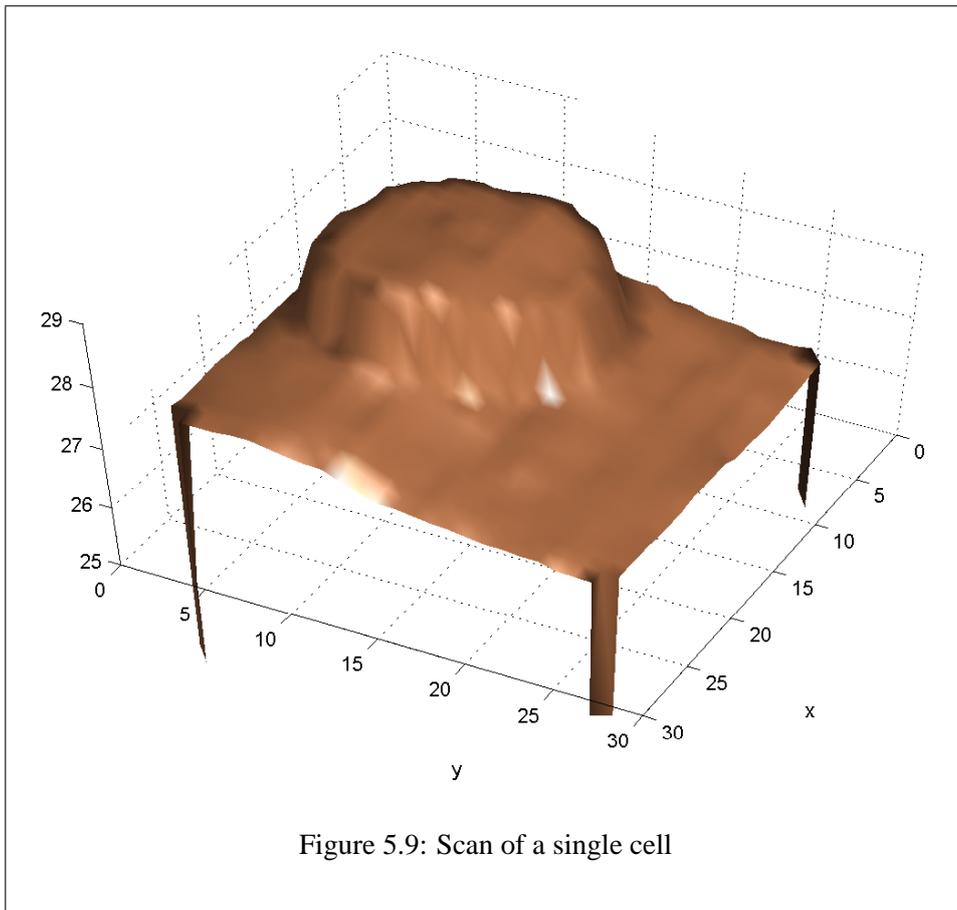
In Fig.5.8 you can see a scan of several red blood cells (erythrocytes) in an area of $60 \times 60 \mu\text{m}$. For comparison Nomarsky phase-contrast pictures are shown in Fig.5.7. This scan took about 6.5 h and surprisingly the tip was still operable thereafter. The step-size was 500 nm and the opening diameter of the used tip was about 400 nm.

The Nomarsky phase-contrast picture was taken after the SICM scan. The fact that both pictures show the same configuration of cells indicates that the SICM did not alter the initial cell distribution.



In the SICM picture the resolution is evidently better than in the phase-contrast picture. Note that the gaps between the cells are clearly recognizable. As opposed to phase-contrast microscopy or similar methods the whole three-dimensional topography of the specimen is recorded.

The results from another scan are illustrated in Fig.5.9. Here a single blood cell is pictured. The area of this scan is $15 \times 15 \mu\text{m}$ and the resolution is 500 nm again. The pipette used for this scan was slightly smaller with a estimated diameter of 300 nm.



