

Chloride versus Protons

Ion Currents in the Cell-Transistor Junction

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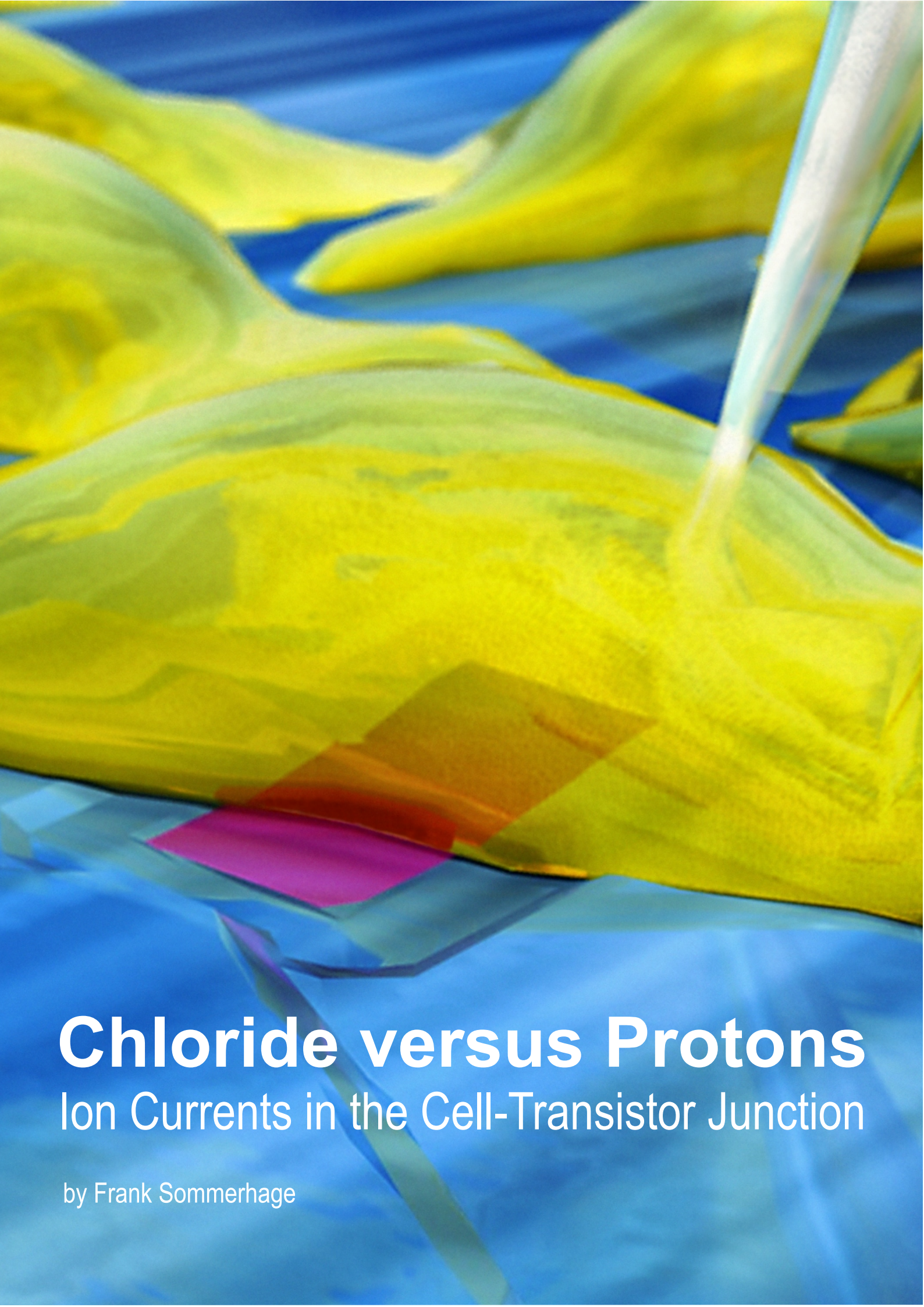
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by Frank Sommerhage

for Mercedes

A student's comment:

What a lot of morons people must have been back in the middle ages? Every morning, they've looked at the dawn and thought they're seeing the sun going around the earth.

Ludwig Wittgenstein's reply to that comment:

Yes, but I wonder what it would have looked like if the sun in fact had been going around the earth? I suppose it would have looked exactly the same...

"You see, what your knowledge tells you you're seeing."

James Burke in THE DAY THE UNIVERSE CHANGED (1985)

Summary

The cell-transistor junction has been investigated throughout the last two decades. Equivalent electric circuits and various models were devised to reproduce measurements and thus increase an understanding of processes within the junction. In parallel, the minute geometry between an individual electrogenic cell and a field-effect transistor was characterized to fine-tune the models. Surprisingly, all past experiments were entirely based on cations, such as sodium or potassium, disregarding potential applications for anions.

In this thesis, the role and influence of anions in the cell-transistor junction was investigated. According to *state-of-the-art* theory in the field, cations and anions have different effects on measurements, since field-effect transistors show a selective sensitivity for small cations. In order to verify the theoretical expectations, a human embryonic kidney cell line was stably transfected with ligand-gated chloride channels, which provided anion currents upon chemical stimulation. As anticipated, a comparison of recordings with field-effect transistors and inert gold electrodes showed no significant differences.

Based on the results obtained from experiments with chloride currents, the recently discovered antiparallel transport of chloride and protons in some voltage-gated chloride channels was investigated. The cell-transistor junction offered the opportunity to reveal the transport of protons in chloride channels since the cation-sensitivity of field-effect transistors induces strong sensor responses upon pH changes. In order to clearly reveal the transport of even smaller amounts of protons, assays were developed to influence pH changes due to buffer concentrations.

Signal modeling and simulation was employed to determine the transport stoichiometry of chloride versus protons in voltage-gated chloride channels. Even advanced models of the cell-transistor junction considered only ion currents carried by one single ion species. Therefore, a new comprehensive model of the cell-transistor junction was developed to simulate electric, electrodiffusive and ion-sensitive processes for multiple ion species. In addition, pH and buffer effects were implemented to simulate the influence of transported protons.

The shape and attachment of cells was analyzed by means of membrane allocation profiling, a new method developed in the course of this thesis. The results were employed to provide accurate geometrical proportions of the junction for the comprehensive model of the cell-transistor junction.

This work unites the fields of cell biology, biophysics and chemistry with microelectronics as well as computational sciences to advance the understanding of cell-transistor junctions. New

detail was provided about the junction's geometry. Further, *state-of-the-art* theories in the field were first confirmed and then improved to develop a comprehensive model of the cell-transistor junction. Finally, the new model was employed to provide insights into the transport stoichiometry of Cl^-/H^+ antiporters.

Zusammenfassung

Die Zell-Transistor Kopplung wurde während der letzten 20 Jahre genauestens untersucht. Die Messergebnisse konnten mittels Simulationen mit elektrisch äquivalenten Schaltkreisen und erweiterten Modellen erklärt werden, was das grundlegende Verständnis der ionisch-elektronischen Kopplung erweiterte. Zur Unterstützung der Simulationsansätze wurden die geometrischen Begebenheiten der Kopplung zwischen Zelle und Feldeffekttransistor durch alternative Methoden genauer charakterisiert. Jedoch basierten alle bisherigen Experimente und die daraus gewonnenen Erkenntnisse ausschließlich auf Kationen, was die Signalbeiträge von Anionen und potentielle Anwendungen vernachlässigte.

Diese Dissertation beinhaltet die Untersuchung der Einflüsse von Anionen auf die Zell-Transistor Kopplung. Die gängigen Theorien gehen davon aus, dass Kationen und Anionen verschiedene Signalkomponenten beitragen, da Feldeffekttransistoren eine selektive Sensitivität für Kationen besitzen. Um die theoretischen Erwartungen zu überprüfen, wurde die Human Embryonic Kidney Zelllinie mit Liganden gesteuerten Chlorid-Kanälen transfiziert, welche unter jeweils elektrischer bzw. chemischer Stimulation Anionenströme liefern. Wie erwartet zeigte ein Vergleich von Messungen mit Feldeffekttransistoren und Goldelektroden im Falle von Chlorid keine signifikanten Unterschiede.

Basierend auf den Resultaten der Experimente mit Chloridströmen wurde der kürzlich entdeckte antiparallele Transport von Chlorid und Protonen in spannungsgesteuerten Chlorid-Kanälen untersucht. Der Zell-Transistor-Kontakt bot die Möglichkeit den Transport von Protonen in Chloridkanälen zu messen, da die Kationensensitivität der Feldeffekttransistoren stark auf pH-Veränderungen reagiert. Zur Messung selbst kleinster Protonenströme, wurden Versuchsprotokolle erstellt, die pH-Veränderungen mit verschiedenen pH-Pufferkonzentrationen beeinflussen. Aufgrund des sehr engen Kontakts der Zellmembran mit dem Transistor bot diese neuartige Messmethode eine sehr hohe Genauigkeit im Vergleich zu den Standardmethoden mit frei manövrierbaren pH-Sonden in Glaspipetten.

Simulationen halfen die Transport-Stöchiometrie von Protonen und Chlorid in spannungsgesteuerten Chlorid-Kanälen zu ermitteln. Selbst moderne Modelle der Zell-Transistor Kopplung berücksichtigten nur Ionenströme, die von einer Ionenspezies verursacht wurden. Es war daher

nötig ein neues umfassenderes Model der Zell-Transistor Kopplung zu entwickeln, das die elektrischen Prozesse, die Elektrodifffusion von Ionen und die Ionen-Sensitivität von Feldeffekttransistoren für mehrere Ionenarten unterstützt. Zusätzlich war die Integration von pH- und Puffereffekten in die Simulationen notwendig, um den Einfluss von transportierten Protonen zu simulieren.

Die Form und Adhäsion von Zellen wurde mit Membran-Allokations-Profilierung, einer neuen Methode, die im Rahmen dieser Doktorarbeit entwickelt wurde, untersucht. Die gewonnenen Erkenntnisse dienen dem umfassenderen Model der Zell-Transistor Kopplung als geometrische Grundlage der Kopplung.

Diese Dissertation integrierte die Fachgebiete Zellbiologie, Biophysik und Chemie mit der Mikroelektronik und Informatik, um das Verständnis der Zell-Transistor Kopplung zu erweitern. Neue Erkenntnisse wurden zur geometrischen Situation der Kopplung gewonnen. Weiterhin wurden derzeitige aktuelle Theorien im Forschungsfeld zunächst bestätigt und dann erweitert, um ein umfassenderes Modell der Zell-Transistor Kopplung zu erstellen. Durch die Anwendung des neuen Modells konnte die Transport-Stöchiometrien von Cl^-/H^+ Antiportern ermittelt werden.

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1. Introduction

The human body has been optimized by evolution throughout millions of years and yet man always had the urge to seek for improvements. Ancient mythologies believed in superior the-
rianthropic entities that were half-man and half-animal, whereas hybrids of humans and deities gradually ruled heroic battles in legends of modern history. Ever since the age of enlightenment, these fantasia of mythical origin turned into realistic body enhancements and were later superseded by machine parts in the course of modern industrialization. The technological climax of the 21st century seeks to unify the human mind with computer networks, inspired by numerous science-fiction authors. However, scientists are still striving to devise a nexus of microelectronic circuits and the central nervous system, while genetic engineering is already at the forefront of performing genuine operator interventions in nature's paragon evolution.

The smallest brick in microprocessor architecture, omnipresent in every piece of computational technology, is the metal-oxide-semiconductor field-effect transistor (MOSFET). These transistors perform millions of digital decisions per second in the form of on-off, one-zero, current or no current. The field effect itself is actually not as digital as it may seem, but can also be employed for analog amplification. In fact, the inventor Julius Edgar Lilienfeld (1930) conceived an early version of the field-effect transistor (FET) as a "*Device for controlling electric currents*" to advance amplification circuits of his day's radio technology (Lilienfeld, 1932, 1933). Unfortunately, his contrivance was ahead of time and had to mature for another thirty years in various research laboratories, until semiconductor-device fabrication preceded and paved the way for more diversity in devices that made use of the field effect.

Quickly developing was a differentiation today known as the electrolyte-oxide-semiconductor field-effect transistor (EOSFET). Replacing the metal by an electrolyte opened up new possibilities for biological and chemical investigations. EOSFETs were initially named ion-sensitive field-effect transistors (ISFETs) because of their remarkable properties as pH sensors and ion-concentration probes (Bergveld, 2003). The field effect in EOSFETs is additionally governed by the concentration of ions in the electrolyte, which react with hydroxyl-groups (surface reaction sites) at the interface of oxide and electrolyte. In the course of this thesis the shorter acronym FET will be utilized to refer to ISFETs. Bergveld (1970) seized on the concept of ion sensitivity and adapted FETs for electrophysiological measurements. He was probably inspired by Hodgkin and Huxley (1952b), who reported merely two decades earlier that the migration of sodium and potassium across the cell membrane generates action potentials in neurons and muscles. Bergveld took the next logical step and investigated mammalian smooth muscles¹ (Bergveld, 1972) as well as insect muscles² (Bergveld et al., 1976) to measure changes in extracellular ion concentrations during action potentials. However, FETs react with cations in general and since Bergveld could not determine the contribution of individual ion species to the measured signals, he ceased his electrophysiological attempts shortly thereafter.

¹ *taenia coli*, smooth muscle from colon of *Cavia porcellus* (Guinea pig)

² *flexor tibialis*, muscle in jumping leg of *Locusta migratoria migratorioides* (African migratory locust)

Almost twenty years later, [Fromherz et al. \(1991\)](#) proceeded on Bergveld's earlier investigations and reported the first neuron-silicon junction. More advanced processing techniques in microelectronic engineering allowed the development of FET arrays on a small scale with very thin gateoxides (the output-modulating part) to primarily detect the electrical components of action potentials via non-ohmic charge transfer, which excelled these devices in the field of extracellular recording ([Offenhäusser and Knoll, 2001](#)). Neurons from various animals were cultured on these FETs and their bioelectric activity coupled into the field effect ([Fromherz et al., 1991](#); [Weis et al., 1996](#); [Sprössler et al., 1999](#); [Ingebrandt, 2001](#)). Analog to Bergveld's devices, the newer FETs recorded a composite of electrical and chemical signals that were hard to interpret. During the following years, attempts to understand the recorded signals revealed electric, electrodiffusive and ion-sensitive processes taking place in the cell-silicon junction. The first is very straight forward and can be explained by pure electrical means ([Gleixner and Fromherz, 2006](#)). The second contribution is an electrodiffusive effect that is mainly governed by the minute geometrical conditions ([Wrobel et al., 2005](#); [Brittinger and Fromherz, 2005](#)). The third share is the ion sensitivity, which is found exclusively in FETs and emphasizes these devices in contrast to metal electrodes, commonly used in electro- and neurophysiology ([Bergveld, 1972](#); [Ingebrandt et al., 2005](#)).

Traditional electrophysiology focusses on action potentials in neurons and cardiac cells, which are carried almost entirely by the cations sodium, potassium and calcium ([Hodgkin and Huxley, 1952b](#); [Neher and Sakmann, 1976](#)). Modern electrophysiology, however, comprises a significantly larger field and clarifies that electrical activity in biology is not thoroughly relying on cations. Electrical activity in fungi and plants manages osmoregulation by means of anion currents and a great variate of anionic bioelectricity can be found in the animal kingdom. In fact, mankind unknowingly used anionic bioelectricity long before [von Helmholtz \(1842\)](#), [du Bois-Reymond \(1848\)](#) and [Bernstein \(1871\)](#) initiated the unveiling of cationic action potentials with their experiments on frog legs. According to [Martin \(2008\)](#), the ancient Greeks released the pain of childbirth and operations with the *narke* or *crampfish* (*Torpedo* ray). In the course of evolution, these electrogenic fish developed the ability to generate electric fields that are by far stronger than those present in nerves or muscles ([Bleckmann and Hofmann, 1999](#)). The *Torpedo* ray emits an electric shock (about 220 V) into the body of other fish to stun his prey or ward of predators. On that account *Torpedo* rays have electroplaques, which are two kidney-shaped electric organs at the base of their pectoral fins. The electroplaques are highly specialized muscle cells that provide remarkable ion currents via channeling transport proteins in their membrane. Upon a change in the electric potential across the cell membrane, these chloride channels³ allow large amounts of chloride to enter the cell. The chloride channel family (CIC) has numerous members throughout all species. The chloride channel, CIC-0, found in the electroplaques of *Torpedo marmorata*, was the first to be purified, sequenced and named ([Dutzler, 2006](#)). [Accardi and Miller \(2004\)](#) found that currents through ecCIC⁴ were not

³ The contribution of protons was found later, thus Cl^-/H^+ antiporters were mistakenly named 'chloride channels'.

⁴ prokaryotic CIC homologue found in *Escherichia coli*

entirely carried by chloride, but also by a simultaneous efflux of protons into the extracellular environment. Subsequently, additional CIC proteins were found to be Cl^-/H^+ antiporters and not Cl^- channels (Accardi et al., 2005; Pusch et al., 2006; Suzuki et al., 2006; Lísal and Maduke, 2009). One CIC protein, whose role has yet to be defined clearly is the chloride channel 1. CIC-1 is located in skeletal muscle cells and involved in setting as well as restoring the resting membrane potential. CIC-1 was long thought to be a chloride-selective ion channel, providing passive pores that allow chloride ions to flow down their electrochemical gradient. A publication from Picollo and Pusch (2005) reporting small extracellular pH changes during CIC-1 activity is still discussed controversially in the field (Pusch et al., 2006; Lísal and Maduke, 2009). Proton currents anti-parallel to chloride currents were usually revealed by little pH probes brought close to the cell membrane or into a patch pipette to measure changes in pH during stimulation (Picollo and Pusch, 2005; Graves et al., 2008; Lísal and Maduke, 2008). However, very small proton currents elude a detection by these means.

The ongoing uncertainty about the function of some CIC family members, Cl^- channel versus Cl^-/H^+ antiporter, is a great opportunity for cell-transistor coupling with FETs. The majority of ion-sensitive FETs are employed as actual pH sensors and can detect the transport of H^+ . Chloride is an anion and should thus not affect the ion sensitivity of FETs (Hajji et al., 2000; Bergveld, 2003). Further, the geometrical situation of a cell-transistor junction is defined by a very small volume in which even a minute amount of protons emitted by the cell can result in large pH changes. Thus, the investigation of CIC-1 chloride channels with FETs would be the next logical step. This step will be taken to determine the transport ratio of protons versus anions in CIC-1 chloride channels. However, such an undertaking arises three major questions, which have to be addressed.

First: What is the geometrical situation of the cell-transistor junction? A tight cell attachment is crucial for the amplitude of measured electrical signals (Regehr et al., 1989; Weis et al., 1996; Sprössler et al., 1999; Ingebrandt et al., 2005). Numerous studies found that the average height of the cleft, the distance between a cell and the substrate it is growing on, ranges from 60 to 100 nm depending on the cell type and substrate properties (Weis and Fromherz, 1997; Braun and Fromherz, 1998; Wrobel et al., 2007). The attached membrane area, facing the surface and thus in contact with the cleft volume, determines the share of overall membrane currents affecting the junction (Ingebrandt et al., 2005). Estimates for the area of the attached membrane area were initially proposed to be 10 %, but were repetitively corrected upwards to 25 % and 33 % of the total membrane area (Weis and Fromherz, 1997; Buitenweg et al., 2003; Ingebrandt et al., 2005). However, for investigations of CIC-1 transport characteristics, the available cleft volume needs to be characterized fairly precise in order to relate changes in pH to proton currents. Since the cell radius can be identified by light microscopy, it seems to be the only reliable factor, whereas the cleft height and the attached portion of the membrane still need to be examined closer in order to conjecture a reliable geometry of the cell-transistor junction.

Second: How will anionic membrane currents act in the cell-transistor junction? This primary question has yet to be answered experimentally. Although cell-transistor coupling exists since almost 40 years, past experiments focused entirely on cationic action potentials (Fromherz, 2005; Ingebrandt and Offenhäusser, 2006). Bergveld's preliminary experiments were conducted with muscles and the following cell-silicon junctions were established with neurons and cardiac cells (Bergveld, 1973; Fromherz et al., 1991; Ingebrandt, 2001; Offenhäusser et al., 2009). Cells, genetically modified to express only one type of cation channel, either sodium or potassium, were cultured on FETs to investigate the influence of individual cation species on measured signals (Wrobel et al., 2005; Peitz et al., 2007). In order to investigate anionic effects, cells exclusively expressing anion channels would have to be coupled to FETs. However, the use of ClC family members for a characterization of FETs and anions would be venturous, since their exact function still seems to be doubtful (Lísal and Maduke (2009)). Therefore, other chloride channels need to be employed in experiments concerning this matter.

Third: How can the two reverse ion currents be regarded? Due to their opposed polarity and inverse direction of migration, chloride and proton currents can not be distinguished by electrical means. The share of proton contributions to total membrane currents in the measured signals can only be determined by the ion sensitivity of the FET. An important characteristic of FETs is that they can not measure the absolute pH, but only a relative change in concentration over time (Hajji et al., 2000; Yuqing et al., 2003; Bergveld, 2003). Assumed, a cell with ClC-1 channels always transports the same amount of chloride and especially protons upon stimulation, the amount of additional H_3O^+ in the cleft volume would be the same as well. Therefore, the ion sensitivity of FETs can be pre-set discretely by the initial pH in the cleft. For a very low initial pH, representing a high concentration of H_3O^+ , additional H_3O^+ released by the cell, would cause a relative small change in pH. Whereas a relative change for higher pH values, with predominantly OH^- instead of H_3O^+ in the cleft volume, would be comparatively larger. However, the transport of protons is not constant for variations in pH, since the electrochemical gradient for protons is affected as well (Chen and Chen, 2001; Bennetts et al., 2007). More promising is an alternative approach. The pH of electrolyte solutions is usually stabilized by a buffer (Cartwright and Shah, 2002). Type and concentration of the pH-buffer determine the amount of H_3O^+ that is neutralized, before a reaction with hydroxyl-groups at the electrolyte-gateoxide interface can contribute to measured signals while the concentration gradient for protons across the membrane is not affected (Yates et al., 1974).

The main objective of this thesis is to determine the interplay of chloride and protons in the cell-transistor junction. However, a significant portion of this thesis will be the characterization of anionic membrane currents and geometrical aspects in the cell-transistor junction and is thusly structured:

Chapter 2 elucidates the theoretical background of this thesis and explains selected topics to the reader who is unfamiliar with the coupling of cells with silicon circuits. It comprises general functions of the electrical activity of cells, a very basic explanation of genetic engineering, standard electrophysiological methods, the general functionality of MOSFETs and ion-sensitive field-effect transistors, selected electrolyte-solids interfaces, basics about pH and buffers and finally an introduction into simulations and models of the cell-silicon junction. Some following chapters will give further insight into their respective field of research.

Chapter 3 describes general materials and methods used throughout this thesis, such as cells and transfected subtypes, culturing protocols, electrophysiological equipment, used FET devices and amplifiers, employed microelectrode recordings systems and the combined setup for bioelectronic coupling. Specific materials or methods exclusively used in a following chapter, will be concerned there.

Chapter 4 introduces a new method to characterize the three-dimensional cell shape and general cell attachment based on surface reconstruction from confocal imaging. The resulting membrane allocation profiles can be used to determine missing parameters for the cleft geometry in cell-transistors junctions. This chapter was published as an article in the journal *Biomaterials* providing a comparison of several protein coatings to promote the adhesion of cells to a substrate (Sommerhage et al., 2008).

Chapter 5 addresses the examination of pure anion currents in the cell-transistor junction. Cells, stably transfected with a ligand-gated glycine receptor, which is also a chloride-selective channel, were cultured on FETs and for comparison also on substrate-integrated gold electrodes. The two resulting cell-sensor systems were used to verify that chloride has no effects on an FET's ion-sensitivity. This chapter was published as an article in the journal *Biosensors & Bioelectronics* (Sommerhage et al., 2010). However, the published article focuses on the opportunity to employ the ligand-gated cell-sensor junction as prototype for biohybrid sensors.

Chapter 6 approaches the coupling of cells expressing CIC chloride channels with FETs. Two different chloride channels were used: CIC-1 and CIC-4. The later one is known to have strong proton-transport capabilities (Picollo and Pusch, 2005). FETs were employed to measure membrane currents of either CIC-1 or CIC-4. The cell-transistor junctions were examined during variations in pH and the concentration of a pH-buffer.

Chapter 7 proceeds on the former chapter by challenging a comprehensive simulation of measured Cl^-/H^+ membrane currents, including representative electrical components, electrodiffusive contributions, pH-buffer interactions and the influences of ion-sensitivity in comparison with inert gold electrodes. Therefore, this chapter addresses the main objective of this thesis and yields an estimate for the transport stoichiometry of chloride versus protons in the chloride channel CIC-1.

Chapter 8 gives an overall discussion of chapters 4 through 7 in the context of this introduction. Further, conclusions will be drawn for the results of this thesis to give an outlook for future projects

concerning anionic membrane currents in cell-transistor coupling and their application in analytical cell biology and the development of biohybrid sensors.

2. Principles of bioelectronic coupling

2.1. From biological membranes to electrically active cells

A membrane is a layer of material which serves as a selective barrier between two phases and remains permeable to specific particles, molecules, or substances (Zydney and Zeman, 1996). Nature's most ubiquitous membrane is a 5 nm thin double layer mainly composed of amphipathic glycerophospholipids, also referred to as phospholipids. In aqueous solutions, lipids screen their hydrophobic tail from the surrounding polar fluid, causing the hydrophilic head regions to associate with the cytoplasmic and extracellular faces of the resulting bilayer. One important role of these membranes is to regulate the movement of materials into and out of cells. The lipid bilayer is fairly permeable to small, uncharged molecules such as oxygen or carbon dioxide, enabling cellular metabolism. However, the inner environment of the bilayer is very hydrophobic, and therefore retards the diffusion of hydrophilic, polar, or ionic compounds across the membrane. Since the import and export of hydrophilic metabolites as well as ions are necessary for a cell's functionality, cells have developed other means to move these compounds across their membranes.

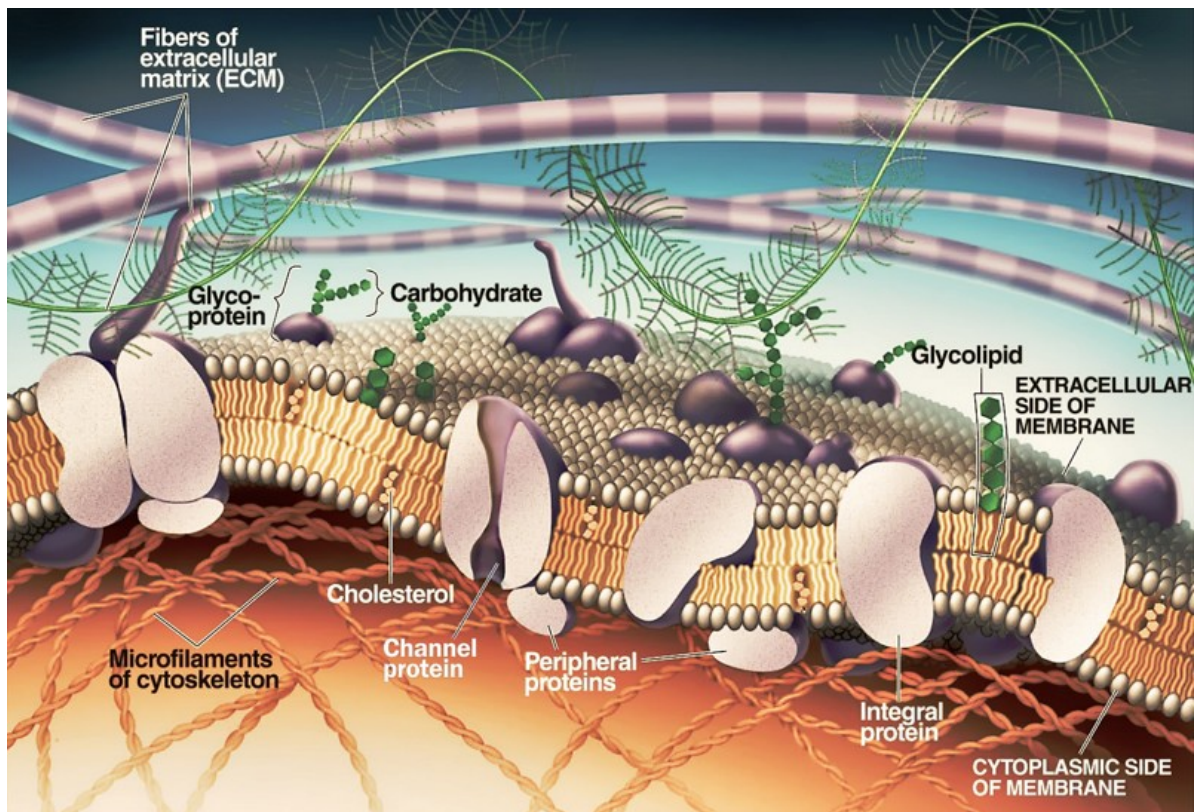


Figure 2.1.: Schematic structure of the cell membrane. The lipid bilayer holds a wide variety of biological molecules, primarily lipids and proteins, which are involved in numerous cellular processes such as cell adhesion, transport or signaling. The figure was adapted from (Pearson and Cummings, 2008)

As depicted in Figure 2.1, numerous proteins are incorporated into the cell membrane. Commonly found are proteins involved in structural integrity and adhesion. Proteins with signaling functions or proteins responsible for transmembrane conveyance are incorporated depending on the specific cell type. Specialized integral proteins span the entire membrane to shuttle hydrophilic

compounds from one side to the other. These specialized proteins add control to the transport process, since their structure, size, or charge selectively determines which compounds enter or leave the cell. Passive transporter proteins with the right size and environment for specific ions are often referred to as ion channels. Ions, migrating through these channels are exposed to two different forces: a chemical driving force, which causes diffusion due to concentration gradients and osmosis⁵, and an electrical driving force, resulting in ion movement because of electrostatic interactions. The net migration of ions is zero, when both forces reach an equilibrium. If the membrane is permeable only for ion species i with valence z_i , its equilibrium potential E_i can be calculated by the NERNST equation:

$$E_i = \frac{RT}{z_i F} \cdot \ln \left(\frac{[i]_{in}}{[i]_{out}} \right) = -\frac{61.5 \text{ mV}}{z_i} \cdot \log \left(\frac{[i]_{in}}{[i]_{out}} \right) \quad (2.1)$$

The potential of -61.5 mV results from combining the gas and FARRADAY's constants R/F with a body temperature of $T = 37^\circ\text{C}$ and conversion from natural to decadic logarithm.

However, in a real cell the membrane is permeable to more than one ion, predominantly for potassium and chloride. If equilibrium is to be reached, the electrical potential across the membrane must balance the diffusive driving forces for both ion species simultaneously:

$$\Delta\Phi_D = E_{K^+} = E_{Cl^-} = \frac{RT}{z_{K^+} F} \cdot \ln \left(\frac{[K^+]_{in}}{[K^+]_{out}} \right) = \frac{RT}{z_{Cl^-} F} \cdot \ln \left(\frac{[Cl^-]_{in}}{[Cl^-]_{out}} \right) \quad (2.2)$$

This equilibrium condition is called DONNAN potential $\Delta\Phi_D$. Inserting the ion charges z_{K^+} and z_{Cl^-} in equation 2.2 allows for a comprehensive simplification of the equation and leads to the DONNAN coefficient r_D :

$$r_D = \frac{[K^+]_{in}}{[K^+]_{out}} = \frac{[Cl^-]_{out}}{[Cl^-]_{in}} \quad (2.3)$$

According to DONNAN, other ions such as sodium or calcium are restricted in their diffusion across the cell membrane, but yet contribute to the overall equilibrium potential $\Delta\Phi_D$. Further, a diverse group of molecules, including proteins, charged amino acids, sulphate and phosphate, is confined within the cell. Most of these molecules provide a single negative charge, whereas others carry up to three negative net charges. On average, each of these molecules has slightly more than 1.2 negative charges (Matthews, 2002), resulting in a strong outward driving force for chloride and

⁵ Osmosis is the movement of water molecules down a water potential gradient (Haynie, 2001).

an inward driving force for potassium. The first DONNAN condition requires electrical neutrality on either side of the membrane with:

$$\sum_i z_i [i]_{in} = 0 \quad \text{and} \quad \sum_i z_i [i]_{out} = 0 \quad (2.4)$$

Ions to consider are chloride, sodium and calcium, which are more abundant outside and also potassium which is strongly enriched within the cell. Exemplary ion concentrations as given in table 2.1 belong to a model cell at perfect equilibrium yielding a comprehensive membrane potential $U_M = \Delta\Phi_D = E_{K^+} = E_{Cl^-} = -87 \text{ mV}$ and a DONNAN coefficient of $r_D = 26$. The second DONNAN condition requires an osmotic balance across the cell membrane stating that the total concentrations of ions on both sides of the membrane are equal (isotonic) and thus no movement of water occurs. The ion concentrations given in table 2.1 sum up to an osmotic balance with 257.5 mOsm on either side of the cell membrane.

	Na^+	K^+	Ca^{2+}	Cl^-	$\text{A}^{1,2-}$	Osmolarity
Cytoplasmic environment	10 mM	130 mM	$\ll^* 0.1 \text{ mM}$	5 mM	112.5 mM	257.5 mOsm
Extracellular milieu	120 mM	5 mM	2.5 mM	130 mM	0.0 mM	257.5 mOsm

Table 2.1.: Intra and extracellular ion concentrations for a model cell at perfect electro-diffusive equilibrium and in osmotic balance. *The Ca^{2+} concentration is usually in the range of micromolar.

Such a cell is a beautiful example of efficiency, existing at perfect ionic as well as osmotic equilibrium and thus in harmony with its electrochemical environment. However, the problem is that the model cell is not an accurate representation of the situation in a real cell. Electrogenic cells are not at equilibrium and must expend metabolic energy to sustain their *status quo*. While no energy is required for passive transport, cellular energy can and actually has to be raised to transport compounds against a concentration gradient or an electrostatic field across the membrane. Various active pumps and exchangers further decrease the concentration of chloride in the intracellular environment, either due to energy consumption or by co-transport with other ions along their respective concentration gradient. Hydrolyzing adenosine triphosphate (ATP), the membrane spanning macromolecule Na^+/K^+ -ATPase pumps sodium and potassium in opposite directions, accumulating sodium outside and potassium inside the cell. Eventually, several ion species experience a steep concentration gradient across the cell membrane. A fairly accurate estimate of the comprehensive membrane potential U_M at rest (not at equilibrium!), can be calculated with the GOLDMAN-HODGKIN-KATZ equation (2.5), which takes permeability coefficients P_i of all monovalent ion species i (Ca^{2+} and $\text{A}^{1,2-}$ are excluded) into account, and delivers the resting potential.

$$U_M = -61.5 \text{ mV} \cdot \log \frac{P_{\text{Na}} \cdot [\text{Na}^+]_{out} + P_{\text{K}} \cdot [\text{K}^+]_{out} + P_{\text{Cl}} \cdot [\text{Cl}^-]_{in}}{P_{\text{Na}} \cdot [\text{Na}^+]_{in} + P_{\text{K}} \cdot [\text{K}^+]_{in} + P_{\text{Cl}} \cdot [\text{Cl}^-]_{out}} \quad (2.5)$$

A cell at rest is polarized (-20 to -100 mV depending on the respective cell) at holding energy to perform a function. Typical purposes of electrogenic cells are signal transduction and information transport. In order to change the membrane potential upon external stimuli, sensory cells seem to change the conductivity of their membrane selectively for specific ion species. Their cell membrane holds gated ion channels which only open for ions when signaled to do so. These signals can be covalent modifications by cellular enzymes, structural changes caused by mechanical forces, conformational changes due to light, changes in pH, binding of a ligand to the channel protein or upon variations of the membrane potential. The functionalities of the latter two are depicted in figure 2.2. Whenever stimuli induce the opening of gated ion channels the cause is transduced into a changing membrane potential. The influx of chloride or efflux potassium hyperpolarizes the cell, whereas the influx of sodium or calcium depolarizes the cell.

In the case of information transport, the membrane potential depolarizes very rapidly and returns back to rest. These action potentials (APs) are facilitated by a brief change in the membrane conductance (Cole and Curtis, 1939). APs occur only in membranes that contain at least two specialized gated ion channels, one for sodium and the other for potassium. These ion channels are voltage-gated and open when the membrane (locally) depolarizes from resting potential to a more positive level, e.g. due to the opening of an activated ligand-gated channel. Past a threshold voltage of $U_T \approx -40$ mV the fast sodium channels open first and only shortly, allowing sodium ions to enter the cell, which further depolarizes the membrane and drives the potential towards the sodium equilibrium ($E_{\text{Na}^+} = 66.4$ mV). While sodium channels are closing already, slow potassium channels open slightly delayed and enabling potassium ions to leave the cell, thereby initiating the repolarization of the membrane. The closing sodium channels are inactivated until the cell reaches its resting potential again. This refractory period limits the frequency at which action potentials can be generated. Potassium channels open slowly, but also stay open longer. Even after the resting potential is reached exiting potassium ions hyperpolarize the cell until the membrane potential is even more negative than the resting potential. When the potassium channels are

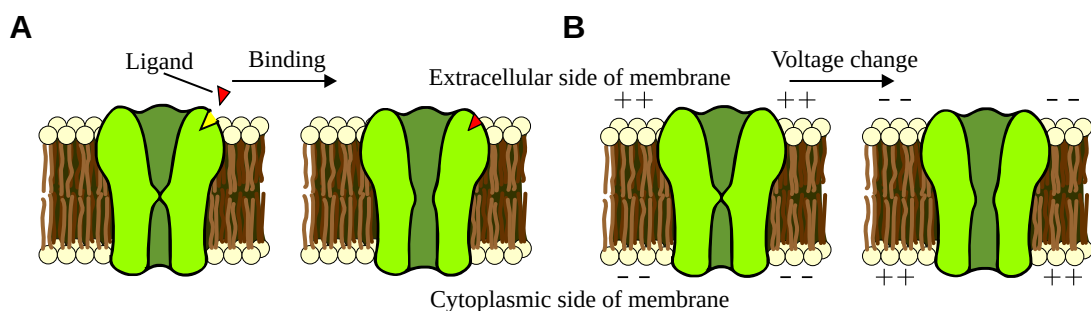


Figure 2.2.: The two main gating mechanisms for ion channels. On the left side the channel is opened by a special molecule called ligand, which binds to a selective spot on the protein. The channel on the right is activated by a potential change across the membrane. The figure was adapted from Penzlin (2005)

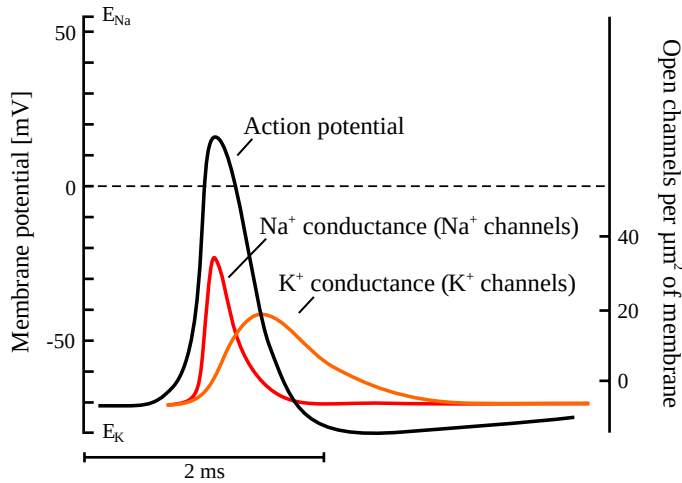


Figure 2.3: An action potential as seen in neurons. The membrane at rest has a potential of about -70 mV. Upon depolarization fast sodium currents drive the potential into the positive range. Slow sodium currents bring the membrane potential back to resting potential and into the refractory phase. Axis for open channels is related to conductances. Figure was adapted from [Kandel et al. \(2000\)](#)

closed, sodium-potassium exchangers, such as ATPase, reestablish the resting potential and prepare the cell for another AP. Whenever a cell fires an AP, it is an *all-or-non principle* of similar shape and magnitude ($\Delta U_M^{AP} \approx E_{Na^+} - E_{K^+} \approx 100$ mV). During information transport, a locally initiated AP depolarizes the surrounding area, resulting in a wave of *local* APs spreading over the membrane with 1 to 5 m/s (without myelin sheath⁶). Besides the above mentioned voltage-gated sodium and potassium channels, other ion channels for several ions species influence the shapes of APs in the respective cell, for instance, calcium or chloride.

[Hodgkin and Huxley \(1952a,b,c\)](#) were the first who explained APs with time-dependent changes in the membrane conductivity for different ions. Using the experimental setup from Cole and Curtis (1939), they inserted one electrode in a giant neuron of the squid *Loligo*, while another electrode remained in the bath solution. They then injected a current into the cell that was modulated with a feedback circuit and thus clamped the membrane potential U_M to a fixed chosen voltage. As no charge could accumulate intracellularly, the injected current represented the ion current I_M passing through the membrane. [Hodgkin and Huxley \(1952b\)](#) observed changes in the AP shape, during variations of intra- and extracellular ion concentrations and thus derived the conductance behavior of selected types of ion channels. Finally, they formulated an electrical equivalent circuit of the cell membrane ([Hodgkin and Huxley, 1952d](#)), as depicted in figure 2.4.

The HODGKIN-HUXLEY circuit represents idealized electrical properties of the cell membrane and channel proteins. The chargeability of the lipid bilayer by ions is represented by the membrane capacitance C_M . Individual equilibrium stages for ion species i are represented by NERNST potentials E_i in series with resistors G_i representing the permeability of ions. In case of sodium and potassium the conductivity G_i is represented by variable resistors. The leak (E_{Leak} and G_{Leak})

⁶specialized nerve fibers contain myelinated axons and can transport information with up to 120 m/s

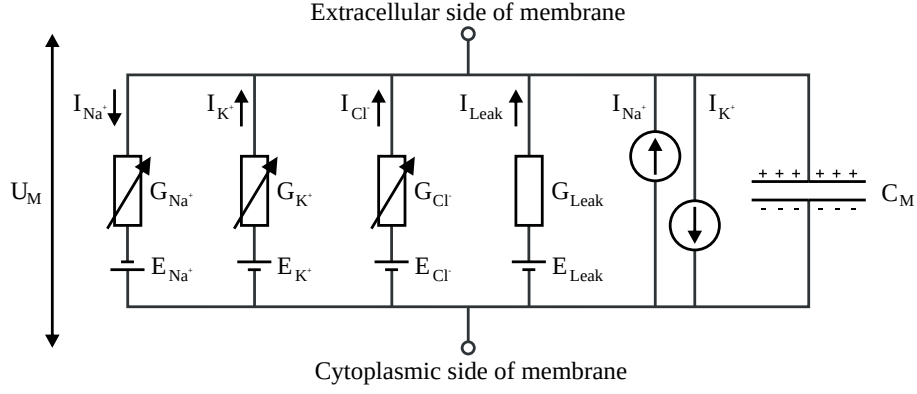


Figure 2.4.: Schematics of the HODGKIN-HUXLEY (1952d) circuit as idealized electrical representation of the cell membrane. The membrane potential U_M is composed of several conductances G_i for ions i in series with electrodiffusive driving forces E_i , further the currents I_{Na}^+ and I_K^+ and finally the membrane capacitance C_M on the far right. The leak includes inter alia ion species not listed.

is a constant and includes the currents and driving forces of calcium and other ions. The current sources I_{K+}/I_{Na+} model ion pumps like ATPase. The net membrane current is calculated by:

$$I_M = \sum G_i (U_M - E_i) \quad (2.6)$$

Since cells vary greatly in size it is convenient to normalize membrane related values by the respective membrane surface: $c_M = C_M/A_M$ and analog $g_M = G_M/A_M$. The specific membrane capacitance (valid for most cells) is $c_{sp} = 1 \mu\text{F}/\text{cm}^2$ and the specific conductance g_{sp} can vary from $100 \mu\text{S}/\text{cm}^2$ up to $1 \text{S}/\text{cm}^2$, depending on the respective cell.

2.2. Genetic engineering

The term genetic engineering was coined in Jack Williamson's science fiction novel "*Dragon's Island*", published in 1951, two years before the scientists Watson and Crick (1953a,b) proposed that DNA could be the medium of genetic information. Today, genetic engineering is the hypernym for biotechnological methods and techniques that provide selective manipulations of the genome and thus give access to biochemical control mechanisms of a living organism. Altered or recombinant DNA can be used to create genetically modified organisms. Accordingly, the term genetic engineering comprises the modification and recombination of DNA sequences *in vitro* and *in vivo* as well as the artificial transfer of DNA from one organisms into another (Madigan and Martinko, 2006). This chapter will give a brief overview about the latter one, the genetic transfection and how it is achieved in four steps. Further information about genetic engineering can be found in Nicholl (2008).

Isolation is achieved by identifying the gene of interest, usually using existing knowledge of the various functions of genes. DNA information can be obtained from cDNA or gDNA libraries, and amplified using PCR techniques.

Insertion of a gene into a vector such as viral vectors, plasmids, bacterial conjugation, or liposomes. Restriction enzymes and ligases are of great use in this crucial step if the gene is inserted into prokaryotic or viral vectors (Roberts, 2005).

Transfection is the process of introducing nucleic acids into cells and typically involves the opening of transient pores or *holes* in the cell membrane, to allow the uptake of material. There are various methods of introducing foreign DNA into an eukaryotic cell. Many materials have been used as carriers for transfection, which can be divided into three kinds: liposomes, nanoparticles, and (cationic) polymers. One of the cheapest methods is transfection by the latter with calcium phosphate, originally discovered by Graham and van der Eb (1973). HEPES-buffered saline solution containing phosphate ions is combined with a calcium chloride solution containing the DNA to be transfected. When the two are combined, a fine precipitate of the positively charged calcium and the negatively charged phosphate will form, binding the DNA to be transported into the target cell. The suspension of the precipitate is then added to a cell culture grown in a monolayer. By a process not entirely understood, the cells take up some of the precipitate, and with it, the DNA.

Selection of genetically modified organisms from those that have failed to incorporate the vector into their genome is recommendable. Transfection can result in unexpected morphologies and abnormalities in target cells, which mostly are an evolutionary disadvantage leading to a successive displacement of actually transfected organisms by the old *wild type*. One method to prevent *wild type* cells to outgrow transfected organisms is to package the genes, so they confer a resistance to certain chemicals such as antibiotics or herbicides into the vector. This, usually toxic, chemical is then applied ensuring that only those cells that have taken up the vector will survive.

2.3. The patch-clamp technique

The patch clamp technique is a refinement of the voltage-clamp experiments that [Hodgkin and Huxley \(1945\)](#) performed over half a century ago. They used electrode contacted capillaries down to 50 μm in diameter during their recordings from squid neurons. [Neher and Sakmann \(1976\)](#) developed the patch clamp technique to record currents of single ion channels under well defined electrolyte solutions on both sides of the cell membrane.

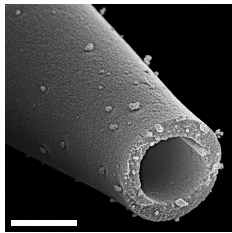


Figure 2.5: Scanning Electron Microscope (SEM) image of a pipette tip (scale bar: 1 μm) used for patch clamp recordings in this work. The pipette was drawn with a P-97 micropipette puller (Sutter Instruments Company, Novato, USA). The filament on the lower left side of the opening increases capillary forces during filling. The image was taken with an LEO 1550 SEM (Carl Zeiss AG, Germany).

This technique uses a fire polished glass pipette with a very small opening at the tip (see figure 2.5) to electrically contact a small *patch* of the cell membrane. This type of pipette is distinct from the sharp microelectrode used to impale cells in traditional intracellular recordings. The pipette, filled with an electrolyte solution, is equipped with a small electrode connected to an amplifier. Slight overpressure is applied to the pipette and electrolyte streams out slowly once the pipette is immersed into solution. The small tip opening induces an electrical resistance of $R_P \approx 5 \text{ M}\Omega$ between the pipette electrode and a bath electrode in the extracellular solution. Approaching the cell membrane R_P rises slightly and a small indentation, caused by the flow of solution, can be observed in the cell membrane. Releasing the excess pressure, pulls a part of the cell membrane into the pipette, causing R_P to rise up to several giga-ohms. This *giga-seal* impedes direct pipette-bath currents and thus enables the measurement of minute currents through individual ion channels, situated in the patched membrane. From this cell-attached configuration (Figure 2.6a), three proximate patch-clamp configurations can be achieved.

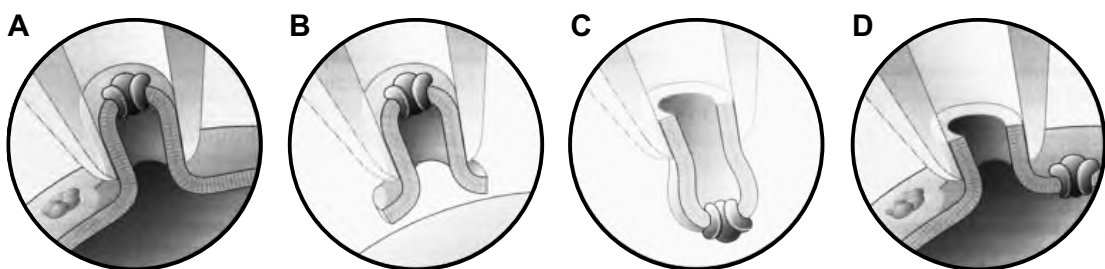


Figure 2.6.: Patch-clamp configurations: cell-attached (A), inside-out (B), outside-out (C) and whole-cell (D). After attaching and sealing of a glass pipette with the cell membrane these configurations can be obtained as describe in the text. The figures were adapted from Numberger and Draguhn (1996).

A pipette in cell-attached mode can be removed from the cell, tearing a small patch out of the membrane that closes up to a spheroid. The unwanted lower part of the membrane disintegrates by surface tension or brief contact with air. This inside-out configuration (Figure 2.6b) is used

for single-channel experiments, where the concentration of ions or other compounds at the inner side of the membrane need to be changed conveniently. Compound effects at the outer part of the membrane are performed in outside-out configuration, where the small membrane spheroid is opened inside the pipette by a brief pulse of depression or voltage (Figure 2.6c). Alternatively to single-channel measurements, the patch pipette can be filled with a solution that closely resembles the cytoplasm. Proceeding from the cell-attached configuration the membrane patch in the pipette can be removed, granting electrical access in the whole-cell configuration (Figure 2.6d). Over time, the cytoplasm will be replaced by the pipette solution and changed physiological conditions need to be considered.

As depicted in figure 2.7a, a great number of variables influence patch-clamp measurements. Most of them can be determined with the help of a small rectangular voltage step $\Delta U_{stim} = 10 \text{ mV}$ applied to the pipette electrode: During cell-attached configuration the *giga-seal* R_{GS} allows an estimation of the pipette capacitance C_P (also known as C_{fast}). The stimulus causes a charge transfer at the thin pipette walls close to the cell, leading to transient currents in I_P at the on and off-sets of ΔU_{stim} . Time constants can be fitted and used for C_P compensation. Similar compensations can be applied after opening the cell and achieving the whole-cell configuration. Analog to the HODGKIN-HUXLEY circuit, the membrane is represented by a capacitance C_M and resistors/conductances are summarized to one common resistance R_M . Assuming the membrane is at rest, the driving forces E_i for individual ion species i can be neglected. ΔU_{stim} causes transient currents in I_P while C_M is charged. The membrane capacitance C_M (also known as C_{slow}) can be compensated and R_P and the resistance of the intracellular environment R_C are usually combined to a series resistance R_S . Changes in the membrane resistance R_M during subsequent experiments then indicate cleared currents I_M over the whole cell membrane due to opening and closing ion channels.

