Doctoral Thesis

Electrophysiology-on-chip monitoring membrane transport in living cells

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ELECTROPHYSIOLOGY-ON-CHIP: 
MONITORING MEMBRANE TRANSPORT IN 
LIVING CELLS

A dissertation submitted to

ETH ZURICH 

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Doctor of Sciences

presented by

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Abstract

The work presented in this thesis focuses on the development of micro total analysis systems (μTAS) for performing non-invasive electrophysiology on living cells. With respect to common electrophysiology techniques, the new methods were designed to allow facilitated sample preparation, simplified automation, lower size requirement and higher potential for parallelization. Three μTAS were developed: two for the investigation of transmembranal solute transport in *Xenopus laevis* oocytes, and one for the investigation of mammalian cell lines.

The first system, the asymmetrical transoocyte voltage clamp (ATOVC), is based on the two electrode voltage clamp (TEVC) technique, but achieves non-invasiveness by placing the electrodes externally instead of inserting them into the cytosol. Via asymmetrical separation of the cell membrane, application of a voltage across the cell results in a current that is mostly dependent on the conductance of the patched membrane. A microperfusion channel underneath the cell allows exposure of activating and deactivating solutions for modulating the transport properties of proteins expressed in the cell membrane.

Experiments on oocytes heterologously expressing the epithelial sodium channel ENaC were conducted within the scope of various zero and hypergravity campaigns under the auspices of the European Space Agency ESA. The results not only demonstrated that amiloride-sensitive currents could be measured accurately, but also confirmed the ATOVC’s suitability for field use where robustness, compactness and autonomous operation were essential. Laboratory-based experiments on oocytes heterologously expressing the sodium/phosphate cotransporter NaPi-IIb demonstrated the ATOVC’s applicability to carrier proteins, while measurements of calcium-dependent currents in thapsigargin-treated oocytes established a good basis for future experiments that investigate mechanotransduction in cells subjected to zero gravity.

The second μTAS employs the detection of changes in surface charge instead of measuring membrane conductance. Based on an ion-sensitive field-effect transistor (ISFET), the method enables the detection of proton concentration in close proximity to the cell membrane in *Xenopus laevis* oocytes. Relying on the distinct proton kinetics at the membrane surface, the sensor allows proton-dependent membrane transport to be monitored continuously. Experiments on the amino acid transporter PAT1 and the sodium/phosphate cotransporters PiT-2 and NaPi-IIb demonstrated the method’s high sensitivity. Furthermore, studies on the electroneutral transport in NaPi-IIC exploited the system’s applicability to assays that cannot be measured with traditional electrophysiology techniques.

Another ISFET-based microsystem was developed for studying membrane transport in mammalian cell lines. In contrast to the oocyte-based system, this μTAS combines multiple ISFET sensors on one chip. Moreover, the sensors are comparable in size with mammalian cells to allow their investigation at a single-cell level. First experiments on C2C12 and MDCK cell lines demonstrated the cells’ viability on chip and laid the technical foundation for studying physiologically relevant cellular processes.
Zusammenfassung

In dieser Arbeit wird auf die Entwicklung von Mikro-Total-Analyse-Systemen (μTAS) für nicht-invasive elektrophysiologische Messungen an lebenden Zellen eingegangen. Im Vergleich zu gängigen Elektrophysiologie-Methoden sollten die neuen Methoden eine vereinfachte Probenpräparation, simplifizierte Automation, niedrigere Gerätegrösse und ein erhöhtes Parallelisierungspotential aufweisen. Drei μTAS wurden entwickelt: zwei Systeme, um den transmembranalen Transport gelöster Stoffe in *Xenopus laevis* Oozyten zu untersuchen und eines für die Untersuchung an Zelllinien von Säugetieren.


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I would especially like to thank Prof. Petra Dittrich and Dr. Ian Forster for guiding me through my PhD, Dr. Alfredo Franco-Obregón for getting my PhD started, Dr. Marcel Egli for the great zero g opportunities, Prof. Yuji Miyahara for the fruitful collaboration and the opportunity to work in his lab, my friends and colleagues from Dittrich lab, Dr. Josep Puigmarti-Luis, Andreas Cavegn, Benjamin Cvetkovic, Felix Kurth, Dr. Dario Lombardi, Conni Hanke, Simon Küster, Philipp Kuhn, Dr. Andreas Jahn, Christian Müller, Pascal Verboket, Manuel Schaffner, Dr. Tom Robinson for all work and non-work related activity, Dr. Tatsuro Goda, Dr. Yasuhiro Maeda, Dr. Akira Matsumoto, Dr. Takumi Sannomiya, Yoshi Endo, Tomoka Kojima, Miyuki Matsuda, Kanami Uchida and her parents for invaluable support and making my stay in Japan unforgettable, Dr. Anne-Kristine Meinild, Dr. Soline Bourgeois, Dr. Chiara Ghezzi, Dr. Olga Andrini and Monica Patti for accepting me as their office-mate and always being nice to me and Mirta Viviani for supply of food that kept me going during the very last moments of writing.

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My most special thanks to my mother, my father and my brothers whose lifelong influence has shaped me into what I am.
**Abbreviations and acronyms**

(in alphabetical order)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-APB</td>
<td>2-aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>ADC</td>
<td>analog-to-digital converter</td>
</tr>
<tr>
<td>ATOVC</td>
<td>asymmetrical transoocyte voltage clamp</td>
</tr>
<tr>
<td>CFD</td>
<td>computer fluid dynamics</td>
</tr>
<tr>
<td>CMOS</td>
<td>complementary metal oxide semiconductor</td>
</tr>
<tr>
<td>CNC</td>
<td>computerized numerical control</td>
</tr>
<tr>
<td>DAC</td>
<td>digital-to-analog converter</td>
</tr>
<tr>
<td>DAQ</td>
<td>data acquisition and control unit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DUT</td>
<td>device under test</td>
</tr>
<tr>
<td>E217βG</td>
<td>estradiol 17β-D-glucuronide</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial sodium channel</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>ESA</td>
<td>European Space Agency</td>
</tr>
<tr>
<td>FEM</td>
<td>finite element method</td>
</tr>
<tr>
<td>FET</td>
<td>field-effect transistor</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAT1</td>
<td>sodium/chloride-dependent GABA cotransporter</td>
</tr>
<tr>
<td>GHK</td>
<td>Goldman-Hodgkin-Katz</td>
</tr>
<tr>
<td>GUI</td>
<td>graphical user interface</td>
</tr>
<tr>
<td>IC</td>
<td>integrated circuit</td>
</tr>
<tr>
<td>IDE</td>
<td>integrated development environment</td>
</tr>
<tr>
<td>ISFET</td>
<td>ion-sensitive field-effect transistor</td>
</tr>
<tr>
<td>I-V</td>
<td>current-voltage</td>
</tr>
<tr>
<td>LDC</td>
<td>large diameter centrifuge</td>
</tr>
<tr>
<td>MCU</td>
<td>microcontroller unit</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine-Darby canine kidney</td>
</tr>
<tr>
<td>MEMS</td>
<td>micro electromechanical system</td>
</tr>
<tr>
<td>MOSFET</td>
<td>metal oxide semiconductor field-effect transistor</td>
</tr>
<tr>
<td>NaPi-IIb</td>
<td>type II sodium/phosphate cotransporter NaPi-IIb</td>
</tr>
<tr>
<td>NaPi-IIC</td>
<td>type II sodium/phosphate cotransporter NaPi-IIC</td>
</tr>
<tr>
<td>NI</td>
<td>non-injected</td>
</tr>
<tr>
<td>OATP-C</td>
<td>human organic anion transporting peptide C</td>
</tr>
<tr>
<td>Opamp</td>
<td>operational amplifier</td>
</tr>
<tr>
<td>PAT1</td>
<td>proton/amino acid cotransporter PAT1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline solution</td>
</tr>
<tr>
<td>PCB</td>
<td>printed circuit board</td>
</tr>
</tbody>
</table>
PDMS  polydimethylsiloxane
PFA   sodium phosphonoformate tribasic hexahydrate
PiT-2 type III sodium/phosphate cotransporter PiT-2
PMMA  polymethylmethacrylate
PTFE  perfluoroethylene
SDS   sodium dodecyl sulfate
SLC20 type III sodium/phosphate cotransporter family
SLC34 type II sodium/phosphate cotransporter family
SOI   silicon on insulator
SPI   serial periphery interface
SPICE simulation program with integrated circuit emphasis
SR    sarcoplasmic reticulum
TEVC  two electrode voltage clamp
TOVC  transoocyte voltage clamp
TRP   transient receptor potential
TRPC  transient receptor potential cation channel
TTL   transistor-transistor logic
USB   universal serial bus
VC    voltage clamp
μTAS micro total analysis system
Research publications and activities