Chapter 6

Proposed improvements

Reprogramming the software: The pictures taken are of good quality but the scanning process takes too much time. To reach a higher scanning speed it will be necessary to re-write the code in C/C++. Matlab is an interpreted language and therefore the bottleneck of the measurement system.

The reprogrammed SICM will scan approximately 20–100 times faster than with the present software. The speed will be improved in two ways: execution speed is dramatically higher and the driver support for the DAQ-card will be better (the DAQ-Toolbox in Matlab requires the program to pause for some tenths of a second after each measurement).

In the course of reprogramming the software a user-friendly graphical user interface should be designed. The Matlab functions will not be obsolete when new software exists. They still have their use for experiments and testing purposes.

A faster computer: The usage of a faster computer will speed up the workflow considerably — especially when working with Matlab. A faster computer will also influence the the scanning speed to a small extent. A DAQ-card with higher sampling frequency will also boost the scanning speed.

Acquisition of a puller: The purchase of a good pipette-puller is required. To get the pulling process under control a lot of research should be invested. A bubble test or some similar method has to be developed to estimate the opening diameter of the produced pipettes. A Laser puller is recommended

for research requiring high resolutions. For less demanding applications a good Brown-Flaming type puller will in be sufficient.

Use of the Computer as function generator: An analog output of the NI-DAQ should be used for generation of the stimulation signal. To get good measurements the voltage should only be turned on while approaching the surface. Additionally the sign of the voltage should be reversed in each scanning point when scanning cells to prevent polarization which could damage the cells.

Construction of a perfusion chamber: To expose the specimen to modified bath solutions a perfusion chamber should be constructed. This is especially a must when trying to investigate ion-channels. Some tips about perfusion systems and different methods to quickly change the bath solution can be found in [ND96]. The perfusion process could also be controlled by the NI-DAQ card.

Experiments:

Calibration The absolute accuracy of the SICM has to be increased by calibrating it with known micro-structures.

Frequency- and step-responses in dependence of the distance to the specimen and the used pipette should be investigated.

Localization of ion channels For this experiment a two pass method can be used. In the first pass ion channels in the cell surface are chemically blocked. The SICM scans the surface as usual to get a picture of the topography of the cell.

In the second pass the ion channels are activated and the SICM follows the previously scanned surface, thus detecting the ion currents and localizing the channels.

Chapter 7

Conclusion

The Project to construct a Scanning Ion Conductance Microscope (SICM) from scratch was initiated in February 2000 by Dr. Ille Gebeshuber. After collecting theoretical background information and literature studies appropriate hardware was selected and ordered. A Finnish exchange student named Perttu Niemelä (University of Oulu) assisted me in this task. In September 2000 the first results were presented at the “WE-Heraeus course Nanophysics” in Dresden [Sch00].

In summer 2001 I was invited to Pisa where most of the actual project was done. A control solution for the time critical approaching process was found and a working prototype was assembled and tested. The work in Pisa was done in cooperation with Prof. Dr. Mario Pellegrini, Prof. Dr. Donatella Petracchi and Prof. Dr. Cesare Ascoli at the *Dipartimento di Fisiologia e Biochimica*.

After being timed out for the duration of my military service the microscope has been re-installed and further enhanced at the *Institute of Chemical Engineering (Technical University of Vienna)* with the help of Prof. Dr. Helmut Stachelberger.

The SICM is operative and its usefulness has been shown. Pictures of red blood cells have been taken as proof of concept. The constructed SICM will be utilized in research projects at the Institute of Chemical Engineering by Prof. Dr. Helmut Stachelberger.

List of abbreviations

AFM	Atomic force microscope
AMFM	Attractive mode force microscope
DIC	Differential phase contrast
EFM	Electrostatic force microscope
FFM	Friction force microscope
MFM	Magnetic force microscope
NI-DAQ	National instruments data acquisition card
SAM	Scanning acoustic microscope
SHFM	Shear force microscope
SICM	Scanning ion conductance microscope
SNOM	Scanning near-field optical microscope
SPM	Scanning probe microscope
STM	Scanning tunnelling microscope
SThM	Scanning thermal microscope
UHV	Ultra high vacuum

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