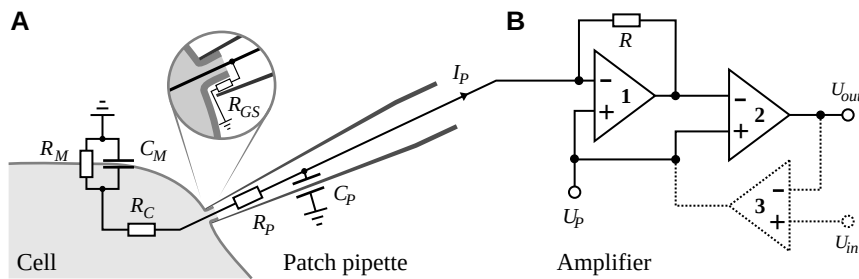


Figure 2.7.: Simplified equivalent electrical circuits for the patch-clamp technique in whole-cell configuration. A: Representative circuits for cell and patch-pipette, B: Amplification circuit for voltage-clamp and current-clamp mode. Symbols and function of cell, pipette and amplifier are explained in the text. The figure was partially adapted from Numberger and Draguhn (1996).

Figure 2.7b represents an amplifier in voltage-clamp mode (only solid circuit), where the pipette voltage U_P is fixed and the injected current I_P equals membrane currents I_M . A feedback loop converts I_P into the voltage U_{out} for read out with an oscilloscope or data-acquisition hardware in a computer. Subsequently, membrane currents I_M need to be calculated by $I_M =$

$I_P = U_{out}/R$. Alternatively to a fixed voltage, the current-clamp mode can be selected (add dotted circuit in figure 2.7b). In current-clamp mode a third operational amplifier converts U_P into a control variable for the second feedback loop. Consequently, the current I_P injected into the cell is fixed to a level that can be set proportionally by the input voltage U_{in} . With $U_P = U_M$ the membrane potential can be monitored during experiments.

Current-clamp recordings are close to the *natural* situation, where ion currents through open channels occur. The injection of negative currents hyperpolarize the cell, while positive currents depolarize. If the depolarization exceeds a neurons threshold voltage U_T , the opening of voltage-gated sodium channels may initiate the generation of an AP.

2.4. Microelectrode arrays

Versatile probes, such as patch-clamp pipettes or impaling electrodes, are available to measure the electrical activity of multiple cells in parallel, however, their number is limited by spatial restrictions. A neat approach is the integration of electrodes into the very substrate on which the cells are cultured. The first “*miniature microelectrode array to monitor the bioelectric activity of cultured cells*” was reported by [Thomas et al. \(1972\)](#) and had 30 platinized gold electrodes of $7 \times 7 \mu\text{m}^2$ in a 2 by 15 array. Today’s microelectrode arrays (MEAs) usually provide 60 or 64 substrate-integrated electrodes of various sizes and materials, organized in an 8 by 8 array ([Pine, 2006](#)).

Combining electrical circuit elements with electrolytes demands the consideration of an electrolytic capacitor formed at the metal-electrolyte interface. At boundaries, liquids behave differently than in their bulk. When polar water molecules get into contact with a metal surface, a space charge builds up that causes the same charge inside the metal ([Berg et al., 2007](#)). The resulting electrical double layer is charged like a capacitor. Ions in electrolytes influence the magnitude of the potential difference across the double layer as well as dissolved metal atoms.

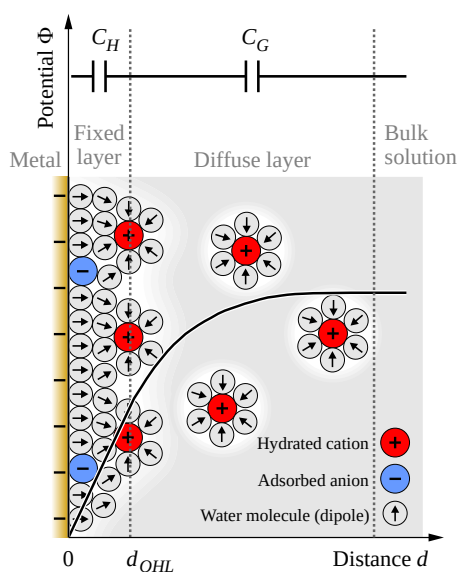


Figure 2.8: A schematic representation of the GOUY-CHAPMAN-STERN model for the electrical double layer of an electrolyte solution at a charged metal surface. Potential variations Φ remain linearly between metal surface and the Outer HELMHOLTZ Layer d_{OHL} . Subsequent potential variations Φ proceed exponentially within the diffuse layer. Changes in Φ are negligible in the bulk solution. A combined capacitance C_I at the interface between metal and electrolyte is represented by the electrically equivalent HELMHOLTZ capacitance C_H and the GOUY capacitance C_G in series. The figure was adapted from ([Butt et al., 2006](#)) and ([Bockris et al., 2000](#))

[Von Helmholtz \(1879\)](#) proposed a fixed charge to be situated close to the electrode surface and in the following decades numerous models were introduced to predict the behavior of double layers. The probably most promising models were suggested by [Gouy \(1910\)](#) and [Chapman \(1913\)](#). [Stern's](#) combination from 1924, the GOUY-CHAPMAN-STERN model, is still a commonly used version that predicts real systems considerably well. However, a few important approximations were made: (1) the solvent is uniform at the atomic scale, (2) ions are treated as point charges, (3) only coulombic interactions are significant, and (4) the electrical permittivity is constant throughout the double layer. Following the equation for a parallel-plates capacitor, the model includes the historical HELMHOLTZ capacitance C_H with a linear potential drop across the Outer HELMHOLTZ

Layer d_{OHL} , formed by ions adsorbed to the metal surface A , as well as solvated yet immobile ions:

$$C_H = \frac{\varepsilon_0 \varepsilon_r A}{d_{OHL}} \quad (2.7)$$

with d_{OHL} of about 0.1 to 0.5 nm and the dielectric constants for vacuum $\varepsilon_0 = 1$ and water⁷ $\varepsilon_r = 74$, respectively. A subsequent diffuse layer of solvated ions is described by the GOUY capacitance C_G and considers a temperature T depending transition into the bulk electrolyte:

$$C_G = \frac{\varepsilon_0 \varepsilon_r A}{\lambda_D} \cdot \cosh \left(\frac{ze_0 U_0}{kT} \right) \quad (2.8)$$

with elementary charge e_0 , ion valence z , BOLTZMANN constant k , and the potential U_0 at a reference electrode in the bath. The DEBEY length λ_D takes inter alia the ionic strength (ions per volume) into account (Bard and Faulkner, 2001). Both capacitances are serially combined in C_I :

$$\frac{1}{C_I} = \frac{1}{C_H} + \frac{1}{C_G} \quad (2.9)$$

For gold surfaces, C_I reaches values up to 40 $\mu\text{F}/\text{cm}^2$, depending on the electrolytic ion concentration (Bard and Faulkner, 2001). A recommendable text book for further information is “*The Electrified Interface*” from Bockris et al. (2000)

⁷ at body temperature (37 °C)

2.5. Open-gate field-effect transistors with ion sensitivity

The basic principle of a field-effect transistor (FET) has been known since [Lilienfeld \(1930\)](#) and [Heil \(1935\)](#) independently described the possibility of controlling the resistance in a semiconducting material with an electric field in their respective US and British patents. Among several other researchers, [Bardeen and Brattain \(1950\)](#) as well as [Shockley \(1951\)](#), described similar mechanisms during the following decades but only the advances in microfabrication and semiconductor technology during the 60's of the last century allowed a practical realization of field-effect devices. The metal-oxide-semiconductor field-effect transistor (MOSFET) is a ubiquitous device that switches bits in computers or amplifies signals in hifi equipment. MOSFETs have a gate (G), drain (D) and source (S) terminal and also a fourth connector (B) called the body, base, bulk, or substrate. Figure 2.9 depicts an n-channel MOSFET consisting of a poorly conducting bulk silicon with a slight surplus of positive charges (holes) and, due to the excess implantation of negative charges (electrons), highly conductive source and drain regions. A device with inverse electron/hole enrichments would be a p-channel MOSFET. The thin but highly resistive gateoxide insulates the semiconductor from the gate electrode.

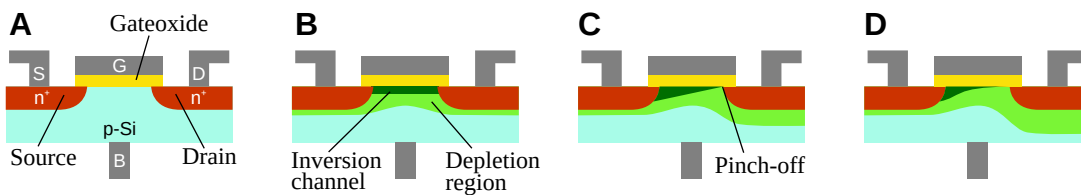


Figure 2.9.: (A) Schematics of a n-channel MOSFET. (B) Inversion layer created but no current flow from drain to source; (C) change from linear to saturated operating region; (D) saturated operating region. The figure is further explained in the text.

A charge separation between gate and bulk influences the oxide-semiconductor interface, which is the core region of the *field effect*. In figure 2.9b a positive potential at the gate electrode pushes holes further into the bulk silicon, forming a highly resistive depletion layer. Increasing the gate-bulk potential difference past the threshold voltage U_T forces even more holes into the bulk leaving free electrons behind, which then establish a conductive inversion layer, the n-channel, at the semiconductor surface. This state is the operating point, describing no current flow between drain and source since the voltage U_{DS} is still zero. Changing the gate and drain voltages (U_{GS} and U_{DS}), with respect to the source potential controls the current I_{DS} through the n-channel. When a potential U_{DS} is applied the channel conducts in the manner of a simple resistor, which results in a linear relationship between the drain current I_{DS} and the drain-source voltage U_{DS} . As long as $0 < U_{DS} \leq U_{GS} - U_T$ (figure 2.9b and c) currents I_{DS} are in a linear region, as depicted in figure 2.10a. When U_{DS} is increased, the voltage U_{GS} between gate and drain diminishes resulting in a constriction of the conducting channel. Figure 2.9d shows the situation past the *pinch-off*

point ($U_{DS} > U_{GS} - U_T$), where the channel thickness becomes zero and the transistor is operating in the saturated region. Then, the current I_{DS} can only be controlled by changes in the gate-source voltage U_{GS} (figure 2.10a). The transfer characteristics show how changes in U_{GS} influence drain-source currents I_{DS} (figure 2.10b), while U_{DS} remains unchanged in saturation.

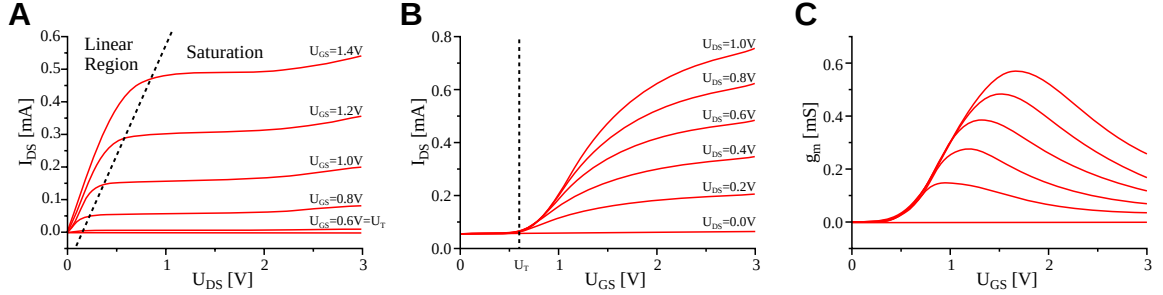


Figure 2.10.: Different plots to characterize a field-effect transistor. (A) Output characteristics $I_{DS}(U_{DS})$ for different U_{GS} . (B) Transfer characteristics $I_{DS}(U_{GS})$ for different U_{DS} . (C) Transconductance calculated as the derivative of the transfer characteristics.

The transconductance g_m (or gain) is an indicator for how effective potential variations at the gate are converted into changes in the drain-source current. For the saturated operating region g_m can be calculated by partial differentiation of I_{DS} :

$$g_m = \left. \frac{\partial I_{DS}(U_{GS})}{\partial U_{GS}} \right|_{U_{DS}=\text{constant}} \quad (2.10)$$

The larger the transconductance g_m , the more prominent are gate-potential changes represented in the drain-source current. For further studies of the *field effect* or the function of MOSFETs, the interested reader may turn to text books specialized in semiconductor technologies.

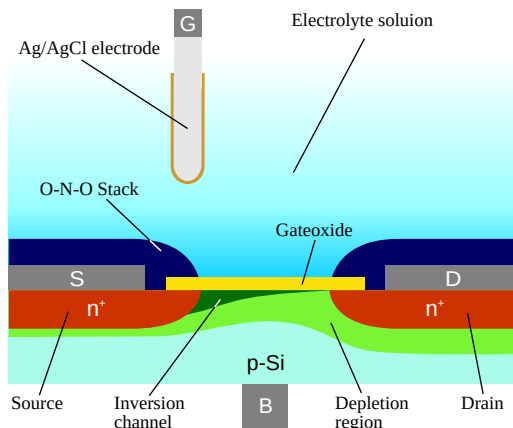
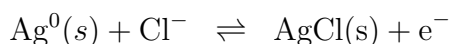


Figure 2.11: Sketch of an ion-sensitive field-effect transistor. Instead of a gate electrode, the gateoxide is in direct contact with an electrolyte solution. A silver-chloride electrode is immersed into the electrolyte representing the former gate contact. Conducting elements of the silicon circuits are protected by a thick stack of varying oxides. All other components are analog to MOSFETs (figure 2.9).

The adaptation for an aqueous environment is the open-gate FET, which originates from the MOSFET structure but differs in many aspects. Open-gate FETs are available in various designs (Inczyedy, 1998), such as Enzyme- FETs (ENFETs), Immuno- FETs (IMFET), insulated gate FETs

(IGFETs), chemical-sensitive FETs (ChemFETs), or ion sensitive FETs (ISFETs). The ISFET concept sketched in figure 2.11 has no metal gate. Instead, the gateoxide is in direct contact with an aqueous electrolyte, which is electronically contacted by a silver-chloride reference electrode as gate equivalent. A several 100 nm thick *oxide-nitride-oxide* stack (ONO-stack) protects conductive elements from the electrolyte and also reduces parasitic capacitances⁸. Potential variations at the gate input are transduced into source-drain currents I_{DS} analog to FET designs with a metal gate. The magnitude of measured gate potentials varies with the present ion concentration according to the NERNST equation (equation 2.1).

In contrast to MOSFETs, ISFETs have two additional interfaces that need to be considered. The first is determined by interactions between the silver-chloride electrode and the electrolyte. An applicable electrode function describes this redox⁹ electrode with the reaction between a silver metal (Ag) and its salt¹⁰:



This reaction is characterized by fast kinetics, which provide high currents through the electrode with the efficiency of the redox reaction given above (dissolution of the metal or cathodic deposition of the silver ions) and is stable in solutions with $\text{pH} = 0$ through 13.5 (Bates and MacAskill, 1978). With respect to a standard hydrogen electrode the standard electrode potential using silver-chloride is $E_{\text{Ag}/\text{AgCl}}^0 = 222.49 \pm 0.13 \text{ mV}$ at 25°C .

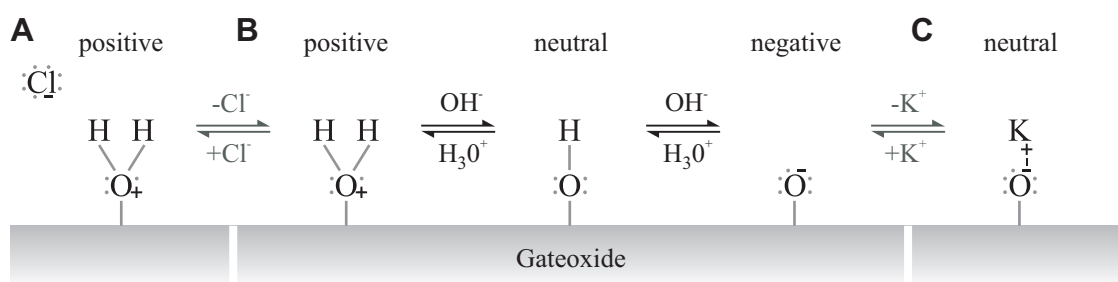


Figure 2.12.: Schematic drawing of reactions between terminating OH hydroxyl-groups (surface reaction sites) on oxide surfaces and considerable ions. (A) Anions do not or neglectably bind to surface reaction sites. (B) Reactions between OH groups and the pH-determining H_3O^+ and OH^- ions of an electrolyte. The acid reaction to the left involves H_3O^+ , K^+ or Cl^- ions and results in positively charged OH_2^+ surface groups, while the base reaction to the right consumes OH^- ions resulting in negatively charged O^- surface groups. To which extent the one reaction exceeds the other is a strong function of the pH and prevails the net surface charge. (C) Other cations, here exemplarily potassium, can compensate the negative charge leading to a neutral surface. This reaction depends on the respective cation concentration.

⁸ capacitive currents are induced directly into the conductive elements during on- and off-sets of e.g. a stimulus pulse

⁹ The oxidation state of atoms is changed (reduction-oxidation).

¹⁰ silver chloride as AgCl , also known as silver(I)chloride

The chemical sensitivity and selectivity of an ISFET are completely depend on the properties of the electrolyte-insulator interface. Facing the electrolyte, the surface of the gateoxide carries a certain charge dependent on the type of oxide and the pH of the solution (Bergveld, 2003). When silanols come into contact with an electrolyte solution, a pH-dependent electrochemical equilibrium is established. The primary chemical reactions occurring at the surface are described by the site-binding model for the electrolyte-oxide interface (Yates et al., 1974). For silicondioxide (SiO_2) the following reactions are present:



Terminating oxygen atoms on SiO_2 surfaces form OH hydroxyl-groups with hydrogen. As illustrated in figure 2.12, these surface hydroxyl-groups are amphoteric and may react with both H_3O^+ and OH^- ions, the pH-determining ions of the electrolyte. The reactions result in either positively charged OH_2^+ groups or negatively charged O^- groups. Depending on the prevailing reaction, which is determined by the pH at the gateoxide (OX) and the bulk (B) electrolyte, a positive or negative net charge, the surface potential Ψ_0 , builds up at the electrolyte-oxide interface:

$$\Psi_0 = 61.5 \text{ mV} (\text{pH}_{\text{OX}} - \text{pH}_{\text{B}}) \quad (2.11)$$

The surface is neutral for one specific pH_{B} value. In case of an SiO_2 surface, this *point of zero charge* is $\zeta = 2$ to 2.5 (Bousse, 1982). Equation 2.12 clarifies how ISFETs are capable of measuring changes in Ψ_0 , e.g. caused by pH changes. As discussed earlier (page 22), the threshold voltage U_T marks the formation of the inversion layer. U_T is the sum of several potentials

$$U_T = E_{RE} - \Psi_0 + \chi_{SD} + \Phi_{IL}^{GO} \quad (2.12)$$

with the potential E_{RE} at the reference electrode, the surface dipole potential χ_{SD} of the electrolyte, and a combination of various potentials Φ_{SI}^{GO} within the gateoxide and the inversion layer (Bergveld and Sibbald, 1988). Upon a pH change of the electrolyte, all contributions in equation 2.12 remain constant, except for Ψ_0 and χ_{SD} . Compared to the surface potential, changes in χ_{SD} are negligible ($\Psi_0 \gg \chi_{SD}$) and the drain-source current I_{DS} can be estimated with a linear function of the surface potential Ψ_0 (van Hal et al., 1996). The pH sensitivity of ISFET devices is reported with $\Psi_0 \approx 30$ to 40 mV/pH (Fung et al., 1986; Bataillard et al., 1987; Bergveld et al., 1997).

The surface reaction sites of oxides are most reactive to the pH-determining ions H_3O^+ and OH. Ions are assumed to adsorb onto the resulting charged surface groups as well, making an additional contribution to the surface potential (Bergveld and Sibbald, 1988; Vlasov et al., 2003). Accordingly, it is believed that the oxide surface potential is altered by the reaction with cations,

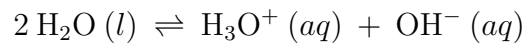
such as sodium and potassium, with negatively charged O^- surface groups as depicted in figure 2.12. In analogy with the pH sensitivity, the potassium sensitivity of the gateoxide is described as

$$\Psi_0 \sim (\text{pK}_{\text{OX}} - \text{pK}_{\text{B}}) \quad (2.13)$$

However, due to a low concentration of positively charged SiOH_2^+ groups (see section 7.2.4), the electrolyte-oxide interface is mostly negatively charged and anions like chloride cannot get close enough to the gateoxide in order to alter the surface potential Ψ_0 , as depicted in figure 2.12.

2.6. pH and buffer characteristics

About one century ago, the concept of pH was first introduced by the Danish chemist [Sørensen \(1909\)](#). The amphoteric nature of water causes a chemical reaction between two water molecules, resulting in the aqueous cation H_3O^+ (hydronium) and the diatomic anion OH^- (hydroxide).



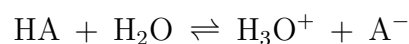
This autodissociation of water is characterized by the dissociation constant K in the following equilibrium expression:

$$K = \frac{[\text{H}^+][\text{OH}^-]}{[\text{H}_2\text{O}]} \quad (2.14)$$

where H_3O^+ is substituted by H^+ . The concentration of undissociated water $[\text{H}_2\text{O}]$ is much larger than the concentrations of hydrogen or hydroxide and thus can be approximated to be constant. Under this premise, the product $K_w = [\text{H}^+][\text{OH}^-]$ needs to be constant to acknowledge equation 2.14. Given the ionization constant of water with $K_w = 10^{-14}$, the concentration of hydrogen $[\text{H}^+]$ determines also the concentration of hydroxide $[\text{OH}^-]$ and both are in equilibrium when $[\text{H}^+] = [\text{OH}^-] = 10^{-7}$. Based on the formulations above, [Sørensen \(1909\)](#) utilized the negative decadic logarithm of the hydrogen concentration

$$\text{pH} = -\log_{10}[\text{H}^+] \quad (2.15)$$

to characterize the acidity ($\text{pH} < 7.0$) or basicity ($\text{pH} > 7.0$) of a solution. Accordingly, acids (proton donors) and bases (proton acceptors) will influence the overall pH of a solution. For instance, an acid HA reacts with water (as base)

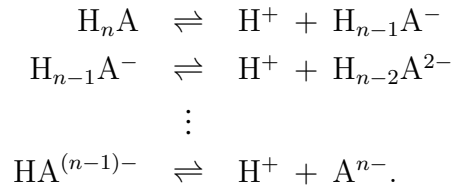


to hydronium and the conjugate base A^- of the acid ([Brønsted, 1923](#); [Lowry, 1923](#)). Combining the virtually constant water concentration with K , the equilibrium constant K_a for such an acid-base reaction is calculated to

$$K_a = K[\text{H}_2\text{O}] = \frac{[\text{H}_3\text{O}^+][\text{A}^-]}{[\text{HA}]} \quad (2.16)$$

Substances with $K_a \gg 1$ are classified as strong acids, since they rapidly transfer all their protons to H_2O , whereas weak acids have an equilibrium constant of $K_a < 1$ and only partially ionize in aqueous solutions. Analog to equation 2.15 for hydrogen, the equilibrium constant K_a can be transformed into pK_a as well. The reaction characteristics of weak acids are often employed to buffer the pH in solutions, as they accept or donate protons when $\text{pH} \approx pK_a$ (Cartwright and Shah, 2002).

In contrast to the monoprotic acid HA , which only donates one proton per molecule, polyprotic acids dissociate in multiple steps and thus donate more than one proton per acid molecule (Harris, 2006). An n -protic acid H_nA reacts in n steps



Each reaction is characterized analog to equation 2.16 by one of n consecutive dissociation constants $K_{a_1}, K_{a_2}, \dots, K_{a_n}$, where $K_{a_1} > K_{a_2}, \dots > K_{a_n}$.

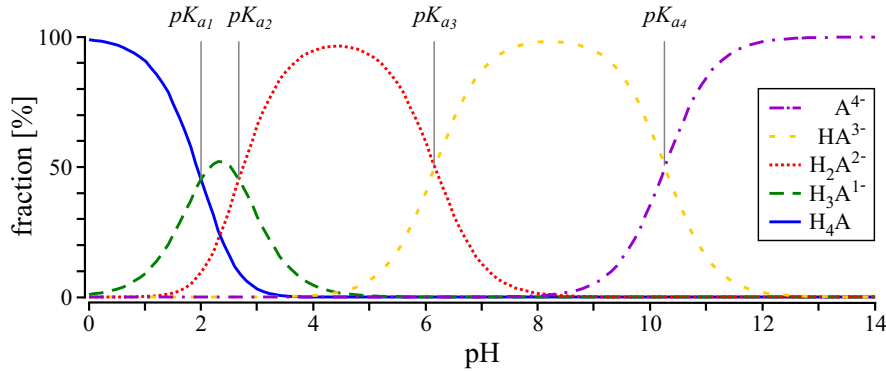


Figure 2.13.: Distribution diagram for polyprotic acids - here exemplarily for the tetraprotic ethylenediaminetetraacetic acid (H_4EDTA) with $pK_{a_1} = 1.99$, $pK_{a_2} = 2.67$, $pK_{a_3} = 6.16$ and $pK_{a_4} = 10.26$. Depending on the proton concentration of a solution, the prevailing acid species (undissociated acid or any acid anion) changes. The pK_a values reflect an equilibrium at each transition.

As illustrated in figure 2.13, partially deprotonated subsets of the polyprotic acid H_nA prevail the solution, depending on the present pH. Described as a function of $[\text{H}^+]$, the fractions α_i of the total acid concentration for individual acid species $i = 0$ through n are calculated to

$$\alpha_i = \frac{\prod_{j=0}^i K_{a_j} \cdot [\text{H}^+]^{n-i}}{\sum_{k=0}^n \left(\prod_{j=0}^k K_{a_j} \cdot [\text{H}^+]^{n-k} \right)}. \quad (2.17)$$

Usually, one of the buffer subspecies dominates the solution. However, when the pH is equal or close to one pK_a two subspecies share the same fraction of the total concentration. At these pH values, the buffer is most effective and has the highest capacity. Depending on the equilibrium constants $K_{a_i}^j$ and the corresponding buffer concentrations C_j the capacity β of one buffer or mixtures of j buffers is calculated by

$$\beta = \frac{\partial n}{\partial \text{pH}} = 2.303 \left(\frac{K_w}{[\text{H}^+]} + [\text{H}^+] + \sum_j \left(C_j \cdot \sum_i \frac{K_{a_i}^j \cdot [\text{H}^+]}{(K_{a_i}^j + [\text{H}^+])^2} \right) \right) \quad (2.18)$$

where ∂n represents an infinitesimal amount of added base and ∂pH is the resulting infinitesimal change in pH (Hulanicki, 1987). The first two terms in equation 2.18 are independent from C_j and reflect the fact that solutions of high (or low) pH are resistant to pH changes. Figure 2.14 illustrates the buffer capacity β of solutions buffered with HEPES for the full pH range from pH = 1 through 14.

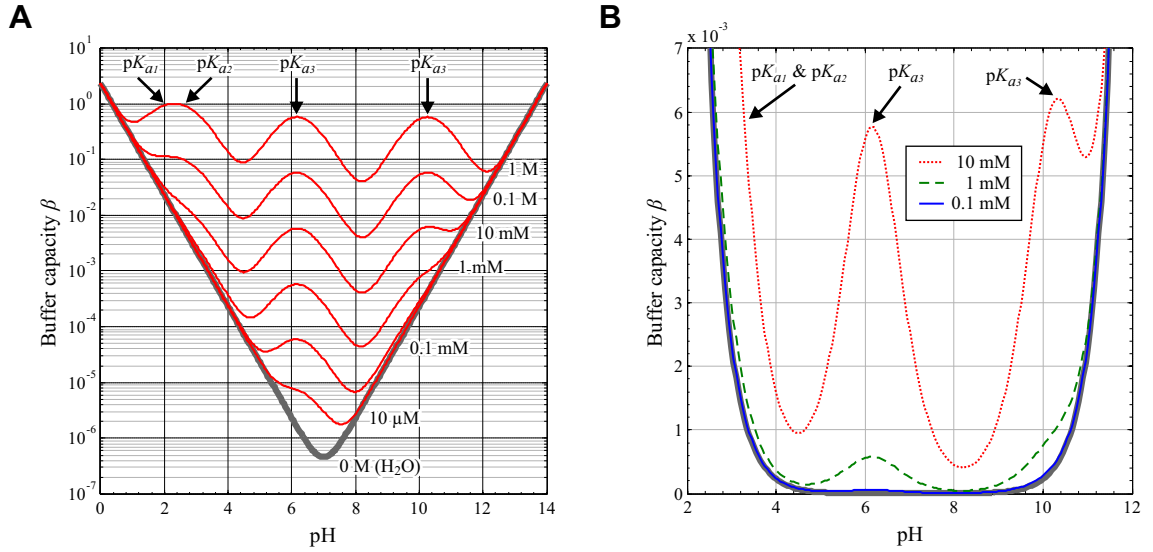


Figure 2.14.: Buffer capacities β for H_4EDTA versus pH are given exemplarily. The thick gray line represents the natural buffer characteristics of water (H_2O) with high or low pH (see equation 2.18). For $\text{pH} = pK_{a_i}$, β reaches a local maximum. (A) Logarithmic β for H_4EDTA concentration from $10 \mu\text{M}$ through 1 M and (B) selected concentrations, linearly scaled β .

2.7. Theories and models in cell-sensor coupling

From electrical equivalent circuits over finite-elements simulations to analytical approaches, various models have been proposed to describe the coupling of cells with electrical sensors. Comprehensive information about cell-sensor models and the investigation of their parameters are summarized in [Fromherz \(2005\)](#), [Ingebrandt and Offenhäusser \(2006\)](#) or [Offenhäusser et al. \(2009\)](#). The point-contact model (PCM) is very commonly used and will be elaborated with some extensions.

The PCM is a simplified electrical circuit where the contact between cell and sensor is reduced to a single point, neglecting most area factors. Starting in 1988, [Regehr et al.](#) refined a model for *loose-patch*¹¹ recordings of patch-clamp experiments ([Stuhmer et al., 1983](#)) to formulate an electrical equivalent circuit for a “*diving-board electrode*”. Indeed, cell-sensor coupling can be seen as a patch-clamp cell-attached configuration where the membrane did not *giga seal* the connection between pipette and bath electrode (see section 2.3). Depending on the area of the respective sensor, the size of a *connected* membrane patch is not merely $1\ \mu\text{m}^2$ (see figure 2.5), but rather several micrometer squared. Consequently, far more than a few ion channels contribute to measurable membrane currents. The membrane patch is thus large enough to require the consideration of HODGKIN-HUXLEY elements (figure 2.4 on page 15). Taking these changes into account [Regehr et al. \(1989\)](#) published an equivalent circuit for the electrical coupling of a cell into the substrate-integrated electrode beneath it. An adaptation for field-effect transistors was suggested by [Fromherz et al.](#) in 1991.

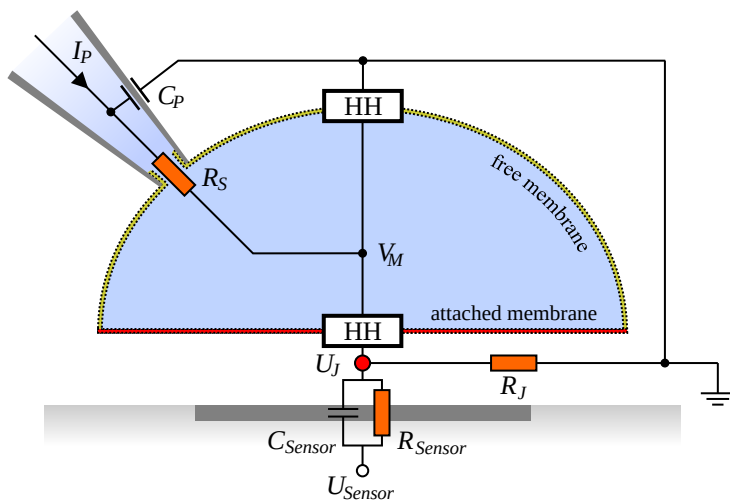


Figure 2.15: The point-contact model adapted from ([Ingebrandt et al., 2005](#)). The cell membrane is divided into a free and an attached membrane, each represented by [HH], HODGKIN-HUXLEY circuits (figure 2.4). Coupling to a substrate-integrated device is condensed into a single (red) point. The (seal or) junction resistance R_J controls how much of the voltage U_J vanishes unmeasured. This figure is explained in the text.

[Fromherz et al. \(1993\)](#) also added parallel patch-clamp experiments to further characterize the cell-sensor contact. Patch-clamp data provides information about a cell’s membrane surface A_M , the actual magnitude of membrane currents/voltages and precise timing possibilities. Including the basic electrical components of a patch pipette, [Ingebrandt \(2001\)](#) proposed an advanced PCM,

¹¹ poor *giga-sealing* and large leak currents between pipette and bath electrode are called *loose patch* (see section 2.3)

which allowed the integration of time-dependent membrane currents I_M and voltages U_M obtained during patch-clamp experiments (Ingebrandt et al., 2005). Figure 2.15 depicts the PCM, including a patch pipette represented by the pipette capacitance C_P and currents I_P injected into the cell. The pipette resistance R_P and the resistance of the intracellular environment R_C are combined into a series resistance R_S (figure 2.7). The cell membrane is divided into two distinct parts, the free membrane (FM) and the attached membrane (AM), both represented by HODGKIN-HUXLEY circuits (see figure 2.4 on page 15). The ratio of AM:FM is about 1 : 2 but can rise close to 1 : 1, depending on cell type and surface modification as shown in chapter 4 (Sommerhage et al., 2008). Currents across the free membrane are technically untraceable for the substrate-integrated sensor. However, their effects on the membrane potential U_M can affect the sensor's signal. In contrast the attached membrane forms a very shallow cleft between cell and substrate that seals the connection to the bath electrode, expressed as junction resistance R_J (or seal resistance). Depending on cell type and surface coating, the cleft height can vary between 60 and 110 nm (Braun and Fromherz, 1998, 2004; Wrobel et al., 2007). The flatter the cleft, the larger R_J , which provides a better coupling of membrane currents I_M or potentials U_M into the sensor:

$$I_{Sensor} = C_{Sensor} \cdot \frac{\partial U_J(t)}{\partial t} + \frac{U_J(t)}{R_{Sensor}} \quad (2.19)$$

with time t , the current I_{Sensor} into the sensor, C_{Sensor} as capacitance of the sensing area and the resistance R_{Sensor} . The junction potential U_J can be related to the intracellular membrane potential U_M by following KIRCHHOFF's circuit laws:

$$I_{Sensor} + \frac{U_J(t)}{R_J} = C^{AM} \cdot \frac{\partial((U_M(t) - U_J(t)))}{\partial t} + \sum_i G_i^{AM} (U_M(t) - U_J(t) - E_i^{AM}) \quad (2.20)$$

with the reverse potentials E_i^{AM} of ion i in the cleft (as opposed to the reverse potentials E_i^{FM} at the free membrane facing the bath solution) and the membrane conductances G_i^{AM} . FETs with high-ohmic gateoxides ($R_{Sensor} > 1 \text{ T}\Omega$) and capacitive currents tending towards zero, I_{Sensor} can be neglected. In the case of an electrode, the current I_{Sensor} is proportional to the measured voltage U_{out} after the first impedance converter (neglecting filter properties) and can be neglected as well to calculate U_J . Provided, the further approximations, that first the junction potential is much smaller than the overall membrane potential ($U_J(t) \ll U_M(t)$) and second, the ion-concentration differences between cleft and bath are negligible ($E_{AM(i)} \simeq E_{FM(i)} = E_{(i)}$) at all times, equation 2.20 can be rewritten:

$$U_J(t) = R_J \left(C_{JM} \cdot \frac{\partial(U_M(t))}{\partial t} + \sum_i G_i^{AM} (U_M(t) - E_i) \right) \quad (2.21)$$

With this rather crude description of U_J , only basic simulations can be performed. One important extension for the PCM is the addition of electrodiffusive potentials. Although neglected for the above mentioned electrical circuit, electrodiffusive components, which are caused by changes in ion concentration in the cleft can take about 25% of measured signals (Ingebrandt, 2001; Brittinger and Fromherz, 2005; Wrobel et al., 2005; Pabst et al., 2007). Figure 2.16 visualizes the concentrational changes in the cleft due to opening ion channels for four distinct states during a voltage step applied by a patch pipette. Different ion concentrations in the cleft and the bulk solution are accompanied by charge differences along the cleft. The result is an electrodiffusive potential between cleft and bulk that is registered by the sensor. A simple compartment model regarding the diffusion of potassium, sodium and chloride can simulate the electrodiffusive situation in the cleft (Ingebrandt, 2001; Wrobel et al., 2005).

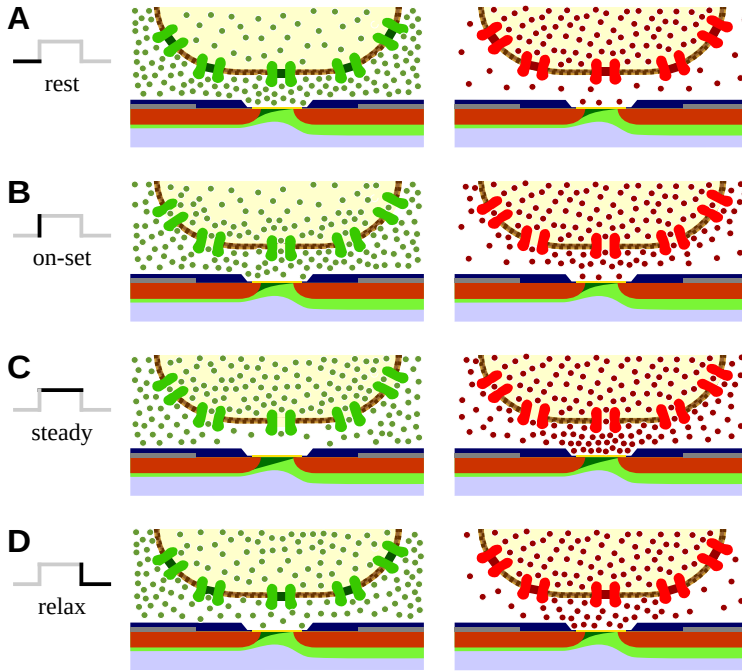


Figure 2.16: Electrodiffusion in the cleft between cell and sensor. Exemplary for HEK293 cells stably transfected with only one ion channel in patch-clamp whole-cell configuration. Electrodiffusion is caused by an accumulation of potassium (left) and depletion of chloride (right) in the small cleft volume. Four distinct states, at rest, pulse on-set, steady state, and relaxation, of the membrane during the applied stimulus pulse are given on the far left.

The cleft is considered as a cylinder with the cell radius r and the height h_J , equivalent to the distance d_J or d_x between cell and substrate or sensor. The cylinder volume V_J and its lateral surface S_J in contact with the bulk solution, are calculated to

$$V_J = \pi r^2 \cdot d_J \quad (2.22)$$

and

$$S_J = 2\pi r \cdot d_J. \quad (2.23)$$

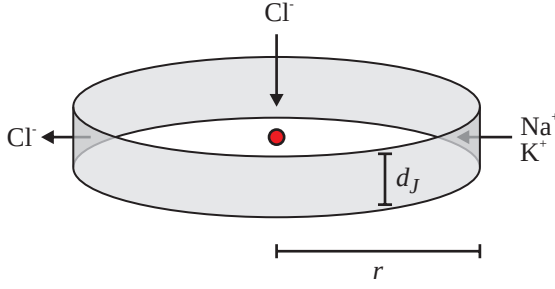


Figure 2.17: Schematics of the cleft geometry and electrodiffusion in the cleft volume between a HEK293 cell and the sensor surface. Cl^- ions diffuse through ion channels into the cleft. To preserve electrical neutrality, the concentrations of sodium and potassium ions in the cleft increase while excess chloride leaves the cleft.

Applying the point-contact simplification correspondingly, ions in the cleft volume are geometrically condensed into one single point. Thus, no radial or vertical dependency of the ion distribution is considered. The influx of ions from the attached membrane area into the cleft is idealized to occur at the center point only. The point-contact model requires ions to diffuse from the center of the cleft to its border and vice versa. The change in concentration c_i^J of ions i induced by electrodiffusion is calculated by

$$\begin{aligned} \frac{\partial c_i^J}{\partial t} &= \frac{1}{V_J} \cdot \frac{\partial n_i^J}{\partial t} \\ &= \frac{1}{V_J} \cdot \left(\frac{\partial n_i^{AM}}{\partial t} + \frac{\partial n_i^S}{\partial t} \right) \\ &= \frac{1}{V_J} \cdot \left(\frac{I_i^{AM}(t)}{e \cdot z_i} + S_J \cdot \Phi_i^J(t) \right) \end{aligned} \quad (2.24)$$

with the number concentrations n_i^J of ions i in the cleft, n_i^{AM} accessing the cleft via the attached cell membrane and n_i^S entering or leaving the cleft via the lateral surface S_J as well as the elementary charge e and the ion charge z_i . The NERNST-PLANCK term

$$\begin{aligned} \Phi_i^S(t) &= \Phi_i^D(t) + \Phi_i^E(t) \\ &= -D_i \frac{\partial c_i^J(t)}{\partial r} - z_i u c_i^J(t) \frac{\partial \Psi}{\partial r} \quad \text{with} \quad u = -D_i \frac{z_i F}{RT} \\ &= -D_i \left(\frac{\partial c_i^J(t)}{\partial r} + \frac{z_i F}{RT} \cdot c_i^J(t) \cdot \frac{\partial \Psi}{\partial r} \right) \end{aligned} \quad (2.25)$$

describes electrodiffusive processes along the cleft radius r and is the sum of a diffusive flux $\Phi_i^D(t)$ and an electrically driven flux $\Phi_i^E(t)$. In electrolytes, the movability u of charges in the electric flux is directly connected to the diffusion coefficients D_i in the diffusive flux. The potential Ψ is mainly induced by the concentration gradients of all considered ions and thus determined by

the potential difference Ψ_{ED} along the cleft radius. It can be approximated by a variation of the GOLDMAN-HODGKIN-KATZ equation (equation 2.5):

$$\Psi_{ED} = \frac{RT}{F} \cdot \ln \frac{D_{Na^+} c_{Na^+}^J + D_{K^+} c_{K^+}^J + D_{Cl^-} c_{Cl^-}^B}{D_{Na^+} c_{Na^+}^B + D_{K^+} c_{K^+}^B + D_{Cl^-} c_{Cl^-}^J} \quad (2.26)$$

with the bulk (or bath) concentrations c_i^B of ions i . Usually, the GOLDMAN-HODGKIN-KATZ equation is valid for membranes. However, equation 2.26 is well suited to approximate Ψ_{ED} for the compartment model (Wrobel et al., 2005).

Depending on cell and expressed ion channels, $c_i^J(t)$ can reach a constant state. Given that only one ion species can permeate the cell membrane, Pabst et al. (2007) proposed an analytical solution to calculate ion concentrations in this *steady-state* situation. The continuity equations, the NERNST-PLANCK equations and the POISSON equation, were coupled in a combined POISSON-NERNST-PLANCK equation system (PNP) to describe the electrochemistry of a system with different ion species. However, for experiments in this thesis, two differently charged ions were migrating across the cell membrane in opposed directions and thus violated a major premise of the PNP equation system.

In order to simulate the recorded signal amplitude of ISFETs, the consideration of one additional effect is inevitable. The silicon-dioxide surface is predominantly sensitive to H_3O^+ ions but also to sodium, potassium, calcium and other positively charged ions (Abe et al., 1979; Hajji et al., 2000). Due to diverse cation diameters and the resulting variations in their stage of hydration, each sensitivity S_i for a cation i is different, and the contribution of individual cation species to the overall ion-sensitivity potential U^{IS} remains complex. In a first approximation, U^{IS} can be estimated by the sum of the individual sensitivity potentials U_i^{IS} .

$$U^{IS} = \sum_i U_i^{IS} = \sum_i S_i \cdot \log \frac{c_i^J}{c_i^B} \quad (2.27)$$

where c_i^J refers to the current ion concentration in the cleft (junction) and c_i^B represents the original ion concentration, which is assumed to be equal to the ion concentration in the bath. Multiple potentials, (1) the solely electrical potential U_J simulated with electrical circuits, (2) the electrode-diffusion potential Ψ_{ED} induced by concentration gradients between cleft and bath, and (3) the subsequent sensitivity potential U^{IS} in case of an ISFET, sum up to the finally measured potentials U_{ISFET} or $U_{Electrode}$.

$$U_{ISFET} = U_J + \Psi_{ED} + U^{IS} \quad \text{and} \quad U_{Electrode} = U_J + \Psi_{ED} \quad (2.28)$$

All potentials are dependent on the cell radius r , which determines the area of the attached membrane A_{JM} , and the cleft height d_J (former junction distance). In the electrical circuit model these variables determine R_J and for the electrodiffusion and ion sensitivities they have an impact on the cleft volume and thus concentrations as well as the distance of charge separation. The potentials U_{ISFET} and $U_{Electrode}$ measured by their respective sensor can further be altered from the eventually obtained potential U_{out} . The assumption that equation 2.19 can be neglected for equation 2.21 is not necessarily valid, since gateoxide and electrode act as filters. Further, most amplifiers (purposefully or not) have filter characteristics, which can alternate the frequency components of the recorded signals and also cause phase shifts. However, the PCM and its extensions give a fairly good simulation result.

3. Materials and methods

3.1. Cell lines and cell culture

Membrane currents for investigations with extracellular sensors were provided by genetically engineered clones of the Human Embryonic Kidney 293 cell line (HEK293). This chapter gives a broad overview of the cell line itself, the derived clones and the objection of their transfection as well as culturing protocols.

3.1.1. Human Embryonic Kidney cell line (wild type)

The cell line Human Embryonic Kidney 293 was generated by transformation of a normal human embryonic kidney cell culture with sheared adenovirus 5 DNA (Graham and van der Eb, 1973; Graham et al., 1977). Twenty years later, a subsequent analysis by Louis et al. (1997) showed that the transformation is essentially an insert, consisting of approximately 4500 nucleotides from the viral genome, incorporated into the human chromosome 19. The origin of the HEK293 cell line is not necessarily a kidney cell, since embryonic kidney cultures may contain almost all cell types of the body. HEK293 cells have many properties of immature neurons, suggesting that the adenovirus transformed a neuronal lineage cell in the original kidney culture (Shaw et al., 2002). However, the actual origin of HEK293 cells is only important for studies, which use HEK293 cells as a model for actual kidney cells, for instance in pharmacological screenings. Experiments conducted in the course of this thesis employ HEK293 cells as a host system for the expression of ion-channel proteins and the subsequent investigation of currents through the ion-channels. HEK293 cells are ideal for the investigation of exogenous ion currents since *wild type* HEK293 cells inherit a very low density of ion channels and thus offer negligible endogenous ion currents. The HEK293 cell line has been widely used in cell biology research. The cells present an extreme transfectability by the calcium phosphate method (see section 2.2).

3.1.2. beag1 clone (voltage-gated potassium channel)

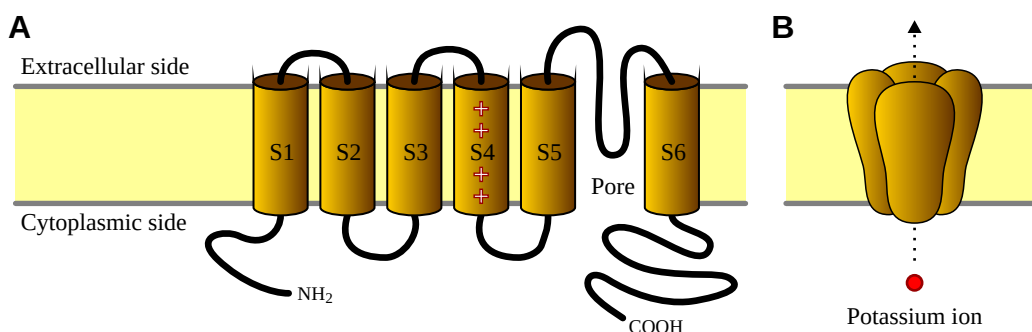


Figure 3.1.: (A) The ether-à-go-go 1 subunit has 6 membrane-spanning α -helices of which segment 4 is the functional element. (B) A total of four of these subunits assemble to functional channels.

Voltage-gated potassium channels represent a very complex class of voltage-gated ion channels (Latorre and Labarca, 1996). Their diverse functional and structural aspects are generated by several families of genes encoding potassium-channel subunits. Most ion channels are assembled of multiple subunits. The *ether-à-go-go* (eag) gene, provides such a subunit of which four are necessary to form a functional pore. Figure 3.1 illustrates one eag subunit with six elements that span the membrane. In the full assembly, segments five and six of each subunit are oriented towards a central pore that is selective for potassium ions. The fourth segment of each subunit is positively charged and influences the conductivity of the pore with respect to the voltage across the cell membrane.

HEK293 cells used in this thesis were overexpressing a voltage gated potassium channel from the bovine retina (beag1). The beag1 gene was extracted from bovine retina and purified for transfections by Frings et al. (1998). HEK293 clones expressing the beag1 potassium channels were used in several studies to characterize the cell-transistor junction (Wrobel et al., 2005; Meyburg et al., 2006). In contrast to AP-generating neurons or cardiac myocyte, these cells provide the opportunity to analyze the junction with only one cationic variable. Further investigations of the cleft height (distance between cell and substrate) were conducted with the beag1 HEK293 cells by Höller (2005) and Wrobel et al. (2007). Proceeding on these initial geometrical studies, examinations of the general morphology of the beag1 HEK293 clone with respect to the cleft in the cell-transistor junction are described in chapter 4 of this thesis.

3.1.3. hClC-1 and hClC-4 clones (voltage-gated chloride channels)

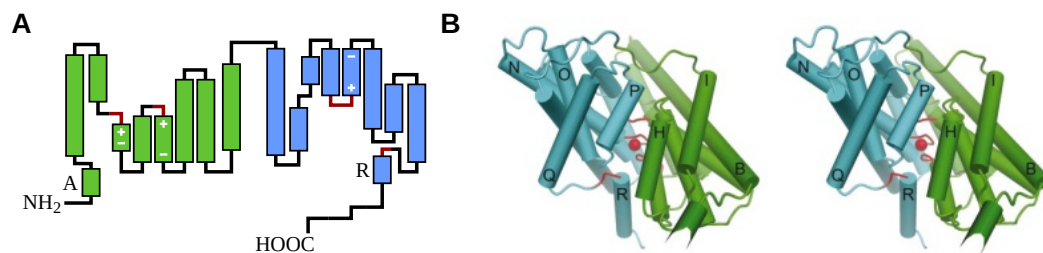


Figure 3.2.: Structure of a bacterial ClC subunit. (A) The α -helices (A-R) are drawn as cylinders with the extracellular region above and the cytoplasmic region below. The two halves of the subunit are green and cyan, and regions forming the chloride selectivity filter are red. Voltage-sensitive segments are indicated with plus and minus. (B) Stereo view¹² of the subunit approached from within the membrane at the interface for the second subunit. The α -helices are drawn as cylinders, loop regions as cords and the chloride ion as a red sphere. The figure was adapted from Dutzler et al. (2002).

Chloride channels are important for setting a cell's resting potential and maintaining proper cell volume (Jentsch et al., 2000). They also display a variety of important physiological and cellular roles that include regulation of pH, organic solute transport, cell migration, cell proliferation and

¹² Convergent stereo viewing (see appendix A.1 on page 146 for cross-eye viewing instructions)

differentiation. The structure of chloride channels differs from other known channels. They consist of two subunits, which contain numerous tilted segments of which some span the membrane entirely and others partially. Further, each subunit has its own pore and conduction channel. Figure 3.2 depicts representatively the bacterial chloride channel StClC, since the structural information of human chloride channels has yet to be discovered. Comparisons of the nucleotide sequences of procaryotic and human ClC channels suggest very similar structural properties (Dutzler et al., 2002). Some members of the human ClC family, which is still poorly understood, are activated by voltage, while others are activated by Ca^{2+} , extracellular ligands and pH among other modulators (Schmidt-Rose and Jentsch, 1997). Voltage-gated chloride channels in humans comprise the genes ClC-1 through 7 as well as ClC-Ka and ClC-Kb. Although they share significant sequence homology, the functional characteristics are quite diverse. Throughout this thesis, transfected HEK293 cell lines, overexpressing either ClC-1 (Fahlke et al., 1996) or ClC-4 (Hebeisen et al., 2003) chloride channels, were brought into cell-transistor junctions. ClC-1 chloride channels regulate the electric excitability of the skeletal muscle membrane by restoring the resting potential faster than in e.g. smooth muscle. Mutations in ClC-1 cause primarily human muscle disorders (Lorenz et al., 1994; Hudson et al., 1995; Trip et al., 2009). The physiological role of chloride channel 4, mostly located in the endoplasmic reticulum, is not well understood but related to renal and neuronal disorders (Schnur and Wick, 1995; Ludwig and Utsch, 2004; Okkenhaug et al., 2006). In 2004, Accardi and Miller discovered the transport of protons through a bacterial homologue of human ClC chloride channels. Picollo and Pusch (2005) published shortly thereafter a proton-chloride antiport in ClC-4 and ClC-5 as well as the indication of a very small proton transport in ClC-1. The motivation to utilize the Cl^-/H^+ antiporters ClC-1 and ClC-4 in this thesis has been elaborated in the introduction (see page 4).