Parts of the work presented in this thesis are based on research publications and activities as outlined below.

Publications


Conference proceedings


**Patent**


**Research stays**

51st parabolic flight campaign of the European Space Agency ESA, 26 October – 5 November 2009, Bordeaux, France.

52nd parabolic flight campaign of the European Space Agency ESA, 26 April – 6 May 2010, Bordeaux, France.


Guest research at the Biomaterials Unit of the National Institute for Materials Science NIMS, 1 July – 30 September 2010, Tsukuba, Japan.

JSPS core-to-core program summer camp for research and English training, 24 – 26 July 2010, Hakone, Japan.

53rd parabolic flight campaign of the European Space Agency ESA, 12 – 21 October 2010, Bordeaux, France.

REXUS/BEXUS selection workshop, 30 November – 2 December 2010, ESTEC Noordwijk, Netherlands.

JSPS core-to-core program summer camp for research and English training, 17 – 19 September 2011, Izu, Japan.

55th parabolic flight campaign of the European Space Agency ESA, 16 – 24 November 2011, Bordeaux, France.


1. Introduction

1.1. Membrane transport proteins

Long before the existence of membrane proteins was discovered, electrical conductance experiments revealed that charged molecules could permeate across biological membranes. The lipid-bilayer of the membrane, however, was considered to be virtually impermeable to charged molecules due to the high hydrophobicity of the lipid chain. This lead to the postulation of embedded units in the membrane that selectively mediate the translocation of ions [1]. Furthermore, not all ions were determined to have the same permeability. These units were later discovered to be ion-specific transport proteins that are expressed in the cytosol and subsequently inserted into the lipid bilayer (Figure 1) [2].

Membrane transport proteins can be seen as gateways that connect the cytosol to the extracellular domain. By doing so, they enable metabolic processes and signal transduction that define the physiological functions in cellular systems. Not surprisingly, the investigation of membrane transport proteins is of utmost importance in fields of science wherever physiological aspects in biological systems are to be understood.

![Figure 1: a) Cartoon of the lipid-bilayer in cells with membrane proteins embedded [2]. b) Schematic side view of the epithelial sodium channel ENaC consisting of the three sub-units α, β and γ. ENaC plays an important role in the taste perception of salt.](image)

1.1.1. Classification of transport proteins

Membrane transport proteins can be classified according to their mode of transport (passive, active) and driving force (entropy, enthalpy) (Figure 2). In passive transport, ions are translocated across the membrane along a gradient of chemical potential. In that case, the concentration of a solute cannot be increased. Active transport, on the other hand, uses energy from a concentration gradient of a secondary ion (entropic energy) or chemical bond (enthalpic energy), which allows a solute to be translocated against its concentration gradient. Secondary active transport is present when entropy is used as the energy source. Primary active transport is
used when enthalpy is used as the energy source. There are various sources of enthalpic energy that transport proteins can use for activation of a transport process, such as dephosphorylation of ATP, oxidation/reduction and photochemical reactions. The capability for transporting solutes against their concentration gradient is a feat which is of vital importance in many physiological processes [2].

The family of transport proteins relying on entropy can be subclassified into channels and carriers. Channel proteins do not transport ions per se, but they provide a continuous pathway between the cytosol and extracellular domain. The driving force in the translocation of ions through channels is thus of electrochemical nature and realized through a diffusion process leading to increased entropy. Carriers on the other hand, do not provide a continuous pathway. They undergo a conformational change once a specific molecule binds to it. One or more solutes may then be translocated across the membrane through a series of conformational changes of the protein. Due to the absence of diffusion processes, the carrier-mediated translocation of ions is independent of the electrochemical gradient across the membrane. Whereas channels can generally be characterized by their ion-specific conductance, carriers have a turnover rate that may be dependent on a number of factors that influence the conformational state of the protein, such as solute concentration, membrane potential and pH [3].

![Figure 2: Classification diagram of transport proteins with some relevant examples.](image)

1.1.2. Study of membrane protein activity

Membrane transport activity can be detected via electrical conductance measurements if a translocation process results in a net transfer of charge. This is usually achieved using voltage clamping on living single cells expressing the membrane protein to be investigated. The *Xenopus laevis* expression system in combination with the two electrode voltage clamp (TEVC) method has established itself as the most popular electrophysiology system.
1.2. The Xenopus laevis expression system

1.2.1. Types of studies

_Xenopus laevis_ oocytes, eggs from a clawed frog native to South Africa, have become widely used for studying ion channels and transporters since their first use as an expression system by Miledi and coworkers [4]. Generally, the studies made possible through the expression system can be divided into five major types. The first type of study involves the isolation of RNA from tissue, such as brain, to characterize the properties of a specific ion channel. The advantage of using _Xenopus laevis_ oocytes is that it provides an environment virtually free of contributions from endogenous transport proteins. The isolation of such responses can then lead to a second type of study, the development of assays for isolating cDNA clones encoding the relevant proteins (expression cloning). Nowadays, these types of studies have become less common due to the large availability of cDNA clones. A third type of study is the correlation of electrophysiological function with structural aspects of the involved molecules. The _Xenopus laevis_ expression system is particularly useful for this type of study due to the usually high signal-to-noise ratios. A fourth type of study is the determination of functional effects that are caused by mutations in the gene of a specific membrane protein. This type of study is particularly useful for understanding the relevant biophysical processes involved in human diseases. The last type of study is found in drug development, where a potential drug is screened against specific ion channels expressed in _Xenopus laevis_ oocytes. Binary (“hit-or-miss”) or dose-response experiments are conducted to determine the efficacy of potential drug candidates [5].

1.2.2. Morphology of Xenopus laevis oocytes

_Xenopus laevis_ oocytes have a spherical shape with a diameter between 0.3 mm and 1.2 mm. Their size depends on the development stage of the oogenesis (Figure 3). Typically, only stage V and VI oocytes are used due to their size and the fact that the development process has produced a phenotype that does not change significantly anymore [6].

The cytosolic contents of the _Xenopus laevis_ oocyte are not distributed equally and are visible as well-separated poles. The dark pole consists of biomass responsible for animal development (animal pole). Its coloration stems from pigments. The light pole consists of biomass used for nutrition of the animal (vegetal pole). The oocyte’s non-transparency is attributed to the presence of a lipid microdroplet emulsion in the cytosol. _Xenopus laevis_ oocytes have a mass density of approximately 1.2 g/cm³ due to their high biomass content. The mass density distribution within the oocyte is not homogeneous, with the vegetal pole having a higher density. This leads to the oocyte’s tendency to orient itself with the animal pole facing up when suspended in aqueous media.
Xenopus laevis oocytes, as opposed to other cell types, do not have their cell membrane directly exposed to the extracellular compartment. A vitelline membrane surrounds the cell membrane which gives the oocyte mechanical stability and alters the accessibility of metabolites to the cell membrane. These features are attributed to the vitelline membrane’s many invaginations. Removal of the vitelline membrane is possible but leaves the oocyte in a fragile state. However, the procedure may be necessary in cases where the experimenter needs to seal the oocyte’s surface tightly against an object (e.g. patch clamping).

The vitelline membrane itself is surrounded by the follicular layer which is, in most cases, removed enzymatically after extraction of the oocytes from the ovaries. Once the oocyte has left the ovaries, the follicular layer loses its functionality and is considered a hindrance to most experimental procedures.

Figure 4: (a) Cartoon of the Xenopus laevis oocyte. The relative scale has been changed for clarity. (b) Micrograph of the cross-section of a stage VI oocyte (magnification factor of 400). SE: Surface epithelium (follicular layer), VE: Vitelline envelope, FC: Follicular cells, CG: Golgi complex [6].
1.2.3. Procedures for using Xenopus laevis oocytes

Oocytes from Xenopus laevis are obtained through surgical removal of the ovaries of female frogs. This procedure does not result in the death of the frog such that it can be repeated many times. However, recovery of the frog must be taken into account which limits the frequency of operations. The frequency at which the experimenter needs fresh oocytes then determines the minimum number of frogs that have to be maintained. For example, if each frog is allowed a recovery time of six months, the maintenance of approximately 12 frogs is necessary if the experimenter needs two batches of oocytes per week.

After removal of the ovaries, the oocytes are usually treated with collagenase to remove the follicular layer that surrounds the oocyte. Mechanical and chemical accessibility to the cell membrane is compromised if the follicular layer is not removed. After the collagenase treatment the oocytes are suspended in Barth’s solution and stored at a temperature between 15 °C and 20 °C. At this stage, the oocytes can be preselected to avoid contamination of healthy oocytes via disintegration of non-viable oocytes.

Since the size of the oocytes depends on their current development stage, the experimenter usually selects stage V and VI oocytes for the studies as handling is facilitated by the larger size. If heterologous expression of a certain membrane protein is required, RNA or cDNA encoding the desired protein is injected into the oocyte. In the case of RNA, injection into the cytosol is done as it can be directly translated by the ribosome. cDNA, on the other hand, is injected into the nucleus as the genetic information needs to be transcribed into RNA which is subsequently released into the cytosol. While injection into the cytosol is a relatively simple procedure, injection into the nucleus generally requires a higher degree of experience and manual skill. A typical setup for microinjection consists of a stereomicroscope and a dedicated injection apparatus with a glass microneedle at the tip (Figure 5). After fabricating the microneedle out of a glass capillary, it is then first filled with a paraffin oil and then with the solution to be injected. The paraffin oil acts as a working fluid between the piston of the injection apparatus and the injection solution. The injection procedure involves penetration of the microneedle into the oocyte and subsequent dispensing of the desired volume [5].

After microinjection, the oocytes are incubated for 2 to 5 days during which expression of the desired membrane protein and its insertion into the cell membrane occurs. The incubation time is dependent on the amount of injected material, biosynthetic rate, incubation temperature and desired expression level. Neither very low nor very high expression levels are desirable which may make optimization of the incubation parameters necessary.

Once the protein has reached its target expression level, the oocyte is then ready for the experiment. In typical electrophysiology experiments, it is not uncommon to observe viability of the oocyte over the course of a few hours, if the oocyte has been of good quality to begin with.

---

1 Automated injection systems exist, but are mostly common to commercial use. E.g: 7. S. F. Graf et al., Fully automated microinjection system for Xenopus laevis oocytes with integrated sorting and collection. *Journal of laboratory automation* 16, 186 (Jun, 2011).
1.2.4. Comparison of the *Xenopus laevis* expression system with other heterologous expression systems for use in electrophysiology

In comparison with other systems there are a few major advantages in the use of *Xenopus laevis* oocytes for electrophysiological studies. First, the physical aspects of the oocyte greatly facilitate many preparative steps. Its size obviates the need for high magnification microscopes or specialized manipulation tools. The robustness of the *Xenopus laevis* oocyte promises a low failure rate during procedures where it is subjected to physical stress (e.g. immobilization, penetration by microelectrode). The *Xenopus laevis* oocyte is also quite tolerant to deviations from ideal incubation conditions. Changes in atmospheric composition, temperature and osmotic stress affect the oocyte’s survival less than in other cell types.

There are also biochemical advantages that are related to the low transmembranal activity present in native *Xenopus laevis* oocytes. Compared to other cell types, *Xenopus laevis* oocytes have a low density of endogenous membrane proteins. This leads to a low non-specific signal in electrophysiological experiments. In whole cell conductance measurements, the experimenter can thus expect a higher dynamic range of a signal specific to a membrane protein expressed in the oocyte, compared to other cell types. Moreover, due to the much larger surface area of the *Xenopus laevis* oocyte, a much higher signal amplitude is obtained at equal membrane protein surface density.

There are not only advantages, but also disadvantages in the use of *Xenopus laevis* oocytes, compared to other expression systems. First, not every membrane protein can be expressed successfully in *Xenopus laevis* oocytes. Second, the efficacy of pharmacological agents in *Xenopus laevis* oocytes has been shown to be lower than in native tissue or mammalian cell lines [5]. This phenomenon is attributed to the presence of the vitelline membrane which may lower the accessibility of solvated molecules to the membrane proteins. Last, and probably the most serious disadvantage is the fact that most assays of interest are not native to *Xenopus laevis*. Therefore, the functional properties of such an assay may be different.
from the native one. For example, a transport protein native to mammalian physiology may exhibit a different turnover rate due to the difference in temperature (20 °C vs. 37 °C) [3].