3.1.4. hGlyR clone (glycine-gated chloride channel)

The glycine receptor (GlyR) is one of the most widely distributed inhibitory receptors in the central nervous system (Lynch, 2004). Being a family member of ligand-gated ion channels, glycine receptors are arranged by five subunits surrounding a central pore. The individual subunits are composed of four α -helical transmembrane segments, where the second segment is facing the pore region and the long N terminus forms the binding site for glycine (Rajendra et al., 1997). Figure 3.3 illustrates the α -helical structure and functional composition of α and β subunits. The embryonic assembly consists exclusively of α subunits, while the postnatal and adult form of the GlyR is a heteromeric receptor composed of α and β subunits. The stoichiometric composition is proposed to be three α_1 subunits and two β subunits or four α_1 subunits and one β subunit (Kuhse et al., 1993, 1995).

In contrast to GABA receptors (γ -aminobutyric acid), glycine receptors are poorly understood and only a few stably transfected cell lines exist. Thus, a new GlyR HEK293 clone, overexpressing

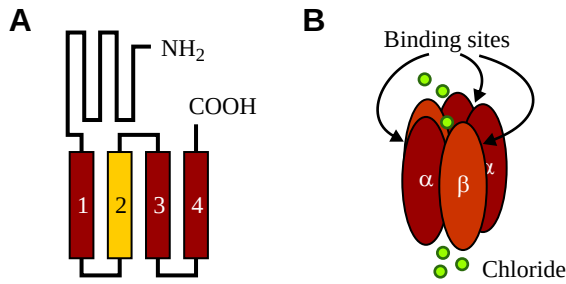


Figure 3.3: The ligand-gated glycine receptor chloride channel. (A) Four transmembrane segments form a subunit with a large extracellular N terminus that forms the glycine binding site in α subunits. The second transmembrane segment of each subunit contributes to the formation of a central pore. (B) A pentameric channel formed by three α and two β subunits.

α_1 subunits, was contributed throughout the course of this thesis. Since glycine receptor channels can be composed entirely of α_1 subunits, HEK293 cells assembled the subunits to fully functional glycine-receptor chloride channels (see chapter 5).

3.1.5. Cell culture

HEK293 cells were easy to culture. They multiplied fast and did not need much attention. Cells were grown in a culture medium (M10) consisting of minimal essential medium (M2279) supplemented with 2 mM glutamine, nonessential amino acids, 10 % (v/v) fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all Sigma-Aldrich Chemie GmbH, Germany). The weekly splitting was maintained according to the following protocol:

- rinsing 2 times with PBS (see section 4.2.1 on page 56)
- incubation with trypsin-EDTA (Sigma-Aldrich) for 5 minutes at 37 °C
- suspension of cells in M10
- centrifugation for 5 minutes at $200 \times g$
- resuspension in M10
- cell counting
- starting next culture cycle by plating cells in new dish (concentration as follows)

Due to the weekly counting the average proliferation rates (table 3.1) have been monitored for all employed HEK293 cells and clones. Cells were plated such that a total of $2 \cdot 10^6$ cells were achieved for the next cell splitting cycle (usually after one week). Culturing media was changed every 2-3 days.

Cell type	wild type	bEAG1	hClC-1	hClC-4	hGlyR
Doubling rate (h)	28.7 ± 1.1	26.16 ± 0.5	25.21 ± 0.3	23.36 ± 0.8	28.01 ± 1.6

Table 3.1.: Proliferation rates of all HEK293 cells and clones used in this thesis.

In case of transfected cells, clones were selected in the presence of 0.9 mg/ml G418¹³ (Sigma-Aldrich Chemie GmbH, Germany) in the culturing media. Occasionally, slight modifications from this protocol were necessary to sustain the used cells. Those changes were elaborated in the respective chapter.

¹³ Geneticin 418 blocks polypeptide synthesis and thus the proliferation of cells

3.2. Setup for bioelectronic coupling

Proceeding on early prototypes (Fromherz et al., 1991; Offenhäusser et al., 1997), setups for cell-sensor junctions have been constantly improved and altered (Ecken, 2002; Weigel, 2006; Meyburg, 2006; Wrobel et al., 2005; Schindler, 2008). A setup configuration, closest to the one used for experiments in this thesis, was described by Ingebrandt (2001). Figure 3.4 depicts a photograph of the bioelectronic setup.

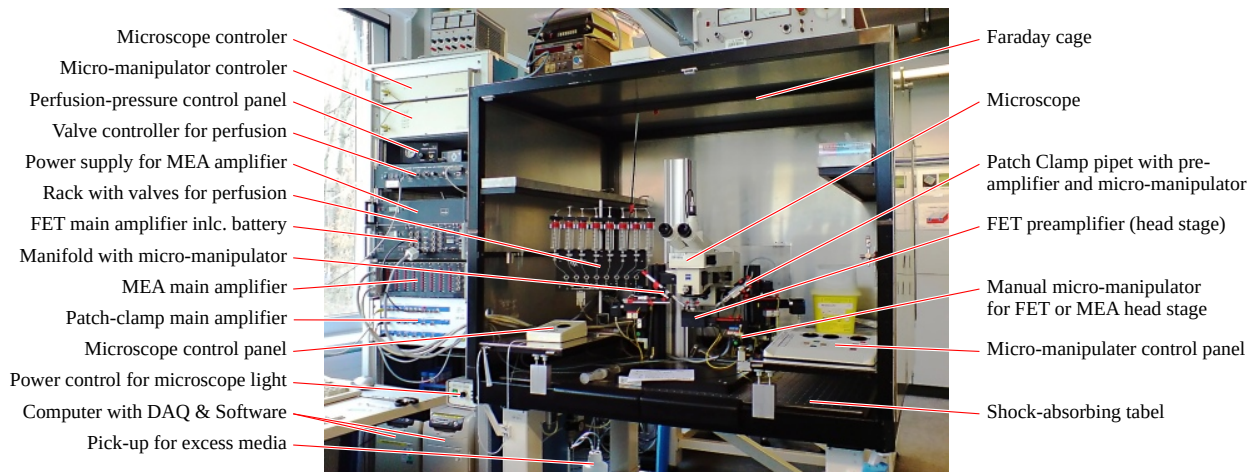


Figure 3.4.: The bioelectronic setup. Preamplifiers were mounted around a microscope on an air-cushioned table in a Faraday cage, close to the actual point of measurement, while main amplifiers and power supply-units were situated externally. Components and their interactions are explained in the text.

The core-setup was build on a vibration isolated workstation (Newport Spectra-Physics GmbH, Germany), electrically shielded by a custom made FARADAY cage. Concerning the optical properties of MEAs (gold electrodes) and FETs (silicon substrate), visual control during experiments was provided by an AxioTech Vario 100 HD upright microscope (Carl Zeiss AG, Germany) equipped with differential interference contrast (DIC) objectives (10 x air, 20 x immersion). Focusing of the microscope was motor-controlled and digital images of cells and sensors were obtained with an AxioCam MRc 5 using the software *AxioVision 4.6* (Carl Zeiss AG, Germany). Two motorized micro-manipulators (Luigs & Neumann GmbH, Germany) were situated on both sides of the microscope to manoeuvre a micro-manifold (left) and a patch-clamp pipette (right) in XYZ-direction. The manifold delivered variations of extracellular solutions (see section 3.2.2 for details), e.g. to chemically stimulate cells. FET and MEA preamplifiers were mounted on hand-controlled XY micro-manipulators (Luigs & Neumann GmbH, Germany) to allow a centering of individual sensors and cells within the microscopic field of view by horizontal panning.

The setup's periphery, including controllers, main amplifiers, computers, power sources and such was located in a 19" rack next to the Faraday cage. Intracellular recording and stimulation of cells was performed with an EPC-9 double patch-clamp amplifier (see section 3.2.1) operated by the software *TIDA 5.45* (HEKA Elektronik GmbH, Germany). The patch-clamp amplifier further

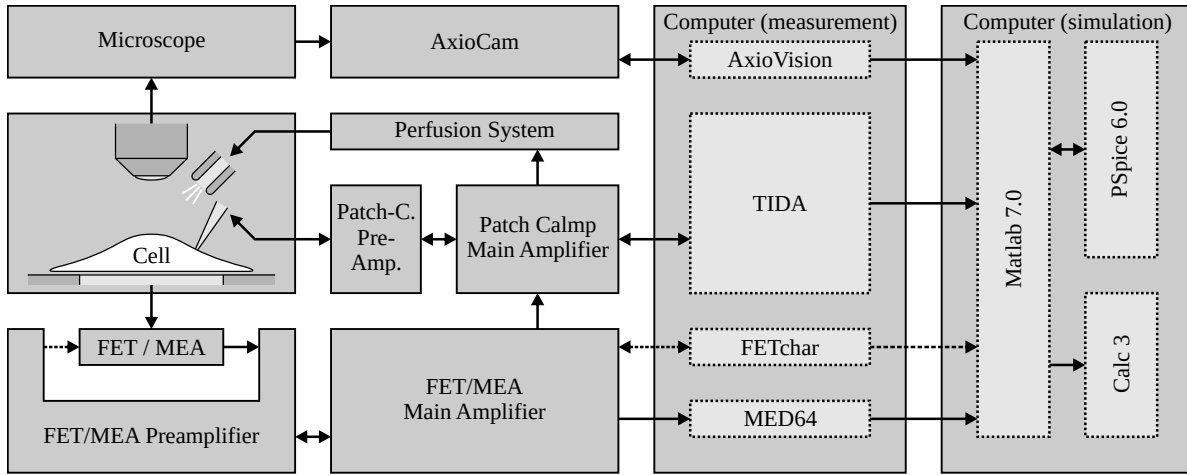


Figure 3.5.: Schematic overview of components for the bioelectronic coupling of cells with FETs or MEAs. Dashed lines are FET specific and were not used during experiments with MEAs. Obtained data was transferred to an off-line PC for further evaluation (section 7.2). The individual components are described in the course of this chapter.

controlled a DAD-8VC perfusion system (ALA Scientific Instruments, USA) at an analog output and received FET and MEA signals at analog inputs. Two computers were assigned with the bioelectronic setup, one controlling patch-clamp experiments and FET characterizations and the other for simultaneous recordings from up to 16 FETs or 64 MEAs. A third office computer was used for subsequent data evaluations and simulations.

Functional elements of the bioelectronic setup and their interactions as well as the subsequent data handling are illustrated in figure 3.5. The setup's key component was the patch-clamp main amplifier, which performed central tasks during experiments. Detailed information about the functionality of FET and MEA specific components as well as other equipment is given in the following sections.

3.2.1. Patch-clamp equipment

Cells located on sensors were contacted with patch-clamp pipettes for an intracellular monitoring of electrical activity in parallel to extracellular recordings with FETs or MEAs. While the general function of the patch-clamp technique is described in section 2.3, this section provides specific information about the employed hardware.

The EPC 9 double patch-clamp amplifier (HEKA Elektronik GmbH, Germany) provided two interfaces for patch-clamp preamplifiers of which only one was used for experiments in this thesis. The preamplifier was mounted on a motorized XYZ-stage (Luigs & Neumann GmbH, Germany).



Figure 3.6: Photograph of the EPC 9 double patch-clamp amplifier and two preamplification head-stages. The main amplifier provided several in- and out-puts to e.g. control a perfusion system or acquire FET or MEA signals in parallel to patch-clamp recordings. All functions were controlled by the software *TIDA*.

Pipettes with resistances $R_P = 5 \pm 3 \text{ M}\Omega$, as shown in figure 2.5, were pulled with a Flaming/Brown P-97 micropipette puller (Sutter Instrument Company, USA) from borosilicate tubing¹⁴ (Hilgenberg GmbH, Germany). The patch-clamp preamplifier was further connected to common ground at the bath electrode. Pressure variations during experiments were applied to a tube sleeve in the preamplifier. Electrical equivalent components of pipettes and cells were monitored and compensated with the software *TIDA 5.45* (HEKA Elektronik GmbH, Germany). Signals from FETs or MEAs were fed into an external input of the EPC 9. All measured signals were processed by a 16-bit AD converter and written to a hard disc drive (see section 7.2.1). Stimulation protocols (written in a *TIDA* specific language) were translated into digital signals and processed by a 16-bit DA converter, yielding currents or voltages to stimulate cells. Stimulation voltages were also assigned to an analog output channel of the EPC 9 to control a perfusion system.

3.2.2. Perfusion system

The majority of experiments in this thesis required a well-timed exchange of extracellular solutions, either to stimulate a cell with specific compounds in the solution or to influence the pH sensitivity of FETs by applying different buffer concentrations. These changes of bath solutions were performed with a voltage controlled perfusion system (DAD-8VC, ALA Scientific Instruments, USA).

As depicted in figure 3.7, the perfusion system was equipped with eight reservoirs for different solutions. A common air pressure of 5 Pa produced a constant stream ($\sim 125 \text{ }\mu\text{l/min}$) of solutions. Valves, opening at defined threshold voltages in steps of 0.5 mV, controlled the flow out of each reservoir. Command voltages were defined in *TIDA* stimulation protocols and applied to an output channel of the EPC 9 patch-clamp amplifier. Supply lines from all eight reservoirs were united in a manifold with 100 μm diameter. The manifold was mounted on a motorized XYZ-stage (Luigs & Neumann GmbH, Germany) to allow a precise positioning of the outlet near a cell. Solution exchanges took place with a delay of $\sim 75 \text{ ms}$ due to a lag volume within the manifold.

¹⁴ 1.5 mm outer diameter and 0.86 mm inner diameter

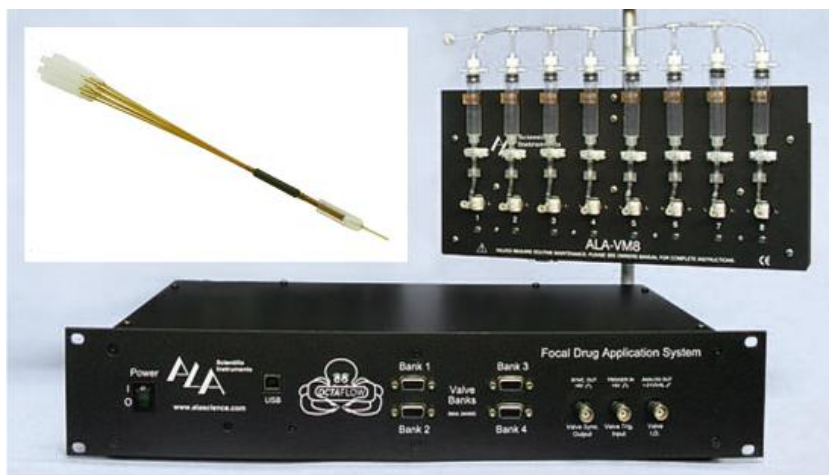


Figure 3.7: The perfusion system consisted of four major components. A rack (upper right) supporting up to eight reservoirs, a controller box (bottom) to open or close voltage-gated valves, a manifold (inset) to merge the eight reservoirs into one outlet and a pressure-control box (not depicted).

3.2.3. Field-effect transistor (FET) arrays and amplifiers

Field-effect transistors for electrophysiological investigations are a very powerful yet rare utility. Bergveld (1973) conducted very preliminary experiments in his thesis and presented “*a field-effect transistor without gate metal*” (Bergveld et al., 1976) later on. However, it took about fifteen years until Fromherz et al. (1991) resumed the concept. Field-effect devices for cell-transistor junctions in this thesis were initially designed by Offenhäusser et al. (1997) and subsequently improved by Sprössler et al. (1998), Krause (2000) and Ingebrandt (2001). Various experiments were realized using these FET devices (Offenhäusser and Knoll, 2001; Ingebrandt et al., 2005; Wrobel et al., 2005; Schäfer et al., 2009). Figure 3.8 depicts a fully assembled FET chip after cleaning (see section 5.2.4). A description of the functionality of the general field-effect and the ion-sensitive FETs is given in section 2.5.

FETs were fabricated with varying gate sizes from $4 \times 2 \mu\text{m}$ up to $16 \times 4 \mu\text{m}$ (Krause, 2000). All FET arrays consisted of 4×4 transistor gates connected by a common source and individual drain contacts. They were arranged with 100 and 200 μm spacing in a square on $5 \times 5 \text{ mm}^2$ silicon chips. A detailed protocol of the FET fabrication and their encapsulation was given in previous publications (Offenhäusser et al., 1997; Sprössler et al., 1998; Krause, 2000; Ingebrandt, 2001; Hofmann, 2010), therefore, only a brief description will be provided here. The $5 \times 5 \text{ mm}^2$ FET chips were glued (EPO-TEK H20E-175, Epoxy Technology Inc., USA) onto a 28 pin DIL-ceramic socket holder and contacts from chip to socket were wire-bonded (Westbond, USA). Conducting elements were protected by a PDMS funnel and further insulated with PDMS inside a glass ring (SYLGARD 182 and DC96-083, Dow Corning, USA). The resulting cell-culture area had a diameter of 3 mm at the chip surface and the ring held approximately 400 μl of culturing media, as shown in figure 3.8a.

The preamplifier provided a ZIF (zero-in-force) socket to host an FET chip. An additional gold pin connected the bath medium to ground potential via a silver-chloride electrode (figure 3.9a). Two lines, $\text{WP} = -U_{GS}$ and $\text{FET} = -U_{GS} + U_{DS}$, supplied the preamplifier with drain, source and gate potentials. FET source-drain currents were amplified 10-fold and converted into voltages by

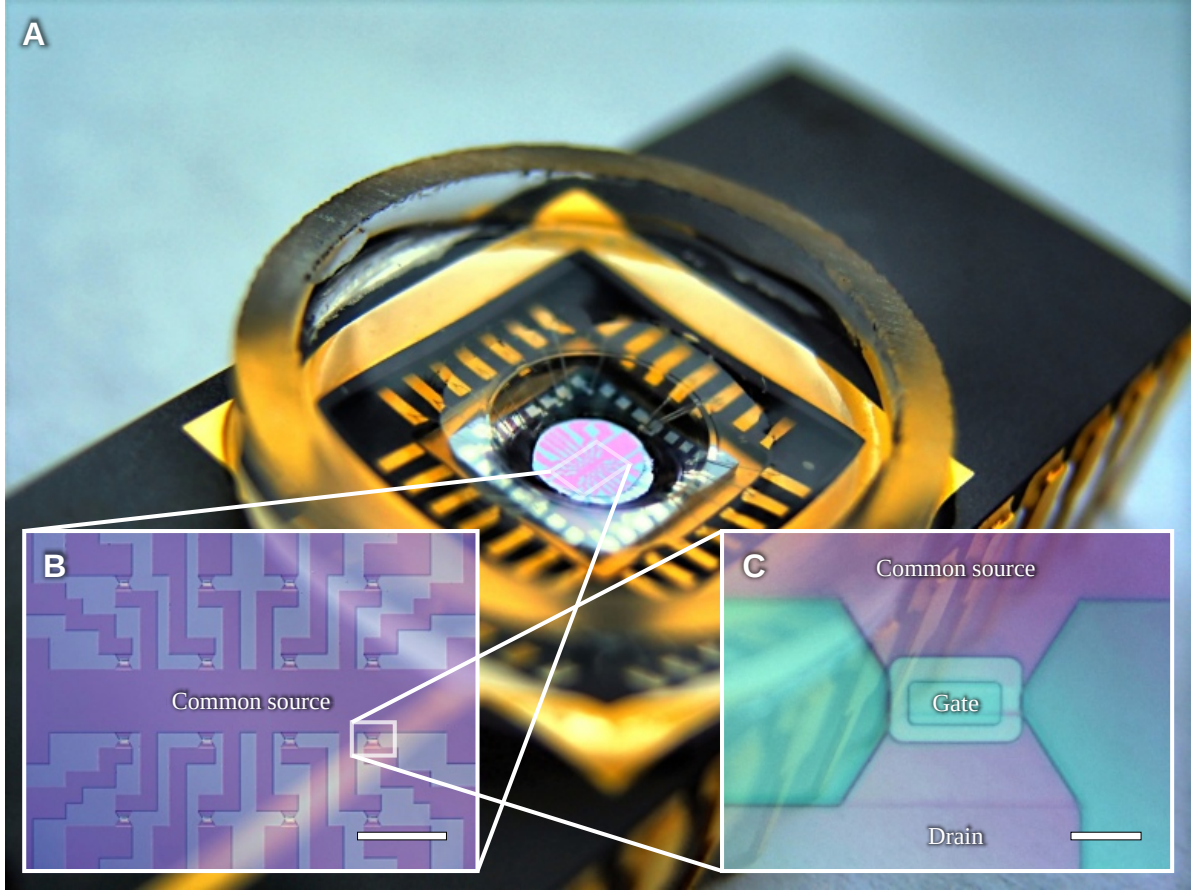


Figure 3.8.: The field-effect transistor chip (A) Photograph of the packaged version, including the actual FET chip with wire bonds to the carrier and a glass ring ($d = 16$ mm) for cell culture. (B+C) Images from differential interference contrast microscopy of the 4×4 gate area with $200 \mu\text{m}$ pitch and a closer view at a single gate structure. The respective scale bars are 200 and $10 \mu\text{m}$

16 operational amplifiers (OP-97F, Analog Devices Inc., USA). Power and data cables connected the head stage with a battery operated main amplifier where the signals were further amplified by a gain of 100. A feed-back loop with the original time constant of $\tau = 550$ ms (Ingebrandt, 2001) was replaced with $\tau = 4.9$ s by Wrobel et al. (2005) in order to provide more recording time for cellular membrane currents during voltage-clamp experiments, yet compensating slowly drifting potentials and stabilizing the baseline. The main amplifier provided 16 BNC (Bayonet Neill-Concelman) connectors at the front side for individual signal pick-off as well as panels to control the chip temperature and display battery information.

The output of the main amplifier was connected to a 16 channel data-acquisition card (PCI 6110, National Instruments, USA) installed in a standard personal computer. Controller connections were established via digital in- and outputs of the PCI 6110. FET chips were characterized with a custom-made software *FETchar* according to procedures described in section 2.5 on page 23. The transconductance g_m was selected in *FETchar* from the first derivative of the transfer characteristics. In order to conduct parallel patch clamp recordings, the electrolyte was kept at ground

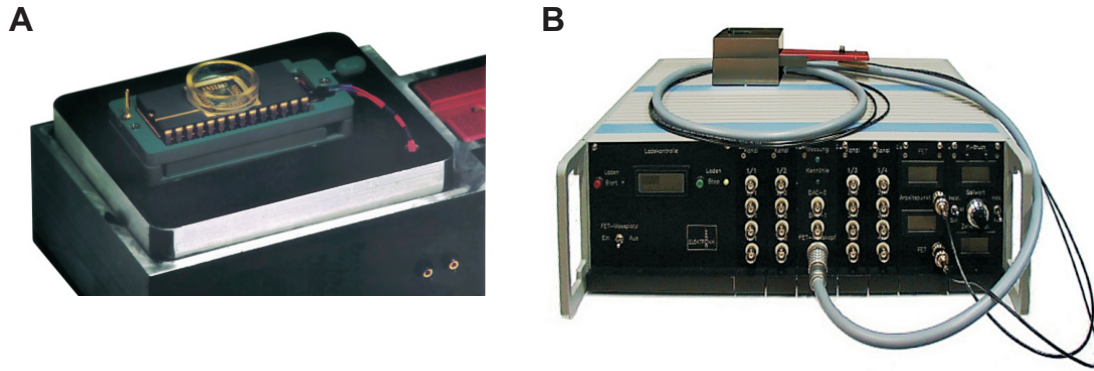


Figure 3.9.: Photographs of the FET amplification equipment. (A) 10-fold preamplifier holding an FET chip. A ground electrode can be connected to the gold pin on the left. The heating element is located beneath the chip build into the ZIF socket. (B) 100-fold main amplifier with 16 pick-off connectors, battery info-panel (left) and heating control (right).

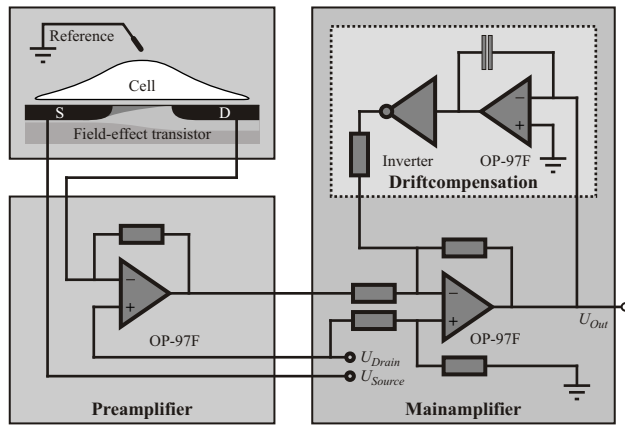


Figure 3.10: A simplified schematic overview of FET amplification circuits. Drain-source currents are pre-amplified and then fed into the main amplifier. The main amplifier contains an inverted feed-back circuit to compensate drift currents. All voltages are set in the main amplifier and forwarded through the head-stage to the FET.

potential with a the silver-chloride bath electrode. The software *MED64 conductor* (Alpha MED Sciences, Japan) was occasionally employed to record from all 16 channels simultaneously. Usually, the software TIDA (see sections 3.2 and 7.2.1) was used to record signals from one particular FET on the chip.

	H ⁺	K ⁺	Ca ²⁺	Na ⁺	Cl ⁻
FET potential $U_{\Delta[i]}^{ISFET}$	$34.0 \pm 2 \text{ mV/pH}$	9.7 mV/pK	6.8 mV/pCa	2.8 mV/pNa	0.0 mV/pCl

Table 3.2.: FET potentials caused by interactions between ions and hydroxyl-groups at the electrolyte-gateoxide interface (Ingebrandt, 2001; Borstlap, 2006).

The ion sensitivity of the utilized FETs (section 2.5) was governed by reactions of cations with hydroxyl-groups at the electrolyte-gateoxide interface. Sensitivities for ion species of interest¹⁵ are listed in table 3.2 (Ingebrandt, 2001). The pH sensitivity was by far stronger than all others and in

¹⁵ Ion species involved in bioelectric signaling of cells.

good agreement with literature values of 30 to 40 mV/pH (Fung et al., 1986; Bataillard et al., 1987; Bergveld et al., 1997).

3.2.4. Microelectrode arrays (MEAs) and amplifiers

Versatile probes are available to measure the activity of multiple electrogenic cells in parallel. However, patch-clamp pipettes or impaling electrodes are limited by spacial restrictions and optical approaches, such as calcium imaging, are limited by their resolution in the time domain. A suitable approach is the integration of extracellular electrodes into the very substrate on which the cells are cultured. The first “*miniature microelectrode array to monitor the bioelectric activity of cultured cells*” was reported by Thomas et al. (1972) and had 30 platinized gold electrodes of $7 \times 7 \mu\text{m}^2$ in a 2 by 15 array. Today’s microelectrode arrays (MEAs) usually provide 60 or 64 substrate-integrated electrodes of various sizes and materials, organized in an 8 by 8 array Pine (2006).

Microelectrodes and amplification systems were initially developed by Krause et al. (2000) as “*extended gate electrodes for extracellular signal recordings*”, where the gate of a junction-gate field-effect transistor¹⁶ (JFET) was extended with a metal wire that ends as an open gold electrode, embedded into a glass substrate. The MEA design as well as amplification steps were later improved by Ingebrandt (2001). Recording electrode diameters were available from 6 to 30 μm and stimulation electrodes from 50 to 100 μm Krause et al. (2000). In contrast to FETs, electrodes need only one contact and their fabrication is more convenient. Sixty-four recording electrodes were organized in an 8×8 array with 100 or 200 μm pitch. In case of stimulation devices, three recording electrodes in the array corners were replaced by larger electrodes. However, stimulation capabilities were not applied in the course of this thesis and mostly used were recording electrodes with a diameter of 10 or 20 μm .

Detailed information about the fabrication process was given in Krause (2000) and an improved packaging by a flip-chip procedure was described by Ingebrandt (2001). Therefore, only a summarized description of the packaging protocol is given here. All contact pads of the $11 \times 11 \text{ mm}^2$ electrode dyes were equipped with conducting glue (EPO-TEK H20E-175, Epoxy Technology Inc., USA) via screen printing. The dyes were then glued to the back of a printed circuit board (PCB) using a polymer flip-chip technology (FINEPLACER-145 APICOB, Finetech Electronic, Germany), and secured with an underfill (EPO-TEK U300, Epoxy Technology Inc., USA). The cell culture area was defined by two glass rings including a PDMS filling (DC96-083, Dow Corning, USA), which held about 500 μl of medium. The full assembly with close-ups of the array and an electrode is depicted in figure 3.11.

¹⁶ JFETs are the simplest type of FETs, e.g. used for amplification in audio equipment (Self et al., 2008).

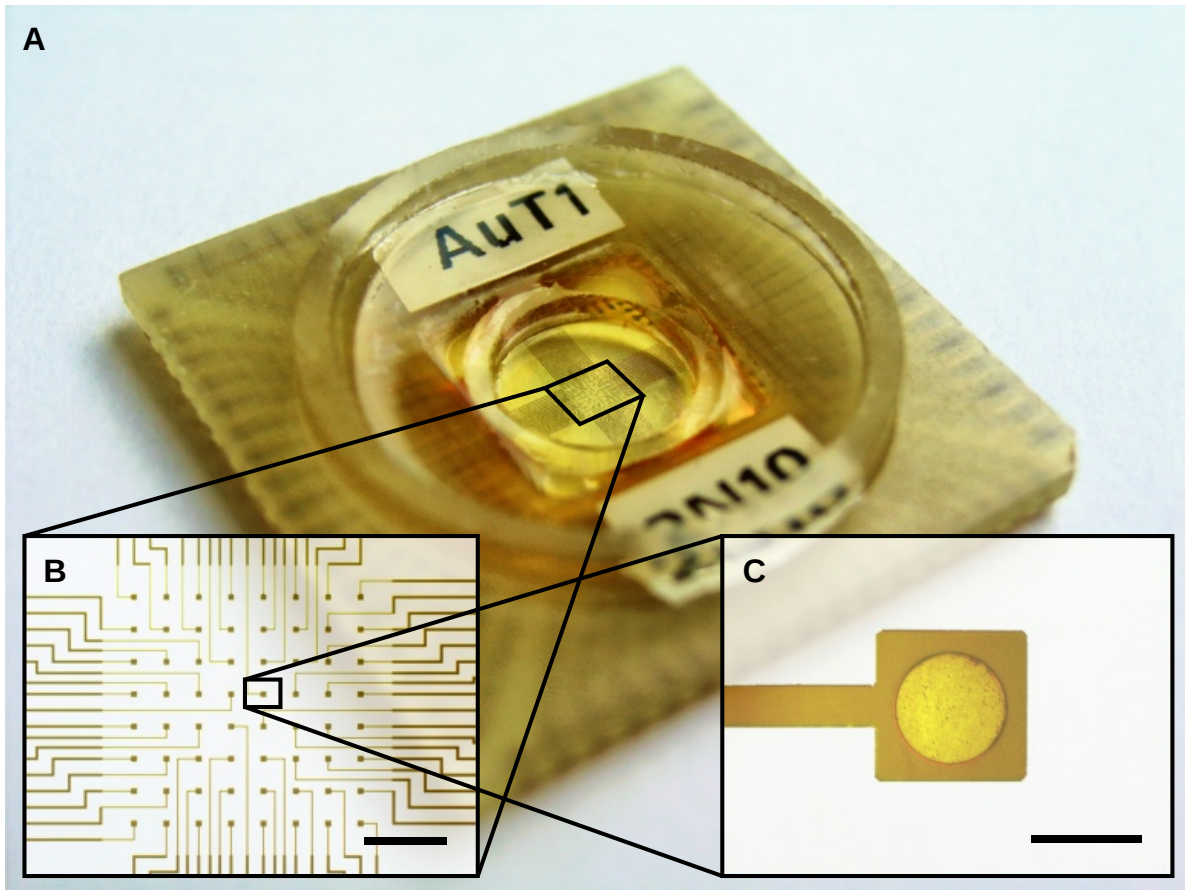


Figure 3.11.: The microelectrode-array chip (A) Photograph of the packaged version, including the glass dye glued to the bottom of the $24 \times 24 \text{ mm}^2$ printed circuit board, a small inner ($d = 7 \text{ mm}$) and a larger outer glass ring ($d = 16 \text{ mm}$) for cell culture. (B+C) Color-adjusted images from differential interference contrast microscopy of the 8×8 electrode area with $200 \mu\text{m}$ pitch and a closer view at an individual $20 \mu\text{m}$ recording electrode. The respective scale bars are 500 and $20 \mu\text{m}$

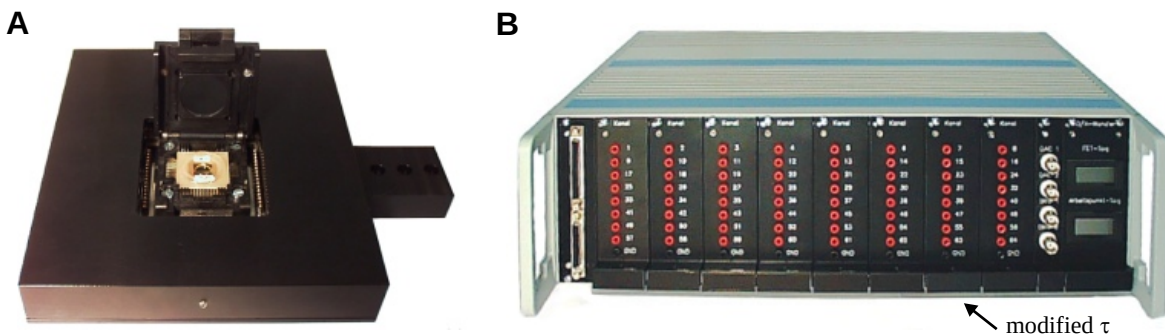


Figure 3.12.: Photographs of the amplification devices for MEAs (A) Preamplifier with 10-fold gain and (B) the 100-fold main amplifier. The seventh drawer has an alternated drift compensation for investigations of membrane currents in steady-state during voltage clamp experiments.

The MEA preamplifier, as revised by [Ingebrandt \(2001\)](#), accommodated the MEA chips in a modified test socket (PLCC68 T+B IC51, YAMAICHI Inc., Germany) and a silver-chloride electrode served as ground (figure 3.12a). It contained 64 low noise JFETs (PMBF5484, Philips Semiconductors, USA) for a 10-fold amplification and operational amplifiers (OP-97F, Analog Devices

inc., USA) for current-voltage conversions. Data (68 Sub D) and power supply (50 Sub D) lines connected the preamplifier to the main amplification stage with a 100-fold gain. A drift component of measured signals was compensated by a feedback loop with the time constant $\tau = 100$ ms [Ingebrandt \(2001\)](#). For experiments in this thesis it was necessary to measure steady-state membrane currents over a longer time course, thus the drift correction had to be alleviated to a slower time constant $\tau = 11$ s (figure 3.13) by replacing capacitances (supplying eight electrodes) on one drawer of the main amplifier (figure 3.12). Drawers could be switched during experiments to provide the modified drift correction for any of the 64 electrodes. The main amplifier output was connected to a data acquisition card (PCI 6803, National Instruments, USA) in a personal computer. The software *MED64 conductor* (Alpha MED Sciences, Japan) was employed to record from all 64 channels simultaneously. The main amplifier provided 64 connectors for an individual pick-off at the front panel (figure 3.12b), e.g. to visualize individual signals with oscilloscopes. During experiments in this thesis, the front-panel output was used to forward signals into an external input of the patch-clamp amplifier.

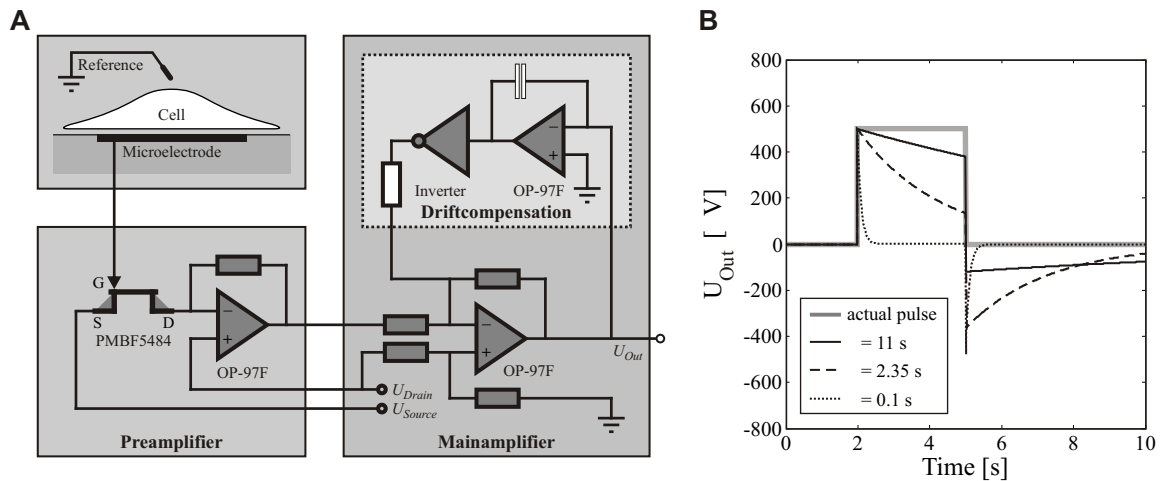


Figure 3.13.: Schematic representation of the MEA amplification setup (A) MEA electrodes are the extension of a JFET gate electrode. After pre-amplification in the head stage, signals were passed on to the main amplifier. Drifting signals were corrected by a feed-back circuit. The time constant τ for the high-pass filter is determined by the resistor and the capacitance in the feed-back circuit (indicated in white). (B) For 8 microelectrodes the original time constant of $\tau = 100$ ms was reduced to $\tau = 11$ s. An intermediate change to $\tau = 2.33$ s was not sufficient enough.

4. Biophysical characterization of the cell morphology for signal modeling

This chapter is based on the following publications:

Sommerhage F., Helpenstein R., Rauf A., Wrobel G., Offenhäusser A., Ingebrandt S. (2008). [Membrane allocation profiling: a method to characterize three-dimensional cell shape and attachment based on surface reconstruction](#). *Biomaterials* 29, 3927-3935.

Wrobel G., Höller M., Ingebrandt S., Dieluweit S., **Sommerhage F.**, Bochem H. P., Offenhäusser A. (2008). [Transmission electron microscopy study of the cell-sensor interface](#). *Journal of the Royal Society Interface* 6, 5(19), 213-22.

4.1. Introduction

Development of techniques for morphological characterization of cells has lead to advances in various fields of research in recent decades. Three-dimensional (3D) imaging of cells and tissues has lead to an enhanced understanding of function, structure and shape (Halbhuber and Konig, 2003; Pygall et al., 2007). In biomaterials research, cell shape is often used as an indicator of biocompatibility and quality of cell-substrate contacts (Ramires et al., 2002; Bacáková et al., 2004; Teixeira et al., 2008; Baschong et al., 2001). Cell response to materials is a complex process involving protein adsorption to the material, cellular attachment to the adsorbed proteins, and a cascade of intracellular signals leading to changes in the cellular behavior (Anderson, 2001). Chemically or physically modifying the surface of various materials is one current strategy to enhance the cellular interaction with the material thereby enhancing the material function. Material biocompatibility and cellular attachment to the material are factors particularly important to implanted devices (Ramires et al., 2002; Walboomers et al., 1998; Heinrich et al., 2007). Generally, cell attachment can be measured in three different ways: the strength of adhesion (Reyes and García, 2003), the distance between cell and substrate (Walboomers et al., 1998; Wrobel et al., 2007), or cell shape while focusing on the ratio of attached to total cell membrane (Buitenweg et al., 2003; Schoen and Fromherz, 2007). If the connection between a material and cells is used for bioelectronic communication, cellular adhesion in terms of the distance and shape becomes very important. Bioelectronic hybrids are compositions of electrically active cells, such as neurons or myocytes, coupled to electronic sensors (Bergveld et al., 1976; Pine, 1980; Gross et al., 1985; Fromherz et al., 1991). In cell-sensor coupling, the communication between cell and sensor strongly increases with the quality of the contact (Regehr et al., 1989; Ingebrandt et al., 2005; Cohen et al., 2008). For instance, the communication quality increases as more cell membrane is attached to the sensor (Wrobel et al., 2005). A common method to characterize cell adhesion in cell-sensor couplings is determining the ratio α_{JM} of the attached membrane A_{JM} (membrane in junction) to the total cell membrane A_M (Buitenweg et al., 2003; Weis and Fromherz, 1997; Vassanelli and Fromherz, 1998; Ingebrandt et al., 2005). Therefore, determining 3D shape of the cellular surface is important to bioelectronic research.

Morphological studies of the cell shape have been performed in various ways. The most commonly used technique is conventional light microscopy, which provides a vertical projection of the specimen on the substrate (Merson-Davies and Odds, 1989; Cayre et al., 1998). Therefore, this technique can not provide accurate information of the 3D shape of a cell and cellular attachment only can be estimated roughly. Another common tool for morphological studies is scanning electron microscopy (SEM). SEM visualizes the specimen from various angles, providing some of the 3D morphological information about cells in a 2D projection. (Bethea and Kozak, 1985; Herrmann et al., 1983). Although SEM was often used to analyze the quality of cell shapes and attachment, it is not capable of providing adequate 3D data for quantitative investigations, e.g. the

cellular surface area, volume or attachment. Also, multiple fixation and drying steps are necessary or recommended for preparation of cellular specimens for SEM observation may cause shape distortion compared to the physiological environment (Gusnard and Kirschner, 1977). Atomic force microscopy (AFM) is a very powerful tool to investigate structural characteristics, especially the surface of tiny objects, at high resolution (Lesniewska et al., 1998; Oberleithner et al., 1996). AFM is typically used to image specimens that are smaller than cells, e.g. adsorbed proteins or chromosomes (Tamayo and Miles, 2002). If employed for cells, AFM provides height maps, which are very helpful for volume or surface estimations to facilitate shape investigations. However, height maps are limited in terms of adhesion evaluation since the attached part of the cell has to be assumed as totally flat with respect to the substrate. To overcome this disadvantage, AFM could be combined with other techniques like total internal reflection microscopy (TIRF) or reflection interference contrast microscopy (RICM). TIRF or RICM can provide detailed 3D information about the attached part of the cell (Axelrod, 1981; Gingell et al., 1985; Curtis, 1964; Izzard and Lochner, 1976), but it does not give any information about the 3D cell shape. Also, TIRF and RICM can only be used for transparent substrates, which limits the applicability to biomaterials or bioelectronic research.

Full 3D information of cell shape, including the attached portion, can be obtained practically independent from the chosen substrate with sectioning techniques (Wouterlood, 2005; Ren et al., 2005). For instance, confocal laser scanning microscopy (CLSM) is capable to measure data for reliable 3D representation of a cell or a small piece of tissue including the basal side of the specimen. Alternatively, in case of very small objects, transmission electron microscopy (TEM) from thin slices can provide the sectioned images (Rollenhagen et al., 2007). For larger specimens like brains, lungs or hearts, MRI or other tomographical methods can provide the volumetric data sets (Cline et al., 1988). However, 3D reconstruction from CLSM data allows detailed examination of the cellular shape in various ways (Wouterlood, 2005; Morgan et al., 1992). Typically, reconstructions are performed either by generating 3D volumes (Chvatal et al., 2007) or 3D (iso-)surfaces (Kubínova and Janacek, 2001; Kubínova et al., 2004). In both cases, the reconstructed specimen can be freely rotated and magnified to facilitate studies on the cell shape or cell-substrate adhesions (Ramires et al., 2002; Strohmaier et al., 1997; Eils and Athale, 2003; Sugawara et al., 2005). Combined with the direct observation of cells, the reconstruction of cells can be utilized to extract cell attributes, e.g. volume, surface area, height, or orientation (Chvatal et al., 2007; Kubínova and Janacek, 2001; Kubínova et al., 2004). However, the characterization of the cellular morphology and the transfer from shape to quality and quantity of adhesion remains a challenge (Fricker et al., 2006).

In this chapter, a method for cell shape analysis from CLSM data is presented. This novel procedure reduces the multidimensional and large data sets from confocal microscopy to single curves: membrane allocation profiles (MAP). The membrane allocation profiling (MAPing) technique uses the 3D image stacks from CLSM. During MAPing, 3D surfaces of cells are sequentially

reconstructed as segments in parallel to the substrate, and the surface areas of individual segments are calculated. Adding up the surface areas of all segments leads to MAPs, which can be used to determine attributes of the cellular morphology. A MAP combines qualitative and quantitative information about cell shape and surface area as a function of cell height. Therefore, this technique can provide information about membrane attachment to a substrate, e. g. as a ratio of attached to total membrane surface. Normalized MAPs from different cells can be visualized to compare differences in shape or cell adhesion to a substrate as demonstrated using HEK293 cells here.

4.2. Materials and Methods

4.2.1. Substrates and surface modifications

Petri dishes (Ø35 mm, Grainer, Germany) were modified by drilling a hole (Ø15 mm) through the bottom and mounting an 80 µm thin glass cover slip (Menzel-Gläser, Germany). Subsequently, only the exposed glass substrates were activated for 20 min with 20 % sulfuric acid at 80 °C, followed by rinsing with bidistilled water, and sterilization with ethanol. Substrates were coated with various proteins to investigate their influence on cell adhesion and morphology: [PLL] 100 µg/ml poly(L)lysine (MW: 150 – 300 kDa) for 30 min in bi-distilled water; [PDL] 50 µg/ml poly(D)lysine (MW: 70 – 150 kDa) for 60 min in HANK's balanced salt solution (HBSS); [LAM] 2 µg/ml laminine (MW: 900 kDa) for 60 min in buffer (in mM: 150 (NaCl), 50 Tris/HCl, pH 7.5); [FIB] 12.5 µg/ml fibronectin (MW: 450 kDa) for 30 min in phosphate buffer solution (PBS, in mM: 137 NaCl, 2.7 KCl, 8.1 Na₂HPO₄, 1.5 KH₂PO₄, pH 7.3); [ECM]: 10 µg/ml extracellular matrix gel for 60 min in HBSS; [PECM] 10 µg/ml poly(D)lysine and 10 µg/ml extracellular matrix gel, both for 60 min in HBSS; [ConA] 0.1 µg/ml concanavalin A (MW: 104 kDa) for 30 min in bidistilled water; no coating control (all from Sigma-Aldrich, Germany). Substrates were incubated with 500 µl of a coating solution at room temperature and then rinsed once (500 µl) with the corresponding diluents (PBS in case of bidistilled water). Quality and thickness of these substrate coatings have been investigated in earlier studies (Wrobel et al., 2007).

4.2.2. Cell culture

For the experiments, Human Embryonic Kidney (HEK293) cells, which stably expressed a voltage-gated potassium channel, were used. The cells were cultured as described earlier (Wrobel et al., 2005). HEK293 cells were grown in minimal essential medium, plus 10 % fetal bovine serum, 2 mM glutamine, 1 % non-essential amino acids, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen, Germany). For image acquisition, cells were seeded at densities of 250 cells/mm² and were cultured for three days at 37 °C and 5 % CO₂.

4.2.3. Staining

After three days in culture, the culture medium was completely replaced by fresh medium containing $1 \mu\text{l/ml}$ calcein (Sigma-Aldrich, Germany). Next, the cells were incubated for another 15 – 20 min under culturing conditions, before the medium was aspirated and replaced by fresh medium again to reduce background fluorescence.

4.2.4. CLSM image acquisition

Calcein stained cells were investigated using a confocal laser scanning fluorescence microscope (LSM 510, Zeiss, Germany) equipped with an argon ion laser, a plan-apochromat objective (63 x/1.4 Oil DIC), and the *Carl Zeiss LSM Imaging* software. Images (average pixel size of all experiments: $87 \pm 27 \text{ nm}$) of adhered cells were acquired in 8 bit (256 grey shades) in 32-78 horizontal sections, using z-steps of $0.37 \mu\text{m}$. The total acquisition time of one image stack varied from about 4-8 min.

4.2.5. Digital image processing and filtering

All sections of a CLSM data set were imported into *Matlab 7.0* (The MathWorks, USA) for further processing using the freely available *LSM-File Toolbox* (Li, 2005). For noise reduction, individual images were 2D median (3×3) filtered separately, before the whole stack subsequently was 3D median ($3 \times 3 \times 3$) filtered (Salvado, 2004).

4.2.6. Statistical analysis

Statistical tests were performed with *Matlab's Statistical Toolbox* and *Microsoft Excel 2003*. For the results of attached membrane ratios from multiple cells ($n = 13$ to 35) a β -distribution is assumed. In contrast to Gaussian distributions, β -distributions are skewed (asymmetric) and have defined boundaries. For attachment ratios, the interval $[0; 0.5]$ was selected, ranging from non-attached cells to 50 % membrane attachment. Ratios were expressed as means and standard deviations corrected by the skewed distribution. Differences among the eight surface modifications were established using an extended WILCOXON rank sum test for equal medians. Statistical significance was designated for $p < 0.01$ (Mann and Whitney, 1947).

4.3. From Image Reconstruction to Morphology

The reconstruction of a cell's shape in 3D from CLSM image stacks is a basic tool for the analysis of cell morphology. During such a reconstruction, image points of equal brightness (grey value

or isovalue) are connected horizontally within an image and vertically in a stack of images to obtain a 3D representation. Various research projects have used this technique (Wouterlood, 2005; Kubinova and Janacek, 2001; Chvatal et al., 2007), but the tremendous amount of data never was compressed for an easy comparison of morphologies of different cells (Fricker et al., 2006). A technique to create curves from CLSM image stacks which represent the cell's shape and attachment to the substrate is presented. First, membrane allocation profiling (MAPing) is demonstrated using geometrical models. After characterizing the method, MAPing is used to measure shape and attachment of HEK293 cells.

4.3.1. Data sets for theoretical investigations

To test the MAPing algorithm, objects were generated to serve as ideal cell models with known geometries in four spherical shapes (sphere, half sphere, and two spherical caps) with the spherical radius $R = 15 \mu\text{m}$. These models were created in *Matlab 7.0* (The MathWorks, USA). The influences of staining and CLSM acquisition were simulated through 3D blurring and white noise. Representative projections of these models are depicted in figure 4.1. Subsequently, the models undergo the same treatment as CLSM data sets from HEK293 cells.

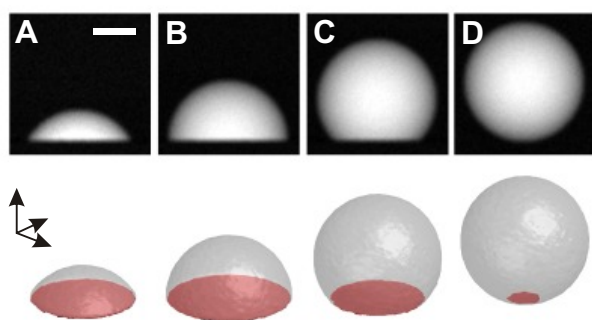


Figure 4.1: Volume x-/y-projections of geometrically defined objects as cell models for the MAPing technique (top). Half transparent 3D reconstructions with attached membranes marked in red (bottom). Scale bars $10 \mu\text{m}$

4.3.2. 3D reconstructions and membrane allocation profiles

Isosurface representations are computed and drawn as small triangles within a volumetric data set (e.g. a stack of CLSM images) according to a given threshold level of brightness (the isovalue). The reconstruction of the half sphere data from figure 4.1b is depicted in figure 4.2a.