1.3. The Two Electrode Voltage Clamp

The concept of the voltage clamp was first introduced in the 1940s by Cole and Marmont. They discovered that it was possible to keep the membrane potential at a set level using two electrodes connected to a feedback control circuit. In 1949, the concept was exploited experimentally by Hodgkin, Huxley and Katz [8]. They performed studies on the electric permeability across the membrane of the giant axon isolated from squid. Even though the existence of ion channels was yet unknown, Hodgkin and Huxley demonstrated the existence of voltage-sensitive units within the membrane. The TEVC was the first implementation of the voltage clamp from which single electrode patch clamping later evolved [9].

1.3.1. The biological membrane as an electrical impedance

The voltage-sensitive units incorporated in biological membranes can be thought of as a voltage source and a variable resistance in series. These units are specific for certain ions such as sodium and potassium. The individual voltage sources represent the difference in electrical potential across the membrane as a result of the concentration gradient of the specific ion. The series resistance represents the internal resistance of the ion-specific unit, or in other words, the capability of contributing to the overall transmembrane potential. It follows the generalized Nernst equation

$$E = \frac{RT}{F} \ln a$$

(1.1)

Where $R$ is the gas constant, $T$ is the absolute temperature and $F$ is the Faraday constant. $a$ is the chemical activity across the membrane. Since we know that the translocation of different ion types is mediated through different ion-specific units, we can express the Nernst equation through the Goldman-Hodgkin-Katz (GHK) voltage equation

$$E_m = \frac{RT}{F} \ln \left( \frac{\sum P_{M^+}[M^+]_{\text{out}} + \sum P_{A^-}[A^-]_{\text{in}}}{\sum P_{M^+}[M^+]_{\text{in}} + \sum P_{A^-}[A^-]_{\text{out}}} \right)$$

(1.2)

where $P$ is the permeability coefficient of a specific ion, analogous to the internal resistance of the corresponding voltage-sensitive unit, $[M^+]_{\text{in}}$ and $[A^-]_{\text{in}}$ are the intracellular and $[M^+]_{\text{out}}$ and $[A^-]_{\text{out}}$ are the extracellular concentrations of the respective cations and anions [1].

The GHK voltage equation is derived from a macroscopic model that describes a mean state of conductance for a certain ion-specific unit. In reality, a single unit is comprised of individual membrane proteins that have a time-dependent on or off state. The state is determined by biophysical processes in the protein. The permeability coefficient is thus a macroscopic quantity that depends on the number of transport proteins and their open
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The permeability for a specific ion can also be expressed in terms of conductance, given by the open probability $O_i$, the intrinsic conductivity $g_i$, and number $N_i$ of the associated transport protein. The total membrane conductance is then given by the sum of all ion-specific conductances. For the total membrane resistance $R_m$, we get

$$R_m = \sum \frac{1}{g_i N_i O_i} \tag{1.3}$$

To complete the electrical model for the cell membrane, the impedance of the lipid-bilayer needs to be added. In principle, the lipid-bilayer is an electrical insulator which can be modeled as a plate capacitor because of its low thickness compared to its total area. Its capacitance $C_m$ can be written as

$$C_m = \frac{\varepsilon A}{d} \tag{1.4}$$

where $\varepsilon$ is the dielectric strength of the lipid-bilayer, $A$ is the total area of the membrane and $d$ is the thickness of the lipid-bilayer. Electrically, this capacitance is connected in parallel with the ion-specific units. As a result, we can describe the impedance of the cell membrane $Z_m$. With $s = i\omega$, we obtain

$$Z_m = \left( \frac{1}{R_m} + sC_m \right)^{-1} \tag{1.5}$$

Measurements on cholesterol-free phospholipid-bilayers determined a capacitance of approximately 0.4 $\mu$F/cm$^2$ (S. Ohki, The electrical capacitance of phospholipid membranes. Biophys J 9, 1195 (Oct, 1969)). Note that even though the cell membrane has a thickness of around 5 nm, the dielectric properties are dominated by the hydrophobic part of the phospholipid-bilayer. $d$ thus obtains a value that corresponds with the thickness of the bilayer.
1.3.2. Basic concept of the TEVC

The ideal voltage clamp (VC) defines the voltage across a biological membrane $V_m$ according to

$$V_m = \frac{V_c}{A_v} \tag{1.6}$$

where $V_c$ is the command voltage and $A_v$ is a constant scaling factor known to the experimenter. The TEVC is one practical implementation of the VC. It gets its name from the fact that there are two electrodes used with different purposes. One electrode serves as a voltage sensing electrode and the other electrode serves as a current passing electrode. The reason for this arrangement lies in the physical nature of ion-sensitive electrodes. First, many practical implementations of ion-sensitive electrodes have a high impedance - as high as several megaohms. This leads to a substantial voltage drop across the electrode if current is passed through it. Since this voltage drop is usually not well defined due to electrochemical processes taking place around the electrode, it is very difficult to use a current passing electrode for stable and accurate measurements. The solution to this problem is the use of a separate electrode for sensing the potential. This is achieved through the use of a pre-amplification stage with very high input impedance. Since virtually no current flows into the preamplification stage, there is no voltage drop across the voltage sensing electrode.
In order to achieve a user-defined transmembrane voltage, current needs to be passed through the current electrode to maintain it. This is achieved by implementing feedback control that compares the sensed voltage with the desired voltage and feeds back a corrective signal through the current electrode. The current injected into the system is monitored to allow accurate membrane conductance measurements. The actual implementation of the TEVC is achievable using analog or digital electronic systems. In principle, the feedback mechanism used in the TEVC is simply a proportional controller so that principles of basic control theory can be employed for the design of the TEVC.

![Diagram of voltage clamp](image)

Figure 7: Basic principle of the voltage clamp used to measure the electric membrane permeability in the giant axon of squid.

### 1.3.3. Typical TEVC setup for investigating *Xenopus laevis* oocytes

The main difference between the original voltage clamp used on the giant squid axon and the one used on *Xenopus laevis* oocytes comes from the electrode arrangement (Figure 8). Insertion of the intracellular electrodes is a more invasive process because the oocyte, as opposed to the giant squid axon, has an enclosing cell membrane. To reduce the disruption of the cell membrane, glass microelectrodes with a very fine tip are typically used. They are produced using a specialized micropipette-puller machine from small-diameter glass capillaries (Figure 9). The constriction at the tip of the glass microelectrode ends with an orifice of a size between 0.3 and 3 μm, which has a direct influence on the resistance of the electrode. Typical values are in the range of 1 to 10 MΩ which creates two major issues:

1. **Large voltage drop across the electrode.** For example, if a current of 10 μA flows through an electrode with a resistance of 10 MΩ, the resulting voltage drop is 100 V. Most integrated active components are not designed for operating voltages higher than 18 V. A high voltage amplifier stage is necessary which complicates the voltage clamp circuit.
(2) **Noise.** Not only does thermal noise increase with increasing resistance, but also the electrode’s proneness to interference from electromagnetic fields and mechanical vibrations increases. As a consequence, the use of Faraday shielding and mechanical decoupling is necessary. Also, the use of a preamplification stage (headstage) situated close to the electrode may be necessary.

Figure 8: a) Schematic setup of the TEVC. In this particular arrangement, two external electrodes are used to account for the access resistance. b) Equivalent electronic schematic of the oocyte/electrode arrangement. $C_M$ and $R_M$ represent the capacitance and resistance of the membrane. $R_{V_{\text{int}}}$ and $R_{I_{\text{int}}}$ represent the resistance of the internal electrodes, $R_{V_{\text{ext}}}$ and $R_{I_{\text{ext}}}$ the resistance of the external electrodes.

Figure 9: (a) Micropipette puller machine (Institute of Physiology, University of Zurich). (b) Image of the tip of a micropipette pulled from a glass capillary with an outer diameter of 1.5 mm.
In a typical TEVC setup for *Xenopus laevis* oocytes, the intracellular electrodes are attached to micromanipulator stages for accurately controlled insertion of the electrodes into the cytosol. This process is usually monitored using a conventional stereomicroscope. Other components usually include a structural element for placement and superfusion of the oocyte, a multi-channel perfusion system allowing the exchange of solution around the oocyte and optionally a temperature control element (Figure 10). Bath electrodes are brought in contact with the solution close to the oocyte to define the reference potential and to provide a return pathway for the current. A voltage clamp amplifier, which usually comes in the form of a rack-mounted device, is connected to the electrodes. Apart from the essential voltage clamp functionality, it usually provides signal recording capabilities (membrane voltage and current) and can often be interfaced to a computer-based data acquisition and control interface.

![Figure 10: (a) Typical TEVC setup with stereo microscope, two micromanipulators and eight solenoid valves for solution exchange. The solutions (not shown) are gravity-fed. Suction is applied at the outlet of the perfusion system to avoid spilling incidents. The entire setup is installed inside a Faraday cage. (b) Close-up photograph showing the central element with installed oocyte. The glass microelectrodes are inserted into the oocyte. This particular setup has temperature control which consists of a thermoelectric element and a temperature probe at the outlet of the perfusion pathway.](image)

### 1.3.4. Ag/AgCl electrodes

Ag/AgCl electrodes are electrochemical elements that are characterized by their capability of transforming ionic current into electronic current and vice versa. This transformation is achieved through a reduction and oxidation reaction at the interface between the ionic solution and the electrode. The redox reaction is characterized through

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

As can be seen, the net redox reaction involves the oxidation of elementary Ag and reduction of Ag⁺. It has a standard potential of +0.80 V (25°C, 1 atm, pH 0, ion activity of 1). Since AgCl is poorly soluble, the solution is saturated with respect to AgCl, meaning that any change in [Ag⁺] is compensated by the change in concentration of its counter ion Cl⁻. At constant temperature,
this results in a constant potential difference at the metal/solution interface, making the Ag/AgCl suitable for DC measurements.

However, changes in electrode potential can occur if the concentration of Cl\(^-\) changes due to electrodiffusion between the solution in proximity to the electrode and the solution interfaced to it. This causes the electrode potential to change over time as a function of the concentration gradient of Cl\(^-\). To minimize this drift, Ag/AgCl electrodes are usually enclosed in a container together with a saturated, or nearly saturated KCl solution. Interfacing to an external solution is then achieved via an ion-selective barrier that limits free diffusion of Cl\(^-\). Since in practice only small ion currents are expected, the Cl\(^-\) concentration next to the Ag/AgCl electrode does not change significantly in relative terms. These ion-selective diffusion barriers can be realized using a ceramic material, an agar bridge or by geometrical constriction of the diffusion pathway (Figure 11). These barriers, however, introduce junction potentials \(E_j\) according to Nernst’s law through

\[
E_j = \frac{u - v}{u + v} \frac{RT}{F} \ln \frac{c_1}{c_2}
\]  

where \(u\) is the mobility of the cations and \(v\) is the mobility of the anions \([\text{II}].\) \(c_1\) and \(c_2\) are the respective concentrations on each side of the diffusion barrier. In practice, the accurate calculation of the junction potential is not trivial, but Equation (1.8) may be helpful in getting a rough estimate to aid in the design of corrective measures, such as electronic voltage offset correction.

Figure 11: Cartoons of ion-sensitive electrodes employing (a) a silicate membrane, (b) an agar bridge and (c) a microsized constriction as a selective diffusion barrier.
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1.4. The ion-sensitive field effect transistor

The ion-sensitive field effect transistor (ISFET) is a sensor system that allows the detection of surface charge in solution via capacitive coupling. In contrast to the TEVC, the ISFET is a completely non-invasive sensor system.

1.4.1. Comparison with the metal oxide semiconductor field-effect transistor

The field-effect transistor (FET) is an electronic device that consists of a semiconducting channel connected to source and drain islands and a gate. The metal oxide semiconductor field-effect transistor (MOSFET) has a gate electrode that is separated from the conducting channel via an electrically insulating material (Figure 12). A voltage on the gate electrode $V_G$ generates an electric field inside the conducting channel which causes its charge density to change due to its semiconducting properties. The ion-sensitive field-effect transistor (ISFET) is a special case of the MOSFET, as the gate voltage is not generated electronically but via chemical charge separation in a solution (Figure 13). The main differences between the ISFET and the MOSFET can be summarized as:

1. The metal gate of the MOSFET is replaced by the solution in contact with the insulator and a reference electrode.
2. The insulator’s uppermost layer usually consists of a material that has sufficient electroactive properties at its surface and at the same time blocks charge from diffusing into the insulator.
3. Ion-selective membranes can be placed over the insulator to alter the ion-selectivity of the ISFET.
4. Encapsulation of the device other than the gate region is necessary to provide a diffusion barrier for the solution.

The large signal parameters of the ISFET are equivalent to the ones of the MOSFET. They are:

- **Drain current $I_D$.** This is the current entering at the source and exiting at the drain. The ease with which this current can flow is determined by the conductance of the channel between source and drain. The drain current is usually externally defined and kept constant.

- **Drain voltage $V_D$.** This is the potential difference going from drain to source. It is related to the drain current and the conductance between source drain according to Ohm’s law.

- **Gate voltage difference $V_G$.** This is the potential difference going from gate to source. It is equivalent to the gate voltage difference in the MOSFET.

For the MOSFET, a voltage bias applied to the gate electrode $V_B$ is characterized as

$$V_B = V_S + V_G$$  \hspace{1cm} (1.9)
where $V_S$ is the voltage going from source to substrate (ground). Due to the presence of solution between the reference electrode and the insulator, the gate bias voltage $V_B$ of the ISFET has additional potential difference contributions according to

$$V_B = V_S + V_G + E_I + V_{ref}$$  \hspace{1cm} (1.10)

where $E_I$ is the potential difference at the liquid/insulator interface and $V_{ref}$ is the reference electrode voltage as a result of the electrochemical potential of the redox reaction (Figure 14).