Instead of traversing the whole data volume, a part of the cell model surface between adjacent image planes is reconstructed by the marching cubes algorithm (Cline et al., 1988; Lorensen and Cline, 1987; Cline et al., 1987). These model segments have two characteristic shapes: reconstructed portions of the attached cell membrane commonly are disc shaped planes while spherical segments of the free membrane are represented as rings (figure 4.2b). Subsequently, the surface area A of each segment is calculated as depicted in figure 4.2c. Membrane allocation profiles were

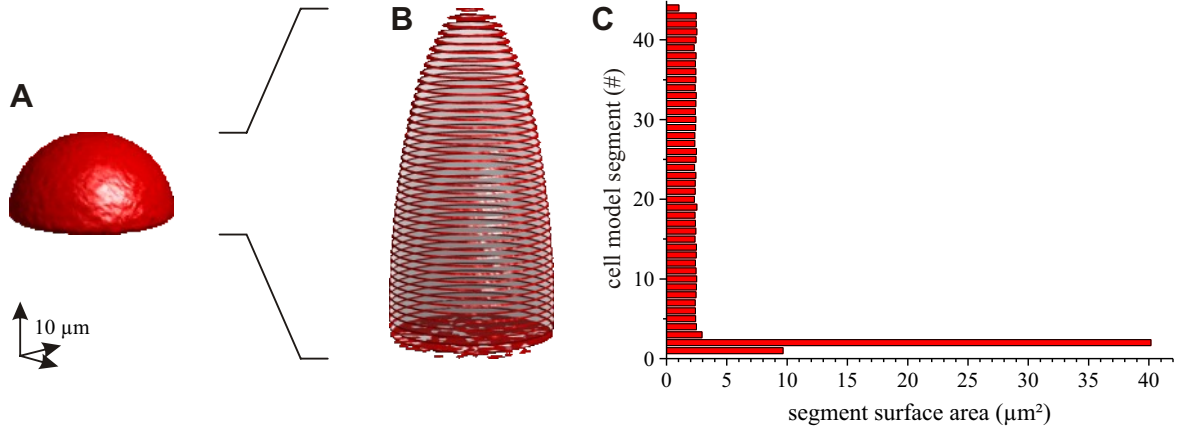


Figure 4.2.: Process of membrane allocation profiling for a half spherical cell model. (A) 3D reconstruction of the cell model. (B) Individual slices from the reconstructed cell model. (C) Surface area of each slice.

obtained by cumulating the surfaces areas A_n of all reconstructed segments, according to equation 4.1:

$$MAP(z) = \sum_{n=0}^z A_n \quad (4.1)$$

where z is the number of cumulated sections and A_n is the respective surface area of each section. Finally, the MAPs are plotted in CLSM z-steps ($0.37 \mu\text{m}$) of the data volume (figure 4.3). This procedure is repeated for all available grey shades (as isovalues) in a data volume (at best from 0 to 255).

4.3.3. Extracting information from MAP

MAPs for the four defined geometries introduced in figure 4.1 are depicted in figure 4.3a. Each curve represents the morphology of one cell model. The curves start with a steep increase blending into a linear gradient (except the whole sphere, figure 4.1d). The height of the steeply increasing part of the curve is the size of the flat attached surface. To characterize MAP curves for spherical objects, a sigmoidal function (MAP-function) according to equation 4.2 is fit to the MAPs:

$$MAP(z) = \begin{cases} \frac{A_{JM}}{1 + e^{\lambda(x+s)}} & \text{if } x < s \\ \frac{A_{JM}}{1 + e^{\lambda(x+s)}} + m(x + s) & \text{if } x \geq s \end{cases} \quad (4.2)$$

where z refers to the vertical position in the 3D data set. Fitting parameters are the slope λ of the sigmoid, the related offset s on the z-axis, and the height A_{JM} of the sigmoid, which is

equal to the area of attached membrane. For values of z larger than the horizontal shift s , a linear gradient with the slope m is added to the domain to symbolize the portion of the free membrane in the MAPs.

For the simulated half sphere model (figure 4.1b), the attached area was $A_{JM} = 1127 \mu\text{m}^2$. A comparison of the simulated MAP and the fitted sigmoid shape for the model is depicted in figure 4.3b.

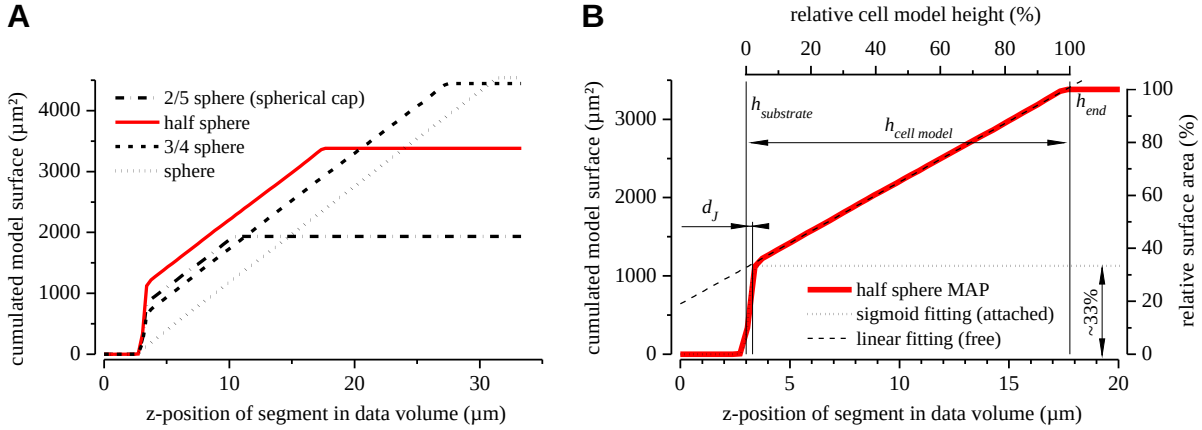


Figure 4.3.: (A) MAPs of geometrically defined cell models display the surface area of attached and free cell membrane. (B) Information extraction from a MAP of a half spherical cell model. $h_{\text{substrate}}$ is the position of the substrate and h_{end} the position of the last part of the cell in the underlying CLSM data volume. The distance d_J symbolizes the average cleft height between cell and substrate.

Start and end positions of the cell in the MAP are defined by deriving the borders of the cell from the fitted MAP-function. The tangential slope at the inflection point of the sigmoid function is $A_{JM} \cdot \lambda/4$. The crossing point $h_{\text{substrate}}$ of this tangential slope with the z-axis (horizontal axis) is set as the substrate level where the cell is attached (figure 4.3b and figure 4.4a) using equation 4.3:

$$h_{\text{substrate}} = s - \frac{4}{\lambda \sqrt{A_{JM}}} \quad (4.3)$$

Theoretically, the average distance d_J between cell and substrate can be estimated roughly with equation 4.4. Note, the estimation of d_J is subject to assumptions: that the substrate is absolutely in parallel to the CLSM sections and that influences of pixel noise are neglected.

$$d_J \approx \frac{8}{\lambda \sqrt{A_{JM}}} \quad (4.4)$$

The highest point of the measured curve represents the total membrane area A_M of the half sphere model, in this case $A_M = 3382 \mu\text{m}^2$ (figure 4.3b and figure 4.4a). The ratio of the attached membrane area in relation to the total membrane area is $\alpha_{JM} = 33.3\%$. To define the end h_{end} of

the cell surface, changes in the MAP that are four orders of magnitude below the whole membrane area A_M are neglected as noise (figure 4.3b and figure 4.4a). Consequently, the cell height is defined by $h_{cell} = h_{end} - h_{substrate}$. MAPing results for the four geometrically defined models are given in Table 4.1.

Since the individual cell models have different surface areas and heights, their respective MAPs are not comparable directly in terms of morphological shape. Thus, the MAP of each is normalized by its total surface area A_M and the cell height h_{cell} as depicted in figure 4.3b (second x- and y-axis).

cell model	$1/3$ spherical cap	half sphere	$3/4$ spherical cap	sphere
attached surface A_{JM} [μm]	807	1127	645	46
total surface A_M [μm]	1935	3382	4445	4542
ratio $\alpha_{JM} = A_{JM}/A_M$ [%]	41.7	33.3	14.2	1.0

Table 4.1.: Surface areas and the resulting ratio for attached parts of geometrically defined cell models (compare figure 4.1).

4.3.4. MAPing versus geometry

Since the surface of defined geometrical objects such as half spheres can be analytically calculated, the MAPing results for the half sphere can be verified with expected mathematical values. Described by horizontal sections, the flat attached area of a theoretical half sphere has the surface of a disc with the half sphere's diameter: $A_{JM} = 707 \mu\text{m}^2$ (for $R = 15 \mu\text{m}$). The dome surface of a half spherical object without the flat section can be calculated with equation 4.5 (figure 4.4b):

$$A = 2\pi \cdot R \cdot h = \pi(r^2 + h^2) \quad (4.5)$$

where R is the radius of the underlying sphere, h is the height, and r is the radius of the spherical cap ($h = r = R$ in case of a half sphere). During MAPing, the dome or spherical cap has to be cut into multiple spherical segments. According to equation 4.6, the surface areas of individual sphere segments are independent from the position of the segment, as long as the segment thickness is constant, e.g. the CLSM z-step (see figure 4.4):

$$\frac{\partial A}{\partial h} = 2\pi \cdot R = \text{constant} \quad (4.6)$$

Consequently, the cumulated surface areas of the segments of a half sphere increase linearly after the instant step by the size of a disc. This mathematical estimation is in accordance to the bars in figure 4.2c and the curves in figure 4.3. Since noise was added to the half sphere model in

figure 4.1b, its surface area is larger than the area of a smooth mathematical half sphere and the instant increase is blurred into a sigmoid s-function. However, the ratios are equivalent: $1/3$ of the half sphere's surface is represented by a flat disc (attached area: $1\pi \cdot R^2$) and $2/3$ are represented by a dome (free area: $2\pi \cdot R^2$).

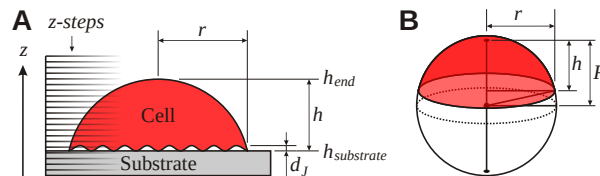


Figure 4.4.: Schematic drawings of geometrical conditions. (A) Cell, substrate and the corresponding variables in a xz- or yz-projection of a CLSM z-stack. (B) Demonstration of spherical caps and the according variables for the calculation of surface areas. The radius of the underlying sphere is R , while r is the radius of the spherical cap and h its height. For a half sphere applies $h = r = R$.

4.4. Results

The membrane allocation profiling (MAPing) procedure has been applied to the measured CLSM image stack of a HEK293 cell. Then this technique has been used to evaluate the influence of substrate modifications with different proteins on the attachment of HEK293 cells measured with the MAPing technique.

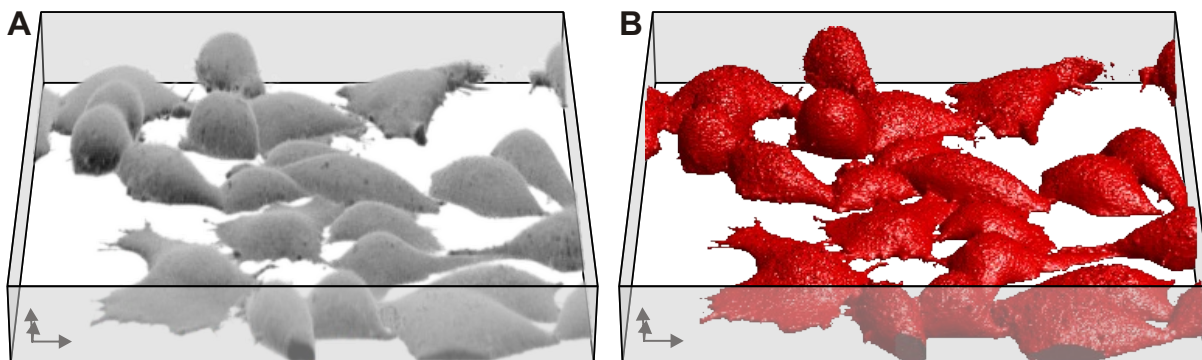


Figure 4.5.: A group of calcein stained HEK293 cells. (A) Inverse 3D volume reconstruction and (B) 3D isosurface reconstruction from CLSM data; scale bars 10 μm.

4.4.1. 3D reconstruction and MAPs from CLSM z-stacks of HEK293 cells

In figure 4.5a, a 3D volume reconstruction (30° projection) of a 3D data volume with multiple cells is depicted. Figure 4.5b shows the corresponding 3D isosurface reconstruction. In the depicted group of cells, various morphologies are present: flat cells, stretched cells, half spherical cells and spherical cells.

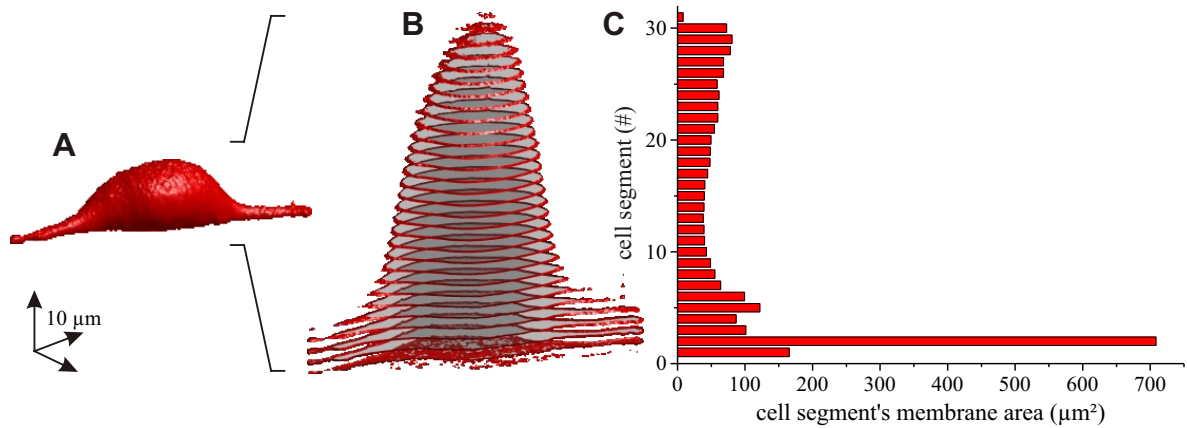


Figure 4.6.: Display of membrane allocation profiling for a HEK293 cell. (A) 3D reconstruction of a HEK293 cell. (B) Individual segments of the cell. (C) Calculated surface areas of the individual slices.

The MAPing process for an individual HEK293 cell is depicted in figure 4.6. The cumulated cell segment areas from figure 4.6c are presented as a MAP in figure 4.7a. In accordance to the MAPs for geometrical models, the parameter fitting for the measured cell MAP, yields the following cell data: The reconstructed surface area for isovalue 39 (grey value) is $A_M = 2591 \mu\text{m}^2$, the cell height is $h = 10.8 \mu\text{m}$, and the attached surface area is $A_{JM} = 893 \mu\text{m}^2$. Therefore, the ratio of the attached membrane is $\alpha_{JM} = 34.5 \%$. Also, a theoretical estimation for the average cell-substrate distance is estimated to be $d_J \approx 24 \text{ nm}$ for this particular cell according to equation 4.4. This value for d_J is in the same order of magnitude as previously published values (Wrobel et al., 2007).

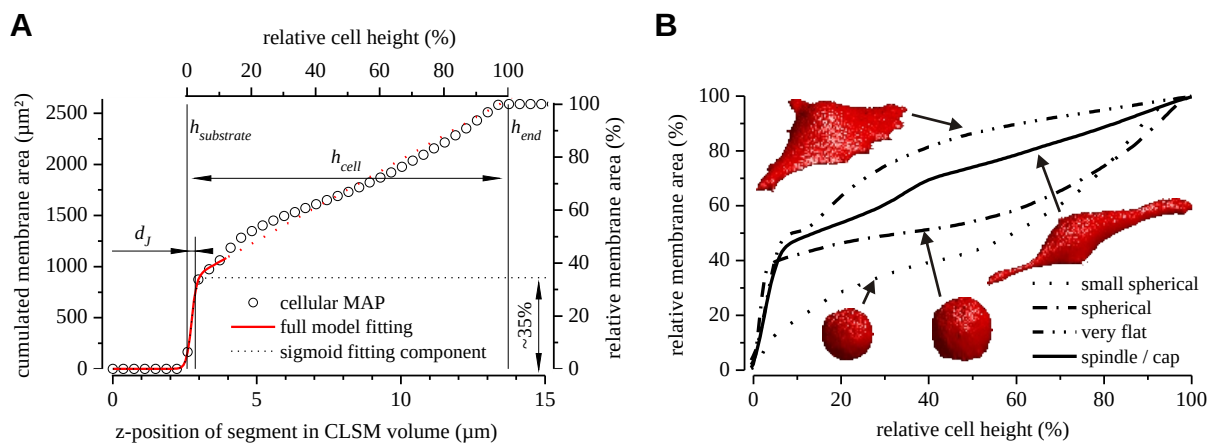


Figure 4.7.: (A) MAP-function (equation 4.2) fitted to the measured MAP of a HEK293 cell. (B) Comparison of measured MAPs from cells with different morphologies. Ratios of the attached membranes: small (possibly dying) 12 %, spherical 38 %, spherical cap or spindle 42 %, very flat 46 %.

MAPs represent cumulated surface areas. Thus, all measured MAPs produce steadily increasing curves. As expected, the curves start with a steep increase of the cellular surface for the attached part of the cell membrane followed by a smaller gradient, which strongly represents the

different shapes of the free cell membrane. For comparison, the resulting MAPs for representative cell shapes are normalized to their corresponding total surface area A_M and height h_{cell} and presented in a superposition in figure 4.7b. Flat cells lead to very high ratios of attached membrane and demonstrate a more logarithmic curve for the free membrane. Cells with the shape of spherical caps lead to high ratios of attached membrane and a fairly linear profile for the free membrane. As the attached membrane decreases, the cells are more spherical, resulting in an exponential increase of the MAP domain representing the free membrane. In the case of incompatible surface coatings (and possibly during apoptosis), cells tend to reduce their level of attachment to very small amounts, approximately less than 10 %.

4.4.2. Influences of isovalue selections

The selection of the brightness threshold (isovalue) has an influence on the final MAP. Therefore, the normalized MAPs were compared for each cell all available isovalues (figure 4.8a). MAPs in the isovalue range from 30 to 39 of the cell in figure 4.6 were very similar (figure 4.8b). To increase the reliability of the results, the 10 similar MAPs (for isovalues 30 to 39) were averaged to decrease the dependence of the reconstruction on the isovalue. In all experiments, at least 7 MAPs for each cell were averaged. To reduce the amount of variation in reconstructed membranes using different isovalues, projections of the reconstructions with different isovalues were examined for each cell as depicted in figure 4.8c.

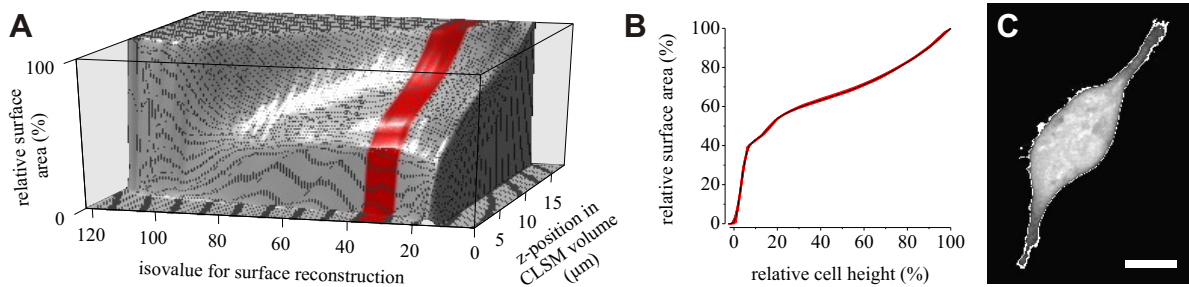


Figure 4.8.: (A) Comparison of MAPs created with different isovalues for one cell. (B) Superposition of similar normalized MAPs for 10 different isovalues. (C) Demonstration of marginal changes in the shape of reconstructions in a z-projection (scale bar 10 μm)

4.4.3. Influences of substrate coatings on the cellular membrane allocation

In this study, the MAPing method was mainly used to determine the ratio of the attached to free membrane. Therefore, HEK293 cells were cultured for three days on substrates with seven different protein coatings and a control of uncoated glass. MAPs were created for $n \geq 13$ cells on each substrate. A summary of the measured membrane ratios is given in Table 4.2.

substrate coating	PDL	PLL	PECM	FIB	ConA	LAM	ECM	control
average ratios $\bar{\alpha}_{JM}$ of attached membrane	44.9 %	44.5 %	42.0 %	39.7 %	38.8 %	38.7 %	37.1 %	31.8 %
standard deviations of attached membrane shifted by the statistical skewness	+0.8 % -5.6 %	+1.0 % -5.1 %	+1.6 % -8.1 %	+2.5 % -8.4 %	+2.0 % -17.8 %	+2.8 % -7.0 %	+3.6 % -9.8 %	+8.5 % -19.6 %
number of cells n	18	32	13	16	15	18	24	35
statistically not distinguishable groups	A	A	B	B/C	B/C	B/C/D	C/D	D
rank-sum test (difference significant for $p < 1e-2$)	PDL	1	4.0e-1	6.9e-3	1.2e-4	4.5e-3	4.8e-6	7.6e-7
	PLL		1	3.8e-3	2.3e-5	2.0e-3	3.7e-7	2.1e-8
	PECM			1	1.3e-1	9.6e-1	2.9e-2	3.6e-3
	FIB				1	2.6e-1	4.8e-1	9.4e-2
	ConA					1	6.8e-2	4.5e-2
	LAM						1	2.5e-1
	ECM							1
	control							

Table 4.2.: Overview of attached to total membrane ratios for seven different protein coatings and the control. Mean values and standard deviations (corrected for skewed distributions) are derived from β -distributions for HEK293 cells shown in figure 4.3. Statistical differences are calculated with the rank-sum test.

The cells develop various shapes and ratios of attachment on these substrates as visible in the MAPs (figure 4.9). Cells growing on uncoated glass were attached very randomly with ratios from 12-40 % (average range in skewed distribution) for the attached part of the membrane surface areas. In contrast, PDL led to the greatest and most consistent membrane allocations with 39-46 % of attached membrane. The measured MAPs of cells on PDL were very close to the presented MAP-function (equation 4.2) with a linear representation for the free membrane (figure 4.3b) indicating a mostly spherical shape. Almost equal ratios were observed for PLL compared to PDL, although PLL coatings yield more morphological variations in the shape of the free membrane. The cells growing on PECM attached with ratios from 34-44 %. FIB and LAM caused similar results with attachments from 31-42 %. A large variation in ratios was observed for cells on ConA as cells attached with 21-41 % of their membrane. ECM led to 27-41 % of attachment. Statistical tests proved that cells growing on LAM and ECM were not distinguishable from the control group on glass (table 4.2). The trend with respect to glass was: PLL > PDL $\gg$ PECM > ConA > FIB > LAM > ECM.

The free membrane domains of the individual MAPs in figure 4.9 strongly varied for all substrate coatings. However, the averaged MAPs for each coating follow a straight line according to the spherical cap model in equation 4.2 (figure 4.3). Cells on LAM and control glass substrates were the exceptions demonstrating very smoothly curved transitions between the attached and free membrane. These results indicate that cells on LAM have large variations in cell height with respect to cells growing on PLL.

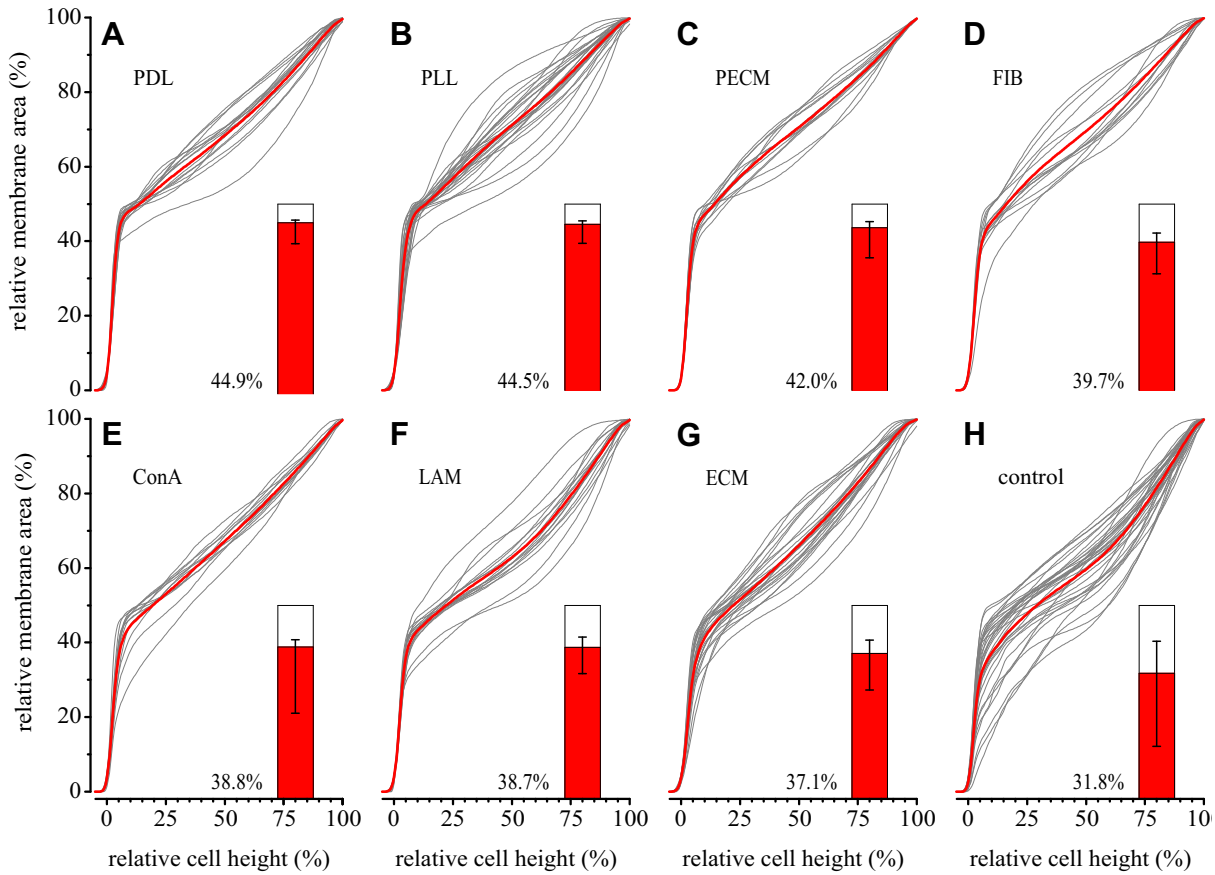


Figure 4.9.: Superposition of normalized MAPs for individual HEK293 cells growing on different substrate coatings. Thick curves represent averages of thin single cell MAPs. Bars show the average ratio of attached to total membrane and empty rectangles the theoretically maximal possible attachment for flat substrates (50 %). For statistical difference see Table 4.2.

4.5. Discussion

4.5.1. Membrane allocation profiling (MAPing)

The surfaces of defined geometrical shapes (figure 4.1) and HEK293 cells observed by CLSM (figure 4.5) were sequentially reconstructed, in order to demonstrate the representation of shape in MAPs. Membrane areas of individual segments were cumulated and revealed MAPs (figure 4.3, figure 4.7 and figure 4.9). According to mathematical and geometrical models, an obvious discontinuity in the measured MAPs led to the conclusion that the surface area of cellular membranes clearly can be split into two compartments. The attached membrane is oriented in parallel to the CLSM images. The surface area of the reconstructed attached cell membrane caused a first steep increase of the MAPs. This instantaneous steep increase is influenced by the irregular shape of the attached cell membrane and noise (figure 4.3). Consequently, the cumulated areas for the attached membrane result a sigmoid shape. At the transition from attached to free membrane, the cell membrane changes orientation with respect to the substrate and also with respect to the CLSM sections.

Thus, smaller membrane areas are reconstructed and added to the MAPs (figure 4.2c and figure 4.6c). In the MAPs, the transition is apparent as an obvious discontinuity (figure 4.3b, figure 4.7, figure 4.8 and figure 4.9). The free membrane domain of MAPs depends on the underlying cellular shape as depicted in figure 4.7b. However, when normalized MAPs are averaged, the MAP domain for the free membrane follows a linear gradient, as provided the transitions from attached to free membranes were similar (figure 4.9a-e and g). When the horizontal position of the discontinuity (due to different cell heights) displays strong variation, the averaged MAPs resulted in a smooth shape (figure 4.9f and h). This effect could be reduced only by normalizing the MAPs by the total membrane area A_M .

4.5.2. Extracting morphological attributes from MAPs

MAPs comprise much information about the cellular morphology and present some of them in a very descriptive way. This analysis yields the following key observations: Determining whether the overall shape of a cell is flat or spherical can be derived easily from the representation of free membrane domain in a MAP plot (figure 4.7b) as an exponential, linear or logarithmic function. Further, the total cell surface area is the greatest value and does not change much at the end of the MAPs. An estimation of the cell height is given in the width of the noticeable rise of the profile. To obtain more reliable values for the cell height and other morphological properties, further analysis is necessary. Due to this observation, a MAP-function (equation 4.2) was fitted to the calculated profiles (figure 4.7a). One difficulty during fitting was the variations in shape of the free membrane. Since the focus of this study was mainly interested in characterizing the attached membrane, the shape of the free membrane was neglected during fitting. Thus, a sigmoid was fitted to the attached membrane domain and only approximated the first $1.5\ \mu\text{m}$ with a linear function for the free membrane domain (figure 4.7a). However, it is possible to extend equation 4.2 with exponential and/or logarithmic sub-functions to fit the free membrane as well.

The most clear information that can be obtained from data fitting is the size of the attached membrane A_{JM} . This parameter is a part of equation 4.2 and is not influenced much by a slanting position of the cell with respect to the CLSM sections or variations in chosen isovalue (figure 4.8). However, the attached membrane value can be affected by the vertical resolution of the CLSM data, especially when the z-stepping is finer than the image resolution. Therefore, the vertical resolution was kept constant at $0.37\ \mu\text{m}$ in the analysis.

To obtain a more accurate value for the cell height, the position of the interface between substrate and cell can be extracted from the MAP-model (equation 4.3). Further, an estimate for the average cleft height d_J can be calculated from the width of the sigmoid slope in the MAP-model (equation 4.4). The obtained distance value of individual HEK293 cells was roughly in the previously reported range (Wrobel et al., 2007). However, the vertical resolution of standard confocal microscopes is at least one order of magnitude lower than d_J and therefore not fine enough to

use the sigmoid slope as clear indicator for the distance between cell and substrate. Furthermore, this estimation requires an absolutely parallel orientation of cell and substrate with respect to the CLSM sections. Therefore, estimating d_J with the MAPing technique leads to theoretical values that need to be verified during further experiments, e.g. where the substrate or the coating proteins are stained with another dye as references. Also, the isovalue has an influence on d_J . Maybe other imaging techniques and structure conserving resolution enhancement tools as well as 3D deconvolution can enhance the data resolution and quality in order to use this capability of the MAP-model.

4.5.3. Influences of data acquisition and 3D reconstructions

The reconstructed surface of spherical objects, including cells, depends on the voxel resolution (pixel and z-step size) as well as the image noise. Especially, noise causes a variation in the free membrane domain of a MAP, resulting not in a linear, but more exponential increase (data not shown). To avoid this effect, it is recommended to use similar image resolutions and a constant z-step during CLSM data acquisition of all specimens. Subsequently, noise reducing techniques are also necessary to prepare the data for isosurface reconstructions. The size of the reconstructed membrane is strongly depending on the chosen isovalue (brightness threshold). Therefore, the isovalues were selected carefully by parallel verification with volume projections (figure 4.8c) to ensure a morphological conformity. Fluorescently labeled substrate proteins or immunostaining of focal adhesions could further improve this technique as an indicator of the substrate plane. However, the focus of these investigations was the relative attachment α_{JM} of the cellular membrane to the substrate. This morphological attribute is stable over a large range of isovalues (figure 4.8b). In some cases slight variations in the free membrane domains were observed in MAPs, but these variations had no effect on the attached membrane domain of the profile (figure 4.9).

4.5.4. Influence of proteins as substrate modifications on MAPs

The MAPing technique allowed the study of differences in the ratio of attached to total cell membrane for cells growing on different coatings. PDL and PLL revealed very similar results on the adhesion of HEK293 cells and are both coating proteins causing the largest areas of attached membrane. Furthermore, it is assumed that PECM caused large areas of attachment as well, due to the existence of PDL in the mixture, especially since ECM alone revealed the smallest ratio of attachment. Cells on FIB grew with smaller ratios of attached membranes. Ratios of cells cultured on ConA spread surprisingly wide, which was not observed for any other coating (except the control). The attachments of cells to LAM and ECM were statistically not distinguishable from the control of HEK293 cells on glass. Cells growing on glass controls showed nearly all possible ratios of attachment and morphologies while other substrates, especially PDL and PLL, caused the HEK293

cells to be attached with a more defined amount of their membrane. As a succinct comparison, it was found that coatings do generally narrow the variance of cellular attachment. As a conclusion, the average of all MAPs for cells cultured on one coating was similar to those of models with the geometry of spherical caps.

The present investigations on the ratio of attached to total membrane area are valuable extension to earlier findings. In a previous study (Wrobel et al., 2007), the cleft height d_J , the average distance between a randomly shaped cell membrane and a planar substrate, was examined by transmission electron microscopy of fixed HEK293 cells. Also, the influences of different surface modifications on d_J were analyzed. PDL, PLL, FIB and PECM caused small distances, while cells on ConA, LAM and the control (ECM was not part of the earlier investigations) were growing in larger distances to the respective substrate. These earlier findings were in accordance to results of the present study on the ratio of attachment. Combining the outcomes of both, the former and the present work, it can be generalized that both poly-lysines, FIB, and the protein mixture PECM are the most promising substrate coatings for bioelectronic hybrids with HEK293 cells.

Other researchers used electrical approaches with alternating polarization of the cell membrane to retrieve areas of attached, free, and whole cell membranes. For HEK293 cells growing on fibronectin, $\alpha_{JM} = 37\%$ ($n = 1$) was estimated from published (Brittinger, 2004) intermediate results, which was in agreement with findings for FIB. Other electrical studies report that snail neurons growing on laminin are attached with $\alpha_{JM} = 22 \pm 6\%$ ($n = 4$) of their membrane (Schoen and Fromherz, 2007). This reported measurement is only about half of the ratio that was estimated for HEK293 cells on LAM. Snail neurons are much larger and their soma morphology is more spherical, which is comparable with the geometrical model in figure 4.1c. The ratio for snail neurons indirectly supports findings for HEK293 cells with the flatter morphology.

However, electrical approaches in general are also dependent on leak currents. Leak currents may result in an underestimated steepness of a linear regression and therefore result in a systematically smaller area for the attached membrane. Moreover, the electrical method can not be used without electrical equipment consisting of electrical sensors as a substrate in combination with a patch-clamp setup attached to the cell under investigation. Thus, this approach is very limited in breadth of application. In contrast, the MAPing technique presented in this article yields an approach to analyze the shape of cells and the level of cellular attachment to planar substrates for a much wider variety of base materials.

4.6. Conclusions

MAPing is a novel method to analyze the cellular shape and the attachment to planar substrates. Using the MAPing technique, influences of different protein substrate coatings on the attachment

of HEK293 cells were characterized. The data demonstrates that the MAPing technique is a powerful tool to examine and compare morphological influences of substrates and substrate modifications. This approach is non-invasive and extracts the information from confocal laser scanning microscopy (CLSM), which is practically independent of the substrate. Therefore, this novel technique presents a new and easy way to study and characterise cellular shape and attachment in vitro with high throughput for representative statistics.

5. Extracellular recording of glycine receptor chloride channel activity as a prototype for biohybrid sensors

This chapter is based on the following publication:

Sommerhage F., Baumann A., Wrobel G., Ingebrandt S., Offenhäusser A. (2010). [Extracellular recording of glycine receptor chloride channel activity as a prototype for biohybrid sensors](#). *Biosensors and Bioelectronics* 26, 155-161.

5.1. Introduction

Sensory systems of animals register the presence of a certain stimulus and convert this event into a logical output signal with a remarkable sensitivity and specificity. Trying to implement these properties into an artificial sensor system it is a challenging task. During the last few decades, biohybrid sensors were designed consisting of chemical and/or biological components coupled to artificial read-out devices. As a proof of principle, insect antennae were connected to electronic sensors and found to detect low concentrations of specific odors in the air (Schroth et al., 1999). Further reducing the biological component in hybrid sensors, prokaryotic and eukaryotic cells were grown on functionalized electrodes, yielding sensors for various purposes (Bentley et al., 2001; Bousse, 1996; Hakkila et al., 2004; Pancrazio et al., 1999). However, hybrid sensors utilizing bacteria frequently faced the problem of being harmed by toxins originating from dead cells (Harms et al., 2006). Drugs and non-lethal compounds were measured in other biohybrid approaches, e. g. monitoring changes in the beat frequency of cardiac myocytes (Ingebrandt et al., 2001; Natarajan et al., 2006). The above mentioned approaches rely on biological sensory systems or receptors that have to be coupled to suitable read-out systems.

For the development of improved or new biohybrid sensors, the technique of genetic engineering offers a promising route (Belkin, 2003; Bousse, 1996; Ding et al., 2008). In order to obtain custom made whole-cell sensors with (pre-)defined repertoires of receptors or ion channels, genetically modified cells cultured on electronic devices can be employed to detect a plethora of chemical or physical stimuli (Roda et al., 2003; Virolainen et al., 2008). Alternatively, cells equipped with luminescent reporters can be grown and studied on optical devices (Bentley et al., 2001). Recently, a cell line was established expressing a serotonin-gated ion channel (5-HT3A) that was used to monitor ligand-dependent membrane currents by transistors (Peitz et al., 2007).

The genetic improvements of biohybrid-sensor designs were also accompanied by a technological progress for the artificial component. The development of biohybrid sensors was mostly based on optical devices or electrodes organized in arrays (Bentley et al., 2001; Belkin, 2003). Due to a more complex fabrication process, the ongoing approach with open gate, ion-sensitive field-effect transistors (FETs), was more challenging as initially expected (Bergveld, 1986). During the last decade, however, several groups presented promising bioelectronic devices using FETs in arrays (Fromherz, 2002; Ingebrandt et al., 2005; Offenhäusser and Knoll, 2001; Poghosian et al., 2009). FETs are different than inert gold electrodes. While unaffected by anions, FETs were proven to be selectively sensitive to cations and thus used to measure cation concentrations, such as pH-changes, in aqueous solutions (Bergveld and de Rooij, 1979; Hajji et al., 2000; Watanabe et al., 1991; Yuqing et al., 2003). FETs were also employed to establish whole-cell biosensors to measure cation currents in combination with electrophysiological recordings (Ingebrandt et al., 2005; Martinoia and Massobrio, 2004; Meyburg et al., 2006; Peitz and Fromherz, 2009). Up to

now, chloride currents of single cells have not been studied with FETs or MEAs. This is surprising because many voltage-, mechano-, and ligand-gated anion channels as well as transporters are involved in balancing a cell's electrical behavior (Hall and Baker, 1977). Needless to say, the study of anionic membrane currents with extracellular sensors would be advantageous.

The interaction of whole-cell sensors expressing defined cation channels with FETs has been studied intensively in recent years and thus became well understood (Ingebrandt et al., 2005; Pabst et al., 2007; Schmidtner and Fromherz, 2006; Wrobel et al., 2005). To challenge and prove the models and hypotheses in cell-transistor coupling, cellular anion channel activity needs to be monitored with extracellular devices. One of the key-statements in cell-transistor coupling theory is that extracellular recordings of cation currents with FETs differ from those recorded with MEAs. FETs inherit an exclusive ion-sensitivity for positive charges that MEAs are lacking. Consequently, extracellularly recorded anion currents were predicted to be identical, regardless whether measured with FETs or MEAs. Thus, integrating a ligand-gated anion channel in a cell-based biohybrid sensor would provide the opportunity to further test cell-transistor simulations.

Receptor channels allowing anions to pass the cell membrane belong to the superfamily of cysteine (cys)-loop ligand-gated ion channels (Jensen et al., 2005). In mammals, two channel families, i.e. gamma-aminobutyric acid receptors (GABA_A and GABA_C) and glycine receptors (GlyR), are known for their mainly inhibitory contribution in neuronal signaling (Enna and Gallagher, 1983; Grenningloh et al., 1987; Rajendra et al., 1997). The native channels were composed of five either identical or highly homologous subunits. This study focused on the GlyR (see section 3.1.4). In native tissue, GlyRs consist of alpha and beta subunits (Jensen et al., 2005; Langosch et al., 1990). While the alpha subunits provide both, the agonist-binding sites to open the chloride channel as well as antagonist-binding sites, the beta subunits preferentially interact with channel-blocking antagonists (Chen et al., 2009; Rajendra et al., 1997). For functional channel activity it is sufficient to express GlyR alpha₁ subunits only (Grenningloh et al., 1990).

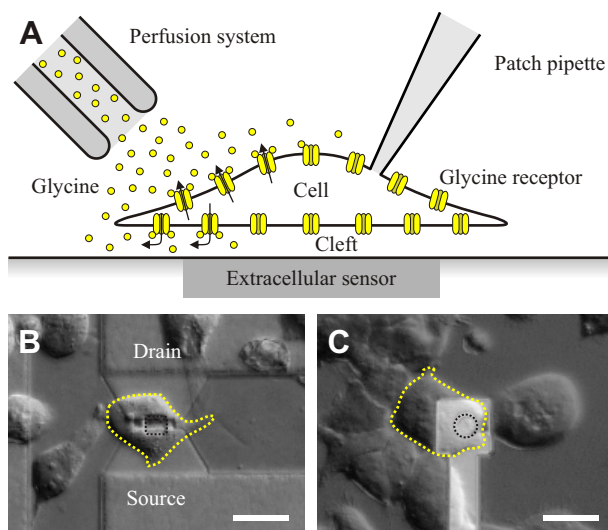


Figure 5.1: Experimental layout: (A) Schematic overview of a biohybrid sensor consisting of a cell, expressing glycine receptors, a perfusion system to apply different concentrations of glycine, and a sensor for extracellular monitoring of electrical cell responses. The patch-pipette is optional and gouges intracellular currents for reference purposes. (B,C) Genetically modified HEK293 cell (yellow border) on transistor gate and gold electrode, respectively (black borders), observed with a differential interference contrast microscope (scale bar = 30 μm).

The prototype for a cell-based biohybrid anion sensor was developed (Fig. 5.1a). Human embryonic kidney (HEK293) cells were stably transfected with the gene encoding the GlyR α_1 subunit and were grown on substrate-integrated micro-devices. Challenging the cells with increasing concentrations of glycine led to the development of chloride currents which could be faithfully monitored with patch-clamp and parallel extracellular recordings. The sensor displayed a working range over three orders of glycine concentrations, including the physiological relevant micro-molar range. Using this device, the role of anions in cell-sensor coupling and the prospect of innovative biohybrid sensors was evaluated.

5.2. Materials and Methods

Supplementary information concerning the multiplication and purification of the human GLRA1 gene, coding for the glycine receptor subunit α_1 , in bacteria is provided in the the appendix A.2 of this thesis.

5.2.1. Cells, transfection, and cell culture

The human GLRA1 gene (Grenningloh et al., 1990) in pRK5 vector was digested by *EcoRI*/*PstI* and subcloned into pBluescript SK(-) vector (Stratagene, USA). After a subsequent digestion, the cDNA was ligated into the *EcoRI* and *XbaI* restriction sites of pcDNA3.1(+) vector (Invitrogen, Netherlands) by standard cloning techniques. Approximately 8 μ g of recombinant plasmid DNA were used to transfect exponentially growing HEK293 cells (ca. 4×10^5 cells/5 cm petri dish) by a modified calcium phosphate precipitation technique (Chen and Okayama, 1988).

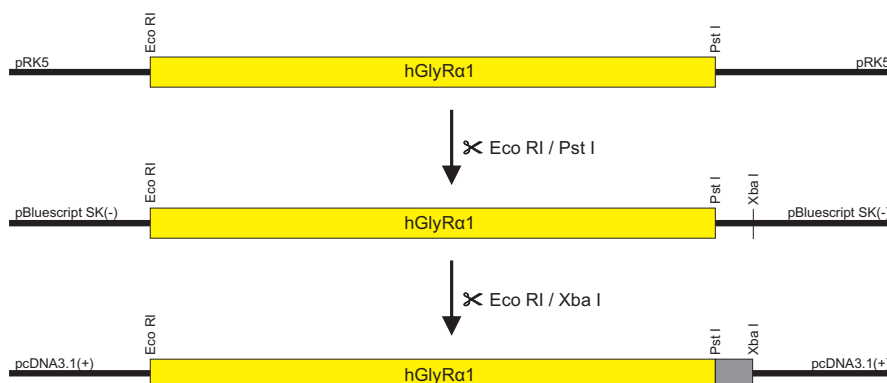


Figure 5.2.: Cloning strategy for the GlyR construct using an intermediate vector. Restrictions points used for the cloning are indicated. Areas coding for GlyR are colored yellow.

Cells were grown in minimal essential medium, supplemented with 2 mM glutamine, 10 % (v/v) fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen, Germany). Stably transfected cell clones were selected in the presence of 1.0 mg/ml of the antibiotic G418

(Sigma-Aldrich, Germany). Isolated cell foci were propagated in medium with 0.8 mg/ml G418. To avoid activation of glycine receptors, the medium was supplemented with 10 μ M picrotoxin (Sigma-Aldrich, Germany).

Cells expressing glycine receptors were split weekly. Briefly, cells were rinsed twice with phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3), followed by incubation with trypsin-EDTA for about 5 min at 37 °C (0.5 mg/ml trypsin; 0.2 mg/ml EDTA, Sigma). Detached cells were suspended in medium and centrifuged for 5 min at 200 $\times$ g. The cell pellet was resuspended in medium and ca. 10⁴ cells were plated on 5 cm petri dishes. According to their growth rate (27 ± 1 hours), cell densities were calculated to obtain 10³ cells/mm² on FETs or MEAs, after 2 to 4 days at 37 °C and 5 % CO₂. Fig. 5.1b depicts a microscopic image of HEK293 cells grown for 3 days on a FET gate structure. The equivalent for an MEA is shown in Fig. 5.1c.

5.2.2. Western blot analysis and immunostaining of cells

Membrane proteins were prepared from wild type and transfected HEK293 cells by standard methods. Briefly, cells were hypotonically lysed in buffer (10 mM NaCl, 25 mM HEPES, pH 7.5, 2 mM EDTA) and protease inhibitors. After centrifugation, the pellet was solubilized in 100 mM NaCl, 25 mM HEPES, pH 7.5, 1 % (w/v) CHAPS and protease inhibitors. About 10 μ g of membrane proteins were separated on SDS-polyacrylamide gels and transferred to polyvinylidene membranes (Immobilon-P, Millipore, Germany) (Laemmli, 1970; Towbin et al., 1979). Membranes were blocked in 5 % non-fat dry milk in Tween-20 containing phosphate-buffered saline (PBT, containing 130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 0.02 % (w/v) Tween-20) and incubated with primary antibodies (dilution 1 : 2000, rabbit polyclonal to glycine receptor subunits α_1 and α_2 ; #23809-100, abcam, United Kingdom). Membranes were washed with PBT and incubated with anti-rabbit secondary antibodies coupled to horseradish peroxidase (dilution 1 : 3000; GE-Healthcare, Germany). Immunoreactive bands were detected with an enhanced chemiluminescence kit (Applichem, Germany). For fluorescence microscopy (Leica TCS, Germany), fixed cells were incubated with primary antibodies (dilution 1 : 2000) and then incubated with secondary antibodies (dilution 1 : 500, goat anti rabbit Alexa 488, Invitrogen, Netherlands).

5.2.3. Substrate-integrated micro-devices for extracellular recordings

In the center of a 5 $\times$ 5 mm² silicon chip, 16 non-metalized field-effect transistor (FET) gates were arranged in a 4 by 4 matrix either with 100 or 200 μ m pitch. The gate oxide area exposed to the electrolyte solution was 80 μ m² with an effective gate width of 16 μ m. FET transfer characteristics were ascertained shortly before measurements, with an average transconductance of $g_m = 0.33 \pm 0.15$ mS. Customized microelectrode arrays (MEAs) and amplifiers provided a data

retrieval from 64 gold electrodes. Electrodes with 10 μm diameter were organized with a pitch of 100 μm or 200 μm , in an 8 by 8 array on $11 \times 11 \text{ mm}^2$ glass chips. On both extracellular devices, the electrical activity of cells was recorded with respect to an extracellular Ag/AgCl electrode at ground potential. Detailed information about FETs, MEAs, and amplification setups can be found in previous publications (Ingebrandt et al., 2005; Krause et al., 2000; Wrobel et al., 2007).

5.2.4. Chip cleaning and surface modification

Chips were cleaned in two subsequent bathing steps with sonication. The first step was for 3 min in 2 % (v/v) Hellmanex II (Hellma, Germany) followed by rinsing in bidistilled water. Only the surface used for cell plating was further treated with 20 % (v/v) H_2SO_4 for 20 min at 80 °C. Devices were rinsed with bidistilled water and kept for 5 min in 70 % (v/v) ethanol. After drying, FET surfaces were coated with 25 μl of 0.1 mg/ml poly(L)lysine (Sigma, Taufkirchen, Germany) for 30 min at room temperature. Accordingly, MEA surfaces were coated with 50 μl poly(L)lysine. Before cell plating, sensor surfaces were rinsed with PBS.

5.2.5. Electrophysiology and bioelectronic coupling

Cells located on top of transistor gates or microelectrodes were contacted with a patch-clamp pipette in the whole-cell configuration as described earlier (Wrobel et al., 2005). Patch pipettes (ca. 5 M Ω) were filled with 145 mM CsCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 10 mM EGTA-KOH, 50 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with NaOH. Clamped to the holding potential of $V_{\text{hold}} = -60 \text{ mV}$, cells were gouged intracellularly for membrane currents with an EPC-9 double patch-clamp amplifier (HEKA Elektronik, Germany). Recordings from extracellular sensors located below the patched cell were amplified and fed into an external input of the EPC-9. Intracellular and extracellular signals were recorded simultaneously at 5 kHz using the software *TIDA* (HEKA Elektronik, Germany). Alternatively, data was recorded with the software MED64 (Alpha MED Sciences, Japan) during high-throughput experiments with MEAs. Cells, sensors, and patch-pipettes were observed with an Axiotech Vario 100 HD microscope (Zeiss, Germany).

The cells were superfused constantly (ca. 125 $\mu\text{l}/\text{min}$) with standard bath solution, containing 12 mM NaCl, 128 mM NaHCO_3 , 5 mM KCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 50 mM glucose, and 15 mM HEPES adjusted to pH 7.4 with NaOH. A stock solution of glycine (1 M) in 15 mM HEPES was adjusted to pH 7.4 with NaOH. Test solutions containing concentrations of 8, 32, 64, 128, 256, 512, and 2048 μM glycine were prepared in standard bath solution. Eight test solutions (seven glycine concentrations and one glycine-free control) were applied to cells, using an DAD-8VC system (ALA Scientific Instruments, USA). The valve control was operated by the software *TIDA*, using an EPC-9 output. The duration of glycine application was 1.5 seconds. Due to a lag volume in the used QMM-8/1 Micromanifold ($\varnothing = 100 \mu\text{m}$, ALA Scientific Instruments, USA), a delay of

$t \approx 75$ ms was observed between initiation of glycine application and the electrical response of the cells. Patch-Clamp, MEA, and FET recordings, were analyzed with *TIDA* and further evaluated in *Calc*, *OpenOffice 3.1*.