Figure 12: Schematic diagram of the MOSFET. (1) drain; (2) source; (3) substrate; (4) gate; (5) insulator; (6) metal contacts; (7) conducting channel [12].

Figure 13: Schematic diagram of the ISFET. (1) drain; (2) source; (3) substrate; (5) insulator; (6) metal contacts; (7) reference electrode; (8) solution; (9) electroactive layer; (10) encapsulant; (11) conducting channel [12].
Section 1.4.2. Small signal behavior of the ISFET

From first-order metal oxide semiconductor transistor theory we know that the drain current $I_D$ in the unsaturated region can be characterized using

$$I_D = \beta \left[ (V_G - V_T) V_D - \frac{1}{2} V_D^2 \right]_{V_G \approx V_G - V_T}$$

(1.11)

where $V_T$ is the threshold voltage, which is the net resulting voltage due to the effects of substrate depletion charge (Figure 15), metal work function, interface states and fixed charges in the oxide [13]. $\beta$ is a geometric device parameter that depends on the charge carrier mobility $\mu$, the capacitance of the insulator $C_{ox}$ and the ratio between the width $W$ and length $L$ of the gate region.

$$\beta = \mu C_{ox} \frac{W}{L}$$

(1.12)

The mutual conductance $S$ between drain and source can also be expressed as a change in $I_D$ as a function of $V_G$. It is equivalent to the product of $\beta$ and $V_D$:

$$S = \frac{dI_D}{dV_G} \bigg|_{V_G=\text{const}} = \beta \cdot V_D$$

(1.13)

For the resistance $R_{ch}$ of the conducting channel between drain and source we can write
\[ R_{eh} = \left. \frac{dV_D}{dI_D} \right|_{V_G = \text{const}} = \frac{1}{\beta(V_G - V_T - V_D)} \] (1.14)

In the MOSFET, \( V_G \) is the input variable and \( V_T \) is kept constant, while in the case of the ISFET, \( V_T \) is the input variable and \( V_G \) is kept constant. We know that \( V_T \) depends on the surface charge of the insulator so that we can write

\[ V_T = V_T^* + E_I \cdot f \] (1.15)

and

\[ \Delta V_T = \Delta E_I \cdot f \] (1.16)

where \( E_I \) is the Nernst potential (see Equation (1.1)) at the insulator surface and \( f \) is an integrity factor that depends on non-ideal behavior (defects) of the materials. From Equations (1.14) and (1.16) we can conclude that the parameters \( V_D, V_G \) and \( R_{ch} \) have to be kept constant if direct readout of the interface voltage at the insulator of the ISFET is desired [14].

Figure 15: Model of the substrate depletion charge at the n-p junction. Shown below are the amplitudes of the charge, the electric field and the voltage as a function of distance perpendicular to the junction plane [15].
1.4.3. pH sensitivity of the ISFET

The surface of a metal oxide can undergo chemical binding due to exposed oxide groups. If a metal oxide surface is exposed to water, the oxide groups may interact with the water molecules and induce hydrolysis (Figure 16). Since the exposed oxygen atom is initially bound to one metal atom only via a single $\sigma$ bond, the oxygen atom is left with six free valence electrons. As a consequence, we can create a site-binding model where the oxygen atom can undergo binding reactions with protons thanks to its electron donating properties [16]. If no proton is bound, the oxygen atom is negatively charged. If one proton is bound, the oxygen atom adopts neutrality. If a second proton binds to the oxygen atom, the group becomes positively charged. The probability of assuming one of these three states depends thus on the availability of free protons in solution. We can therefore expect a dependence of the surface charge density at the oxide surface on the pH of the solution. Application of the Nernst equation for the proton surface charge density

$$E_H = \frac{RT}{F} \ln \frac{\sigma_H}{\sigma_{H^{2pc}}}$$

(1.17)

where $\sigma_{H^{2pc}}$ represents the proton zero point charge density. It is the proton surface density at which the surface has no net charge. From the above equation, we can then calculate the pH sensitivity of the sensor. Since we know that

$$\frac{\sigma_H}{\sigma_{H^{2pc}}} = a_H = 10^{-pH}$$

(1.18)

for the ideal pH sensitivity at 20 °C, we obtain

$$E_H = -58.2 \frac{mV}{pH} \mid_{T=20^\circ C}$$

(1.19)

The above equation is valid in the region where the surface is not saturated with protons. It is therefore important to choose a metal oxide with sufficiently large proton buffering capacity within the desired pH detection range [17].

The binding and unbinding of protons on the metal oxide surface can be described via Langmuir’s adsorption model [18]. It states that the rates of adsorption $r_{ad}$ and desorption $r_d$ depend on the respective binding rate constants $k_{ad}$ and $k_d$, the partial pressure of free protons $p_H$ and the surface concentration of free sites $[S]$ and occupied states $[H_{ad}]$, as expressed through

$$r_{ad} = k_{ad} p_H [S]$$

(1.20)

and

$$r_d = k_d [H_{ad}]$$

(1.21)
These equations assume that protons can diffuse freely and that there are no inelastic interactions between them (see Section 1.6.8). In reality this is not the case and therefore Equations (1.20) and (1.21) should not be used for accurate calculations.

![Diagram of site-binding model of the oxide-water interface]

**Figure 16**: Site-binding model of the oxide-water interface

### 1.4.4. Temperature sensitivity of the ISFET

The temperature sensitivity of the ISFET is directly related to the Nernst equation. Therefore, the sensitivity of the ISFET scales linearly with temperature. At a pH value of 7, we obtain

\[
\frac{d}{dT} \left( \frac{RT}{F} \ln a_H \right) = -1.39 mV / K \left. \right|_{pH=7}
\]

(1.22)

Considering the pH sensitivity of an ideal ISFET (-58.2 mV/pH at 20 °C), the influence of temperature must be taken into account if it is not kept constant. In such cases, the pH sensitivity can either be corrected through acquisition of the temperature signal or through electronic compensation [19].

### 1.4.5. Range of detection of the ISFET sensor

Due to the solution’s ionic nature, the electric field length of a charge is limited by the self-shielding properties of an electrolyte. It can be characterized via the Debye length \( \kappa^{-1} \) by

\[
\kappa^{-1} = \sqrt{\frac{\varepsilon_0 \varepsilon_r k_B T}{2 N_e e^2 I}}
\]

(1.23)

With the ionic strength \( I \) given by

\[
I = \frac{1}{2} \sum_{i=1}^{N} c_i z_i^2
\]

(1.24)

where \( z_i \) is the charge number for a specific ion with its corresponding concentration \( c_i \). From the above equations we can deduce the detection distance \( \lambda_d \) of the sensor surface which is equivalent to the Debye length. For a typical physiological solution containing 100 mM NaCl (\( \varepsilon_r = 80 \) for water) at 20 °C we obtain [20]

\[
\lambda_d \approx 1 \text{ nm}
\]

(1.25)
\( \lambda_d \) is an important parameter for the design of an ISFET sensor. If, for example, ions were able to diffuse into the insulator with a penetration distance of more than \( \lambda_d \), electroactive events at the insulator/liquid interface would not be detected by the ISFET sensor. It is therefore very important to choose an insulator material with low permeability to electrolytes.

1.5. State-of-the-art in microsystem and automation technology for measuring membrane transport in single cells

1.5.1. Lab-on-a-chip technology

With the introduction of microtechnology within the various fields of life sciences, a new generation of microdevices for chemical and biological analysis emerged (Figure 17). Such microsystems, most often called micro total analysis systems (\( \mu \)TAS) or Lab-on-a-Chip, were introduced to expand the possibilities in chemical and biological analysis and to overcome limitations of traditional analytical methods [21]. The most beneficial contributions of \( \mu \)TAS can be summarized as:

1) **Miniaturization.** This is an inherent property of \( \mu \)TAS. It is achieved through the use of lithographic techniques or precision robotic tools. As a consequence, this increases the potential for high system portability and reduced sample consumption.

2) **Integration.** Thanks to common microfabrication techniques, sensor systems and micro electromechanical systems (MEMS) can be integrated with microfluidics. This enables sensing and manipulation on a microscopic level.

3) **Predictability.** \( \mu \)TAS allow for a high degree of precisely-defined integration. In addition, chaotic advection is not present in microfluidic systems. These aspects greatly enhance the predictability of the \( \mu \)TAS, so that the design process is less based on trial and error.

4) **Speed.** As many sensing techniques have two-dimensional detection, downscaling of a system can shorten the time needed for the analysis. This is due to the higher surface-to-volume ratio and shorter diffusion distances compared to larger systems.

5) **Cost.** Due to the manufacturing methods used for mass-production of \( \mu \)TAS, high volumes with low cost per unit can be achieved.

The potential of \( \mu \)TAS for performing electrophysiology on living cells has been exploited in the recent past with significant success. A number of voltage clamp techniques as well as other methods for monitoring membrane transport in *Xenopus laevis* oocytes and mammalian cells have been developed.
1.5.2. Chip-based voltage clamp techniques

There has been continued interest in improving voltage clamp techniques with regard to ease of operation and throughput. Planar patch clamp methods have been developed to avoid the delicate and labor-intensive use of micropipettes. For mammalian cells, a number of microchip-based planar patch clamp systems has been realized. The first system was presented by Schmidt and coworkers. It consisted of a hole etched into a silicon nitride membrane onto which a polydimethylsiloxane (PDMS) layer was deposited. Self-integrating channel-forming peptides in combination with giant unilamellar vesicles were successfully used for measuring microscopic membrane currents [23]. Fertig and coworkers presented a patch clamp method based on a planar glass chip into which holes were etched using gold ions (Figure 18). They used CHO cells overexpressing calcium-activated potassium channels [24]. A planar patch clamp method specific to *Xenopus laevis* oocytes was developed by Klemic and coworkers (Figure 19). They fabricated electrodes out of micromolded PDMS and measured potassium currents in oocytes expressing *Shaker* channels [25]. Chip-based versions of the planar patch clamp for mammalian cells were developed which rely on a lateral arrangement of the cell trapping (Figure 20). These systems simplify the fabrication process and allow the use of microfluidic channels for access to the cytosol [26-29].
Figure 19: Planar patch clamp method for *Xenopus laevis* oocytes. It features a suction manifold for immobilization and patching of the cell and a perfusion pathway for solution exchange [25].

![Planar patch clamp method](image)

Figure 20: SEM images of a patch clamp device with multiple lateral trapping sites. (g) shows the upper layer of the device before bonding (upside down) and (h) shows the trapping hole after bonding. (i) shows the SU-8 mold of the access channel for the trapping hole [29].

![SEM images](image)

Cucu and coworkers introduced the first voltage clamp method for *Xenopus laevis* oocytes that allowed currents across the entire cell to be measured (Figure 21). The new method, referred to as transocoety voltage clamp (TOVC), involves the immobilization of the oocyte over a large orifice such that the membrane impedance is divided into two equal
fractions. The group conducted AC voltage clamp experiments to detect changes in transoocyte impedance upon activation and deactivation of potassium channels [30].

Figure 21: (A) Equivalent electronic schematic of the TOVC. The arrangement divides the impedance of the oocyte membrane into two separate impedances. (B) Nyquist plot of the transoocyte impedance when subjected to 90 mM NaCl Ringer solution on each side [30].

A chip-based macropatch clamp system for *Xenopus laevis* oocytes has been developed by Dahan and coworkers (Figure 22). They successfully integrated planar macropatch clamping, Ag/AgCl electrodes and a microfluidic solution exchange system into a single µTAS. Measurements on the epithelial sodium channel (ENaC) demonstrated the system’s capability of rapid, automated solution exchange at the cell membrane. Another important aspect of the system is its non-invasive methodology. Instead of disrupting the cell membrane to gain electrical access to the cytosol, it relied on the self-permeabilizing properties of ENaC [31, 32].

Figure 22: (a) Multifunction PDMS microchip for macropatch voltage clamp measurements on *Xenopus laevis* oocytes. (b) The cross-section view of the chip reveals its multi-layered structure [32].

1.5.3. Alternative electrophysiology microdevices

A few microtechnology-based systems for investigating membrane transport without the use of voltage clamping have been developed. Dharia and coworkers described a method for mapping the impedance of the cell membrane in *Xenopus laevis* oocytes (Figure 23). They used a multi-
layered μTAS where they integrated electrode arrays arranged in a circular fashion around the oocyte membrane. Results showed that the density of expressed membrane transport proteins was not homogeneous and especially that there were significant differences between the vegetal and animal pole of the oocyte [33].

A radically different method for monitoring membrane transport in *Xenopus laevis* oocytes was developed by Sakata and coworkers. Instead of characterizing current-voltage relationships, they used a surface charge detection method based on an ISFET. By configuring the ISFET in a source-drain follower mode, the method allows the direct readout of the Nernst voltage generated at the sensor surface as a result of chemical charge separation. To demonstrate the change of surface potential upon modulation of membrane transport, the researchers performed studies on uptake of estrone-3-sulfate in cells heterologously expressing the human organic anion transporting peptide C (OATP-C). In a second experiment involving estradiol 17β-D-glucuronide (E217βG) uptake, the method proved to be sensitive enough for discriminating between mutant variants of OATP-C [34].
1.5.4. Robotized electrophysiology systems

A number of robotized electrophysiology systems has emerged in the commercial sector of electrophysiology. High-throughput systems based on planar patch clamping often use arrays which are often compatible with 96-well plate technology such that existing pipetting robotics can be used without further modification. Manufacturers of such systems include Sophion Bioscience (QPatch), Molecular Devices (PatchXpress), Cytocentrics (Cytopatch). A comparison of these systems was done by Dunlop and coworkers, laying out the advantages and disadvantages of each system [35]. Screening systems designed for high-throughput electrophysiology on *Xenopus laevis* oocytes include the POETs™ platform OpusExpress 6000A by Axon Instruments and the Roboocyte and Hiclamp instruments by Multichannel Systems [36-38].