5.2.6. Identifying the seal resistance

The seal-resistance R_J is an indicator for the quality of attachment and also for the coupling of currents into the sensor. R_J can be estimated by applying a rectangular stimulus pulse of $V_{stim} = 100$ mV with the patch electrode in whole-cell configuration. The stimulus leads to a quick charge transfer at the cell membrane, resulting in transient peaks in extracellular recordings. The amplitude of this transient artifact is determined by R_J , which shields the cell-sensor contact against ground potential. In an electrical equivalent circuit model, the seal resistance R_J can be fitted until the transient artifacts in simulation and measurement match (Ingebrandt et al., 2005; Wrobel et al., 2005).

5.2.7. Statistics

Statistics were performed with *Calc*, *OpenOffice 3.1*. Assuming Gaussian distribution, values were expressed as means with corresponding standard deviations. For correlations, logarithmic trend lines were fitted to measured or calculated values over glycine concentrations.

5.3. Results

5.3.1. Expression of glycine receptors in HEK293 cells

The expression of glycine receptor α_1 subunits (GlyRA1) in HEK293 cells was examined by immunostaining and western blot analysis (Fig. 5.3a). Membrane proteins were prepared from four stably transfected cell lines (1B3, 1B5, 2B2, 2C2), transiently transfected HEK293 cells, and non-transfected HEK293 cells. A single band of ca. 48 kD was detected in transfected cells with the GlyR-specific antibody. The same molecular weight was previously reported for GlyRA1 (Grenningloh et al., 1990). According to the band intensities, clone 2C2 seemed to express the highest amount of GlyRA1. No protein was labeled in the wild type control. Immunofluorescent staining of fixed cells from clone 2C2 showed that GlyRA1 protein was located in cell membranes as well as intracellularly (Fig. 5.3b).

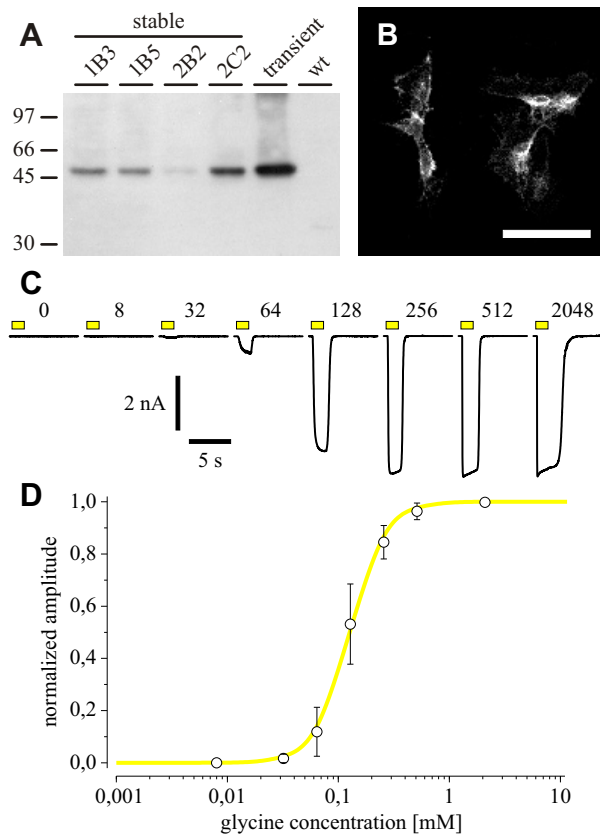


Figure 5.3: Characterization of GlyRA1 in stably transfected HEK293 cell lines. (A) Western blot of membrane proteins from four independent cell lines, transiently-, and non-transfected HEK293 cells. A band of ca. 48 kD is specifically stained. (B) Immunostaining of cells from clone 2C2. The GlyR is located in cell membranes and cytosol. (C) Electrophysiological characterization of GlyR clone 2C2 during application of glycine. Patch-Clamp measurements were performed in voltage-clamp whole-cell configuration. Glycine concentrations are given in μ M. (D) Dose-response curve for intracellular recordings fitted to the Hill equation ($EC_{50} = 140 \mu$ M and $h_c = 2.8$, $n = 9$).

5.3.2. Electrophysiological characterization of cell lines

The stably transfected cell lines were electrophysiologically tested for the presence of functional glycine receptors. Whereas no membrane currents were observed in wild type cells in the presence of up to 10 mM glycine, cell line 2C2 expressed the highest density of glycine-gated chloride channels. Chloride currents increased with rising glycine concentrations. Membrane currents I_M of up to 5 nA were observed when cells were stimulated with 2 mM glycine (Fig. 5.3c). As depicted in Fig. 5.3d, a normalized dose-response curve was constructed from maximal current amplitudes ($n = 9$ cells) in the presence of 7 glycine concentrations. Fitting the data to the Hill equation (Hill coefficient of $h_c = 2.8$) resulted in an EC_{50} of 140 μ M glycine for half-maximal current activation. Therefore, clone 2C2 was used for further bioelectronic coupling with FETs and MEAs.

5.3.3. Monitoring anion currents intra- and extracellularly

To characterize cell-sensor hybrids, cells located on FET gates ($n = 17$) and MEAs ($n = 7$) were contacted with patch-clamp pipettes in the whole-cell configuration for parallel current recording. The average membrane capacitance of GlyR clones was $C_M = 17.3 \pm 7.7$ pF. According to a specific membrane capacitance $c_{sp} = 1 \mu$ F/cm² and $\alpha_{JM} = 40\%$ as the ratio of attached to total cell membrane area, the average cell diameter was $d_{cell} = 29.1 \pm 5.9 \mu$ m (Gentet et al.,

2000; Sommerhage et al., 2008). Thus, the cells were able to cover substrate-integrated sensors completely (Fig. 5.1b and c). A tight attachment of cells to their substrate is essential for cell-sensor coupling as it thereby allows membrane currents to be measured by substrate-integrated sensors. The seal-resistance R_J is an indicator for the quality of attachment and also for the coupling of currents into the sensor, i.e. the larger R_J , the larger the measured signal (Ingebrandt et al., 2005; Wrobel et al., 2005). The average seal resistance of GlyR clones ($n = 24$) was fitted to $R_J = 0.3 \pm 0.2 \text{ M}\Omega$, whereas $R_J = 0.8 \pm 0.3 \text{ M}\Omega$ ($n = 10$) of non-transfected HEK293 cells was much larger.

In contrast to the patch-clamp recordings which showed a glycine-dependent, non-inactivating current in the presence of the ligand, the extracellular recordings with FETs as well as MEAs looked different (Fig. 5.4a). On both extracellular devices, capacitive peaks were registered shortly after the application of glycine, which paralleled the onset of current traces measured with the patch clamp electrode. The different shapes could not be attributed to specific glycine concentrations or to the device used for the measurement. However, although the current traces differed from those obtained by patch clamp, we it was found that the recordings performed with FETs and MEAs were very similar. During the initial phase of stimulation with glycine, capacitive peaks with negative amplitude were present in 75 % of all measurements. Only positive components (Fig. 5.4a-1) were observed in 25 % of extracellular measurements. 54 % of recordings had a capacitive peak in addition to positive components (2) and 13 % had capacitive peaks only (3). In 8 % of extracellular recordings, capacitive peaks were observed in parallel to negative components (4). The peak amplitudes of capacitive components in extracellular recordings correlated with the applied glycine concentration ($R^2 = 0.96$). Variations of signal shapes did not correlate with the respective seal resistance R_J . Control recordings with wild type HEK293 cells did not show responses to glycine in intracellular as well as extracellular recordings (Fig. 5.4b).

The capacitive peaks of extracellular recordings had great similarity with the first derivative of the patch-clamp recordings. As shown in Fig. 5.5, the negative peak of the numerically derived patch data superimposes the extracellular recordings. However, the extracellular peaks occurred shortly delayed ($\Delta t = 80 \pm 22 \text{ ms}$) compared to the onset of the intracellular recordings.

5.3.4. Detecting glycine with biohybrid sensors

In a high-throughput approach, GlyR-mediated anion currents were recorded with 64 gold electrodes during stimulation with glycine, as shown in Fig. 5.6a. In order to mark the time point right before application of different glycine concentrations, an intended artifact was caused in extracellular recordings. As depicted in Fig. 5.6b, extracellular recordings from GlyR cells in the presence of glycine were clearly distinguishable from glycine-free controls and resembled signals with shape 2, observed during initial experiments (Fig. 5.4a). In contrast to recordings with parallel patch-clamp, the positive drift of $V_{drift} = 37.4 \pm 3.3 \text{ }\mu\text{V}$ returned back to base line before the

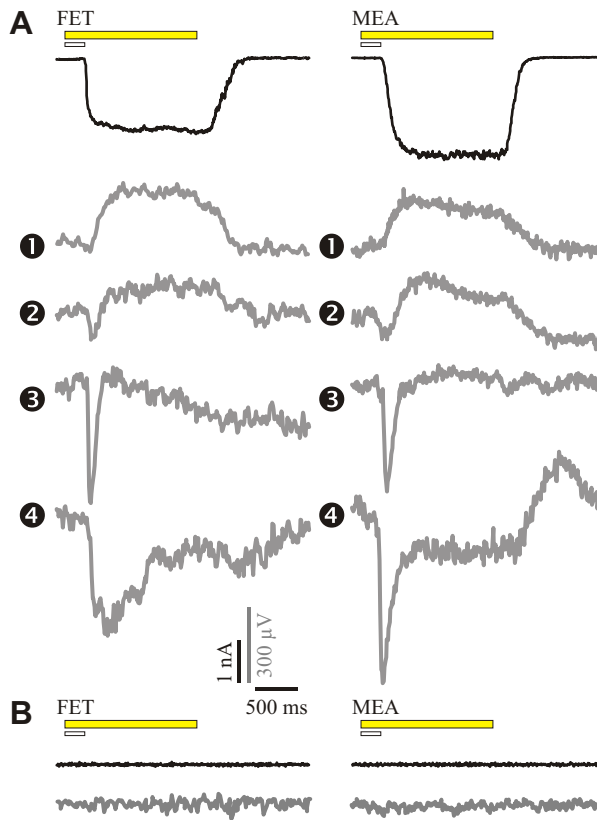


Figure 5.4: Results of bio-electronic coupling: Representative intra- (black) and extracellular (gray) recordings from HEK293 cells on FETs and MEs, respectively. In order to reduce noise 50 data points were averaged over time. Stimulation with 512 μM glycine is marked with a yellow box, the lag of 75 ms is marked with an white/empty box. (A) Recordings of glycine-induced chloride currents from GlyR cell line have various shapes and contain positive, capacitive, and negative components (1-4). (B) Recordings from wild type HEK293 cells which do not respond to glycine applications.

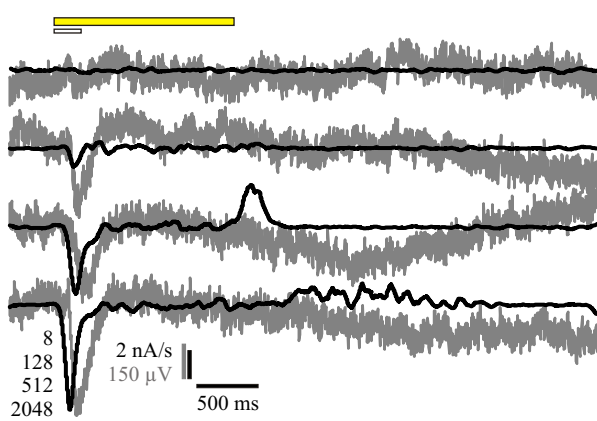


Figure 5.5: Superposition of extracellular FET recordings (gray) and the first derivative of intracellularly measured currents (black). The stimulation period with glycine is marked with a yellow box. The white/empty box represents the lag time of 75 ms, before a cellular response occurs. Glycine concentrations are indicated.

stimulation with glycine actually ceased. Drift amplitude or shape did not correlate significantly with the applied glycine concentration ($R^2 = 0.25$). However, the time difference between application of glycine and the occurrence of a small negative peak $\Delta V_{peak} = 6.8 \pm 0.3 \mu\text{V}$ (triangles in Fig. 5.6) nicely followed the trend of rising glycine concentrations ($R^2 = 0.78$), i.e. the higher the glycine concentration the faster this peak occurred.

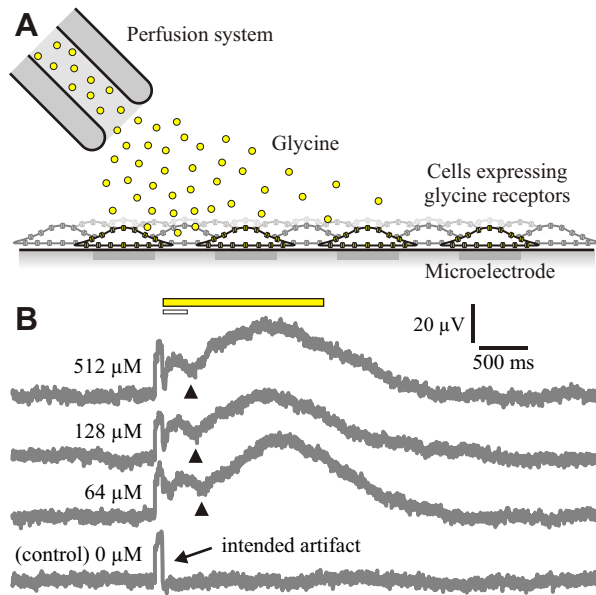


Figure 5.6: Prototype of a whole-cell biohybrid sensor. (A) Schematics, showing multiple cells growing on an MEA. (B) Recordings obtained without simultaneous patch-clamp contact. An intentional artifact has been induced to mark the time point of glycine application. The stimulation with glycine is given as a yellow bar. The white/empty box marks the lag time of 75 ms. Black triangles mark negative peaks, which might be putative indicators for different glycine concentrations.

5.4. Discussion

This study describes the development of an anion-selective biohybrid sensor. In order to generate chloride currents, cell lines were established that constitutively expressed functional glycine receptors. The dose-response curve determined for cell line 2C2 had an EC_{50} value of 140 μM for glycine. This is in good agreement with published data, where EC_{50} values from 20 to 300 μM glycine were reported for various cell types expressing ionotropic glycine receptors (Chen et al., 2009; Grenningloh et al., 1990; Rajendra et al., 1997).

5.4.1. Relevance of anion currents in bioelectronic coupling

Anion currents are new in cell-sensor coupling and had yet to be investigated. Using the GlyRA1 receptor cell line, it was possible to experimentally proof model predictions used in cell-sensor simulations (Fromherz, 2006; Pabst et al., 2007; Wrobel et al., 2005). According to established models, signal shapes recorded with FETs are expected to be like those of MEAs, only if membrane currents are carried by anions. On both, FETs and MEAs, the recorded signals in fact had similar shapes, amplitudes, and durations. Similar signal shapes strongly suggest common coupling processes in cell-sensor communication. In contrast to anions, cation currents cause different signal shapes in FETs and MEAs, since FETs are electrochemically sensitive to cations. This causes additional potentials in extracellular recordings. Therefore, the presented results confirm predictions of models, which encounter side-binding processes of cations at the transistor gate (Pabst et al., 2007; Wrobel et al., 2005). Experiments with cells expressing anion channels cultured on substrate-integrated sensors will certainly improve the understanding in cell-transistor coupling.

5.4.2. Characterization of extracellularly recorded signals

The GlyR cell line was successfully cultured on chips with substrate-integrated micro-devices. With an average diameter of about 30 μm , cells covered microelectrodes and the gates of field-effect transistors (Fig. 5.1b and c). During repeated perfusion with different concentrations of glycine the cells remained firmly attached. Patch-clamp recordings clearly showed that the GlyR was functional and conducted large, glycine-dependent chloride currents. Microelectronic devices in contact with the cells successfully traced the GlyR-mediated cellular anion currents with various shapes (Fig. 5.4a). Since MEAs and FETs recorded signals with similar shapes from transfected cells and neither intracellular nor extracellular control recordings (Fig. 5.4b) from wild type HEK293 cells showed a response to the applied glycine concentrations, pH effects on cells or sensors were excluded as cause for the positive components in extracellular recordings. It is known that besides large membrane currents, a seal resistance of about 1 $\text{M}\Omega$ in the contact plane between cell and sensor, promotes successful coupling of membrane currents into extracellular devices (Gleixner and Fromherz, 2006; Wrobel et al., 2005). The influence of different seal resistances on recorded extracellular signals has been reported by Jenkner and Fromherz (1997) as well as Cohen et al. (2008). They observed changing amplitudes of capacitive components or whole signal inversions caused by varying seal resistances. It is thus likely that extracellular recorded signals of GlyR currents were alternated by variations in the seal resistance as well. However, signal shapes could not be correlated unequivocally with seal resistances. Compared to other cells, the GlyR clones tended to have smaller seal resistances, hampering an interpretation of signal shapes. Wild type HEK293 cells provided almost three times larger seal resistances. For brain stem neurons, Ingebrandt et al. (2005) reported $R_J = 0.6 \pm 0.3 \text{ M}\Omega$. HEK293 cells, stably transfected with the bovine EAG potassium channel, were reported to have seal resistances of $R_J = 0.5 \pm 0.2 \text{ M}\Omega$ (Wrobel et al., 2005). However, HEK293 cells expressing serotonin-gated ion channels sparsely sealed with $R_J = 0.12 \pm 0.07 \text{ M}\Omega$ (Peitz et al., 2007).

An obvious similarity between extracellular recordings that contained capacitive components and the numerical derivative of the patch-clamp data was observed (Fig. 5.5). Such similarities were reported previously (Fromherz, 2002; Grattarola and Martinoia, 1993; Ingebrandt et al., 2005). The first, negative peak of the derived patch-clamp data matched extracellularly recorded signals of shape 3 (Fig. 5.4a). However, the signals were shifted by an average of 80 ms. A delay between intracellular and extracellular recordings of similar experiments has been reported by Peitz et al. (2007) and was attributed to a slowed diffusion of ligands into the cleft between cell and substrate. The delay due to diffusion into the cleft is calculated to $t_d = j_0 \cdot r^2 / D$ in cylinder coordinates, with the reciprocal of the first zero of the Bessel function $j_0 = 0.42$ as a constant, the cell radius r and the diffusion coefficient D of the ligand. Applying the average cell radius of $r \approx 14.5 \mu\text{m}$ and the diffusion coefficient for glycine $D_{\text{Gly}} \approx 10^{-5} \text{ cm}^2/\text{s}$, a delay of $t_d = 87.4 \text{ ms}$ was obtained. This estimated value was in very good agreement with the delay that was observed

in the data. Refined simulations implementing ligand binding, unbinding, and diffusion as well as ion concentrations and currents might help understanding the delay with more accuracy.

During the characterization phase with patch-clamp contact, glycine concentrations from 64 μM to 2 mM were applied extracellularly. Responses to glycine concentration of 32 μM or less could not be distinguished from noise in extracellular recordings. More recently, improved versions of FETs or MEAs were claimed to have better signal-to-noise ratios and therefore could further increase the sensitivity of the anion-selective biohybrid sensor towards lower ligand concentrations. However, glycine concentrations over three orders of magnitude, including physiologically relevant glycine concentrations, were measured reliably.

5.4.3. Measuring glycine with a prototype for cell-based biohybrid sensors

GlyR clones growing subconfluently on MEAs were perfused with varying glycine concentrations. Collecting data exclusively from extracellular sensors, the recorded signal shapes resembled capacitive peaks with positive components, as observed when the cells were held in the whole-cell patch-clamp configuration (compare Fig. 5.6b and Fig. 5.4a shape 2). Amplitudes of capacitive components were much smaller than during parallel patch-clamp contact. Furthermore, signal shapes showed a positive drift that returned to baseline before the stimulation with glycine was terminated. Without the patch pipette, cells were not constantly clamped to $V_M = -60 \text{ mV}$. Under these more *natural* conditions, the membrane potential might have changed rapidly during glycine application, possibly due to the large chloride currents. Neither the amplitude of positive components nor the amplitude of capacitive components sufficiently correlated with the applied glycine concentrations. Only the timing between glycine application and the occurrence of capacitive peaks were found to depend on the applied glycine-concentration. Future investigations with low-noise devices for extracellular recordings will focus on a detailed analysis of the signals' temporal correlation. However, this prove-of-principle study has shown that biohybrid sensors in fact can be operated without a patch clamp connection and that a correlation between an applied ligand concentration and the extracellularly measured chloride currents is possible.

5.4.4. Limits, improvements and capabilities of cell-based biohybrid sensors

The versatility of a biohybrid sensor that relies on the activity of ligand-gated ion-channels, strongly depends on the fast, precise and reproducible application of the ligand-containing solution. The perfusion system provided an almost instant change of the test solutions for cells located close to the small manifold. However, in the high-throughput approach with 64 electrodes, cells further away from the superfusion source developed slowly rising chloride currents. Consequently, many extracellular signals had smaller amplitudes and eventually vanished in noise. These observations

ask for improved devices which allow a parallel and rapid solution exchange for all cells growing on the chip.

Smaller signal amplitudes were generally observed in high-throughput experiments with prototype sensors as opposed to characterizations with individual cells in patch-clamp whole-cell configuration on sensors. These differences between patched-clamped and non patch-clamped cells, expressing ligand-gated channels were also observed by [Peitz et al. \(2007\)](#). Large membrane potentials, artificially applied by the patch-pipette, maintained membrane currents that do not occur under natural conditions. The capacitive components of the sensor cell line also had larger amplitudes when parallel patch-clamp recordings were performed. One approach to increase the membrane potential without employing patch-clamp pipettes could be a steeper chloride gradient between the cells cytosol and the bath solution.

The contact between cells and electronics could also lead to improved signals. Higher seal resistances would increase the coupling quality of chloride currents into extracellular devices. More specialized surface coatings or topological improvements could support a tighter cell attachment, e.g. due to more focal adhesions. Estimated seal resistance were taken as an indicator for the coupling quality between cell and electronic device in experiments. A representative circuit model was fitted to a transient capacitive spike, which was an artifact in extracellular data caused by a voltage step applied intracellularly with the patch-clamp electrode ([Ingebrandt et al., 2005](#); [Wrobel et al., 2005](#)). In the high-throughput approach, however, the GlyR cell line was not stimulated electrically but by the application of glycine. Thus, seal resistances and coupling qualities could not be determined for this prototype. [Schäfer et al. \(2009\)](#) investigated the surface adhesion of individual HEK293 cells by impedance spectroscopy. In future experiments with biohybrid sensors, this technology could be used to estimate the seal resistance of cells without any patch-clamp contact ([Fromherz et al., 1999](#); [Kiessling et al., 2000](#)).

5.5. Conclusion

The prototype for a cell-based biohybrid sensor was generated by coupling a genetically modified cell line to microelectronic devices. HEK293 cells were stably transfected with cDNA encoding human glycine receptors. The sensor was established by plating these cells on microelectrode array and field-effect transistor chips. Both combinations were equally capable to detect cellular, glycine-dependent chloride currents. While the cells registered glycine concentrations over four orders of magnitude, the electronic read-out hardware monitored cellular responses to glycine concentrations over three orders of magnitude, including physiologically relevant ones. Improving the electronic components towards better signal to noise ratios, may help to increase the detection limit of the sensor well below 64 μM of glycine.

Taking advantage of anion currents, this study challenged *state-of-the-art* theories of cell-sensor coupling, which were exclusively based on cation currents. Demonstrating that recordings from FETs and MEAs have the same shape when anions carry the coupling process, strongly support cell-transistor models which include cation side-binding effects. Forthcoming experiments and refined modeling approaches with anions in cell-sensor communication will certainly advance the understanding of cell-electronics interaction.

6. Monitoring voltage-gated Cl^-/H^+ -antiporter activity with FETs

Parts of this chapter are in preparation for publication:

Sommerhage F., Ingebrandt S., Offenhäusser A. Monitoring voltage-gated Cl^-/H^+ antiporter activity with ion-sensitive field-effect transistors. *Article in preparation.*

6.1. Introduction

The importance of sensor arrays in biological research has increased during recent years as they provide multiple recording sites for the collection of consecutive data on the same specimen. Field-effect transistor (FET) and microelectrode arrays (MEAs) were designed to gather information about the electrical activity of multiple cells or cell networks. The non-invasive character of the sensor arrays allowed the collection of data over several weeks yielding insights into network behavior and information processing among the cells. The gain of long-term capabilities and special resolution was a trade off for less information about individual cells since the substrate integrated FETs and MEAs were only able to record a fraction of the extracellular potential. Consequently, researchers seeking for high information-content during investigations of a cell's electrical behavior were not attracted to FETs or MEAs. Interestingly, a side-effect of ion-sensitive FETs offers great opportunities to help solving a variety of ambitious biological questions.

Ion-sensitive FETs are mostly employed to measure the pH of electrolytes. The underlying functional mechanism is attributed to surface reactions between the pH-determining ions in the electrolyte and hydroxyl-groups at the sensor surface, known as the gate or gateoxide. A very common gate material is an insulating layer of silicon dioxide (SiO_2) that is just several nanometers thin. Terminal oxygen atoms on the SiO_2 surface, in contact with electrolytes, form hydroxyl-groups until a pH-dependent electrochemical equilibrium is established. The primary chemical reactions at the electrolyte-oxide interface are described by the site-binding model (Yates et al., 1974). Besides hydrogen, other cations interact with deprotonated and thus negatively charged hydroxyl-groups as well. Bergveld (1970) used ion-sensitive FETs to gather information about changes in ion concentration of electrophysiologically important cations, such as sodium, potassium and calcium. However, the interaction of other cations is not as prominent as the actual reaction of hydrogen with the gateoxide (Bergveld, 2003). In contrast, anions do not react or interact with positively charged silanols at the sensor surface. Therefore, FETs represent a promising tool to answer questions in current ion-channel research which is - among other quests - seeking for insights into Cl^-/H^+ antiporters, also known as chloride channels (CIC).

Members of the CIC gene family were found to be present in species ranging from bacteria to humans facilitating numerous functions (Lísal and Maduke, 2009). During recent years, intense studies revealed important biophysical and physiological properties of these membrane proteins including the stabilization of membrane potential, excitation, regulation of the cell volume, fluid transport, protein degradation in endosomal vesicles and possibly cell growth in mammals (Suzuki et al., 2006). CIC proteins were considered pure voltage-gated chloride channels which assemble functional complexes from two subunits, each with its own pore for the ion transport. During recent years, research projects revealed more and more CIC proteins that were Cl^-/H^+ antiporters conveying chloride into the cell and protons out of the cell (Accardi et al., 2005; Lísal and Maduke, 2009; Pusch et al., 2006; Suzuki et al., 2006). Two chloride-channel antiporters were selected to

be the subject of experiments in this article. CIC-4 is localized to the endoplasmic reticulum and may contribute to the pathogenesis of neuronal disorders but the exact physiological role is unknown (Okkenhaug et al., 2006). In contrast, the physiological role of CIC-1 is well known as it regulates the resting potential in human muscle and also processes in the central nervous system (Aromataris and Rychkov, 2006). The outward rectifying currents in CIC proteins rule out a detailed reversal-potential analysis, which is usually performed to evaluate the stoichiometric transport of different ions (Friedrich et al., 1999; Scheel et al., 2005). CIC-4 has been studied intensely with pH-sensitive dyes revealing a Cl^-/H^+ transport stoichiometry of 1.6 ± 0.7 (Scheel et al., 2005). Investigations with pH sensors, brought close to cells expressing CIC-1, indicated slight changes in pH during electrical stimulation. However, the Cl^-/H^+ -exchange stoichiometry has yet to be determined (Picollo and Pusch, 2005).

The transport of protons across the cell membrane affects the cytoplasmic and the extracellular pH, at least in the intermediate vicinity of a cell. However, tools to measure the pH activity of cells are limited and time consuming as proton transporters have to be activated for 10 to 30 minutes to induce a firm change in pH. Detailed experiments requiring several hours of membrane depolarization certainly lead to severe cell stress and fatigue. The extended experimental duration rises from the respective sensor design where comparatively large volumes are the subject to proton migration. Picollo and Pusch (2005) placed pH-sensitive microelectrodes very close to the cell membrane and detected an acidification of the extracellular environment during activation of antiporters. Protons need to diffuse several micrometers into a glass pipette to affect the pH electrode and allow measurements. Using an optical solution Scheel et al. (2005) measured the fluorescence emission of a pH sensitive dye, which was added to the intracellular patch solution, and observed a cytoplasmic alkalization. In this approach, the pH of an entire cell has to be changed before the emission of light changes significantly. The key for faster pH measurements in electrophysiology is therefore (i) bringing the pH sensor very close to the cell and (ii) incorporating it into a very small volume.

As illustrated in figure 6.1, cells can be cultured directly on FET-based pH sensors (Fromherz et al., 1991; Ingebrandt et al., 2001; Sommerhage et al., 2010). The junction of cells and FETs has been investigated throughout the last 15 years and is now well understood (Braun and Fromherz, 1998; Ingebrandt et al., 2005). In essence, the contact can be characterized by a very flat cylindrical cleft volume located between the cell and the FET (Wrobel et al., 2005). This tight geometrical situation of the cleft allows the measurement of minute pH changes due to protons released by the cell. FET sensitivities to pH changes range from 30 to 40 mV/pH (Bataillard et al., 1987; Bergveld et al., 1997; Fung et al., 1986). Due to their marvelous pH sensitivity and the widely proven compatibility with cultured cells, FETs are ideal for the investigation of Cl^-/H^+ antiporter activity.

Human embryonic kidney (HEK293) cells, stably expressing either CIC-1 or CIC-4 Cl^-/H^+ antiporters, were cultured on ion-sensitive FETs to measure pH changes due to proton transport

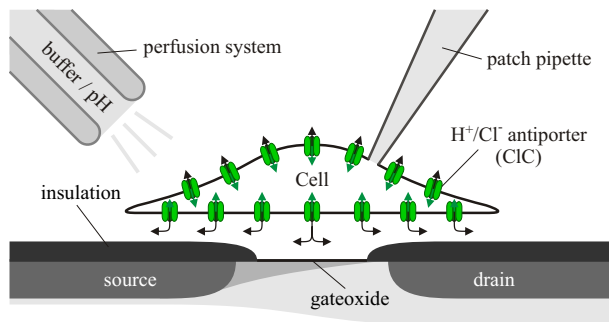


Figure 6.1: Schematic overview of the experimental setup to measure Cl^-/H^+ transport in chloride channels (Cl^- green and H^+ black), including a cell in whole-cell patch-clamp configuration growing on an ion-sensitive field-effect transistor. The perfusion system provides solutions with different buffer concentrations or bath pH to modulate the effect of protons on the pH .

across the cell membrane. As depicted in figure 6.1, cells were approached with patch-clamp pipettes in voltage-clamp configuration allowing a controlled stimulation of antiporters and intracellularly recording of membrane currents. In order to reveal the transport of protons in CIC proteins active antiporters were monitored by FETs, while the effective pH change in the bath solution was modulated by different pH-buffer concentrations or the initial pH of the bath.

6.2. Materials and Methods

6.2.1. Ion-sensitive field-effect transistor (FET) chips

FET chips, as depicted in figure 6.2, provided 16 transistor gates arranged in a 4×4 matrix in the center of a $5 \times 5 \text{ mm}^2$ silicon chip. Each FET had an effective gate width of $16 \mu\text{m}$ and the gate oxide (SiO_2) exposed to the electrolyte solution covered an area of $80 \mu\text{m}^2$. A silver-chloride electrode, setting the bath electrolyte to ground potential, served as a common gate contact for the FETs. Transfer characteristics of FETs were determined shortly before experiments to set an optimal operation point. The average transconductance was $g_m = 0.4 \pm 0.2 \text{ mS}$ and the average pH sensitivity of FETs was $U_{\text{pH}} = 34 \pm 2 \text{ mV/pH}$. Detailed information about the general function of the used FET devices and amplification systems can be found in previous publications (Ingebrandt et al., 2005; Offenhäusser et al., 1997; Sprössler et al., 1998).

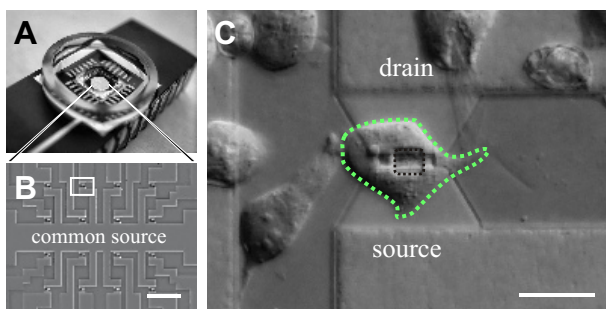


Figure 6.2: Ion-sensitive field-effect transistors. (A) Photography of the encapsulated chip. The culture ring has an outer diameter of 16 mm and holds about $500 \mu\text{l}$ of media/electrolyte. (B) 16 transistor gates in a 4×4 array (scale bar = $200 \mu\text{m}$) and (C) a HEK293 cell growing on an FET gate observed with a differential interference contrast microscope (scale bar = $30 \mu\text{m}$).

6.2.2. Chip cleaning and surface modifications

Chips were cleaned by sonication for 3 min in 2 % (v/v) Hellmanex II (Hellma, Germany). Subsequently, this step was repeated with bidistilled water. Only the surface used for cell plating was further treated with 20 % (v/v) H_2SO_4 for 20 min at 80 °C to remove remaining organic materials and activate silanols at the gate oxide. Devices were rinsed with bidistilled water and kept for 5 min in 70 % (v/v) ethanol. After drying, FET surfaces were coated by incubation with 25 μl of 0.1 mg/ml poly(L)lysine (Sigma, Taufkirchen, Germany) for 30 min at room temperature. Before cell plating, sensor surfaces were rinsed with a phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.3).

6.2.3. Cell culture

Human embryonic kidney (HEK293) cells were grown in minimal essential medium containing 10 % fetal bovine serum (v/v), 2 mM glutamine, 1 % non-essential amino acids, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (all from Invitrogen, Germany). The culture media for HEK293 clones, stably expressing voltage-gated Cl^-/H^+ antiporters, was further supplemented with 0.8 $\mu\text{g}/\text{ml}$ (Invitrogen, Germany). Respective clones were either CIC-1 (Fahlke et al., 1996) or CIC-4 (Hebeisen et al., 2003). Culture media was exchanged every 2-3 days and cells were split weekly by a standard HEK293 protocol. According to the average proliferation rate of the respective cell type (CIC-1: 25 ± 1 hours, CIC-4: 23 ± 1 hours, wild type: 29 ± 1 hours), cell densities were calculated to obtain $2 \cdot 10^4$ cells/ mm^2 on FET devices after 2 to 4 days in culture (37 °C, 5 % CO_2). Figure 6.2c depicts a microscopic image of HEK293 cells cultured on an FET structure.

6.2.4. Bioelectronic coupling for pH measurements

The culturing media in FET devices was exchanged with standard bath solution containing 3 mM KCl, 140 mM NaCl, 5 mM MgCl_2 , 1 mM CaCl_2 , 50 mM glucose and 10 mM HEPES adjusted to pH 7.4 (all from Sigma, Germany). Only individual cells, located directly on top of transistor gates, were contacted with a patch-clamp pipette in whole-cell configuration as described earlier (Sommerhage et al., 2010; Wrobel et al., 2005). Patch pipettes ($\sim 5 \text{ M}\Omega$) were filled with 145 mM KCl, 5 mM NaCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM EGTA, 50 mM glucose and 10 mM HEPES, adjusted to pH 7.4 (all from Sigma, Germany). Measurements with the ion-sensitive FET located below a patched cell were amplified and fed into an external input of an EPC-9 double patch-clamp amplifier (HEKA Elektronik, Germany). Intracellular membrane currents and corresponding extracellular FET signals were recorded simultaneously at 30 kHz using the software *TIDA* (HEKA Elektronik, Germany). During experiments the patch-pipette, cells and FETs were observed with an Axiotech Vario 100 HD microscope (Zeiss, Germany). Cells were superfused ($\sim 125 \mu\text{l/s}$) with test solutions containing different buffer concentrations or pH by a DAD-8VC system (ALA

Scientific Instruments, USA). Before cells were stimulated via the patch-pipette, a lag time of 30 seconds was granted to assure the diffusion of a new test solution into the small cleft volume.

Buffer assay

Three bath solutions with different buffer concentrations were applied to modulate the pH-effect of protons released into the small cleft volume. As illustrated in figure 6.3 (left), higher buffer concentrations reduced the amount of free H_3O^+ in the extracellular solution resulting in fewer reactions at the gate surface of the FET. In contrast, the buffer capacity (2.14 on page 29) of a low buffer concentrations was soon saturated with H^+ and excess H_3O^+ reacted with silanols at the FET's gateoxide. Experiments were started with cells in standard bath solution with 10 mM HEPES (CAS: 103404-87-1, Sigma, Germany). After contacting cells with a patch-clamp pipette, the extracellular solution was exchanged to alternate the HEPES concentration to 0.1 mM, 1 mM or de novo 10 mM in random order. For each HEPES concentration a voltage step of $U_{\text{Step}} = 120 \text{ mV}$ was applied for the duration of $\Delta t_{\text{Step}} = 500 \text{ ms}$. The pH value in the small cleft volume decreased during this pulse, resulting in a higher amplitude of the FET signal. Immediately after the stimulation pulse, the cleft pH increased in less than one second back to the value of the surrounding bath electrolyte depending on diffusion effects and the geometrical situation (Wrobel et al., 2005). In order to reduce noise in FET signals, the voltage stimulation was repeated 15 times before another test solution was applied.

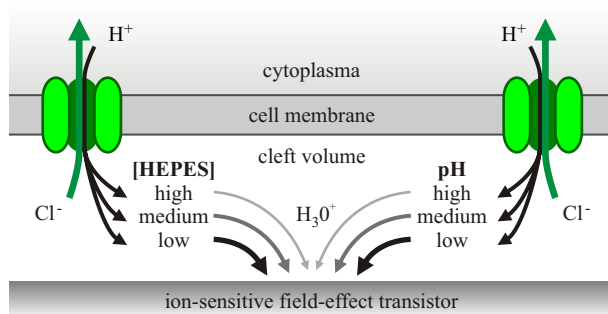


Figure 6.3: Illustration of pH-influencing processes in the cleft volume between cell and transistor gate. (left) The HEPES concentration affects the amount of H_3O^+ which can react with the gate oxide. (right) The initial pH in the bath solution determines the effective pH change during Cl^-/H^+ antiporter activity.

pH assay

In this alternative approach (figure 6.3 right) experiments were conducted with pH variations of the bath solutions. The logarithmic nature of pH (equation 2.15) modulates the nearly constant proton transport. Low pH values represent high H_3O^+ concentrations and additional H^+ released by the cell will only slightly affect the pH. In contrast, high pH values represent a low concentration of H_3O^+ in the extracellular environment. Additional protons released by the cell will therefore have a relatively large impact on the pH. Five different pH values, 5.4, 6.4, 7.4, 8.4 and 9.4, were applied to the cells. Experiments with different pH values were unfortunately distorted by the buffer capacity of HEPES (page 29) which was determined by the bath pH. Consequently,

this alternative approach was less reliable. The patch-clamp contact was established in standard bath electrolyte with pH 7.4. Subsequently, the bath solution was exchanged to present one of the experimental pH values in random order. Analog to experiments with HEPES, cells were stimulated via the patch pipette.

6.2.5. Interpretation of FET signals and pH

Repetitively activated antiporters carry each time roughly the same amount of chloride and protons across the membrane and therefore lead to the same shape in FET recordings. However, if the buffer concentration is alternated the pH in the cleft will be different and thusly lead to different amplitudes in FET recordings. Similar effects are expected for the initial bath pH where a certain amount of protons, released by the cell, have consequently different impacts on the pH in the cleft volume. FET signals are complex and influenced by electrical, chemical and geometrical factors (Ingebrandt et al., 2005; Wrobel et al., 2005). In order to convert FET signals into pH values a pH-independent signal-component, based on a purely electronic coupling between cell and FET, needs to be extracted. During the last two decades, electrical equivalent models of the cell-transistor junction were brought close to perfection and allow a reliable determination of the electrical component in FET signals (section 2.7). Electrical shares of the FET signals were simulated with the point-contact model, where electric processes in the three-dimensional cleft geometry are simplified and compacted into one point. The necessary parameters for this model are derived from information obtained during patch-clamp. Detailed information about the simulation can be found in section 2.7 or earlier publications (Regehr et al., 1988; Fromherz et al., 1993; Wrobel et al., 2005; Ingebrandt et al., 2005). After subtraction of electrical components, signals are mostly determined by ion-sensitive pH effects. Only a small fraction represents charges due to concentration differences of individual ion species between the cleft volume and the surrounding bath electrolyte (Wrobel et al., 2005).

6.2.6. Statistical analysis

Statistical tests were performed with standard functions in *Calc*, *OpenOffice 3.1*. Numbers are given as mean $\pm$ standard deviations of Gaussian distributions.

6.3. Results

The H^+ transport in two selected ClC antiporters was investigated with ion-sensitive FETs. Wild type HEK293 cells or the respective ClC-protein expressing clones ($n = 8$) were individually cultured on FET devices. Equally sized cells (ClC-1: $C_M = 18.1 \pm 3.4$ pF and ClC-4: $C_M = 18.3 \pm 2.4$ pF) located on FET gates were approached with patch-clamp pipettes in whole-cell

configuration. Stimulation protocols were repetitively presented to clamped cells, however, with alternating concentrations of HEPES or different pH in the bath electrolyte. Cells remained firmly attached during superfusion with experimental solutions which allowed a successful recording with substrate-integrated FETs.

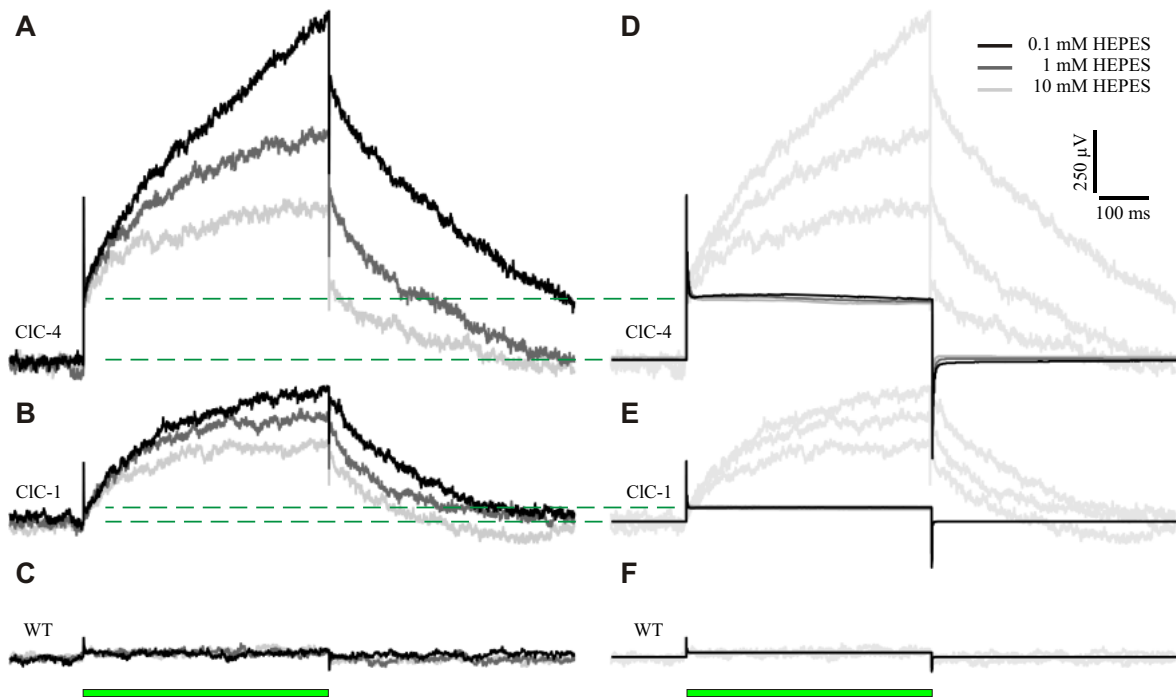


Figure 6.4.: (A-C) FET recordings (average of 15 traces) from HEK293 cells with three different HEPES-buffer concentrations in the extracellular solution. The green bar marks the stimulation period of 500 ms with 120 mV, applied intracellularly via the patch electrode. (D-F) Simulations of pH independent, electrical components for each HEPES concentration. Simulated traces are mostly identical.

Representative FET recordings for the three different HEPES concentrations are superimposed in figure 4. FET signals rose quickly during the applied stimulus. The signals usually approached a steady state towards the end of the depolarization and slowly returned back to zero after pulse deactivation (Fig. 6.4a and b). Analog control experiments with wild-type HEK293 cells showed no change in FET traces (Fig. 6.4c). The variation of HEPES concentrations in the bath solution had no significant influence on intracellularly measured membrane currents of $I_M = 0.4 \pm 0.1$ nA on average for CIC-1 and CIC-4. However, FET recordings from CIC clones in bath solutions with 0.1 mM HEPES resulted in significantly higher amplitudes than recordings with 1 mM and recordings with 10 mM HEPES had the smallest FET amplitudes. These differences in FET-amplitudes correlated with the respective HEPES buffer concentration and were therefore attributed to an acidification of the small cleft volume between cell and sensor during Cl^-/H^+ antiporter activity. Measurements with CIC-4 cells and 0.1 mM HEPES in the bath electrolyte showed strongly increasing trends, which occasionally did not reach a steady state within the stimulation duration (Fig. 6.4a). Most pH changes, reported here, were below 1. Yet, strongly increasing FET signals

might indicate large pH changes past the effective buffer-capacity range (6.55 – 8.55) for HEPES (2.14, page 29).

Ion-sensitive FETs measured a composition of purely electrical currents across the attached cell membrane and a pH dependent, ion-sensitive component that was influenced by the respective HEPES concentration. The electrical components were almost constant for all three HEPES concentrations (Fig. 6.4d-f) and thusly pH independent.

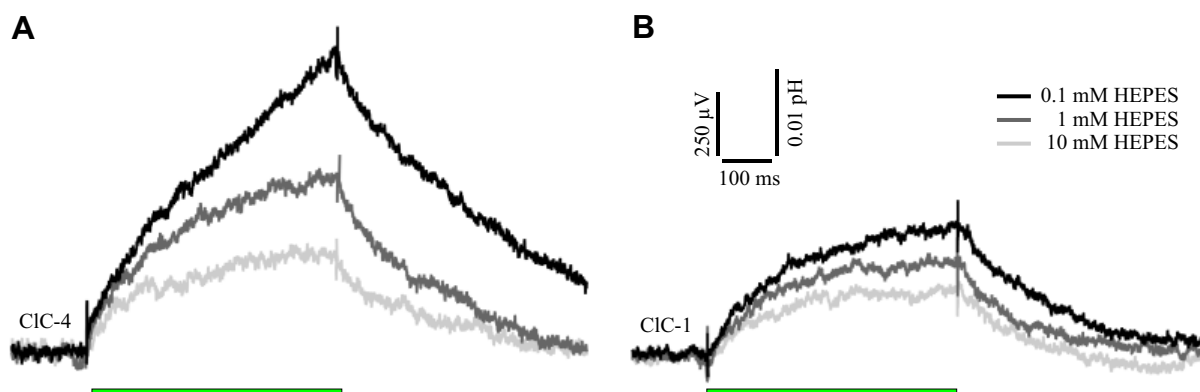


Figure 6.5.: Influence of HEPES on FET recordings and pH in cleft volume. FET recordings were cleared from constant electric components. These adjusted FET signals represent pH changes in the cleft volume. (A) pH changes for CIC-1 and (B) for CIC-1.

FET amplitudes in addition to the electrical component were attributed to pH effects. Figure 6.6a and 6.6b depict the adjusted, solely pH-dependent FET signals (without electrical component) respectively recorded from CIC-4 and CIC-1 cells. The amplitudes at the end of the stimulus in adjusted FET signals were averaged for each CIC type and plotted versus the HEPES concentration in figure 6.6c. FET signals from experiments with CIC-4 cells showed, in general, higher signal amplitudes than the respective recordings from CIC-1 cells. Since intracellularly measured membrane currents were similar for the two CIC clones, differences between the respective signal amplitudes were attributed to individual Cl^-/H^+ transport ratios. CIC-4 induced pH changes were 1.9 ± 0.2 times larger than those of CIC-1. Scheel et al. (2005) estimated that CIC-4 transported about 2 protons per 3 Cl^- ions. Applying the here found difference between the two antiporters, CIC-1 transported only 1 proton per 3 Cl^- ions. This was in agreement with earlier findings for the transport of protons in CIC membrane proteins, where larger transport ratios were estimated for CIC-4 (Picollo and Pusch, 2005).

An alternative approach was performed with changes of the initial pH in the bath solution. Recordings from CIC-4 superfused with five different pH values are superimposed in figure 6.7a. Analog to results with changes in the buffer concentration, FET amplitudes correlated with test solutions providing different pH values. The expected non-linear effect of changed buffer-capacities for the respective pH value was not observed (compare figure 2.18). However, the measured signals showed very small differences in amplitudes, suggesting small changes in pH, which were probably caused by the relatively high HEPES concentration of 10 mM. In contrast, only 1 mM

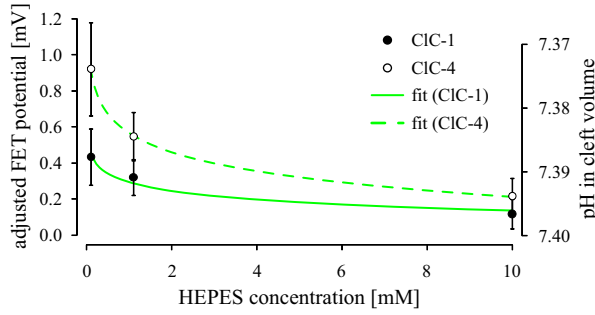


Figure 6.6: Averaged, adjusted FET signals over HEPES concentrations. The conversion into pH is given with a secondary axis on the right, starting at the bath pH of 7.4. Logarithmic fits (green lines) illustrate the putative effects of other HEPES concentrations in the cleft volume on recordings with FETs.

was used for experiments with CIC-1. The respective recordings are superimposed in 6.7b where FET amplitudes rose with the initial pH value and in a non-linear manner. As expected, differences in signal amplitudes were smaller for low pH values and larger for high pH values. The constant amount of protons added to the cleft was therefore modulated by the initial bath pH. Influences of the variable buffer capacity further amplified the effect for low and high pH values.

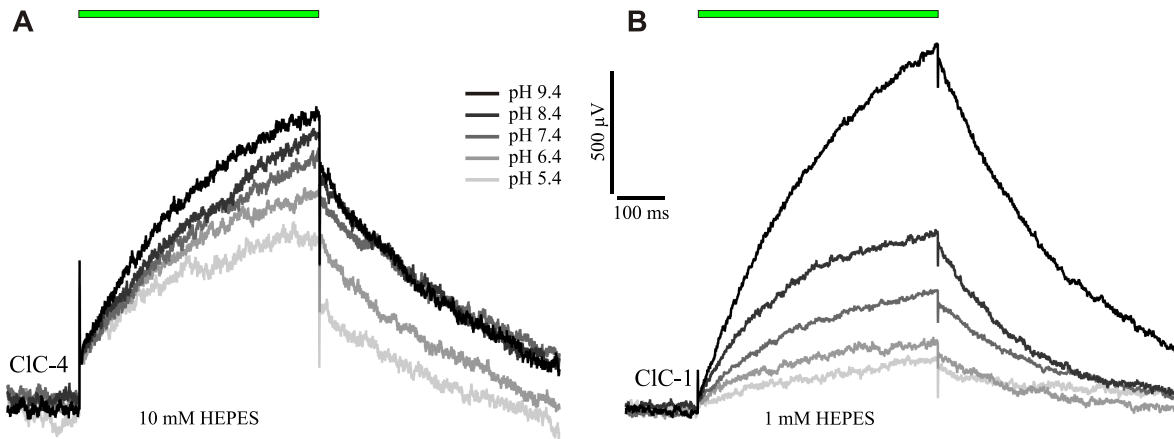


Figure 6.7.: FET recordings from CIC protein expressing HEK293 cells for variations in electrolyte pH and the modulating effect of HEPES on the FET ion sensitivity. The green bars are analog to Figure 4. Results for (A) CIC-4 with 10 mM HEPES and (B) with 1 mM HEPES to emphasize pH effects with respect to fluctuations in Cl^- membrane currents mediated by CIC-1 antiporters.

6.4. Discussion

Ion-sensitive field-effect transistors (FETs) were employed to study the transport ratios of protons versus chloride in Cl^-/H^+ antiporters. The antiporters, also known as chloride channels (CIC), were expressed by HEK293 cells growing on FET devices, specifically built for cell culture purposes (Fig. 6.1 and 6.2). Cells were carefully chosen to provide similar membrane capacitances C_M (cell size), membrane currents I_M and qualities of attachment to obtain comparable results. The most variable factor, the quality of attachment, was partially compensated by removing the pH-independent component from FET signals. Reliable data was obtained by averaging

the pH measurements, which were depending on the cleft volumes between the sensor and the analyzed cell. FETs detected pH changes within some 100 ms, outperforming conventional optical or electrical approaches (Fig. 6.6).

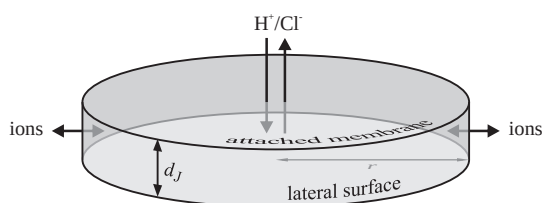


Figure 6.8: Schematic representation of the cleft volume, limited by the attached membrane on top and the substrate (including integrated FETs) at the bottom. The lateral surface surrounds the cleft volume with the cell radius r and allows diffusion of ions from and into the bath. Cl^-/H^+ transport is mediated through the attached membrane, which also determines the average cleft height d_J .

The greatest advantage of substrate-integrated FETs is the proximity to the ion-channel of interest (Fig. 6.1) and the small size of the sample volume subject to membrane currents (Fig. 6.8). A cell with radius $r \approx 15 \mu\text{m}$ and an average cleft height $d_J \approx 75 \text{ nm}$ (junction distance) yields a cleft volume in the range of 10^{-14} liters. The pH effect of protons released by the cell into this tiny volume is significant. Due to the flat geometry with sub-micron height, ions easily accumulate or deplete while antiporters are active yielding an amplification of pH effects (Ingebrandt et al., 2005; Sommerhage et al., 2008; Wrobel et al., 2007). In the case of Cl^-/H^+ antiporters, the concentration of chloride decreases in the cleft while protons accumulate and thus induce an acidification of the cleft volume. However, the open nature of the cleft volume (Fig. 6.8) allowed H_3O^+ , the ionized HEPES-buffer molecules as well as other ions to diffuse slowly along the cleft into the bath volume or vice versa. Thusly, not all protons accumulated in the cleft volume hampering a direct translation of measured pH changes into the amount of protons transported in CIC molecules. The Cl^-/H^+ -transport ratio in CIC-1 was therefore not calculated directly from pH measurements. However, the FET recordings from CIC-1 could be compared to those from CIC-4 transporters which had been characterized in the past (Scheel et al., 2005). The resulting ratio for the proton transport in CIC-1 was about 1 H^+ per 3 Cl^- . The calculated ratio of 1.9 ± 0.2 had a low standard deviation, compared to direct measurements for CIC-4, where the standard deviation was at 50 % of the average value. Since the presented results were based on this previously published, highly variable data, the derived transport ratio for CIC-1 is likely to be smaller than 1 H^+ per 3 Cl^- .

According to current models of cell-transistor contacts, the steady state in measured FET signals reflects a stable state of diffusion where only ions able to pass the cell membrane are moving along the cleft, while all other ions rest (Wrobel et al., 2005). Pabst et al. (2007) introduced an analytical method for ion channels transporting only one ion species. Modifications of such analytical models could provide detailed, quantitative information about the proton transport directly from FET signals.

Common protocols for the investigation of transport stoichiometries in membrane proteins, such as the analysis of reversal potentials, had to be ruled out as currents mediated by ClC transporters/channels were strongly rectified (Friedrich et al., 1999; Scheel et al., 2005). Instead, pH changes were measured in the intracellular or cell-adjacent extracellular environment. However, typical probes for pH measurements struggle particularly with large volumes subject to pH changes and/or an (intracellular) environment with unknown pH-buffer characteristics. Glass pipettes, containing minute pH electrodes, provide the possibility to use defined electrolytes. However, protons need to diffuse into the pipette and affect the pH of a rather large volume compared to that of a cell (Piccolo and Pusch, 2005). Optical measurements where pH-sensitive dyes are injected into the cell are distorted by the unknown buffer characteristics of the cytosol or effects of different cellular compartments (Scheel et al., 2005). The here introduced FET-supported method provides amazingly small volumes with known electrolytes, which can be easily exchanged to modify buffer characteristics.

Besides adjustments of the buffer concentration in the bath, the initial pH in the bath solution was modified, which modulated effective pH changes in the cleft during Cl^-/H^+ -exchanger activity as well. In fact, the buffer and pH changes were combined and the results presented in figure 6.7 demonstrate the flexibility of FET-supported ion-channel investigations. However, comparing the results for experiments with changed bath pH remains challenging. The buffer capacity is dependent on two factors, the buffer concentration and the present pH of a solution (see equation 2.18 on page 29). Since experimental solutions for changes in the initial pH also contained a certain concentration of HEPES, the buffer capacity was different for each of the five initial pH values. The transport of H^+ in ClC-1 and ClC-4 proteins was clearly indicated by the correlation of amplitudes in FET recordings with changes in the initial bath pH. However, according to (Chen and Chen, 2001), the chloride gating of ClC-1 is influenced by extracellular pH. Therefore, pH changes in the bath solution were not just modulating the ion-sensitivity of the FET, but also regulated the Cl^-/H^+ -exchanger activity directly. The results in figure 6.7 represent a general proof of principle as the buffer capacity was not constant for the five initial pH values. In future experiments, the initial pH value needs to be set as the pK of the present buffer in the solution. Different buffers, with their respective pK could then be employed to change the initial pH accordingly and yet keep the buffer capacity constant. Then FET devices could be used to investigate gating-effects of the extracellular pH on Cl^-/H^+ exchanges as well.

Substrate-integrated, ion-sensitive FETs further offer the possibility to probe the transport of other cations with lower sensitivities. Bergveld (1972) employed FETs to extracellularly measure the transport of cations during action potentials in insect and mammalian muscle. However, the measurements reflected multiple cation-currents through various ion channels, hampering an interpretation. The here presented measurements were performed with cells expressing predominantly one specific ion-channel of interest yielding a very promising tool for ion-channel research. The construction of artificial bi-lipid membranes (e.g. black lipid membranes) on FET devices

(Atanasov et al., 2005), teemed with ion-permeable channel-proteins could improve the still variable quality of cell attachment as well as further increase the purity of analyzed ion-channels.

6.5. Conclusions

A fast and reliable method for the investigation of proton transport in chloride channels is presented. The substrate-integrated FETs detected pH changes in the close vicinity of cells expressing either ClC-1 or ClC-4 membrane proteins. The findings were further backed up by repetitive measurements using the same cells presented to multiple concentrations of pH buffers in the bath solution. The respective FET recordings showed increasing pH changes for decreasing buffer concentrations. The experiments were in agreement with published results, indicating a prominent H^+ transport in ClC-4 antiporters and a smaller, but clearly measurable, contribution of H^+ to currents in ClC-1 proteins. In contrast to earlier studies, which monitored intra- or extracellular pH fluctuations in the range of hours, the here presented FET-supported method reacts to pH changes in far less than one second and clearly indicates the transport of protons in chloride channels within less than 5 minutes per experiment.

7. Modeling ion currents and buffer reactions in the cell-sensor junction

This chapter is partially based on the following publications:

Ingebrandt S., Wrobel G., Zhang Y., Meyburg S., Schindler M., **Sommerhage F.**, Borstlap D., Offenhäusser A. (2005). [Investigation of extracellular signal shapes recorded by planar metal micro electrodes and field-effect transistors.](#) *Sensors, 2005 IEEE*, 611-616.

Wrobel G., Zhang Y., Krause H. J., Wolters N., **Sommerhage F.**, Offenhäusser A., Ingebrandt S. (2007). [Influence of the first amplifier stage in MEA systems on extracellular signal shapes.](#) *Biosensors and Bioelectronics* 22(6), 1092-1096.

Pabst M., Wrobel G., Ingebrandt S., **Sommerhage F.**, Offenhäusser A. (2007). [Solution of the Poisson-Nernst-Planck equations in the cell-substrate interface.](#) *European Physical Journal E: Soft Matter and Biological Physics* 24(1), 1-8.

7.1. Introduction

The investigation of cell-sensor junctions using FETs or MEAs in the last chapter was supported by simulations composed of several sub-models. The implementation of multiple ion fluxes across the cell membrane into the model and calculations of buffer reactions were a key-element of simulations in this thesis. An introduction into pH and buffer calculations was given in section 2.6. Electric circuit and electrodiffusion sub-models were theoretically elucidated in section 2.7. Further discussions and descriptions of the respective algorithms will be conducted here.

The primary rational for the assembly of a comprehensive model describing the cell-sensor junction was a quest for the transport ratio of chloride versus protons in the human skeletal muscle antiporter CIC-1 (Pusch et al., 2006; Lísal and Maduke, 2009). Another motivating factor was certainly the desire to gain a better understanding of cell-sensor junctions in general. Despite Hodgkin and Huxley's finding that the electric activity of cells was carried out by ions, electrogenic processes and their coupling into sensors was traditionally conceived as entirely electric and thus supposed to be explained solely by rather simple electric equivalent circuits (Hodgkin and Huxley, 1945; Thomas et al., 1972; Neher and Sakmann, 1976; Fromherz et al., 1991; Joye et al., 2008). However, in the case of cell-transistor junctions the purely electric approach was not sufficient enough. Several factors influenced the amplitude and shape of extracellularly recorded signals:

1. The cell itself, including type, size, shape, age, expression of ion channels and so forth (Ingebrandt et al., 2005; Fromherz, 2006).
2. The nature of used electrolytes such as the ingredients, electrical conductivity, capacitance or homogeneity (Brittinger, 2004; Wrobel et al., 2005).
3. The contact between a cell and the sensor describing the location and area of contact as well as the surface modifications, which influence the tightness of contact (Buitenweg et al., 2003; Wrobel et al., 2007; Sommerhage et al., 2008; Cohen et al., 2008).
4. The sensor itself especially its type (transistor, electrode, SQUID¹⁷, ...), gate oxide or metal, modifications in the surface structure or material, general geometry, impedance and noise (Maher et al., 1999; Ingebrandt et al., 2005; Joye et al., 2009; Eick, 2010).
5. The analog signal processing, comprising gain, bandwidth or the behavior outside of cutoff frequencies (Wrobel et al., 2007; Schindler, 2008).
6. The data sampling properties, including the sampling rate, aliasing and digital signal processing.

The influence of each point above can be significant or neglectful depending on the other factors. Commonly used point-contact models (PCM) ignored most of the factors mentioned above

¹⁷ Semiconductive quantum interference device to measure minuscule changes in magnetic fields

and were thus subject to enhancements such as area-contact models, which allow a better adjustment of the contact between cell and sensor (Fromherz and Vetter, 1992; Buitenweg et al., 2003; Joye et al., 2008). Hybrid models moved the interpretation of cell-sensor junctions away from purely electric circuits towards biophysical solutions. Ingebrandt (2001) introduced the concept for an extended PCM in which the ion-sensitivity of FETs played a major role for the final signal shape. Wrobel et al. (2005) and Brittinger (2004) brought the concept to life and added electrodiffusion to the PCM. Their approach still used the traditional PCM to simulate electrical signal components, whereas a geometrically reduced electrodiffusion model determined the migration as well as accumulation and depletion of ions in the cleft between cell and sensor. The obtained changes in concentration induced a potential difference between cleft volume and bath, which was also caught by the substrate-integrated FETs in real experiments.

In the following, the extended point-contact model (Ingebrandt, 2001; Wrobel et al., 2005) was generalized to be used for recordings from FETs as well as MEAs. The electrodiffusive model was extended to allow the active¹⁸ change in concentration for more than just one ion species, which was mandatory for the estimation of Cl^-/H^+ -transport ratios. Further extensions incorporated buffers and pH effects to fully and accurately reproduce the effects of ion-sensitivity *in silico*. Geometrical parameters, such as the cell radius or the attached surface area, were applied globally for both the electrical and the electrodiffusive model. Variable or uncertain model parameters were optimized and fitted by an evolutionary strategy to adjust simulated signal shapes to those of actual measurements. All adjustments combined enabled the hybrid model of the cell-transistor junction to estimate the transport ratio of chloride versus protons in ClC antiporters.

7.2. Implementation of the point-contact model and extensions

The experimental data was stored in a binary file format, as used by the software *TIDA 5.45* (HEKA Elektronik GmbH, Germany) for patch-clamp setups (section 3.2.1). The general simulation platform was *Matlab 7.0* (MathWorks Inc., USA). A routine to automatically import RAW data from *TIDA*'s binary files into the *Matlab* workspace is presented in section 7.2.1. While most models were implemented in *Matlab*, electrical simulations of the cell-sensor junction were performed with the point-contact model (PCM) in *PSpice 6.0* (MicroSim Corporation, USA). The necessary interface to mediate data between the two programs is described in section 7.2.2, followed by descriptions of the remaining sub-models to simulate the cell-sensor junction in greater detail.

7.2.1. Importing experimental data

All measured data was recorded with *TIDA* and stored to a hard disk drive. The reverse-engineered structure of a binary *TIDA* data-file is depicted in figure 7.1 and included the stimulation patterns

¹⁸ induced by voltage-gated currents across the cell membrane

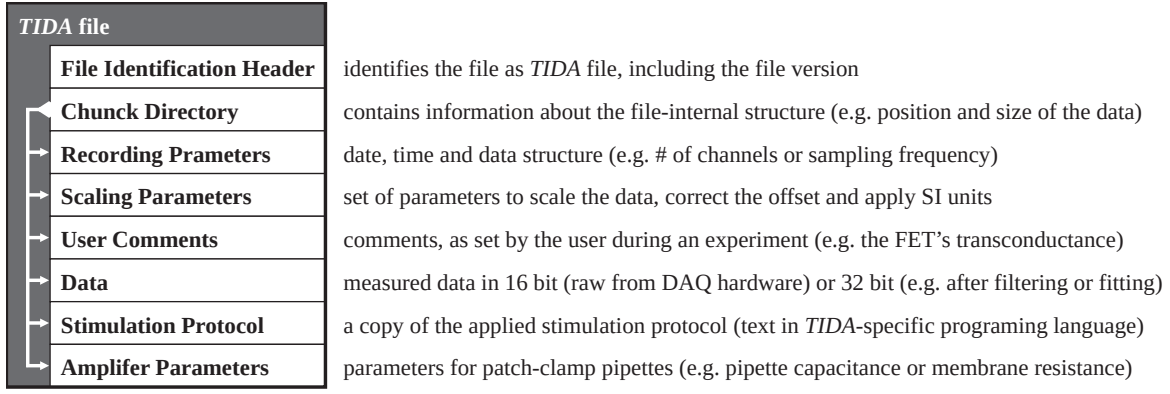


Figure 7.1.: Structural organization of *TIDA* files. The data section included all measured signal traces as an array over time

to set the cell's membrane potential, the resulting membrane currents, the out put from FET or MEA amplifiers as well as numerous parameters of the patch-clamp contact and control voltages for the perfusion system (compare figure 3.5).

The imported data was subject to further processing, where SI prefixes of the imported data were removed by scaling to SI base units (V, A, Ω , F, ...). RAW data from extracellular sensors was further scaled by a factor addressing an FET's transconductance (figure 2.10) or the data-inversion of MEA data. The necessary factors were assigned during the respective experiment and stored as a user-defined parameter within *TIDA* files. For FETs the user-variable g_m was set to the actually measured FET transconductance in mS whereas $g_m = -1$ was assigned during experiments with MEAs to represent the inversion of the MEA amplifier (see section 3.2.4). In the majority of experiments, cells were repetitively stimulated with the same pattern which provided redundant data to increase the signal-to-noise ratio by averaging. An offset of averaged signals from extracellular sensors and intracellularly obtained membrane currents was removed as well.

Using an automated routine to import *TIDA*'s data files into the *Matlab* environment, reduced the risk of errors as usually caused by the user during the manual assignment of parameters and variables (Sommerhage, 2009b). An automated import further allowed the convenient processing and handling of large data amounts as well as sorting data by chosen criteria.

7.2.2. A Matlab-PSpice interface for electrical equivalent circuits

An interface for the automated exchange of data between the programs *Matlab* and *PSpice* was developed in the course of this thesis (Sommerhage, 2009a) to facilitate highly accurate simulations with electrical equivalent circuits. As depicted in figure 7.3a, the *MPS Interface* allowed the import of circuit designs as well as the assignment of parameters to its components in *Matlab*, whereas the actual simulation took place in *PSpice*. The interface further provided the subsequent import of *PSpice*-simulation results back into the *Matlab* environment (Sommerhage, 2008).

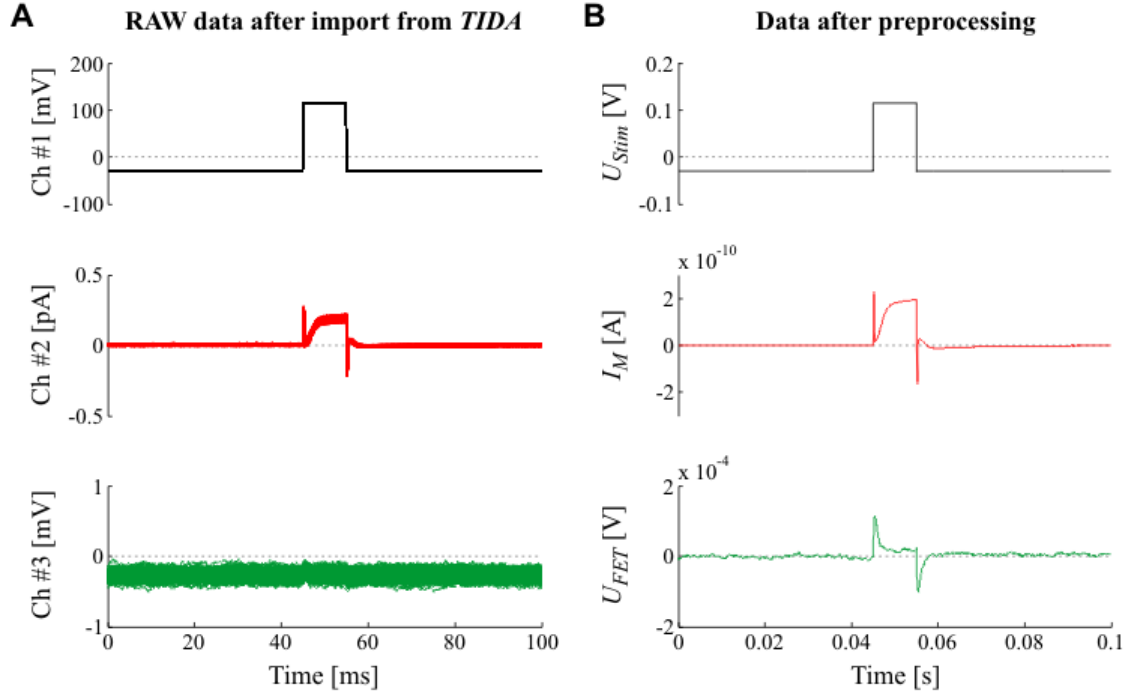


Figure 7.2.: Data traces imported from TIDA. (A) RAW data and (B) after pre-processing.

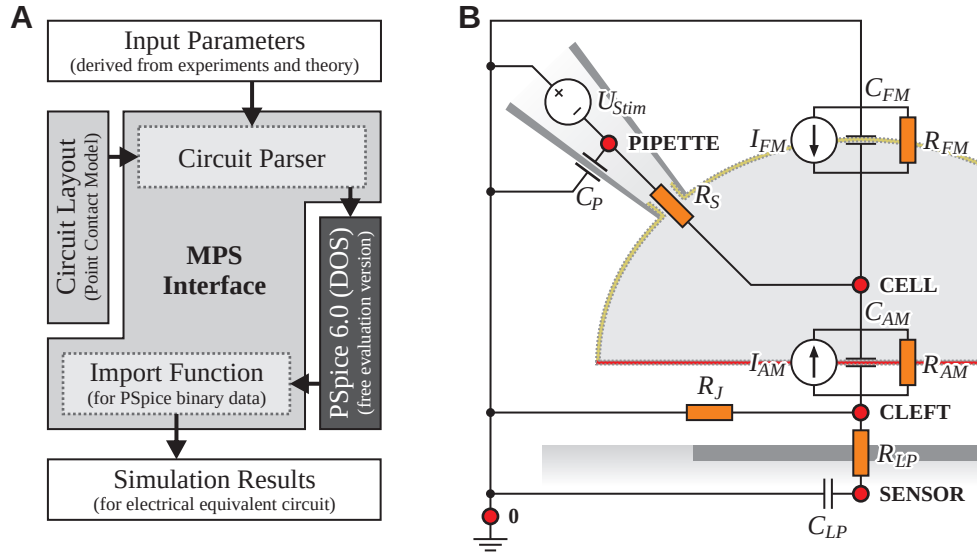


Figure 7.3.: (A) Schematic of data flow in the MPS interface to simulate electrical equivalent circuits of the cell-sensor junction. (B) A slightly modified Point-Contact-Model (compare figure 2.15). The circuit nodes PIPETTE, CELL, CLEFT, SENSOR and 0 (for ground) are indicated with red dots. An exemplary net list is given in table 7.1.

As depicted in figure 7.3b, simulations of the cell-sensor junction were performed with a slightly modified PCM (compare section 2.7). Input parameters were imported from TIDA, further processed to meet simulation standards and then assigned to individual components in the electrical circuit.

Circuit element	1 st node	2 nd node	set-value
U_{Stim}	0	PATCH	PWL file "V_STIM.PWL"
C_P	0	PATCH	7.1 pF
R_S	CELL	PATCH	31.2 M Ω
R_{FM}	0	CELL	23.0 M Ω
C_{FM}	0	CELL	14.1 pF
I_{FM}	0	CELL	PWL file "I_FM.PWL"
R_{JM}	CLEFT	CELL	34.5 M Ω
C_{JM}	CLEFT	CELL	9.4 pF
I_{JM}	CLEFT	CELL	PWL file "I_AM.PWL"
R_J	CLEFT	0	0.1 M Ω
R_{LP}	CLEFT	SENSOR	10 T Ω
C_{LP}	0	SENSOR	20 pF

Table 7.1.: An exemplary node net-list for the Point-Contact-Model as used by *PSpice*. The variables R_J , α_{JM} and l_{JM} were fitted to optimize the simulation output and match the measured FET data (compare figure 7.4)

- Pipette specific parameters were designated directly from imported data. The stimulation voltage U_{Stim} was defined as a *piecewise linear* source (PWL) linked to a file containing time-dependent voltages, which were previously measured during the actual experiment. This instructed *PSpice* to use a particular voltage at each time step during simulations. The pipette capacitance C_P was identical with *TIDA*'s pipette-compensation parameter and could be assigned directly. A series resistor R_S available in *TIDA*'s RAW data, representing the pipette resistance R_P and the resistance R_C of the cytoplasmic environment (figure 2.7) was compatible with the PCM and thus assigned without changes.
- Parameters characterizing the cell had to be scaled since the cell membrane was divided into a free and an attached area (see figures 2.7 and 7.3b). At first, the total membrane area was derived from a compensation factor in the *TIDA* file, which represented the cell's membrane capacitance C_M . According to Gentet et al. (2000), HEK293 cells have a specific membrane capacitance of $c_{sp} \approx 1 \mu\text{F} / \text{cm}^2$. This information allowed an estimation of the entire membrane area A_M with equation 7.1.

$$A_M = \frac{C_M}{c_{sp}} \quad (7.1)$$

As a result of experiments in chapter 4, the area of the attached membrane A_{JM} was found to be approximately 40 % of A_M (Sommerhage et al., 2008), with the ratio

$$\alpha_{JM} = \frac{A_{JM}}{A_M} \quad (7.2)$$

of attached to total membrane. *TIDA*'s RAW data contained the membrane specific parameters C_M and R_M . According to KIRCHHOFF's circuit laws, these electrical components were scaled by α_{JM} , yielding C_{JM} and R_{JM} , whereas the components C_{FM} and R_{FM} in the free membrane were the results of scaling with

$$\alpha_{FM} = \frac{A_{FM}}{A_M} = 1 - \alpha_{JM}. \quad (7.3)$$

The ratio of attached to total membrane area was initially set to $\alpha_{JM} = 2/5$ and later fitted during simulations to match the geometrical conditions of the respective cell.

- Membrane currents were time-dependent data within *TIDA* files that required some corrections for simulation purposes. *TIDA* offered a leak compensation g_L , which automatically subtracted stimulation-dependent leak currents across the cell membrane.

$$I_L = g_L \cdot U_{Stim} \quad (7.4)$$

In most cases, the I_L was not distributed evenly across the whole cell membrane. One major cause for leakage was minor cell damage due to the patch pipette at the free membrane of the cell, whereas the attached membrane remained nearly leak free. In order to compensate for this imbalance, leak currents I_L were recalculated using equation 7.4 and then scaled by the variable factors l_{JM} and l_{FM} . Membrane currents I_M were scaled by α_{JM} as well as α_{FM} . All currents across the free and the attached membrane were then combined to I_{JM} and I_{FM} and were stored as PWL data in files. Similar to the stimulation voltage U_{Stim} , the circuit components I_{JM} and I_{FM} were defined as PWL and linked to those files.

- The junction resistance R_J remained unknown during experiments and had to be fitted in subsequent simulations. However, using a frequency-dependent impedance analysis, [Fromherz et al. \(1993\)](#) was able to define a specific junction conductance g_J with respect to the attached membrane area A_{JM} :

$$g_J = \frac{G_J}{A_{JM}} \quad (7.5)$$

During stimulation on- and off-sets, extracellularly measured signals show capacitive transients that are scaled by the inverse of g_J . [Ingebrandt \(2001\)](#) defined the peak height U_J^0 of these transient voltages as

$$U_J^0 = \frac{1}{g_J} \left(c_{sp} \cdot \frac{\partial U_{Stim}}{\partial t} \right). \quad (7.6)$$

The derivative of U_{Stim} with respect to t basically represents charging of the cell membrane by a voltage step U_{Step} via the patch pipette. Considering an exponential filter effect due to the time constant $\tau = R_S \cdot C_M$ in the circuit, the derivative can be written as

$$\frac{\partial U_{Stim}}{\partial t} = U_{Step} \cdot \frac{1}{R_S \cdot C_M} \cdot \exp\left(\frac{-t}{R_S \cdot C_M}\right). \quad (7.7)$$

The exponential part of equation 7.7 disappears for $t = 0$. Inserting equation 7.7 into equation 7.6 and solving the resulting equation for g_J thus leads to

$$g_J = \frac{1}{U_J^0} \left(c_{sp} \cdot \frac{U_{Step}}{R_S \cdot C_M} \right). \quad (7.8)$$

Entering equation 7.8 into equation 7.5 and solving it for the unspecific junction conductance G_J results in

$$G_J = \frac{1}{U_J^0} \left(c_{sp} \cdot \frac{U_{Step}}{R_S \cdot C_M} \right) \cdot A_{JM}. \quad (7.9)$$

According to equation 7.2, A_{JM} is a part of $A_{Membrane}$, which in turn is obtained by equation 7.1. Applying these conclusions to equation 7.9 reveals

$$\begin{aligned} G_J &= \frac{1}{U_J^0} \left(c_{sp} \cdot \frac{U_{Step}}{R_S \cdot C_M} \right) \cdot \frac{C_M}{c_{sp}} \cdot \alpha_{JM} \\ &= \frac{U_{Step} \cdot \alpha_{JM}}{U_J^0 \cdot R_S}. \end{aligned} \quad (7.10)$$

Since the junction conductance G_J represents the inverse of R_J , the junction resistance can be obtained by

$$R_J = \frac{U_J^0 \cdot R_{Series}}{U_{Step} \cdot \alpha_{JM}}. \quad (7.11)$$

The peak height U_J^0 can be easily obtained from the measurements. However, the amplitude of U_J^0 was further influenced by a lowpass in the amplification setups. R_J obtained with equation 7.11 was therefore used as the initial value. During simulations, the value of R_J was adjusted such that the peak height in the simulation matched the peak in measured data (figure 7.4).

- The amplification units for extracellular recordings were limited in their frequency range. During the design phases of the employed FET and MEA setups, bandpass ranges were attuned with frequencies occurring in electrogenic cells (Offenhäusser et al., 1997; Ingebrandt, 2001). The high-pass circuits for self-compensation had low-frequency limits of $f_c^{FET} \approx 0.03$ Hz in the FET setup and $f_c^{MEA} \approx 0.01$ Hz in the MEA setup. Those values were low enough to be neglected during simulations with electric equivalent circuits¹⁹. However, the upper cut-off frequencies, caused by the cascading amplification stages, had a 3 dB point at 5 kHz in the FET setup and thus cut off the peaks of capacitive responses needed to estimate R_J . Therefore, the low-pass characteristics of the FET setup were modeled with a resistor $R_{LP} = 10$ T Ω and a capacitor $C_{LP} = 20$ pF in the *PSpice* circuitry. The filter characteristics of the MEA setup were more challenging (see section 2.4) and had to be fitted for every experiment, since the individual impedance of each electrode contributed differently to the cutoff features (Wrobel et al., 2007).

The *MPS Interface* parsed the PCM circuit and automatically assigned the provided input parameters for a simulations with *PSpice*. Exemplary circuit definitions were listed in table 7.1. The assigned parameters were defined from theoretical conclusions, directly transferred from the experiment or were slightly modified and then applied (see above for details).

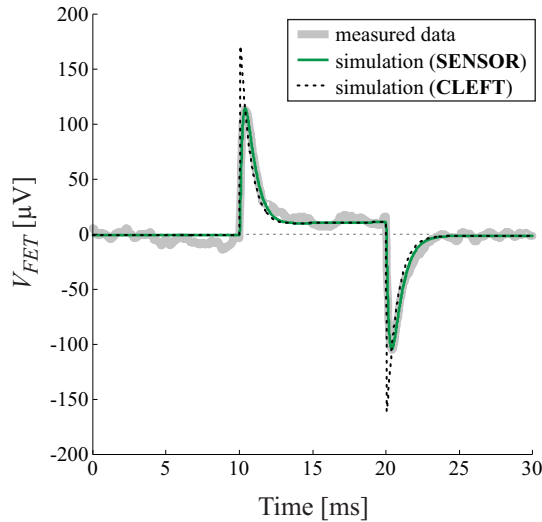


Figure 7.4: Result of PCM simulations. Differences between the voltages simulated for the cleft and simulations of signals filtered by the amplification setup are clearly visible. The average error of this simulation with respect to the measured data was 4.75 μ V

The simulation time-frame was automatically defined according to the experimental time frame. In order to process the model, *PSpice* was called in a hidden DOS thread and subsequent results were automatically imported into the *Matlab* environment for further evaluation. Figure 7.4 depicts the results of a simulation with electrical equivalent circuits for the cell-sensor contact, according to parameters in table 7.1, superposed with measurements.

¹⁹ drift-compensations were included after calculations regarding the ion-sensitivity

7.2.3. Electrodifusion in the cell-transistor junction

Following the PCM simplification, charges of ions in the cleft volume were considered to be only present at one singularity in the center of the cleft (see section 2.7). Ions were able to enter or leave the cleft either across the attached cell membrane above or via the lateral surface along the cleft side (figure 7.5a). In order to migrate from the bath solution into the cleft or vice versa ions were bound to electrodiffusion, whereas migration across the cell membrane was treated as instantaneous. The rather slow electrodiffusion within the cleft caused an accumulation or depletion of individual ion species at the point-contact, resulting in a small voltage drop Ψ_{ED} between cleft and bath. The potential difference was yet significant enough to influence FET and MEA recordings. Since the electrodiffusive components were nonlinear, they were simulated according to the flow depicted in figure 7.5c.

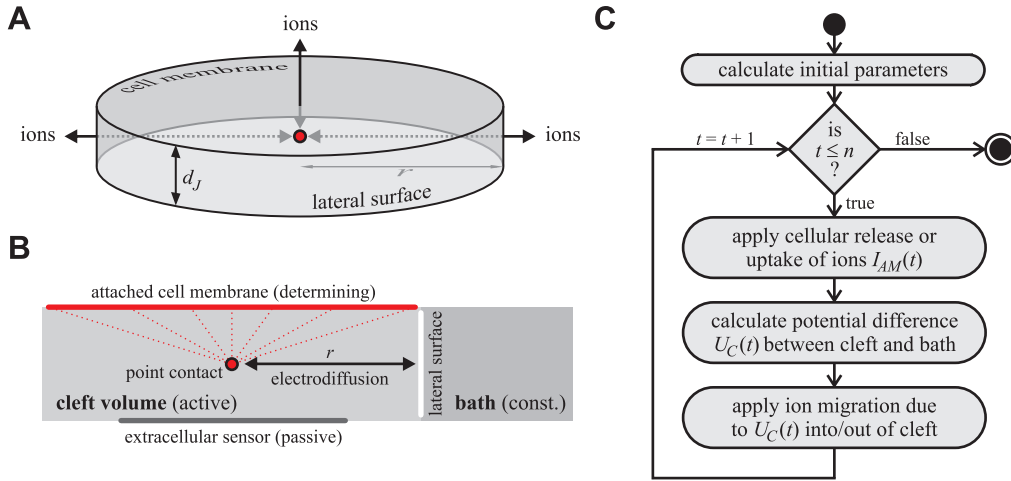


Figure 7.5.: (A) Schematics of the cylindric cleft geometry with radius r and height d_J . Ions can access the cleft either via the attached cell membrane A_{JM} or the lateral surface S_J . Between those two areas of access, ions are bound to electrodiffusion. (B) Compartments of the electrodiffusive PCM extension. (C) Activity diagram for the calculation of potentials caused by the accumulation and depletion of ions and the resulting diffusion along the cleft radius for each time-step t out of n .

Initial parameters concerning the geometrical situation of the cleft, the concentration of ions in each compartment as well as the effects of ion currents across the attached membrane, were defined prior to simulations:

- Parameters describing the cleft dimensions were defined first. Literature values and theoretical estimations were combined to estimate the average cleft height with $d_J \approx 75$ nm (Wrobel et al., 2007). Since d_J could not be monitored or measured during experiments, the cleft height was subject to fitting during parameter optimization. The cleft radius r was calculated to

$$r = \sqrt{\frac{A_{JM}}{\pi}} \quad (7.12)$$

assuming a circular shape. Depending on the ratio of attached to total membrane α_{JM} , the area A_{JM} was calculated from equations 7.1 and 7.2. Subsequently, the cylinder volume V_J of the cleft and its lateral surface S_J were calculated according to equations 2.22 and 2.23 (page 32).

- Ionic concentrations in the cleft c_i^J and the bath c_i^B were applied according to the respective extracellular solution. All other ingredients, including divalent²⁰ ions, were neglected during simulations. Positive and negative charges were chosen such that the overall charge of ions was zero in order to provide electroneutrality. Table 7.2, representatively lists the default concentrations of ions.

Ion species	Na^+	K^+	Cl^-
concentration c_i^J in mM	5	140	145

Table 7.2.: Typical initial ion concentrations for the cleft and the bath in general.

Due to the obvious dimensional difference between cleft ($V_J \approx 50 \mu m^3$) and bath ($V_B \approx 250 mm^3$), ion concentrations in the bath compartment were considered constant during simulations and served as a passive donator or acceptor of ions. Ionic concentrations in the cleft volume were initially the same as in the bath solution ($c_i^J = c_i^B$ for $t = 0$), but were then altered with each simulation step t due to the electrical activity of the attached cell membrane.

- Membrane currents I_M from patch-clamp experiments represented ion currents across the whole membrane A_M . However, only ions passing the attached portion A_{JM} of the membrane were able to enter or leave the cleft. Based on the assumption that ion channels were evenly distributed across the cell surface, I_M was scaled by the ratio α_{JM} . Since I_{JM} represented charges over time, the number of charges N_Q^{AM} moving across the attached membrane was calculated to

$$N_Q^{AM} = \frac{I_{JM} \cdot dt}{e} \quad (7.13)$$

with the elementary charge e and the minimal time-step dt . The latter was determined by the sampling frequency of input data (usually 20 kHz). If I_{JM} was carried by only one ion species, equation 7.13 was applied directly. However, if I_{JM} comprised two (or more) moving ion species, the technical direction of currents for all ion species had to be signed equally:

- both ion species are either positive or negative and migrate into the same direction across the membrane (e.g Na^+ and K^+ moving inwards)

²⁰ The GOLDMAN-HODGKIN-KATZ equation (page 12) was solved assuming only monovalent ions ($|z_i| = 1$)

- both ion species have opposed charges and pass the membrane into opposed directions (e.g. Cl^- moving inwards and H^+ moving outwards)

The amount N_i^{AM} of an ion species i was calculated from the total number of charges N_Q^{AM} by scaling with the quotient q_i and the ion's valence z_i . The product of $q_i \cdot z_i$ determined an ion's share of the total amount of charges. Using the AVOGADRO constant N_A , the number concentration n_i^{AM} of an ion species influencing the cleft was calculated to

$$n_i^{AM} = \frac{N_i^{AM}}{N_A} = \frac{q_i \cdot z_i \cdot N_Q^{AM}}{N_A} \quad \text{with} \quad \sum_i q_i \cdot |z_i| = 1. \quad (7.14)$$

Depending on positive or negative membrane currents I_M , the ion valence z_i determined the direction²¹ of movement as well. The quotient q_i was assumed to be (A) constant over time and (B) constant for different voltages U_{stim} across the membrane. Initially, q_i was defined according to literature values and later fitted during optimization.

Using the definitions and calculations above, simulations were initiated for $t = 0$. Actual simulations, of ion accumulation and depletion in the cleft including electrodiffusion, were started with $t = 1$, according to figure 7.5c. Simulated changes in concentration are listed in figure 7.6.

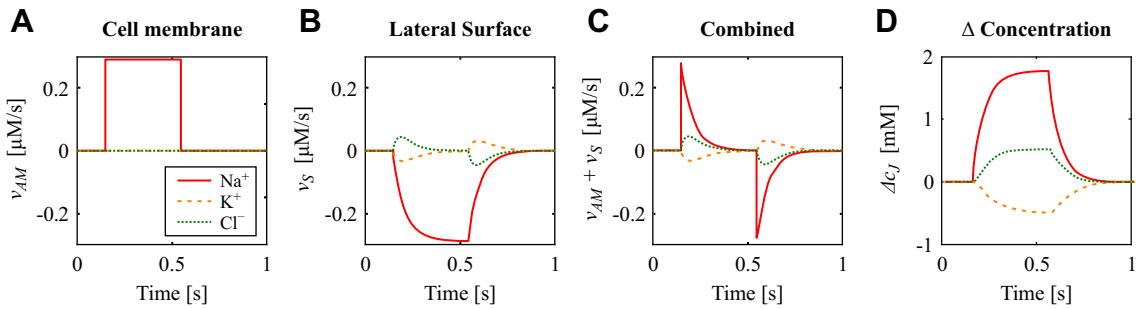


Figure 7.6.: Simulation of changes in ion concentrations in the cleft for an exemplary cell with radius $r = 15 \mu\text{m}$ and a junction distance of $d_J = 100 \text{ nm}$. (A) Changes in ion concentrations in the cleft volume over time due to membrane currents of $I_M = 500 \text{ pA}$ carried by potassium only. (B) The changes in concentrations due to electrodiffusion across the lateral surface. (C) Overall rates v of change due to membrane currents and the flux across the lateral surface. (D) Simulated changes in ion concentrations in the cleft.

1. The amount $n_i^{AM}(t)$ of ion i passing the attached cell membrane was calculated using equation 7.14 and then added to the previous ion concentration $c_i^J(t-1)$ in the cleft

$$\dot{c}_i^J(t) = c_i^J(t-1) + \frac{n_i^{AM}(t)}{V_J} \quad (7.15)$$

²¹ determining the addition to or subtraction from ions in the cleft volume

with respect to the cleft volume V_J . The variable $\dot{c}_i^J$ was an intermediate result, which did not represent the final ion concentration for the current calculation step t .

- Each ion carried a charge z_i . Thus, the resulting difference in concentrations between cleft and bath ($\dot{c}_i^J(t) \neq c_i^B$) lead to a potential difference. $\Psi_{ED}(t)$ was calculated with equation 2.26 (see page 34), a modified GOLDMAN-HODGKIN-KATZ equation, where the permeability coefficients P_i were exchanged by diffusion coefficients D_i (table 7.3).

Ion	Na ⁺	K ⁺	Cl ⁻
D_i [$10^{-5}\text{cm}^2/\text{s}$]	1.33	1.96	2.02

Table 7.3.: Diffusion coefficients D_i of selected ions (al Baldawi and Abercrombie, 1992; Lide, 2002)

Figure 7.7 depicts an exemplary simulation of potential differences during the course of a stimulus pulse. The corresponding fluxes and differences in concentrations were listed in figure 7.6.

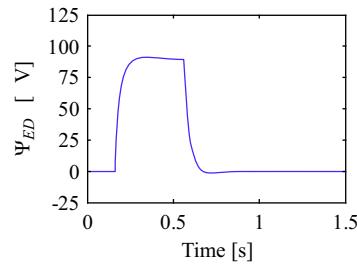


Figure 7.7: Simulation for an exemplary cell with radius $r = 15 \mu\text{m}$, a junction distance of $d_J = 100 \text{ nm}$ and membrane currents of $I_M = 500 \text{ pA}$. The simulated potential difference is caused by unequal ion concentrations in cleft and bath (see figure 7.5d), and drives the electrodiffusion of ions.

- The concentration gradient between cleft and bath lead to a diffusive flux of ions seeking for an equilibrium. In parallel, the potential difference $\Psi_{ED}(t)$ caused an additional electrically driven flux. The comprehensive area-specific flux of ions $\Phi_i^S(t)$, across the lateral surface S_J of the cleft, was implemented according to equation 2.25 using the NERNST-PLANCK term

$$\Phi_i^S(t) = -D_i \left(\frac{\dot{c}_i^J(t) - c_i^B}{r} + \frac{z_i F}{RT} \cdot \dot{c}_i^J(t) \cdot \frac{\Psi_{ED}(t)}{r} \right) \cdot dt \quad (7.16)$$

with the molar gas constant R , the absolute temperature T and the FARADAY constant F . The ionic flux was employed to calculate the number concentration of ions $n_i^S(t)$ entering or leaving the cleft via the lateral surface S_J :

$$n_i^S(t) = \Phi_i^S(t) \cdot S_J \quad (7.17)$$

Finally, $n_i^S(t)$ was added to the intermediate ion concentration $\dot{c}_i^J(t)$ for each ion i , yielding the current concentration

$$c_i^J(t) = \dot{c}_i^J(t) + \frac{n_i^S(t)}{V_J} \quad (7.18)$$

for step t .

The next calculation cycle with $t = t + 1$ was initiated with step 1 until t reached the end of the simulation time-frame. Currents across the cell membrane changed ion concentrations c_i^J in the cleft actively and instantaneous during simulations, whereas the ionic flux Φ_i^S included the diffusion coefficients D_i . Thus, an equilibrium was not reached within one simulation step t and charges could build up within the cleft.

7.2.4. pH and buffer reactions in the cleft

The electrical activity of ClC-transfected HEK293 cells (see section 3.1.3 and chapter 6) was accompanied by the release of protons into the cleft volume. The additional protons interacted with buffer molecules and affected the pH within the cleft. Since FETs are particularly sensitive to pH changes (see section 2.5), proton-buffer reactions had to be a part of simulations. Adjustments were made for the initialization of simulations, declaring the concentrations and diffusion coefficients of ion species, as well as for the calculation of the pH in the cleft volume.

Concentrations

Ion species	Na ⁺	K ⁺	Cl ⁻	H ₃ O ⁺	OH ⁻	H ₂ A	HA ⁻	A ²⁻	SiOH ₂ ⁺	SiOH	SiO ⁻
concentration [M]	0.140	0.005	0.145	10 ^{-pH^B}	10 ^{-(14-pH^B)}	$\alpha_i^{\text{HEPES}} \cdot C_{\text{HEPES}}$			$\alpha_i^{\text{SiOH}} \cdot C_{\text{SiOH}}$		

Table 7.4.: Initial ion concentrations for simulations including buffer effects. The variables pH^B and C_{HEPES} were determined by the respective experimental plan, whereas C_{SiOH} was set according to literature values. The concentrations of step-wise dissociating species were calculated according to their respective pK_a values using α -functions.

The standard electrolytic salts (table 7.2), the water components H₃O⁺, OH⁻ and all involved buffer molecules were added to the simulation as ingredients of the bath solution (table 7.4). Two buffers were present in the cleft volume, (i) HEPES²² dissolved in the electrolyte and (ii) SiOH localized at the electrolyte-oxide interface (figure 2.12). The respective concentrations of (ionized) subspecies were applied as pH-dependent α -fractions of the total concentrations (equation 2.17 on page 28). Charges were associated according to the protonation-state of the respective molecule.

²² 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with C₈H₁₈N₂O₄S

HEPES was introduced by Good et al. (1966) as one out of twelve buffers for cell culture. The distribution diagram for HEPES (figure 7.8a) clearly shows the two equilibrium constants, $pK_{a1} = 3$ and $pK_{a2} = 7.55$. Concentrations of individual subspecies in the cleft volume were determined by the total concentration of HEPES in the bath solution. Three different HEPES concentrations (0.1 mM, 1 mM and 10 mM) were used for experiments in this thesis.

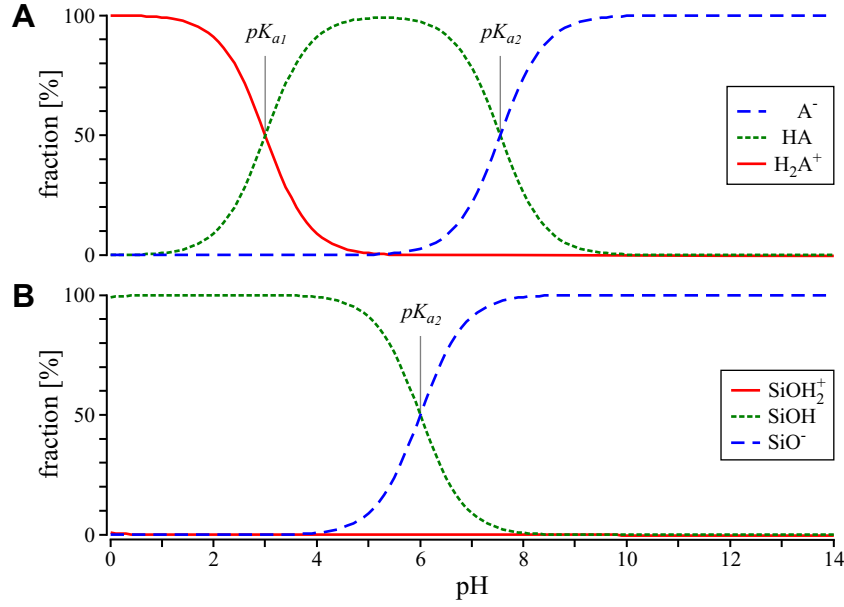


Figure 7.8.: Distribution diagrams for buffers involved in cleft pH. (A) Dissociation of the weak acid HEPES (HA) versus pH. (B) Stepwise ionization of silanols at the electrolyte-oxide interface ($pK_{a1} = -2$)

The SiOH buffer was immobilized at the substrate surface and characterized by two equilibrium constants (van Hal et al., 1995). SiOH had one $pK_{a1} \approx -2$ far outside the typical pH range for water-based solutions, while $pK_{a2} \approx 6$ was reported to be within range for biological pH variations (figure 7.8b). The approximations of pK_{a2} were challenging for simulations, as it was so close to the pK_{2a} values for HEPES (Harame et al., 1987; van Hal et al., 1995). In order to calculate the concentration of SiOH, the amount of silanols in the cleft

$$N_{Si}^J = \pi r^2 \cdot N_{Si} \quad (7.19)$$

was determined via the cell radius r . The density of surface reaction-sites for SiO_2 was mostly reported to be $N_{Si} \approx 5 \text{ nm}^2$ (Harame et al., 1987; Grattarola et al., 1992; van Hal et al., 1996; Liu and Dutton, 2009). However, some studies estimated with $N_{Si} \approx 0.5 \text{ nm}^2$ much less silanol groups at the surface (Lichtenberger and Fromherz, 2007; Veenhuis et al., 2009). The concentration of silanols c_{Si}^J in the cleft volume was calculated to

$$c_{Si}^J = \frac{N_{Si}^J}{N_A \cdot V_J} \quad (7.20)$$

with the AVOGADRO constant N_A and the cleft volume V_J . Inserting equation 7.19 and equation 2.22 (page 32) for the cleft volume into 7.20, leads to

$$c_{Si}^J = \frac{\pi r^2 \cdot N_{Si}}{N_A \cdot \pi r^2 \cdot d_j} = \frac{N_{Si}}{N_A \cdot d_j} \quad (7.21)$$

clearly showing that the concentration of SiOH buffer in the cleft volume was determined exclusively by the cell-substrate distance d_J . A larger cell covered more substrate and thus included more surface reaction-sites, however, the cleft volume increased as well compensating the addition of SiOH. Figure 7.9 shows the concentration c_{Si}^J as a function of the junction distance d_J , according to equation 7.21.

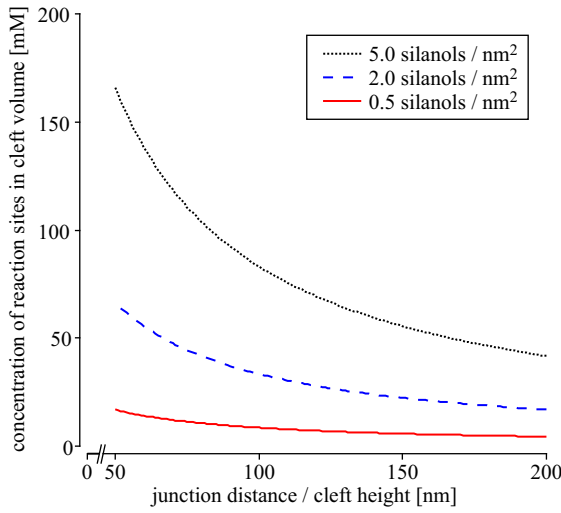


Figure 7.9: Concentration of silanols c_{Si}^J in the cleft volume V_J as a function of the cleft height d_J for three different densities of silanols N_{Si} at the electrolyte-oxide interface.

The presence of the dissolved HEPES buffer and the immobile SiOH buffer at the electrolyte-oxide interface lead to a combined buffer capacity β . Depending on the concentration of HEPES, surface reaction-sites SiOH or the actual pK_{a2} of SiOH, the overall buffer capacity β showed significant differences. Exemplarily buffer capacities, according to equation 2.18 on page 29, were listed in figure 7.10.

Diffusion coefficients

Ion species	Na ⁺	K ⁺	Cl ⁻	H ₃ O ⁺	OH ⁻	H ₂ A	HA ⁻	A ²⁻	SiOH ₂ ⁺	SiOH	SiO ⁻
D_i [10^{-5} cm ² /s]	1.33	1.96	2.02	9.31	5.38	0.51	0.51	0.51	0.25	0.25	0.25

Table 7.5.: Diffusion coefficients including buffers. The diffusion of protons was considerably faster than other cations, HEPES molecules diffused about one order of magnitude slower than H⁺ and silanol groups at the electrolyte-oxide interface were immobile. Transport coefficients for H⁺ between SiOH reaction-sites were initially defined to be slightly slower than the diffusion of HEPES (see text).

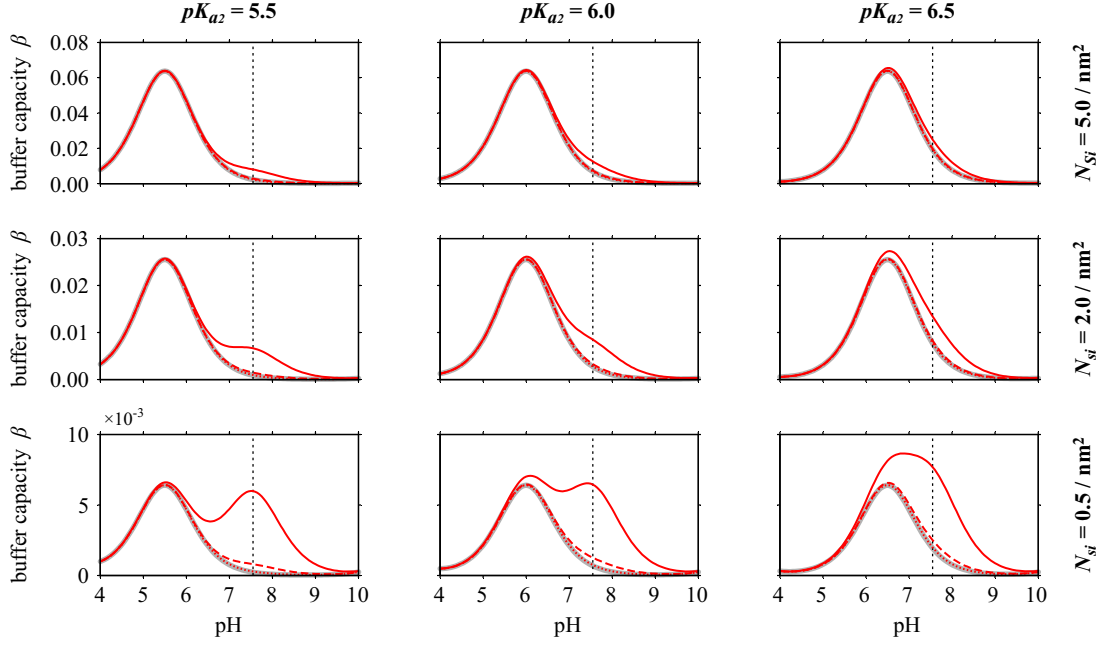


Figure 7.10.: Buffer capacities in the cleft volume, depending on the amount of silanol reaction-sites on the electrolyte-oxide interface (thick gray line), the actual pK_{a2} of the interface and the three different HEPES concentrations, applied during experiments: 0.1 mM (dotted), 1 mM (dashed) or 10 mM (solid). The dotted vertical line indicates the default bath pH of 7.4.

Defining the diffusion coefficients of hydronium and hydroxide was challenging. The actual molecules diffuse rather slow or virtually not at all, whereas hydrogen atoms can be passed on very fast among water molecules. [Ruff and Friedrich \(1972\)](#) investigated the transfer-diffusion rates of H^+ in water and found $D_{H_3O^+} = 9.307 \cdot 10^{-5} \text{ cm}^2/\text{s}$ and $D_{OH^-} = 5.38 \cdot 10^{-5} \text{ cm}^2/\text{s}$. Since [Intharathap et al. \(2006\)](#) confirmed these results, the transfer-diffusion rates were implemented as diffusion coefficients for simulations in this thesis.

[Kovbasnjuk et al. \(2000\)](#) determined the diffusion coefficient of HEPES in water. The diffusion of individual HEPES ions is certainly dependent on their present state of protonation. However, only an overall coefficient of $D_{\text{HEPES}} = 5.1 \pm 0.76 \cdot 10^{-6} \text{ cm}^2/\text{s}$ was provided and diffusion was thus assumed to be equal for all HEPES dissociation states.

The silicon oxide surface provided the second buffer in the simulated system. As the reaction sites were presumed to be immobile, the diffusion of SiOH and its (de)protonated subspecies should be set to zero. Nevertheless, proton diffusion within the plane of the electrolyte-oxide interface could not be neglected. Obviously, protons did not actually diffuse in the cleft volume (cm^2/s) but were rather transferred from one silanol group to the next at the surface (cm/s). Therefore, D_{Si} was not a traditional diffusion coefficient. In order to keep simulations consistent, calculations were based on the assumption that protons bound to the FET surface were still subject to electrodiffusion in three dimensions with the diffusion coefficient D_{Si} .

Additional computing steps during simulations

Additional calculations were implemented between steps 1 and 2 (section 7.2.3 on page 112). Membrane currents I_{JM} across the attached membrane influenced the concentration of protons $\dot{c}_{H_3O^+}^J(t)$, which affected the pH in the cleft. According to Hicks and Gebicki (1986), the reaction rate for HEPES to donate H^+ to OH^- was $k = 5.1 \cdot 10^9 \text{ l}/(\text{mol} \cdot \text{s})$. Therefore, the reaction rate k was faster than the diffusion of any pH related ion (7.5). The rate k was also faster than the sampling frequency of measurements and thus the time step $dt \geq 3 \cdot 10^4 \text{ Hz}$ of simulations. Hence, the new pH balance was calculated before electrodiffusion was taken into account.

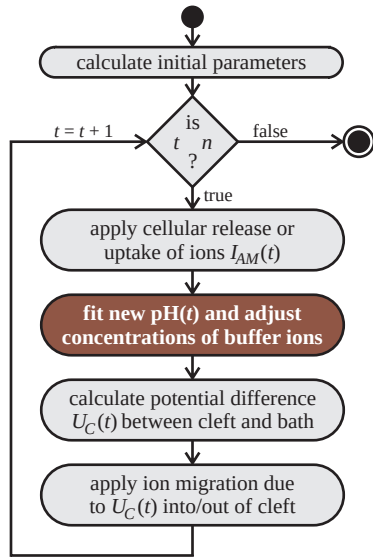


Figure 7.11: The algorithm for the pH equilibration (red box) was included into simulations after the addition of ions migrating across the attached cell membrane and before the calculation of electrodiffusion.

A four step algorithm was implemented, performing the following calculations, to correct the concentrations $\dot{c}_i^J(t)$ of pH-determining ions i in the cleft for each electrodiffusive iteration t :

1. HEPES diffused freely between cleft and bath. Further, due to the charged nature of HEPES, when conjugated with or without H^+ , some ions were driven out of or drawn into the cleft. Consequently, the total concentration of HEPES was not constant for each time step t and needed to be calculated to

$$\dot{c}_{\text{HEPES}}^J = \dot{c}_{\text{H}_2\text{A}^+}^J + \dot{c}_{\text{HA}}^J + \dot{c}_{\text{A}^-}^J. \quad (7.22)$$

The concentration $\dot{c}_{\text{SiOH}}^J$ of surface reaction-sites remained constant.

2. The total concentration of binding sites already occupied by H^+ (acid), whether conjugated with water, HEPES or hydroxyl groups at the substrate, was calculated to

$$\dot{c}_{\Sigma \text{ acid}}^J = \dot{c}_{\text{H}_3\text{O}^+}^J + 2 \dot{c}_{\text{H}_2\text{A}^+}^J + \dot{c}_{\text{HA}}^J + 2 \dot{c}_{\text{SiOH}_2^+}^J + \dot{c}_{\text{SiOH}}^J \quad (7.23)$$

with 2 H^+ in H_2A^+ and SiOH_2^+ . Similarly, the total concentration of all free H^+ -acceptors (base) was calculated to

$$\dot{c}_{\sum base}^J = \dot{c}_{\text{OH}^-}^J + 2 \dot{c}_{\text{A}^-}^J + \dot{c}_{\text{HA}}^J + 2 \dot{c}_{\text{SiO}^-}^J + \dot{c}_{\text{SiOH}}^J \quad (7.24)$$

where 2 free sites were available in A^- and SiO^- .

3. Due to the addition of protons to the cleft, represented by a concentration change in H_3O^+ in the simulation and the possible diffusion of HEPES the concentrations of buffer subspecies were not correlated with the current pH in the cleft. In order to restore an equilibrium among the pH related compounds, the concentration of protons and the pH dependent α -fractions of buffer subspecies had to be reorganized. The following reactions of H^+ were considered to reorganize the cleft pH by fitting:
 - direct reaction of hydronium with hydroxide to water
 - pH depended dissociation of HEPES
 - pH depended protonation of SiOH

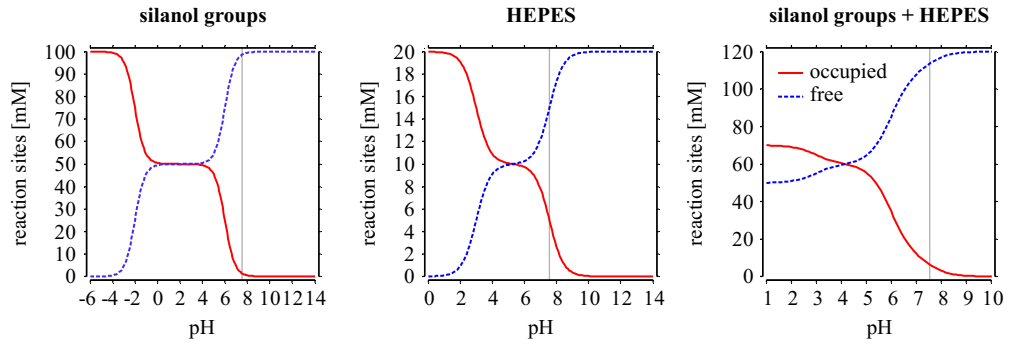


Figure 7.12.: Occupied (red) and free reaction sites (blue) in HEPES and SiOH subspecies as functions of pH. Vertical line indicates standard pH of 7.4 in bath. (A) Concentration of occupied or free H^+ -acceptors in surface reaction sites for an effective concentration of 50 mM SiOH. (B) Concentrations of H^+ -acceptors for 10 mM HEPES. (C) Combined concentrations of H^+ -acceptors in SiOH and HEPES.

The pH defined the concentrations of H_3O^+ and OH^- . Further protons were present in the cleft, associated with HEPES buffer molecules and surface reaction-sites. Figure 7.12 shows the occurrence of H^+ and free H^+ -acceptors in buffer molecules (without those in water) as functions of pH.

Putative pH^{fit} were assigned to the cleft volume during fitting. In order to evaluate the quality of a pH^{fit} value, the sums of H^+ related binding sites $c_{\sum acid}^{fit}$ and $c_{\sum base}^{fit}$ (analog to equations 7.23 and 7.24) were calculated to

$$c_{\sum acid}^{fit} = 10^{-\text{pH}^{fit}} + (2 \alpha_{\text{SiOH}_2^+} + \alpha_{\text{SiOH}}) \cdot \dot{c}_{\text{SiOH}}^J + (2 \alpha_{\text{H}_2\text{A}^+} + \alpha_{\text{HA}}) \cdot \dot{c}_{\text{HEPES}}^J \quad (7.25)$$

where α -fractions were calculated according to equation 2.17 (page 28) as functions of pH^{fit} . Similarly, the total concentration of all free H^+ -acceptors was calculated to

$$c_{\sum base}^{fit} = 10^{-(14-\text{pH}^{fit})} + (2 \alpha_{\text{SiO}^-} + \alpha_{\text{SiOH}}) \cdot \dot{c}_{\text{SiOH}}^J + (2 \alpha_{\text{A}^-} + \alpha_{\text{HA}}) \cdot \dot{c}_{\text{HEPES}}^J. \quad (7.26)$$

The simulated concentrations/amounts of occupied $c_{\sum acid}^{fit}$ and free $c_{\sum base}^{fit}$ binding sites for H^+ were dependent on pH^{fit} and the buffer concentrations and thus in accordance with the α -fractions for the current pH. Nevertheless, simulated concentrations of binding sites had to resemble the current situation in the cleft, where specific concentrations of $\dot{c}_{\sum acid}^J$ and $\dot{c}_{\sum base}^J$ were present. The respective differences between simulated binding sites in the cleft and the binding sites proposed by the fitting routine were calculated as follows:

$$\begin{aligned} d_{acid} &= c_{\sum acid}^{fit} - \dot{c}_{\sum acid}^J \\ d_{base} &= c_{\sum base}^{fit} - \dot{c}_{\sum base}^J \end{aligned} \quad (7.27)$$

The parameter pH^{fit} was alternated until the difference between simulated and fitted binding sites was smaller than 10^{-40} , which is close to

$$d_{acid} = d_{base}. \quad (7.28)$$

Since d_{acid} represented H^+ and d_{base} was the equivalent for OH^- , excess H^+ and OH^- were equal and reacted to water. Consequently, d_{acid} and d_{base} were excluded in the ongoing simulation and the momentary pH of the cleft volume was set to the current pH^{fit} .

4. The concentrations $\dot{c}_i^J(t)$ of pH related ion species i were updated according to the new pH, fitted in the previous step.

Subsequently, effects of electrodiffusion were computed as described earlier (step 2 on page 112). The migration of ions along the cleft included pH determining ions and thus had an influence on the charge balance between occupied and free binding sites for H^+ as well.

7.2.5. Ion sensitivity of field-effect transistors

FETs inherited a strong pH sensitivity caused by reactions between hydroxyl groups at the sensor surface and hydronium or hydroxide in the electrolyte (Bergveld, 2003). The ion sensitivity of FET was not limited to hydrogen but also included other small cations such as potassium, calcium or sodium. In contrast, anions had no effect on the ion sensitivity. This was due to the fact that the concentration of positively charged hydroxyl groups $SiOH_2^+$ was virtually zero for $pH > 2$. At $pH = 7.4$, the fraction of the total concentration of positive reaction sites was $\alpha_{SiOH_2^+} = 15.24 \cdot 10^{-12}$ or less than $10^{-7} \%$, whereas the negatively charged SiO^- groups represented more than 96 % of surface reaction-sites (compare figure 7.12 or figure 7.8). Hence, the cation sensitivity for FETs with SiO_2 as gate-oxide. These cation effects on FET measurements needed to be considered during simulations of the cell-transistor junction. Since concentrational changes in the cleft were rather small (see section 7.2.3), the individual cation sensitivities were considered linear among physiological concentrations. Changes in the measured FET potential, due to ion sensitivities were calculated according to equation 2.13. Equation 2.27 describes potentials induced by the FET's ion-sensitivity as

$$U^{IS} = \sum_i U_i^{IS} = \sum_i S_i \cdot \log \frac{c_i^J}{c_i^B} \quad (7.29)$$

with the sensitivity S_i and concentration c_i^J of ions i .

7.3. Model characterization and optimization

Several sub-models were the basis for the simulation of cell-silicon junctions (sections 7.2.2 through 7.2.5). This section describes the initial calibration and verification of the model with selected data sets and the optimization of most implemented complex multi-parametric functions. Evolutionary algorithms represented one strategy that had proven to be very successful in optimization throughout the history of this planet (Schittkowski, 2002).

7.3.1. Evolutionary algorithm for parameter fitting and model optimization

Evolutionary algorithms like evolution in nature, are based on replication including minor mutations, followed by recombination and subsequent selection to promote a *survival of the fittest*.

They are comprised of genetic algorithms, genetic programming, evolutionary programming and the evolutionary strategy. Since the latter one was used in this thesis, it will be briefly described in this section. For a detailed description of the evolutionary strategy or general information about evolutionary algorithms, the interested reader is asked to turn to publications of [Nachtigall \(2002\)](#) or [Deb \(2009\)](#).

The evolutionary strategy (ES) describes an optimization with regard to multiple objective model-functions aiming at a simultaneous improvement and are described by the following terminology:

$$(\mu/\rho + \lambda)^\gamma - \text{ES}$$

with μ setting the number of individuals surviving after selection, ρ describing the number of parents needed²³ to create offspring by recombination, λ giving the number of children and γ determining the number of generations ([Rechenberg, 1994](#)). The evolutionary strategy (section [7.3.1](#)) was ideal to optimize the model output such that the simulated FET or MEA signals resembled actually measured data as close as possible. Model parameters, whose values were not directly derivable from the experiments or literature, were fitted by a $(15/1+1)^{500}$ -ES. A single-population evolutionary strategy using $\mu = 15$ parameters sets of which only the best $\lambda = 1$ was selected to compete against 14 new and slightly mutated children. Mating was not used. Results after $\gamma = 500$ generations were only accepted if the parameters were within theoretically meaningful ranges.

Two exemplary data sets, obtained from FET measurements with HEK293 cells presented to either 0.1 mM HEPES or 10 mM HEPES, were employed to (a) characterize the model and (b) evaluate the parameter optimization with an evolutionary strategy. A changed value due to the evolutionary strategy was applied equally to the simulation of each data set, optimizing the model for both data sets simultaneously. The quality q of a parameter set was evaluated by the least squares method between simulation and the corresponding measurement:

$$q = \frac{\sum_{i=1}^n \sum_{j=1}^t (y_j^{M_i} - y_j^{S_i})^2}{t \cdot n} \quad (7.30)$$

where t is the number of measured and simulated points, y^M is the measured data, y^S is the corresponding simulation and n is the number of data sets fitted simultaneously. The smaller q , the better the parameter set used to produce y^S . A list of variable model parameters and their value ranges can be found in table [7.6](#).

²³ Mating of two sexes is the highest number of recombination found in nature. In evolutionary theory, however, this number is not limited and can be three or more.

	Parameter	Default Value	Range
Seal resistance	R_J	1 M Ω	[100 k Ω , 5 M Ω]
Ratio of AM and total membrane	α_{JM}	0.4	[0.1, 0.5]
Leak ratio	l_{JM}	0.5	[0.0, 0.75]
Cleft height	d_J	75 nm	[50 nm, 200 nm]
Ratio of Cl ⁻ vs. H ⁺ currents	S_{CLH}	2	[1, 25]
Equilibrium constant	K_{a2}^{Si}	6	[5, 8]
Diffusion coefficient ($\times 10^{-5}$)	D_{Si}	0.25 cm ² /s	[0.05 cm ² /s, 0.5 cm ² /s]
Binding sites	n_{Si}	5 nm ⁻²	[0.1 nm ⁻² , 10 nm ⁻²]

Table 7.6.: List of variable model parameters subject to fitting by the evolutionary strategy.

The seal resistance R_J , the ratio of attached to total membrane α_{JM} and the leak ratio l_{JM} were fitted in a first run to match the electrical equivalent circuit to the capacitive peak in measured data (see figure 7.4). All other parameters were subsequently fitted for the simulation of pH, electrodiffusion and ion-sensitivity. The essential parameter S_{CLH} to describe the stoichiometry of Cl⁻/H⁺-antiporters was initialized with literature values and further optimized during fitting. The cleft height d_J was adjusted to scale the cleft volume and influence the concentration of ions. Initial parameters for the buffer-characteristics of silanol groups were obtained from literature (see page 114) and then optimized during fitting as well. The diffusion coefficient for protons within the electrolyte-oxide interface could not be found in the literature. Therefore, the initial value for D_{Si} was assumed to be slightly slower than the diffusion of HEPES and initialized with $0.25 \cdot 10^{-5}$ cm²/s.

7.3.2. Fitting parameters of the electric equivalent model

Figure 7.13 shows simulation results with optimized parameters for the purely electric component of the cell-silicon junction superposed with the respective FET measurements. The seal resistance $R_J = 0.57$ M Ω , the ratio of total to attached membrane area $\alpha_{JM} = 0.43$ and the contribution of the leak to currents across the attached membrane $l_{JM} = 0.12$ were simultaneously fitted to both the experimental data with 0.1 mM and 10 mM HEPES.

The simulated transients matched actually measured transient (figure 7.13a). Differences in the peak height did not result from variations in the seal resistance R_J , but were perfectly simulated with measured patch-clamp parameters. The membrane capacitance C_M , the membrane resistance R_M and the series resistance R_S were subject to small fluctuations over time. Especially the latter one can determine the peak height significantly, as R_S (also named access resistance) influences the injected stimulus pulse (see section 7.2.2). During this experiment, R_S dropped within 10 minutes from 42.76 M Ω to 17.72 M Ω and thus provided a better access resistance for the stimulus pulse.

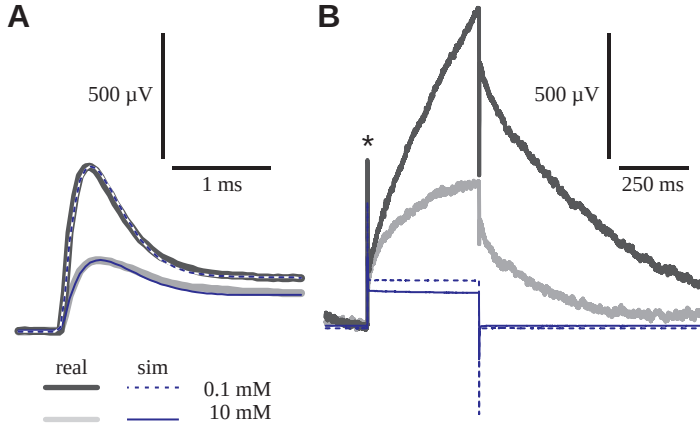


Figure 7.13: Superposition of experimental data (grey) and PSpice-simulations (blue). Detailed view of peak transients (A) and full traces (B). The asterisk marks the transient peak shown in (A).

Simulated electrical FET-signal components for the full experimental time length, past the initial transient peak (figure 7.13b), underestimated the measured data by far. These differences between simulations with electric equivalent circuits, such as the PCM, and actual measurements were observed in the past (Wrobel et al., 2005; Ingebrandt et al., 2005; Brittinger, 2004).

7.3.3. Fitting parameters for pH and electrodiffusion

The missing potentials in figure 7.13b were attributed to electrodiffusive potentials and the effects of pH changes on the ion-sensitivity of FETs. Those effects were fitted with their respective HEPES concentrations. Figure 7.14 exemplarily shows changing model parameters during evolutionary fitting. After about 150 generations, changes slowed down and parameters became more stable.

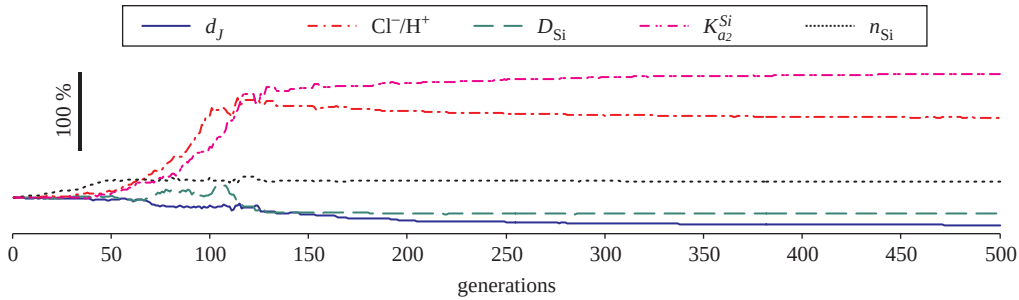


Figure 7.14.: Changing model parameters during evolutionary fitting. The changes were normalized to their initial value (table 7.6).

Table 7.7 lists fitted parameters for the electrical equivalent circuit and for electrodiffusion after evolutionary fitting for 500 generations. All parameters were well within the allowed ranges. The optimized ratio of Cl^- versus H^+ was twice as large as the stoichiometry reported by (Scheel et al., 2005). The equilibrium constant K_{a2}^{Si} for the silanol groups was fitted to be almost 25 % larger than published values (van Hal et al., 1995) and further was with 7.4 exactly at the set bath pH. The amount of silanol binding-sites n_{Si} was found to be only about half of published amounts.

The optimized parameters determined simulated processes within the cleft volume. The results of cleft-internal changes will be listed in the following.

Parameter	Fitted value
Seal resistance R_J	0.57 M Ω
Membrane ratio α_{JM}	0.43
Leak ratio l_{JM}	0.12
Cleft height d_J	64 nm
Ratio of Cl^-/H^+ S_{ClH}	3.53
Diffusion coefficient D_{Si}	$0.17 \cdot 10^{-5} \text{ cm}^2/\text{s}$
Equilibrium constant $K_{a_2}^{\text{Si}}$	7.40
Binding sites n_{Si}	2.85 nm^{-2}

Table 7.7: Fitted model parameters for the simulation of FET measurements from HEK293 cells expressing ClC-4 antiporters in the presence of 0.1 mM and 10 mM HEPES at pH 7.4. The first two parameters were fitted to the transient peak in electrical components. All other parameters were fitted during the simulation of electrodiffusive components and effects of ion-sensitivity.

A depolarization of the membrane potential activated Cl^-/H^+ antiporters (ClC-4), which changed the concentration of ions in the cleft between cell and FET (figure 7.5 on page 110). The rates v of change in ion concentrations were calculated using measured membrane currents I_{JM} , according to section 7.2.3. Figure 7.15a depicts the antiporter-determined rate v_{JM} of change for the concentrations of hydrogen and chloride in the cleft volume. The differences in concentration between cleft and bath induced the electrodiffusion of ions across the lateral surface from the bath into the cleft and vice versa. The corresponding rates v_S of change in ion concentrations are shown in figure 7.15b. Sodium, potassium and chloride diffused across the lateral surface of the cleft volume to compensate the difference between the loss of negative charges due the transport of chloride into the cell and the addition of positive charges in the form of protons to the cleft.

During the on-set of stimulation sodium and potassium diffused out of the cleft, whereas they diffused into the cleft as soon as the chloride transport across the cell membrane ceased. Protons, transported across the cell membrane reacted with pH-buffers in the cleft. Molecules of the HEPES buffer (HA and A^-) were subject to electrodiffusion in the cleft, whereas protons bound to the SiOH buffer were only able to diffuse within the electrolyte-oxide interface.

Since the diffusion of most ions was slower than the transport of ions across the cell membrane, certain ion species accumulated or depleted in the cleft. The simulated changes in concentration for all considered ion species are listed in figure 7.16. Simulated changes in concentrations of ions were within the range of earlier reported changes (Wrobel et al., 2005; Brittinger and Fromherz, 2005; Pabst et al., 2007). Compared to the simulated data for 0.1 mM HEPES, the respective concentration of chloride, sodium and potassium in the case of 10 mM HEPES changed at least two times less (figure 7.16a). In the case of 0.1 mM HEPES, changes in pH were about two times larger than for 10 mM HEPES. The changes in concentration of dissociated buffer ions (figure 7.16c and d) suggest that the curve shapes and amplitudes are strongly dependent on the provided membrane currents I_{JM} (compare figure 7.15). This observation was further supported by the

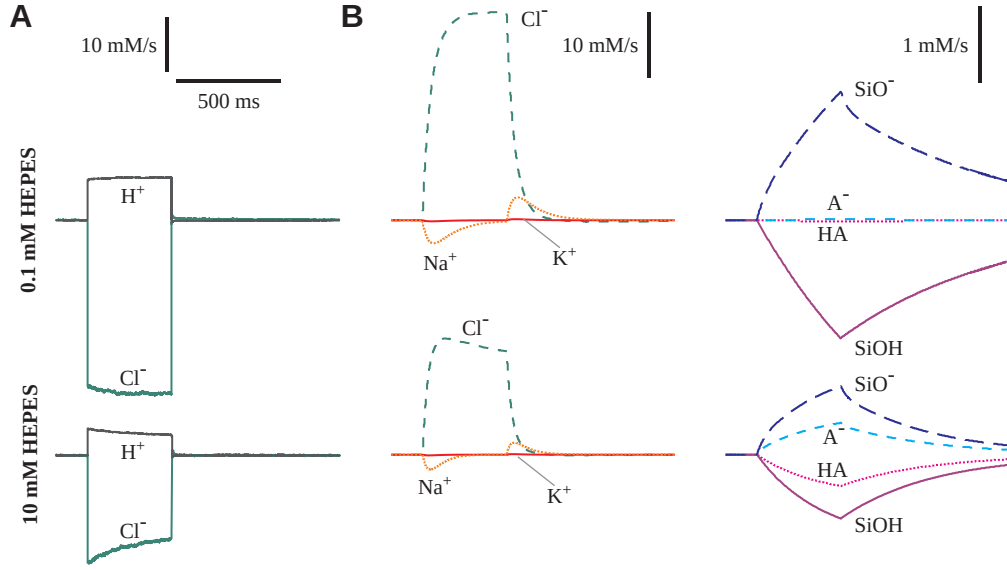


Figure 7.15.: Simulated rates v of concentration change due to membrane currents (A) and electrodiffusion in the cleft (B) according to parameters in table 7.7. Other ion-species were subject to electrodiffusion as well (see figure 7.16), however, their rates v were too small to be represented in (B). Simulation results for 0.1 mM (top) and 10 mM HEPES (bottom).

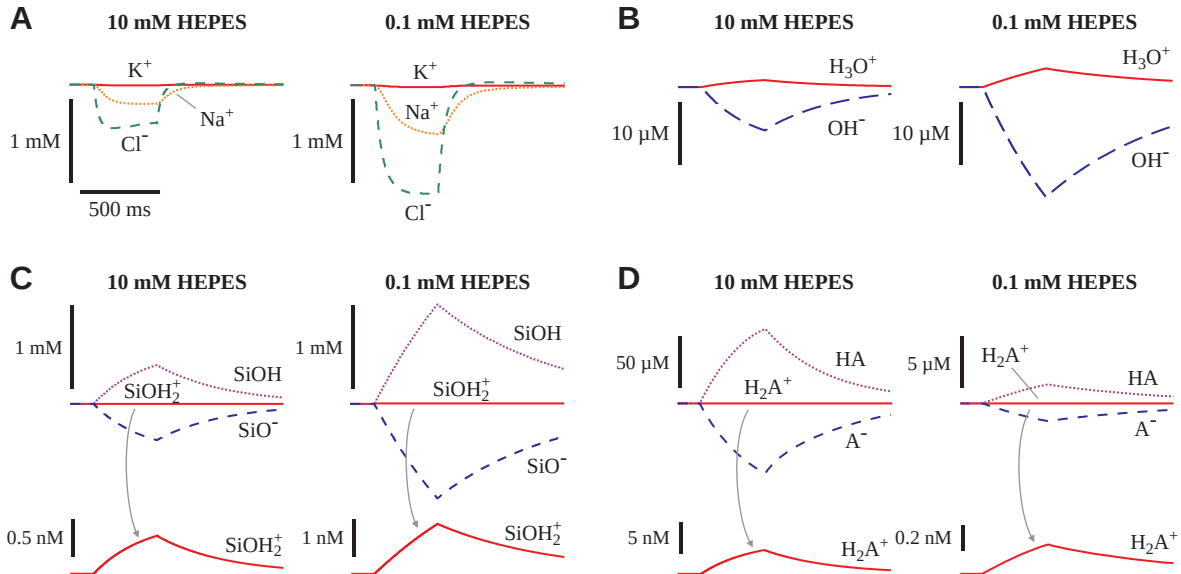


Figure 7.16.: Changes in ion concentrations during ClC-4 activity: (A) Sodium, potassium and chloride, (B) pH determining ions hydronium and hydroxide, (C) buffer ions at the electrolyte-oxide interface and (D) HEPES-buffer ions. Note different scale bars.

calculated potential difference due to concentration differences of ions inside the cleft volume and the bath (figure 7.17).

The dissociation of buffer molecules during the activation of Cl^-/H^+ antiporters is depicted in figure 7.18a. The cleft pH (figure 7.18b) changed by one-tenth of a percent from initially 7.4 to 7.387 in the case of 10 mM HEPES and from 7.4 down to 7.369 with only 0.1 mM HEPES. Simulating the ion-sensitivity of FETs for pH changes resulted in a potential change of about

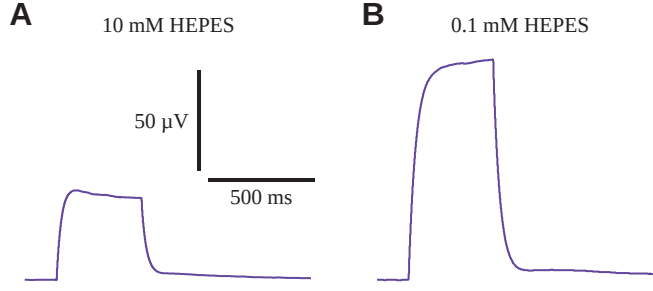


Figure 7.17: Calculated potential difference due to concentration differences between cleft and bath for 0.1 mM HEPES and 10 mM HEPES.

500 μ V and 1 mV for the respective HEPES concentrations (figure 7.18c). Potential changes due to the sensitivity of FETs for potassium ions were negligible.

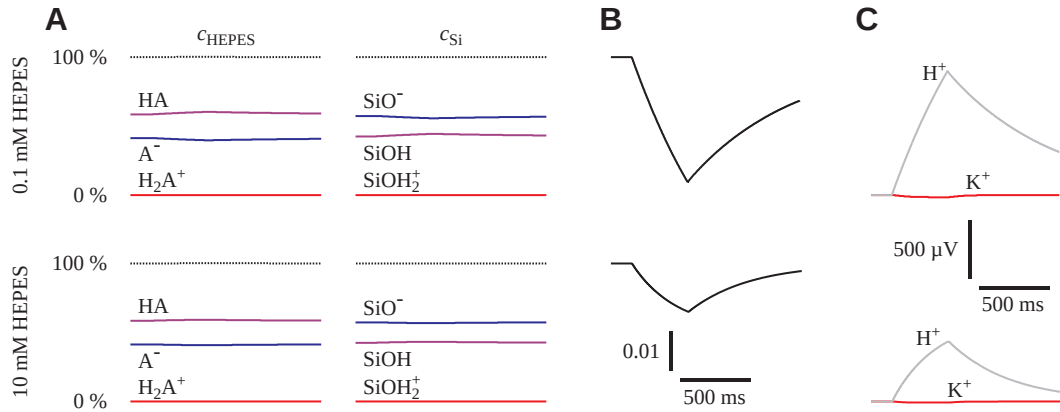


Figure 7.18.: Simulated pH and buffer effects in the cleft volume: (A) dissociation of conjugated HEPES buffer molecules (left) and conjugated SiOH at the electrolyte-oxide interface (right). (B) Change in pH during ClC-4 activity. (C) Additional FET potentials due to the cation sensitivity for hydrogen and potassium.

Figure 7.19 shows the sum of all simulated FET potentials for the respective HEPES concentration superposed with the actual FET measurements. The model successfully simulated FET signals in duration and amplitude. Only small disagreements in the kinetics were found for the higher HEPES concentration (see arrow heads). A reasonable cause could be the diffusion constant for HEPES, which had to be assumed to be equal for all three protonation states. Compared to sodium, potassium and chloride, HEPES was a rather large molecule and might have diffused slower in the narrow cleft volume than stated in standard literature.

7.3.4. Reviewing model parameters

Fitting with too many variable parameters can cause over-fitting²⁴. In order to gain a better understanding of the importance of specific model parameters, the results in table 7.6 were questioned by excluding the Cl⁻/H⁺ stoichiometry S_{ClH} , the equilibrium constant K_{a2}^{Si} and/or the number of

²⁴ multiple optimal parameter-sets can be found, whose values are often contradictory

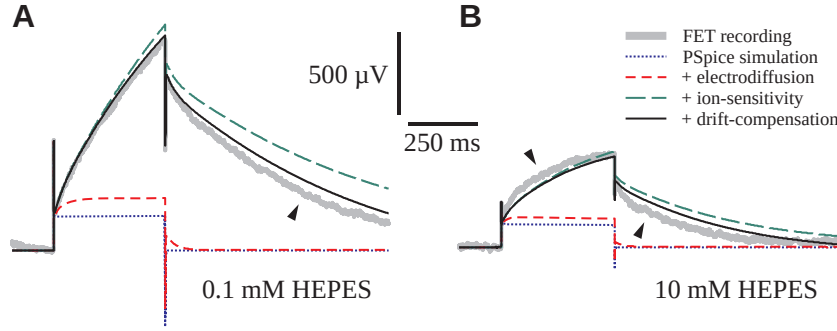


Figure 7.19.: Simulated FET signals for measurements with CIC-4 HEK293 cells, consisting of electrical and electrodiffusive components as well as effects of pH and ion-sensitivity. Simulations also include the drift-compensation as high-pass filtering with $\tau = 4.9$ s. Arrow heads indicate differences between simulation and measured data. (A) 0.1 mM HEPES, (B) 10 mM HEPES.

silanol groups n_{Si} from otherwise equal fitting processes. Table 7.8 lists the obtained parameters after evolutionary optimization for 500 generations.

Run	Fixed parameters	Parameters				
		d_J [nm]	S_{ClH}	D_{Si} [$\times 10^{-5}$ cm ² /s]	$K_{a_2}^{Si}$	n_{Si} [nm ⁻²]
1	none	64.3372	3.5267	0.16944	7.3998	2.848
2	$K_{a_2}^{Si} = 6$	56.9611	3.5351	0.17450	-	18.49
3	$n_{Si} = 5$	98.0069	2.0664	0.18515	6.9697	-
4	$n_{Si} = 5$; $K_2^{Si} = 6$	31.9792	17.841	0.13131	-	-
5	S_{ClH}	99.1788	-	0.18448	7.0221	4.8607
6	S_{ClH} ; $n_{Si} = 5$	96.1100	-	0.18557	6.9925	-
7	S_{ClH} ; $K_{a_2}^{Si} = 6$	77.5082	-	0.18459	-	27.1889
8	S_{ClH} ; $K_{a_2}^{Si} = 6$; $n_{Si} = 5$	simulation not successful				

Table 7.8.: List of alternative model parameters obtained by fitting with restrictions. Outliers were marked in red.

The first row in table 7.8 contains all fitting results from the previous section (page 124). The following rows list alternative results obtained by superseding certain parameters with literature values during the fitting process. In runs 1 through 7, the simulated curves resembled the measured data similarly (exemplarily figure 7.19). No parameters could be found in run 8 to reproduce the measured data. The fixation of K_2^{Si} to its literature value 6 lead to unrealistic parameters (run 2, 4 and 7) during fitting or even corrupted a successful simulation (run 8). The outcome of the remaining optimizations suggest that $K_{a_2}^{Si} = 7$ (run 3, 5 and 6). The results from run 3, 5 and 6 were in general very similar and thus provide a meaningful set of parameters for the underlying data sets. For all following simulations and optimizations the default equilibrium constant $K_{a_2}^{Si}$ was set to 7, the number of silanol groups n_{Si} was implemented with 5 per nm² and $0.185 \cdot 10^{-5}$ cm²/s was applied as the diffusion coefficient for protons within the electrolyte-oxide interface. The most

variable parameters were therefore the cleft height d_J and the stoichiometry of the Cl^-/H^+ antiport $S_{\text{Cl}/\text{H}}$.

7.3.5. Reproducibility of fitted results

The evolutionary strategy was powered by randomized mutations in parameters, gradually leading to a local optimum in the parameters space. Depending on the initial parameter set, fitting can end up with different results and yet look perfect in quality. One important step to avoid such a variability is the application of physically meaningful parameter ranges (see table 7.6). In order to further test the reproducibility of fitting-results the initial parameters were altered, offering the evolutionary strategy to approach an optimum from different directions. Table 7.9 lists fitting results for varying start parameters.

Run	Initial parameters		Fitted parameters	
	d_J	q_{ClH}	d_J	q_{ClH}
1	100 nm	2.0	76.6557 nm	1.9948
2	100 nm	2.0	76.6125 nm	1.9949
3	200 nm	5.0	76.5199 nm	1.9951
4	200 nm	0.5	76.8201 nm	1.9948
5	50 nm	1.0	76.6087 nm	1.9973

Table 7.9.: Reproducibility of fitting results for different initial input parameters.

The fitting results were stable with respect to varying initial parameters. Out of five test runs, the cleft height was fitted to be $d_J = 76.4 \pm 0.5$ nm and the stoichiometry was fitted to be $S_{\text{ClH}} = 1.995 \pm 0.001$. The standard variations offer enough safety to assume the same results within a physically reasonable parameter space.

7.3.6. Fitting unknown data sets with pH variations

Results from experiments with variations in the initial pH were employed to exemplarily test the model on unknown data sets. ClC-4 transfected HEK293 cells were cultured on FETs. The cells were presented to one out of five different pH values, before Cl^-/H^+ -antiporters were activated by stimulation via a patch clamp pipette (see section 6.2.4). Fitting all five data sets in parallel (similar to earlier HEPES fittings) did not lead to successful result. Therefore, each data set was fitted individually. In addition to the standard fitting parameters (table 7.6), also the initial pH value was treated as a variable. The underlying rationale lies in the exchange of bath solutions, which could only be performed partially while the cells were contacted with the patch clamp pipette. The final pH was therefore very likely to be influenced by the previous pH in the bath. Figure 7.20 shows the simulation results in comparison with actual measurements.

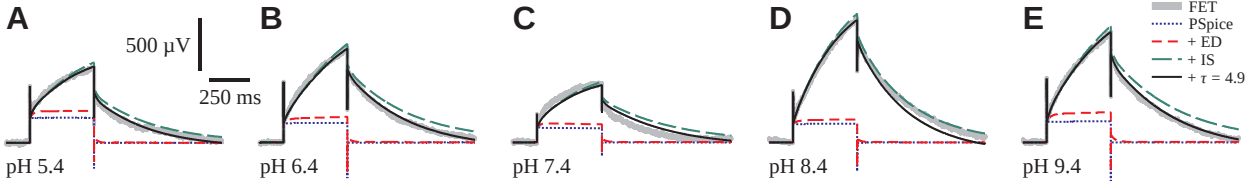


Figure 7.20.: Simulated FET signals for measurements with CIC-4 HEK293 cells, consisting of electrical and electrodiffusive components as well as effects of ion-sensitivity. Simulations also include high-pass filtering with $\tau = 4.9$ s. (A-E) Indicated pH values represent intended initial pH, not the fitted pH.

In terms of signal-shape reconstruction, the simulation results were perfectly acceptable. Different signal amplitudes were traced back to variations in membrane currents. The corresponding parameters are listed in table 7.10.

Parameter	(intended) initial pH				
	5.4	6.4	7.4	8.4	9.4
Seal resistance R_J [$M\Omega$]	0.50333	0.61162	0.59546	0.62062	0.59075
AM ratio α_{JM}	0.28441	0.29861	0.29969	0.27783	0.28798
AM leak ratio l_{JM}	0.19837	0.22062	0.18602	0.18764	0.18468
Cleft height d_J [nm]	92.1635	76.1013	73.4443	76.5825	80.5641
Cl^-/H^+ -stoichiometry S_{ClH}	5.5442	2.0082	2.0214	2.0046	2.6599
fitted pH	6.6466	6.7228	7.5917	7.9039	6.8541
pH error	1.2466	0.3228	0.1917	-0.4961	-2.5459
previously intended pH	9.4	8.4	media	7.4	6.4

Table 7.10.: Fitted parameters for simulations with varying initial pH. (Rows 1-3) parameters for electrical equivalent model. (Rows 4-6) parameters for electrodiffusion and pH. (Rows 7 and 8) additional information of experimental plan.

Although the fitting was performed for each parameter set individually, results for the electrical equivalent circuit were in good agreement. The average seal resistance was fitted to be $R_J = 0.58 \pm 0.05 M\Omega$, the average ratio of attached to total membrane was fitted to be $\alpha_{JM} = 0.29 \pm 0.01$ and the leak ratio was fitted to be $l_{JM} = 0.20 \pm 0.02$. The respective standard deviations were small enough to assume successful fitting.

Fitting results for simulations comprising electrodiffusion, pH and ion-sensitivity resulted in partially contradictive parameter sets. According to the fitted parameters, the cleft height d_J increased for pH values smaller as well as larger than 7.4. Since neither the seal resistances R_J nor the attached membrane ratios α_{JM} reflected this trend, the results have to be interpreted carefully. Especially the values for the Cl^-/H^+ -stoichiometry showed large differences for pH 5.4 and 9.4. A pH-dependence of CIC-4 activity was not reported in literature. More likely was the influence of extra charges at the gate surface due to the pH changes with 34.2 mV/pH, which changed the transconductance g_m (see section 2.5) and thus measured signals. Future experiments with changing initial pH should consider frequent verifications of the transconductance. Another plausible

explanation could be influences of binding kinetics between silanol groups and H^+ , affecting the signal shape of measured FET signals. On the biological side, progressing cell damage could have lead to unexpected results, as measurements for pH 5.4 and 9.4 were performed last in an experiment lasting about 45 minutes with multiple exchanges of the complete bath solution. Finally, the model itself could have caused the unexpected results, since simulations at low buffer capacitances were difficult to solve numerically. The average fitted parameters, excluding pH 5.4 and 9.4, were $d_J = 75 \pm 2 \text{ nm}$ and $S_{ClH} = 2.01 \pm 0.01$.

Differences between the intended initial pH and the eventually fitted initial cleft pH were probably caused by the experimental plan, where the bath pH was changed in the following order: $7.4 \rightarrow 8.4 \rightarrow 6.4 \rightarrow 9.4 \rightarrow 5.4$. Culturing media was removed before the first solution with pH 7.4 was applied. Presumably, the intended pH values were not established, as the respective preceding solutions could not be removed completely. A shifting of intended pH values towards the previous one supports this conclusion (table 7.10).

7.3.7. Sensitivity analysis of model parameters

Due to fluctuations in membrane currents I_M during the course of experiments, a purely amplitude- and shape-based interpretation of FET measurements from transfected HEK293 cells presented to HEPES or initial pH variations was difficult or not possible. However, the simulation-aided interpretation lead to fitted parameter sets, which were very conclusive and gave valuable insights into diffusion processes and pH reactions within the cleft as well as effects of the ion-sensitivity of FETs. The transport stoichiometry S_{ClH} was determined with great precision.

The sensitivity test, described in the following, gave further information about the influence of changes in input parameters on the shape and amplitude of the simulated curve. Tested parameters were the HEPES concentration and the initial pH in the cleft as well as the cleft distance d_J , the Cl^-/H^+ -stoichiometry S_{ClH} , the ratio of attached to total membrane α_{JM} and membrane currents I_M . Since parameters obtained from real measurements fluctuated too much, a representative artificial parameter set was created to test the model. Selected parameters, listed in table 7.11, were analyzed individually for their influence on the simulated shape and amplitude despite the fact that some of them were dependent on each other. For instance, the seal resistance R_J for the electric equivalent circuit correlated to the cleft height d_J and cell radius r in the model for electrodiffusion and pH.

Figure 7.21 depicts FET-signal simulations with five different HEPES concentrations in the bath/cleft volume. Simulations with the highest HEPES concentration (figure 7.21A) reached a steady state at the end of the stimulation period. However, the biocompatibility of 1 M HEPES would be questionable. A reduction of the HEPES concentration by one order of magnitude caused prominent changes (about $500 \mu\text{V/pHEPES}$) in the FET-signal amplitude (compare panels A, B and C), whereas the HEPES concentrations used during experiments in this thesis (panels C-D)

Parameter	Default value	Parameter	Default value
C_P	7 pF	HEPES	10 mM
R_S	20 M Ω	pH	7.4
C_M	21 pF	S_{ClH}	2
R_M	14 M Ω	d_J	75 nm
l_{JM}	5 %	D_{Si}	$0.185 \cdot 10^{-5} \text{ cm}^2/\text{s}$
R_J	75 M Ω	K_s^{Si}	7
I_M	0.5 nA	n_{Si}	5 nm^{-2}
α_{JM}	2/5		

Table 7.11: Model parameters calculated for an artificial cell with radius $r = 15 \text{ }\mu\text{m}$. Parameters on the left are mostly determining the electric component of FET signals, whereas parameters on the right influenced processes for electrodiffusive, pH and ion-sensitive signal components.

had barely any influence on the amplitude of simulated FET-signals ($5 \text{ }\mu\text{V/pHEPES}$). This prominent effect was due to the second buffer, up to 100 mM silanol groups at the electrolyte-oxide interface, which virtually became the only buffer in the cleft once HEPES drops below 10 mM (compare figure 7.10). Therefore, the large differences in signal amplitudes measured in chapter 6 or simulated in section 7.3.3 had to be attributed to different membrane currents I_{JM} .

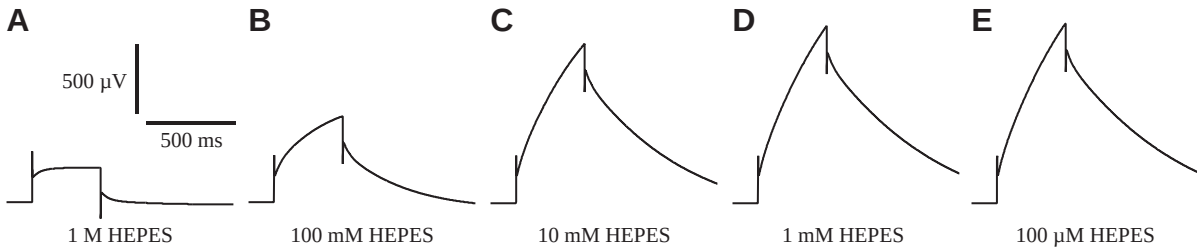


Figure 7.21.: Sensitivity analysis for HEPES concentrations. Simulated FET signals consist of electrical and electrodiffusive components as well as effects of pH and ion-sensitivity. Simulations further include high-pass filtering with $\tau = 4.9 \text{ s}$. (A-E) HEPES concentrations as indicated.

Simulations with varying initial pH in the cleft influenced the buffer capacity as well. Modifications of the HEPES concentrations only influenced the HEPES buffer capacity, whereas the initial pH also determined the capacity of the immobile SiOH-buffer in the cleft. Figure 7.22 shows simulations for five different initial pH values. The pH values 5.4 and 9.4 resulted in extremely high FET amplitudes and further had very different shapes. The simulation for pH 5.4 showed a steep almost linearly increasing shape, while the shape for pH 9.4 was rather rounded. The different curve shapes are related to the buffering effects of water, while the capacity of HEPES and SiOH buffers was minimal. The addition of protons released by the cell into a cleft with pH 5.4 had a smaller impact on the logarithmical calculated pH than the addition of the same amount of protons into a cleft with pH 9.4. The smallest simulated amplitude was observed for pH 7.4, where both buffers, HEPES with $K_{a2} = 7.55$ and SiH with $K_{a2} = 7.0$, were close to their respective equilibrium.

Other variable parameters were investigated for their influence on measured signal shapes as well. Figure 7.23 depicts simulations with variations in four selected parameters. None of the

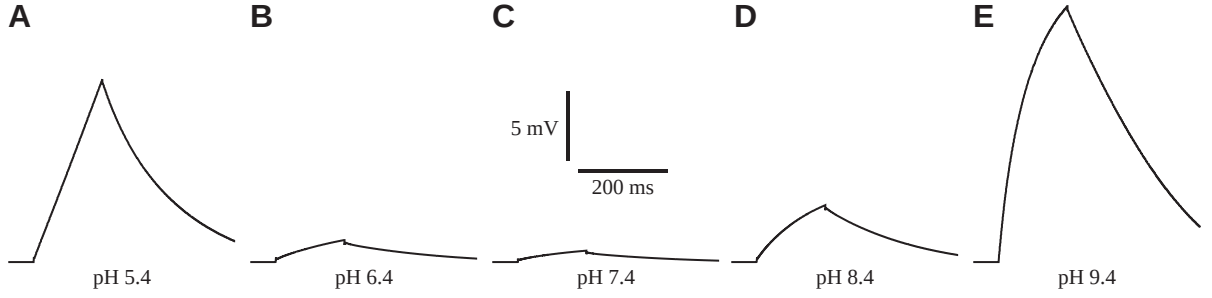


Figure 7.22.: Sensitivity analysis for the initial pH. Simulated FET signals consist of electrical and electrodiffusive components as well as effects of pH and ion-sensitivity. Simulations further include high-pass filtering with $\tau = 4.9$ s. Indicated pH values represent initial pH.

signals reached a steady state towards the end of the stimulation period, illustrating the involved pH and buffers with slow diffusion coefficients. As expected, membrane currents I_M had a significant influence on the amplitude of simulated FET signals. For the artificial cell, an increase in 0.5 nA added almost 1 mV to the amplitude of FET signals. A modulation of the antiporter stoichiometry S_{ClH} was similarly effective. Reducing the stoichiometry by a factor of two (between 0.5 and 5.0), results in an extra 500 μV for FET signals. This strong influence allowed a secure fitting of the Cl^-/H^+ -stoichiometry during evolutionary optimization. However, for higher stoichiometries the influence on the FET amplitude became weaker. The ratio of attached to total membrane α_{JM} and the cleft height d_J had a rather minor impact on the signal amplitude with about 10 μV /percentile and 1 $\mu\text{V}/\text{nm}$, respectively.

How sensitively would MEA recordings react to changes in the Cl^-/H^+ -transport stoichiometry? The question arises as recordings with FETs were enhanced by the ion-sensitivity for small cations. Figure 7.24 shows simulations without the effects of ion-sensitivity for five different S_{ClH} . Assuming a linear dependence, the sensitivity was approximately 1 $\mu\text{V}/1$. In real MEA-measurements, including errors and noise, the small increment would be too small to safely determine the Cl^-/H^+ -transport characteristics. In comparison with figure 7.23B, changes in the signal shape were inverted and limited to the beginning of the anyways difficult to calculate slope. FET measurements are thus mandatory to determine the transport stoichiometry of Cl^-/H^+ -antiporters.

7.4. Results and discussion

After a thorough calibration of the model simulation parameters were fitted to FET recordings from HEK293 cells, stably expressing CIC-1 chloride channels (see section 3.1.3 and chapter 6). Model parameters were fitted for recordings from cells presented to various pH or HEPES conditions. Table 7.12 lists the averaged results of all model optimizations with CIC-1 antiporters.

Based on their measurements, Picollo and Pusch (2005) concluded that the transport ratio of CIC-1 antiporters had to be multiple times slower than that of CIC-4. Unfortunately, they were

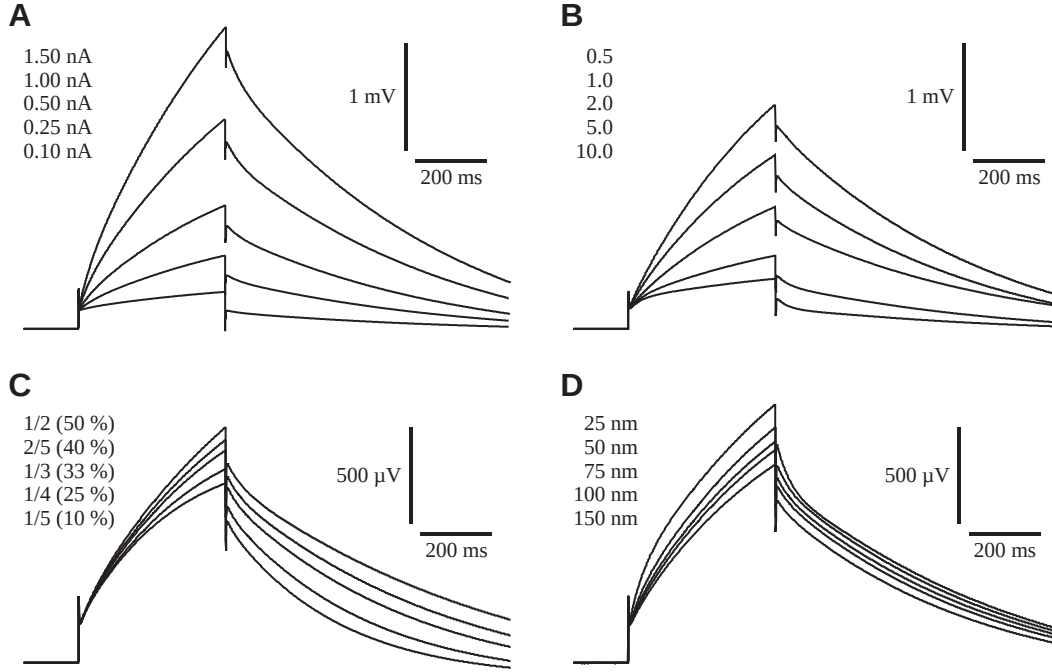


Figure 7.23.: Sensitivity analysis of (A) membrane currents I_M , (B) stoichiometry S_{C1H} , (C) ratio of attached to total membrane α_{JM} and (D) cleft height d_J . Simulated FET signals consist of electrical and electrodiffusive components as well as effects of pH and ion-sensitivity. Simulations further include high-pass filtering with $\tau = 4.9$ s. The listed parameter changes are sorted by the respective signal amplitude.

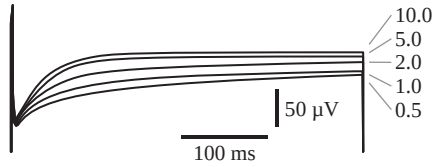


Figure 7.24: Sensitivity analysis of the antiporter stoichiometry on simulated MEA signals (compare figure 7.23B). Simulations consist of electrical and electrodiffusive components as well as effects of pH, however, do not include ion-sensitivity.

not able to estimate an actual ratio. According to the simulations in this thesis, the transport of protons in CIC-1 was 5 times slower as in CIC-4 and thus in good agreement with expectations in literature. The small standard deviation of less than 5 % assured that the transport stoichiometry of CIC-1 antiporters was robustly fitted to be 10 Cl^- per 1 H^+ . The average fitted seal resistance in the electric component of simulated signals was $R_J = 0.4 \pm 0.1 \text{ M}\Omega$ and correlated with fitting for the cleft distance, which was in average 100 nm. Assuming a rough linear²⁵ trend, the cleft height d_J depended with $1/3$ of a nm per $\text{k}\Omega$ on R_J . CIC-1 transfected HEK293 cells attached with about one third of their membrane to the substrate, suggesting a half-sphere as approximated cell shape. Figure 7.25 depicts exemplary simulation results.

The model reproduced measured traces successfully. Simulations with pH 7.4 and 10 mM HEPES were represented most accurately, although the rising slope following the on-set and the falling slope right after the off-set of stimulation pulses could not be met perfectly. Simulations of the falling slope were especially difficult, since differences to the actual measurements were found

²⁵ The actual trend is non-linear.

S_{ClH}	R_J	d_J	α_{JM}
9.985 ± 0.417	$0.421 \pm 0.118 \text{ M}\Omega$	$103 \pm 42 \text{ nm}$	0.33 ± 0.03

Table 7.12.: Average biologic model parameters obtained by fitting of $n = 15$ simulated FET signals to recordings from CIC-1 transfected HEK293 cells. Results were initially averaged for each cell individually.

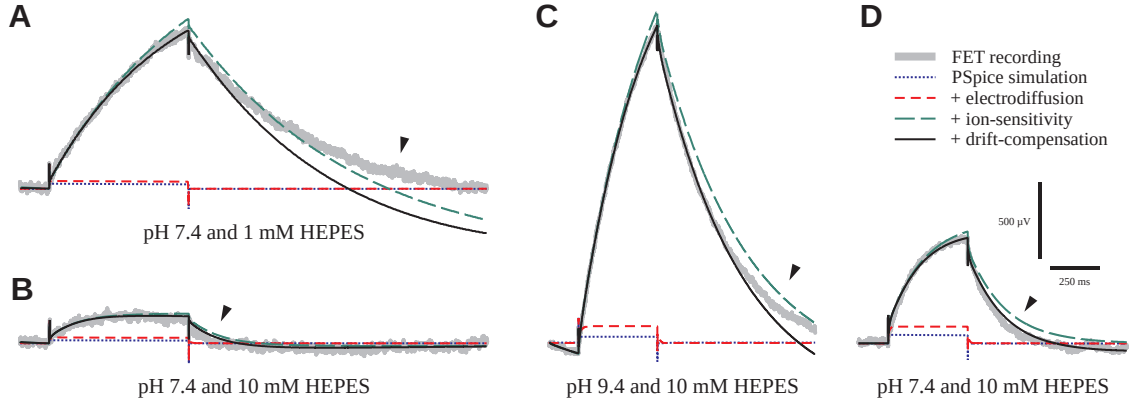


Figure 7.25.: Four exemplary simulation results of FET recordings from CIC-1 HEK293 cells after parameter optimization. (A+B) Simulation of data with longer stimulation pulse (700 ms) from the same cell in the presence of either 1 mM or 10 mM HEPES at pH 7.4. (C) Simulation of data with 400 ms stimulation duration and 10 mM HEPES at pH 9.4. (D) Simulation of CIC-1 activity for the duration of 400 ms in the presence of 10 mM HEPES at pH 7.4. Arrowheads mark disagreements between simulation and real data.

in all cases. Using lower HEPES concentrations or initial pH values different from 7.4 further increased the disagreement between simulations and measurements, particularly towards the end of traces, where signals met the zero line (compare figure 7.25A and C).

$K_{a_2}^{\text{Si}}$	n_{Si}	D_{Si}
6.71 ± 0.48	$5.79 \pm 1.62 \text{ per nm}^2$	$0.12 \pm 0.08 \cdot 10^{-5} \text{ cm}^2/\text{s}$

Table 7.13.: Average SiOH-buffer parameters obtained by fitting simulated signals to FET recordings. Results were initially averaged for each FET-chip individually.

Parameters describing the buffer characteristics of FETs were initially averaged per chip²⁶. The results per chip were then averaged to be listed in table 7.13. The buffer characteristics of the electrolyte-oxide interface were fitted with small variations among individual chips. The equilibrium constant $K_{a_2}^{\text{Si}}$ for $\text{SiO}^- \rightleftharpoons \text{SiOH}$ was fitted to be 6.7 ± 0.5 and was thus slightly higher than the value 6 given in literature. The amount of silanol groups n_{Si} was on average fitted to be 5.8 ± 1.6 per nanometer, which was among the higher values found in literature. A definite reason for variations in fitted buffer characteristic could not be found. However, slight variations in processing conditions for chips from different wafers or even variations on one wafer were identified as a possible cause. A further influence could be the application and density of surface modifying proteins (mostly poly-D-lysine) or the days in culture before measurements.

²⁶ In some cases multiple cells were patched on one chip (16 FETs/chip)

The diffusion coefficient for protons in the electrolyte-oxide interface D_{Si} was fitted to be $0.12 \pm 0.08 \cdot 10^{-5} \text{ cm}^2/\text{s}$ for the electrodiffusive component in simulations. The large standard deviation was mostly induced by differences among simulations with variations of the initial pH in the bath/cleft. As described earlier (page 116), the diffusion of protons was actually a transport within a plane at the surface. This transport was facilitated between silanol groups, which probably allowed a faster transport the more protons were bound to the surface. The fastest diffusion rates ($0.01 \cdot 10^{-5} \text{ cm}^2/\text{s}$) were fitted for experiments with pH 5.4, whereas the slowest ($0.22 \cdot 10^{-5} \text{ cm}^2/\text{s}$) were fitted for experiments with pH 9.4. This trend suggests a reduction of $0.05 \text{ cm}^2/\text{s}$ per pH. However, experiments performed in this thesis were designed to determine the transport ratio of Cl^-/H^+ -antiporters and the variation of parameters in table 7.13 should be evaluated in separate experiments, customized for these questions.

In an alternative approach, the Cl^-/H^+ -antiporter activity in HEK293 cells was recorded with MEAs which were not ion-sensitive. The pH effects, only found in FETs, were thus lacking in MEA recordings. In order to simulate MEA recordings, the following modifications were applied to the model:

- A STERN capacitance C_{dh} (see section 2.4) was added in parallel to R_{LP} to the point-contact model.
- Both the STERN capacitance C_{dh} and the lowpass capacitance C_{LP} were included into the fitting process.
- The concentration c_{Si}^J of silanol groups within the cleft volume was reduced by the area of one electrode (equations 7.19 - 7.21).
- Any equations to calculate the ion-sensitivity were removed from the simulation process.
- The time constant for drift compensations was set to $\tau = 11 \text{ s}$ (see figure 3.13 on page 51).

Figure 7.26 shows an exemplary simulation result for MEA recordings. The variables describing pH effects and ion-sensitivity were still included in simulations. However, the MEA recording provided no limiting information about pH processes in the cleft. Therefore, the almost perfect match between simulation and recording in figure 7.26 was achieved due to overfitting. Lacking the ion-sensitivity, the model had multiple additional variables to adjust the simulated shape without physical limitations. Evidence for overfitting was found in the resulting parameter sets after optimization. The Cl^-/H^+ stoichiometry, for instance, was fitted to be $S_{ClH} = 0.4$ (2 Cl^- per 5 protons), which was evidently not in agreement with opinions found in literature or any earlier shown simulation results for FET recordings.

Lacking an ion-sensitivity MEAs did not detect pH changes, which were the only measurable evidence for an antiport of chloride and protons in ClC channel proteins. MEA recordings were useful tools for a general comparisons of FET recordings with sensors lacking ion-sensitivity, but

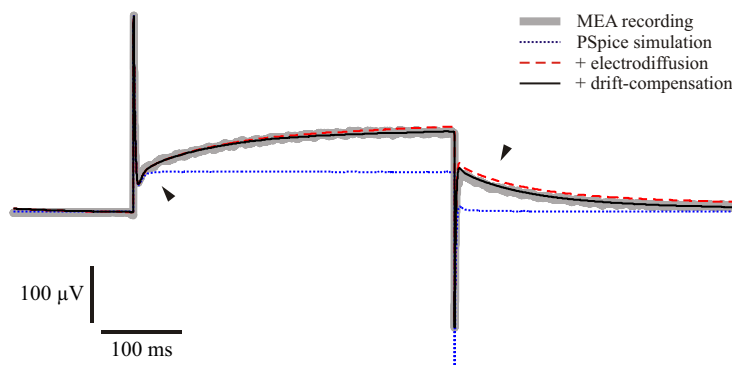


Figure 7.26: Exemplary simulation of MEA recordings, including electric components (blue), electrodiffusive components (red) and a drift-compensation (black). Effects of ion-sensitivity are not present in MEA recordings. Small disagreements between simulation and recording are indicated by arrows.

a subsequent signal simulation and parameter fitting was not able to determine the transport ratio for CIC antiporters.

7.5. Conclusion and outlook

A comprehensive model for the simulation of FET- or MEA-recordings from transfected HEK293 cells has been introduced. The model allows the input of membrane currents carried by two (or more) different ions, encompasses electrical and electrodiffusive components, calculates pH or buffer reactions and predicts ion-sensitive responses in the case of FETs. Simulations with this model provided valuable information about changes in ion-concentrations within the cell-transistor junction, pH changes during CIC antiporter activity, potential differences between cleft and bath due to differences in concentration as well as buffer characteristics of the electrolyte-oxide interface. The model has been characterized, optimized and calibrated with FET recordings from CIC-4 transfected HEK293 cells. Subsequently, the model estimated the Cl^-/H^+ -transport stoichiometry of CIC-1 antiporters with great success. According to the simulation and fitting results, the average stoichiometry was 1 proton for 10 chlorides. This result was 5 times larger than the transport ratio in CIC-4 channels and thus in very good agreement with approximations in literature. In fact, the presented results were the first data-based estimations for the transport stoichiometry of CIC-1 antiporters. Further experiments with variations in the stimulus voltage or extracellular ion concentrations in the bath could be conducted to affirm the results. The fitting results also suggested a transfer-coefficient for protons within the electrolyte-oxide interface and proposed that this transfer-coefficient might be pH dependent. New experiments to verify the buffer characteristics of SiO_2 (and other oxides) as gate-material in EOSFETs would help narrowing down variable parameters in the model. The presented simulations were entirely based on monovalent ions, due to the use of the GOLDMAN-HODGKIN-KATZ equation in the calculation of electrodiffusion-driving potentials. Alternatives, such as the POISSON equation, could allow the inclusion of divalent ions into the model to simulate the effects neuronal action potentials in the cell-transistor junction. The presented point-contact model with its current extensions could be employed for the evaluation of transport stoichiometries in other CIC family members. Analog experiments with MEAs did not

collect sufficient information to estimate Cl^-/H^+ -transport characteristics, since the ion-sensitivity was necessary to measure pH changes in the cell-transistor junction.

8. General survey and outlook

This thesis was a study of anions and protons in the cell-transistor junction with the objective to determine the transport stoichiometry of chloride versus protons in chloride-channel antiporters. In order to achieve the objective, a pH enhanced, extended point-contact model of the cell-transistor junction has been developed.

The quality of a comprehensive model, including pH and buffer reactions, is strongly dependent on an accurate representation of the cleft volume. In order to provide the model with detailed information about the junction geometry, the contact situation between cells and substrates was investigated. Therefore, membrane allocation profiling (MAPing), has been developed as a new method to analyze the shape of cells and their attachment to planar substrates. In this technique, three-dimensional image stacks of stained HEK293 cells were created by means of confocal microscopy. The MAPing procedure reduced the multidimensional and large data sets from confocal microscopy to single curves: membrane allocation profiles (MAPs). Such a MAP combined qualitative and quantitative information about cell shape and surface area as a function of cell height.

The reconstructed surface of a cell depended on the voxel resolution as well as image noise. A subsequent noise reduction was necessary to prepare the data for isosurface reconstructions. The size of the reconstructed membrane strongly depended on the chosen isovalue (brightness threshold). Fluorescent labeling of substrate proteins or immunocytochemistry of focal adhesions would clearly indicate the substrate plane and thus help finding the cell membrane during reconstruction. However, the investigations of the relative attachment of the cell membrane to the substrate was stable over a large range of isovalues.

Using the MAPing technique, the effects of eight different protein substrate coatings on the attachment of HEK293 cells were successfully characterized. According to the MAPing results, cell shapes tended to show characteristics of a spherical cap. The results further demonstrated that HEK293 cells generally attach with larger partitions of their cell membrane than previously assumed and published. Depending on the respective substrate coating, the examined cells attached with up to 45 % of their membrane. Previously published values were obtained using an electrical approach with alternating polarization of the cell membrane. The measurements were probably influenced by leak currents and thus resulted in a systematically smaller area for the attached membrane.

Evidently, substrate-integrated sensors, such as field-effect transistors (FETs) and multielectrode arrays (MEAs), were able to detect almost 50 % of total membrane currents. Therefore, the outcome of this image-based study had an important impact on simulation: cell coupled stronger into sensors in biohybrid experiments and the stoichiometry of Cl^-/H^+ antiporters was estimated with more certainty. The MAPing technique further provides the capabilities for numerous attachment studies in the field of biomaterials, including the cell-transistor junction with other cell types such as neurons or cardiac myocytes.

The study of anions in cell-sensor junctions was essential because anions and cations can have different effects, depending on the selected sensor type. While all ions have the same effects in recordings with MEAs, most FET inherit a specific sensitivity for anions. Traditional electrophysiology and cell-sensor coupling were exclusively performed with cations. Therefore, HEK293 cells were genetically engineered to express chloride currents by stable transfection with cDNA encoding human glycine receptors. The receptors were also chloride channels opening depending on the glycine concentration in their vicinity. The dose-response curve determined for the newly generated cell clone had an ED_{50} value of 140 μM for glycine, which was in good agreement with literature values.

A prototype for a biohybrid sensor was constructed by culturing the transfected cells on FETs and MEAs. Cells directly located on sensors were additionally contacted with patch-clamp pipettes during an initial characterization of the biohybrid sensor. Both sensors were equally capable of detecting glycine-dependent chloride currents across the cell membrane. Four different groups of signal shapes were observed in recordings from either cell-sensor combination. The shapes consisted of negative spikes, which were in most cases accompanied by positive or negative plateaus. A definitive cause for the variations in signal shapes could not be identified. Plateau-forming influences due to pH effects were ruled out since FETs and MEAs recorded signals with very similar shapes as well as the fact that recordings from wild-type HEK293 cells with either sensor did not contain any signals upon stimulation with glycine. However, signals consisting exclusively of spikes were very similar to the first derivative of the corresponding intracellularly measured membrane currents. A time shift between extracellular recordings and the intracellular derivative was probably caused by diffusion of glycine into the cleft before a significant amount of glycine receptors were activated at the attached membrane.

Preliminary test runs with the patch-clamp free, stand-alone version of the biohybrid sensor always resulted in recordings with positive plateaus which contained a rather weak spike component. The location of the spike correlated to the applied glycine concentration. Differences between the stand-alone version of the sensor and the initial characterization were most likely caused by the clamped membrane voltage, which provided a constant driving force for chloride as opposed to rapidly changing membrane potentials in non-clamped cells of the prototype. A stronger concentration gradient for chloride across the cell membrane would induce stronger or longer chloride currents upon stimulation and thus enhance the amplitude of spikes in extracellular recordings.

Other surface coatings or treatments would probably increase the coupling between cell and sensor and also lead to higher signal amplitudes. The perfusion system provided an almost instant exchange of the test solutions for cells located close to the small manifold. However, in the high-throughput approach with 64 electrodes, cells further away from the superfusion source developed slowly rising chloride currents. Consequently, many extracellular signals had smaller amplitudes and eventually vanished in noise. Improvements on the signal-to-noise ratio of sensors would enable the chloride-selective single-cell biohybrid sensor to detect even lower glycine concentrations. Nevertheless, the said observations suggest the use of other delivery options which allow a parallel and virtually immediate exchange of ligand-containing solution for all cells growing on the chip.

The conducted experiments with a biohybrid sensor set the stage for custom made sensors with genetically engineered cells expressing exactly the receptor of interest. Future developments of biohybrid sensors will most likely split at this point and either reduce or enhance the biological part. For the latter, a cascading amplification step could be implemented into the cell. Receptors that use second messengers, such as cyclic nucleotides, inositol trisphosphate or calcium, can open hundreds of ion-channels per detected ligand. Even minute amounts of a compound, down to single molecules, could be detected with this enhanced biohybrid sensor. Alternatively, the cell could be reduced to a bilipid membrane teeming with receptor-channels of interest only. Less variability due to internal cell processes and a longer lifetime would be among the advantages of such devices.

Taking advantage of anion currents, *state-of-the-art* theories of cell-sensor coupling were confirmed. Using the new glycine-receptor expressing cell line, it was possible to experimentally prove model predictions used in cell-sensor simulations. Recordings from FETs and MEAs showed the same shape when anions carried the coupling process and thus strongly supported cell-transistor models which include cation side-binding effects.

Based on the ion-sensitivity of FETs, two experimental assays were developed to detect the anti-parallel transport of anions and protons in chloride channels. Therefore, the buffering capabilities of the cell-transistor interface were modulated. The first approach employed a series of different bath pH values to relatively influence the impact of transported protons on the cleft pH. Unfortunately, strong changes in the bath pH significantly influenced the transconductance of FETs and further changed the buffer capacities. Both effects complicated an interpretation of FET measurements since changes in recorded amplitudes had multiple causes. In order to avoid at least a variation in the capacity of the dissolved buffer, future experiments could employ different buffers for each initial pH value and set the pH to the equilibrium constant of the respective buffer.

The more promising assay employed different concentrations of the pH buffer in the bath solution to modulate the amount of protons reacting with the gate oxide. FET recordings showed increasing signal amplitudes for decreasing buffer concentrations and thus indicated pH changes. The experiments suggested a prominent H^+ transport in ClC-4 antiporters and a smaller but clearly measurable contribution of H^+ to currents in ClC-1 proteins. The findings were in agreement with

published results and current opinions in the field but a quantitative estimation of the transport stoichiometry, purely based on signal amplitudes, was not possible. Earlier studies monitored intra- or extracellular pH fluctuations with small pH-probes in closed compartments, such as the cell soma or externally attached pipettes, and thus were able to estimate stoichiometries. As opposed to the earlier studies in the range of hours, the newly developed FET-supported method reacted to pH changes in far less than a second, but was also subject to equilibration due to the electrodiffusion of ions between the cleft and the bath. Ratios for the antiport of chloride versus protons based on measured data alone were thus underestimated. The presented assay needed to be supported by simulations or analytical models in order to offer quantitative results and provide reliable estimations of transport ratios.

Combining multiple findings in this thesis, a comprehensive model of the cell-transistor junction was developed to reproduce extracellularly measured CIC antiporter activity with substrate-integrated sensors *in silico*. A physically accurate range for the attachment of cells to a substrate was obtained with MAPing. The fact that FET- and MEA-recordings were virtually identical when only anions carried the coupling process was seen during experiments with HEK293 cells expressing glycine receptors. The results also emphasized the importance of the side-binding effects in FETs in order to distinguish proton currents from chloride currents. Experimental assays for a clear identification of the proton transport in chloride channels were developed and tested in experiments with CIC-1 and CIC-4 transfected HEK293 cells. A comprehensive model of the cell-transistor junction was achieved by combining a classic point-contact model with several enhancements, such as electrodiffusion, buffer effects, pH, and ion-sensitivity. The evolutionary strategy was then employed to fit model parameters and match simulations with real measurements. Initially the model was calibrated with measurements from CIC-4 cells, whose transport ratio had been estimated in the past. The model was further tested for over-fitting and verified with unknown data sets. A subsequent sensitivity analysis of critical parameters showed that the developed model was able to robustly estimate the transport ratio of chloride and protons in FET measurements. MEA recordings, in contrast, did not provide sufficient information about processes in the cleft volume to identify the transport of protons and thus lead to inconclusive fitting results. The simulations showed that the cell-transistor contact was very well explained by the conceived model. Using this newly gained understanding the antiport stoichiometry of ions in CIC-1 proteins was determined to be 10 : 1 (Cl^- vs. H^+). The result was in agreement with published opinions, which stated that the proton transport in CIC-1 had to be much lower than in CIC-4.

The presented model and experimental layout could be easily employed to reveal the transport of protons in other CIC-family members and determine the respective stoichiometries. Future experiments could alter the concentration gradient of chloride between the intra- and extracellular compartments in order to gain an even better understanding of ion-currents in the cell-transistor junction. More detailed stimulation protocols on the electrophysiological part of the experiments

would provide a greater understanding of the proton-transport in chloride channels. Since pH-dependencies were reported for ClC-1, it would also be plausible that the transport ratio changes with respect to the applied stimulation pulse.

Besides the Cl^-/H^+ -transport ratio, valuable parameter ranges for the buffering characteristics of silicodioxide were found as well. The buffering effects of glass surfaces were usually neglected in simulations, since large volumes strongly decreased the concentration of buffering molecules. Taking the minuscule cleft geometry into account, the surface to volume ratio changed significantly and concentrations of up to 100 mM silanol-buffer had to be expected. According to the results from evolutionary fitting, the second equilibrium constant and the amount of silanols per area seemed to be slightly higher than stated in literature. A third parameter, the transport-diffusion coefficient, for the lateral migration of protons within the electrolyte-oxide interface had to be implemented to successfully reproduce measured signal shapes. This coefficient seemed to be dependent on the bath pH and thus the protonation state of silanol groups. The found parameter values should be investigated closer in specifically designed future experiments.

Using gate materials with other pH sensitivities, such as silicon-nitride or tantalum-pentoxide, could increase the accuracy of stoichiometric estimations. The employment of gate-oxides with equilibrium constants that are different from the bath pH would further enhance the impact of changes in the concentration of buffers dissolved in the electrolyte. Customized ion-sensitivities, especially tailored towards selected cations (or even anions), and slight modifications to the presented model would provide the basis to analyze and characterize other antiporters or transporters by means of FETs.

Cell-transistor junctions were very well described by a simple electric equivalent model in conjunction with extensions accounting for electrodiffusion and ion-sensitivity. Uncertain parameters, such as the ratio of attached membrane to total membrane, were determined in customized experiments and implemented into the model. Other parameters were based on literature or estimated by evolutionary fitting. The presented investigation of proton-transport ratios in chloride channels had a rather exotic experimental plan, which needed two further extensions for the model: two ions with opposing charges traveling into opposing directions across the membrane and pH effects within the cleft volume. Further experiments with cells expressing anion channels cultured on substrate-integrated sensors will certainly improve the understanding of cell-transistor coupling. A possible future target for simulations with the presented model could be the implementation of two cation-currents in the cell-transistor junction as found in neuronal action potentials. The cleft geometry was assumed to be constant in the presented model. However, it might be mandatory for future models to account for changing cleft heights if contracting cells, such as cardiac myocytes, become the subject of interest. Those simulations would also include three or more different cation-currents across the cell membrane, of which one is divalent. Parts of the present equations for electrodiffusion were limiting simulations to monovalent ions. Future projects should look out

for replacements of those equations in order to allow simulations with all physiologically relevant ion-currents in the cell-transistor junction.

A. Appendices

A.1. 3D cross-eye viewing instructions

Human vision uses several cues to determine relative depth in a perceived scene (Rolfé and Staples, 1986), but only stereopsis or retinal / binocular disparity provides the experience of realistic perceive depth when viewing Magic Eyes, Autostereograms, 3D movies and stereoscopic photos. If an object is far away, the disparity of that image falling on both retinas will be small. If the object is close or near, the disparity will be large. By using two images of the same scene obtained from slightly different angles, it is possible to triangulate the distance to an object with a high degree of accuracy.

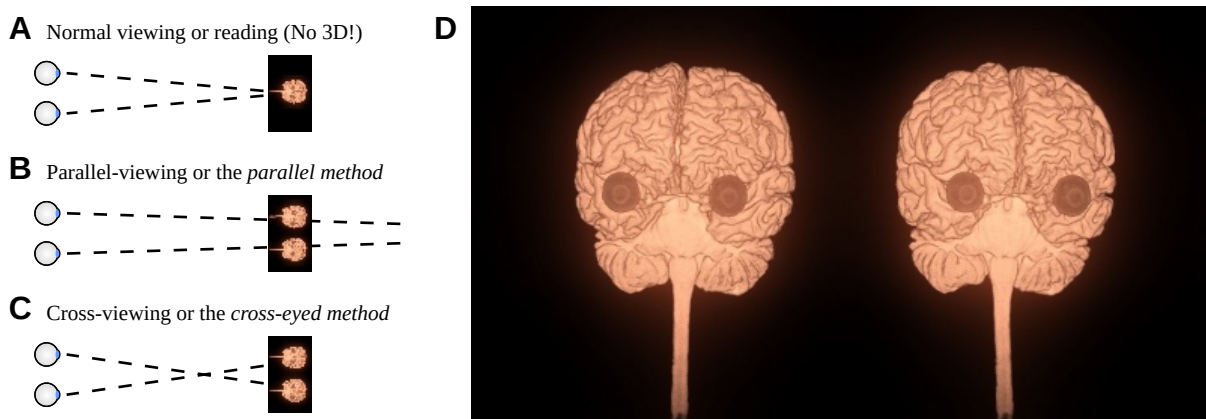


Figure A.1.: 3D viewing examples. (A) Normal view, (B) parallel view, (C) cross view and (D) cross view example (rendered MRI data from the author of this thesis). The brain on the left shows the perspective for the right eye and the brain on the right shows the perspective for the left eye, thus cross view.

Figure A.1 depicts free-viewing²⁷ in contrast to standard viewing. In order to see a 3D image, each eye needs to get a different view. Figure A.1D shows the same object from two slightly different perspectives side-by-side. Using the cross-eye method (figure A.1C), a viewer can overlap the pictures to gain perspective depth.

- Place figure A.1D (screen or book) directly in front of you, at about arm's length
- Keep your head level horizontally, tilting prevents merging the images
- Hold a pen (or finger) right between your eyes and stare at its tip
- Move the pen (or finger) slowly towards the stereo pair of images in the back
- You will start seeing four blurry brains behind the pen's (or finger's) tip
- Continue until the center two of the four brains overlap and you see only three
- At some point the center image will pop into focus and be in 3D

²⁷ observation of three dimensional scenes without special equipment

A.2. Multiplication and purification of the gene coding for the glycine receptor subunit α_1

This section contains supplemental information for chapter 5. The gene coding for the glycine receptor subunit α_1 (sequence shown in Fig. A.2) was multiplied in *E.Coli* bacteria. Protocols for culturing the bacteria and purifying the gene of interest are listed here.

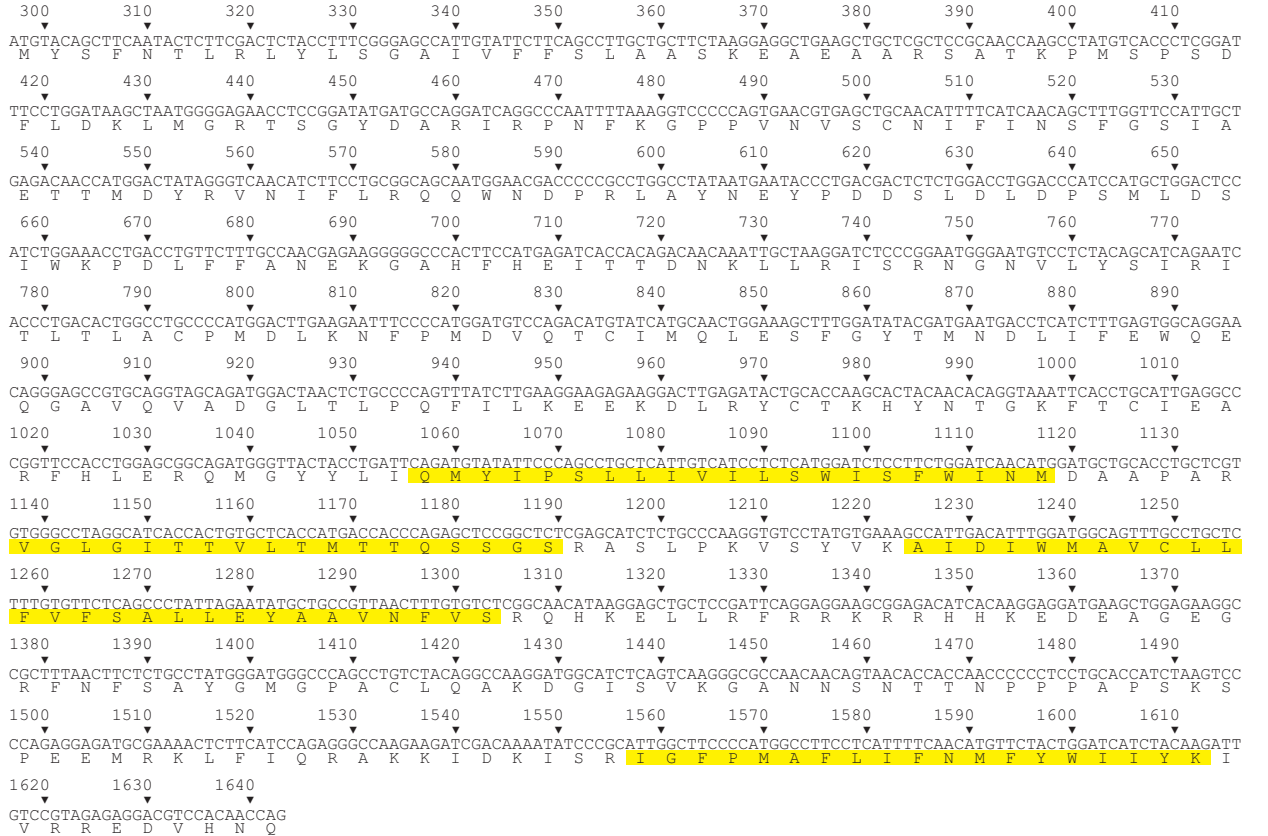


Figure A.2.: Nucleotide and deduced amino acid sequences of the human glycine receptor subunit α_1 cDNA. The putative membrane-spanning segments M1 to M4 are highlighted.

A.2.1. Vectors and bacteria strains

In contrast to eucaryotic cells (e.g. animal cells) bacteria have their genetical information in circular DNA, so called plasmids. Used in genetic engineering they are called vectors. They are *transport vehicles* to transfer genes from one organism to another. The following vectors have been used in this present work.

pRK5

was gently provided by C. M. Becker (University of Erlangen, DE). The plasmid contained the cDNA of 6LRA1, which encloses the human glycine receptor α_1 subunit (hGlyR α_1) with START

and STOP in 1425bp (Becker, 1995).

pBluescript SK(-)

from Stratagene (La Jolla, CA, USA) was a temporary sub clone for the hGlyR α 1 cDNA.

pcDNA3.1(+)

from Invitrogen (Groningen, NL) was applied for the actual transfection and expression of hGlyR α 1 cDNA in HEK293 cells

Since plasmids or vectors are a part of bacteria and procaryotic cells grow very fast (about 20 min. doubling rate) they are used to manifold/copy vectors for genetic engineering. Two strains of *Escherichia Coli* (*E.coli*) from Invitrogen (Groningen, NL) have been used to enrich the plasmid-DNA.

XL1-Blue *E.coli* for pBluescript with these genotypes:

recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻, m_K⁺) supE44 relA1 lac [F' proAB lacI^qZ Δ M15 Tn10 (Tet^R)]

TOP10F' *E.coli* for pcDNA3.1 with the following genotypes:

F' [lacI^q Tn10 (Tet^R)] mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 deoR araD139 Δ (ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG

Bacteria cell culture

E.coli were cultured with constant movement in Luria-Bertani (LB) medium (10 g/l bactotrypton; 5 g/l yeast extract; 10 g/l NaCl) at 37°C for at least 8 hours before passaging. Ampicillin (0,1 mg/ml) was added for a selection based on plasmid coded resistance for antibiotics. In case of LB-agar plates 15 g/l agar were admixed to the LB medium before it was poured in sterile petri dishes (25-30 ml / dish). All media were autoclaved for 20 min at 121°C. Liquids were stored at room temperature, the agar plates at 4°C.

Production of competent bacteria cells

Competence is the ability of a cell to take up extracellular molecules from its environment, typically super-coiled plasmid DNA. It can be realized by electrophoresis, lysis or other methods. In this thesis competent cells were created by using a slightly changed CaCl₂-method (Mandel and Higa, 1970).

E.coli were incubated in 50 ml LB medium at 37°C up to an OD₆₀₀ of 0.4. During the following steps cells, vessels and media solutions were cooled on ice. After reduction of the cells metabolism on ice for 10 min, they were centrifuged (5000 g; 10 min; 4°C). The pellet was suspended in 1 ml of 100 mM CaCl₂ and filled up to 25 ml. A second cooling step for 20 min on ice was applied. Then the cells were once more centrifuged, suspended in 1 ml of 100 mM CaCl₂ containing 25%

glycerine and filled up to 5 ml. After 2 additional hours on ice the cells were stored in aliquots at -80°C. If required they could be thawed and immediately provided with super-colloid DNA for uptake.

A.2.2. DNA preparation and cloning

Molecular cloning of nucleic acids refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it *in vivo*. Cloning of any DNA fragment essentially involves four preparation steps: fragmentation, ligation, transfection, and screening/selection. They are elucidated in the following, as well as miscellaneous intermediate steps for cleaning and separation.

Plasmid preparation

Living bacteria have been used to reproduce vectors which contained the genes coding for the glycine channel subunit α_1 . The genes were then present in plasmids inside the recombinant bacteria. Used work stages to extract the multiplied vectors out of the cells are given in the following two paragraphs.

Mini plasmid preparation

This procedure was applied according to [Birnboim and Doly \(1979\)](#). 5 ml LB-medium (with Ampicillin) were inoculated with a colony of recombinant bacteria and incubated overnight at 37°C. The bacteria out of 1.5 ml culture liquid were centrifuged (18000 g; RT; 1 min), resuspended in 100 µl Solution I (50 mM Glucose; 25 mM Tris/HCl pH 8.0; 10 mM EDTA pH 8.0) and lysed in 200 µl solution II (0.2 M NaOH; 1% SDS). Proteins and highly molecular DNA were precipitated by adding 150 µM of solution III (3 M KAc pH 4.8). The precipitates were centrifuged at RT (18000 g; RT; 3 min). Soluble proteins in the plasmid containing supernatant were extracted with phenol/chloroform (1:1) and chloroform. Subsequently they have been precipitated in 1 mM ethanol (absolute) and washed with 70% ethanol. The nucleic acids were solved in 20 µl TE buffer (1 mM EDTA; 10 mM Tris/HCl pH 7.5) as soon as they have been dried in vacuum (max. 1 min).

LiCl plasmid preparation

250 ml bacteria solution were centrifuged (3.000 g; 4°C; 10 min) and the supernatant discarded. The bacteria were resuspended in 10 ml solution I and lysed in 20 ml solution II. Proteins and highly molecular DNA were precipitated in 20 ml solution III and centrifuged (5000 g; 4°C; 10 min). The plasmid DNA containing supernatant was filtered, the nucleic acids were precipitated with 25 ml isopropanol and centrifuged (5000 g; 4°C; 5 min). The pellet was resolved in 2.9 ml

sterile bidistilled water. Afterwards, 5 ml of 4 M LiCl solution and 0.1 ml (1 M Tris/HCl; pH 7.5) were added to fractionally precipitate the RNA, followed by centrifugation (5000 g; 4°C; 10 min). 2.5 volumes of ethanol (total) were added to the supernatant and centrifuged again (5000 g; 4°C; 19 min). The precipitate was resuspended in 500 µl TE buffer and remaining RNA was digested by 2 µl RNase mix (Ambion; 10 U / µl) for 45 min at 37°C. The DNA containing suspension was washed two times with phenol/chloroform (1:1) and two times with chloroform to extract remaining proteins. The DNA has been precipitated by 3 volumes of ethanol and 1/10 volume of 3 M LiCl, centrifuged (18000 x g; 4°C; 5 min) and washed with 70% (v/v) ethanol. The dried DNA was resuspended in up to 500 µl TE buffer.

Cleaning and separation of nucleic acids

Bacterial DNA achieved from plasmid preparations is mixed with smaller cellular components and particles. To obtain the pure DNA coding for the desired protein several washing and separation steps are needed. Related protocols are given in this section.

PHENOL/CHLOROFORM-EXTRACTION

Impurities of DNA solution with e.g. proteins or membrane fragments were removed by extraction with phenol/chloroform. DNA solutions were mixed with an equal volume of TE-saturated phenol and chloroform (1:1) solution. The phases were mixed (Vortex) and separated by centrifugation (18000 g; RT; 3 min). The aqueous phase was removed and remaining Phenol diminished by the same volume of Chloroform. Subsequently the DNA has been precipitated with Ethanol.

ETHANOL PRECIPITATION

The DNA solution was adjusted to a LiCl-concentration of 0.3 M and mixed with a three times higher volume of ethanol (total). This making-up can either be centrifuged directly or the DNA can be precipitated at -20°C. The centrifugation (18000 g; 4°C; 30-120 min; Sigma 2K15) formed a DNA pellet. The duration of centrifugation is adjusted to the amount of DNA (lesser DNA requires longer centrifugation). The DNA pellet was washed with 70% Ethanol and resolved in TE-Puffer after drying in vacuum.

AGAROSE-GEL-ELECTROPHORESIS

The DNA fragments were separated in vertical agarose gel system to analyze their size and qualitative amount. Gels were made out of 0.75% - 2% (w/v) agarose in TBE (50 mM Tris/Borat pH 7.8; 1 mM EDTA pH 8.0). The agarose concentrations depended on the fragment sizes. Before setting the probes in gels they were mixed with 1/5 volumes of 5x probe buffer (100 mM EDTA; 20% (w/v) ficoll 400; 0.01% (w/v) bromophenol blue; 0.01% (w/v) xylem cyanol). The running

buffer was TBE. To separate the fragments 112 V and 121 mA were applied (GPS 200/400, Pharmacia). The gel was stained by the addition of 1 µg /ml ethidium bromid. DNA fragments were visualized with UV-light ($\lambda = 302$ nm).

ISOLATION OF DNA FRAGMENTS

Ethidium bromid stained DNA fragments, visualized in UV-light were isolated after activation with UV-light [Heery 1990]. Positions of DNA fragments in the gel could be identified in a transilluminator (TI 2, Biometra). DNA bands were cut out and eluted by centrifugation (4500 g; RT; 4 min) on silanized glass cotton.

Cloning of proteins

The reproduction of DNA coding for the glycine channel subunit α_1 took place in bacteria. Protocols preceding the actual introduction of DNA into the bacteria are described in the following paragraphs.

DEPHOSPHORYLATION OF VECTOR PLASMIDS

To prevent self- ligation of the DNA, the 5'-phosphate residues were removed using alkaline phosphatase (Roche Applied Science). Therefore, 5 µg of vector DNA, 10 µl 10x phosphatase buffer (500 mM Tris/HCl pH 8.5; 1 mM EDTA) and 2 U alkaline phosphatase were filled up with H₂O to 100 µl and stored for 60 min at 37°C. The reaction was stopped by adding 10 µl EGTA (0.2 M) for 10 min at 65°C. The DNA was extracted with phenol/chloroform, precipitated with ethanol or separated by gel electrophoresis, respectively and subsequently eluted from the agarose gel (as described earlier in this section).

DNA RESTRICTION

1 µg DNA fragment was digested with 1-3 U restriction enzymes (*EcoRI*, *PstI* or *XbaI*) for 90 min at 37°C in buffer as recommended by the producer. Restriction reactions, which contained vector DNA out of mini-preparations, were additionally mixed with RNase-cocktail (Ambion, UK).

LIGATION OF INSET DNA WITH PLASMIDS

To ligate inset DNA fragments into cloning vectors, both were mixed in a molar ration of 3:1. The reaction was performed with T4 DNA ligase (1 U) in ligation buffer (60 mM Tris/HCl, pH 7.5; 5 mM MgCl₂; 1mM DTE and 1 mM ATP) for 180 minutes at RT.

TRANSFORMATION OF *E.coli*

The ligation reaction was added to 100 µl of competent bacteria. The transformation reaction was maintained for 30 min on ice. Selection for plasmid uptake was induced by inoculation of bacteria on LB-Ampicillin plates and incubation over night at 37°C.

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A.3.4. Symbols

A	surface (area)
$A^{1,2-}$	organic anions
A_n	surface area of the n^{th} CLSM z-stack
A_{FM}	surface area of the free cell membrane
A_{JM}	surface area of the substrate-attached cell membrane (junction membrane)
A_M	surface area of the cell membrane
c_i	concentration of ion i
c_M	membrane capacitance normalized by area
c_{sp}	specific membrane capacitance ($1 \mu\text{F} \cdot \text{cm}^{-2}$)
C_j	concentration of compound j
C_{FM}	capacitance of free membrane
C_G	GOUY capacitance
C_H	HELMHOLTZ capacitance
C_I	combined capacitance (C_H and C_G) between metal and electrolyte
C_{JM}	capacitance of attached membrane (membrane in junction)
C_M	membrane capacitance
C_P	capacitance of patch pipette walls
d	diameter
d_{acid}	difference in occupied proton acceptors
d_{base}	difference in free proton acceptors
d_J	junction distance (synonyms are h_J or d_x)
d_{OHL}	outer HELMHOLTZ layer
dt	time step
e	elementary charge ($1.60 \cdot 10^{-19} \text{ C}$)
$E_{\text{Ag/AgCl}}^0$	standard electrode potential of a silver-silverchloride electrode
E_i	equilibrium or NERNST potential for an ion i
E_{RE}	potential at reference electrode
EC_{50}	half maximal effective concentration
f_c	cutoff frequency
F	FARADAY constant ($96\,485 \text{ C} \cdot \text{mol}^{-1}$)
g	standard gravity
g_{sp}	specific membrane conductivity
g_J	specific junction conductance
g_m	transconductance or gain of an FET
g_M	membrane conductance normalized by area
G_i	membrane conductivity for an ion i
G_J	junction conductance

h	height
h_c	HILL coefficient
h_{cell}	cell height
i	ion species
i	iterator
I_{DS}	drain-source currents (FET)
I_{FM}	currents across free membrane
I_{JM}	currents across attached membrane (membrane in junction)
I_M	membrane current
I_P	currents through patch pipette
j	iterator
j_0	first zero of bessel function
k	BOLTZMANN constant ($1.38 \cdot 10^{-23} \text{ J} \cdot \text{K}^{-1}$)
k	iterator
k	reaction rate
K	dissociation constant
K_a	equilibrium constant of an acid
K_W	dissociation constant of water
K_{a_i}	i^{th} equilibrium constant
l_{FM}	leak ratio for free membrane
l_{JM}	leak ratio for attached membrane (membrane in junction)
m	linear slope
n	number (of experiments)
n_i	number concentration of ion/compound i
N_A	AVOGADRO constant ($6.02 \cdot 10^{23} \text{ mol}^{-1}$)
N_Q	number of charges
N_i	number of ions of a species i
P_i	permeability coefficient of an ion i
Q	charge
r	radius (of a cell)
r_D	DONNAN coefficient
p	statistical significance
q_i	quotient of ion species i
R	gas constant ($8.31 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$)
R	resistor
R	radius of a sphere
R^2	coefficient of determination
R_C	resistance of intracellular environment
R_{FM}	resistance of free membrane

R_{GS}	gigaseal resistance
R_J	junction resistance or seal resistance
R_{JM}	resistance of attached membrane (membrane in junction)
R_P	resistance of patch pipette tip
R_S	series resistance or access resistance
s	vertical offset of a cell in CLSM z-stack
S_i	ion-sensitivity of an FET for an ion i
S_{ClH}	transport stoichiometry for chloride versus protons
S_J	lateral surface of a cylindrical volume (cleft volume)
t	time
t_d	delay due to diffusion
t_{stim}	duration of stimulation
T	temperature (in KELVIN)
u	ion movability
U_0	reference potential
U_{in}	input voltage
U_{out}	output voltage
U_M^{AP}	difference in min/max membrane potentials during an action potential
U_{DS}	drain-source voltage (FET)
U_{GS}	gate-source voltage (FET)
U^{IS}	FET-potential due to ion-sensitivity
U_J^0	transient-peak potential
U_J	junction potential
U_M	membrane potential
U_P	potential difference between patch pipete and bath
U_{Step}	voltage step for stimulation
U_T	threshold voltage
v	rate of change
v_{JM}	rate of change due to currents across attached membrane
v_S	rate of change due to electrodiffusion across S_J
V_B	bath volume
V_J	cleft volume
y^M	measured data
y^S	simulated data
z	vertical position in z-stack from CLSM
z_i	valence of an ion i
α_i	fraction i of dissociation
α_{FM}	ratio of free membrane to total membrane

α_{JM}	ratio of attached (junction) membrane to total membrane
β	buffer capacity
γ	number of generations (ES)
ϵ_0	dielectric constant in vacuum ($8.85 \cdot 10^{-12} \text{ A} \cdot \text{s} \cdot \text{V}^{-1} \cdot \text{m}^{-1}$)
ϵ_r	relative dielectric constant (water)
ζ	point of zero charge
λ	maximal slope of sigmoid curve (MAP)
λ	number of children (ES)
λ_D	DEBEY length
μ	surviving individuals (ES)
ρ	mating individuals (ES)
τ	time constant (usually for drift compensation)
Φ	potential
Φ_i	flux of ion i
Φ^D	potential due to diffusion
Φ^E	potential due to electrodiffusion
Φ	potential
Φ_D	DONNAN potential (see equilibrium potential E_i)
Φ_{SI}^{GO}	sum of potentials within an FET
Ψ_0	surface potential
Ψ_{ED}	electrodiffusive potential (along junction cleft)
χ_{SD}	surface dipole potential of an electrolyte

A.3.5. Abbreviations

A	surface
AD	analog to digital
AFM	atomic force microscopy
AM	attached membrane
AP	action potential
ATP	adenosin triphosphate
beag	bovine ether-à-go-go (potassium channel)
B	bath
B	bulk / body / base / substrate (FET)
BNC	Bayonet Neill-Concelman (connector type)
cDNA	complementary deoxyribonucleic acid
ChemFET	chemically sensitive field-effect transistor
CIC	chloride channel
ConA	concanavalin A
CLSM	confocal laser scanning microscopy
G	drain (FET)
DA	digital to analog
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DOS	operating system (disk operating system)
eag	ether-à-go-go (potassium channel)
ECM	extracellular matrix
EDTA	tetraprotic ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ENFET	enzyme field-effect transistor
EOSFET	electrolyte-oxide-semiconductor field-effect transistor
ES	evolutionary strategy
FIB	fibronectin
FET	field-effect transistor
FM	free membrane
gDNA	genomic deoxyribonucleic acid
G	gate (FET)
G418	geneticin 418
GlyR	glycine receptor
GABA	gamma-aminobutyric acid
hGlyRA1	human glycine receptor subunit alpha ₁
HBSS	HANK's balanced salt solution

HEK293	human embryonic kidney cell line 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH buffer)
HH	HODGKIN-HUXLEY circuit
IGFET	insulated gate field-effect transistor
IMFET	immuno field-effect transistor
ISFET	ion-sensitive field-effect transistor
J	junction between cell and sensor (also, cleft or cleft volume)
JFET	junction-gate field-effect transistor
LAM	laminin
LB	lysogeny broth
LP	low-pass
LSM	laser scanning microscopy
M10	culture medium for HEK293 cells
MAP	membrane allocation profile
MEA	multielectrode array
MOSFET	metal-oxide-semiconductor field-effect transistor
MPS	Matlab-PSpice (software interface)
MRI	magnet resonance microscopy
MW	molecular weight
OD	optical density
ONO	oxide-nitride-oxide (SiO_2 , Si_3N_4 , SiO_2)
PBS	phosphate buffered saline
PC	personal computer
PCM	printed circuit board
PCM	point-contact model
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
PDL	poly(D)lysine
PECM	mixture of poly(D)lysine and extracellular matrix
PLL	poly(L)lysine
PWL	piecewise linear (source)
RICM	reflection interference contrast microscopy
RNA	ribonucleic acid
RNAse	ribonuclease
S	source (FET)
SI	international system of units (<i>système international d'unités</i>)
SEM	scanning electron microscopy
SQUID	superconducting quantum interference device
TEM	transmission electron microscopy

TIRF	total internal reflection
TRIS	tris(hydroxymethyl)aminomethane
US	united states (of America)
wt	wild type
WP	work point (German <i>Arbeitspunkt</i>)
ZIF	zero-in-force

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