All of the systems mentioned so far share the disadvantage of being too large for installation on a typical laboratory bench. A benchtop-style system for performing automated patch clamping of mammalian cells is the Port-a-Patch designed and manufactured by Nanion Technologies. It is, however, only a single-channel device, which makes it unsuitable whenever high data throughput is demanded [39].
1.6. Kinematic properties of a fluid and solute transport inside a microchannel

µTAS often incorporate microfluidic systems to benefit from the advantages described in Section 1.5.1. In such systems, bulk motion of a fluid is restricted by its viscosity, preventing the streamlines from being chaotic and thus allowing them to be predicted mathematically.

1.6.1. Shear stress and viscosity

To understand the behavior of liquid motion inside a microchannel, it is important to know which are the fundamental forces that are involved. It is evident that an intrinsic cohesive force must exist that keeps liquids from disintegrating. These force interactions exist also on a molecular level which can be comprised of either purely electrostatic interactions (e.g. benzene) or a combination of electrostatic and chemical interactions (e.g. protic liquids). On a macroscopic scale, these forces can be characterized using viscosity as a bulk property of a liquid phase. In principle, viscosity is merely a result of shear stress $\tau$ within a liquid. In Newtonian physics, it is defined as

$$\tau = \frac{F}{A}$$

(1.26)

Where the vector of the force $F$ is parallel to the surface area $A$. To characterize the shear stress in liquids, one could start by considering a rectangular block element in the Cartesian coordinate system. When subjected to shear stress, the block will deform into a 6-sided prism (Figure 26). If we now regard the structure as being comprised of infinitely thin sheets which slide past each other with varying velocity (no velocity for the lowermost and maximum velocity for the uppermost), we can immediately assume the following proportionalities (in orthogonal coordinates):

![Figure 25: (a) Port-a-patch system by Nanion Technologies. (b) Port-a-patch system with internal perfusion option [39].](image-url)
\[
\tau \propto \frac{1}{\Delta y} v_x
\]  
(1.27)

where \( \Delta y \) is the height of the block and \( v_x \) is the scalar component of the velocity vector parallel to the stress vector. For calculating the shear stress in a liquid, we can therefore use:

\[
\tau = \mu \frac{v_x}{\Delta y} = \mu \frac{\partial v_x}{\partial y}
\]  
(1.28)

where \( \mu \) is a liquid-specific proportionality factor, called dynamic or absolute viscosity. Note that the internal volume of the block is preserved when deformed under shear stress. In reality, this is not the case as all materials compress or expand when put under pressure. To account for these effects, we need to make Equation (1.28) dependent also on the mass density \( \rho \). Thus, the kinematic viscosity \( \eta \) is introduced, employed wherever liquids are considered compressible:

\[
\eta(\rho) = \frac{\mu}{\rho}
\]  
(1.29)

However, in typical microfluidic systems where water at constant temperature is used, the assumption of incompressibility bears reasonable accuracy when fluid motion problems are solved.

![Figure 26: Deformation of a rectangular block under shear stress. In 3D space, the resulting geometric structure is a 6-sided prism. The scalar component of the velocity parallel to the shear stress is linearly dependent on \( y \) and equals zero for \( y = 0 \).](image)

1.6.2. Laminar flow and the Reynolds number

Liquid flow mechanics inside a microchannel have several characteristics that make them different from those observed in the macroscopic world. Probably the most important characteristic is that the viscous forces become more dominant with decreasing channel dimensions. If these forces are significantly higher than the ones forcing the fluid through the channel (drag forces), laminar flow is observed. The conditions for laminar flow are thus
dependent on the kinematic viscosity of the liquid, the flow velocity $v$ and the hydraulic diameter $D_H$ of the channel cross-section. To determine whether the flow inside a closed channel is laminar or turbulent, the Reynolds number $Re$ can be used:

$$Re = \frac{\rho v D_H}{\mu}$$  \hspace{1cm} (1.30)

The hydraulic diameter depends on the shape of the cross-sectional profile of the channel. For a circular duct, $D_H$ is equivalent to its diameter. For a non-circular duct, where all sides are of equal length, the hydraulic diameter can be characterized as

$$D_H = \frac{4A}{P}$$  \hspace{1cm} (1.31)

where $A$ is the cross-sectional area and $P$ is the perimeter of the duct. For a wide duct, where the width is much longer than the height, the characteristic dimension is approximately twice the height.

For fluid flow in a pipe, $Re$ values below 2300 predict laminar flow and values above 4400 predict turbulent flow. Values between 2300 and 4400 are considered to be in a transition region where both laminar and turbulent flow can occur. In this case, factors relating to the inner wall surface (e.g. roughness) determine the flow behavior [40]. Since $Re$ depends on both $v$ and $D_H$, laminar flow can also exist in large environments (Figure 27).

![Figure 27](image)

Figure 27: Laminar flow is not exclusively inherent to microfluidic systems, but is also observed in large-scale phenomena such as in glacier formation [41].

1.6.3. The Navier-Stokes equation

In principle, Equation (1.30) is a consequence of the Navier-Stokes equation describing the motion of fluids. Consistent with Newton’s second law of motion, the Navier-Stokes equation
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assumes that the total momentum of the fluid is conserved and that transfer of momentum can occur between infinitesimal elements within the fluid:

\[
\rho \left( \frac{\delta \vec{v}}{\delta t} + \left( \vec{v} \cdot \nabla \right) \vec{v} \right) = -\nabla p + \nabla \cdot \mathbf{T} + \vec{f} \tag{1.32}
\]

where \( \rho \) is the density of the fluid and \( \vec{v} \) is the velocity vector of an infinitesimal element of the fluid. \( p \) is the pressure, \( \mathbf{T} \) is the stress tensor and \( \vec{f} \) represents body forces such as gravity. In principle, the left hand side describes fluid motion and the right hand side describes the involved forces. The first term on the right hand side is the divergence of pressure, such as the pressure drop along a fluid pathway. The second term describes the viscous forces (shear stress) as a consequence of the cohesive forces between the elements (molecules) of the fluid.

If the fluid is assumed to be non-compressible, the stress term can be rewritten such that it depends solely on the constant dynamic viscosity \( \mu \) and the velocity vector \( \vec{v} \). Moreover, the volume is conserved which means that the scalar product of the Laplace operator and the velocity vector equals zero:

\[
\rho \left( \frac{\delta \vec{v}}{\delta t} + \left( \vec{v} \cdot \nabla \right) \vec{v} \right) = -\nabla p + \mu \nabla^2 \vec{v} + \vec{f} \tag{1.33}
\]

\[\nabla \cdot \vec{v} = 0\]

The Navier-Stokes equation is often used for solving liquid flow problems in computer fluid dynamics (CFD) simulations. The finite element method (FEM) is usually employed which discretizes user-defined geometry in two- or three-dimensional space. In most cases, the assumption of incompressibility of the liquid is sufficient. In principle, CFD can solve liquid flow problems with arbitrary complexity. Before solving the model, the user needs to define the physical properties (density and viscosity) of the bulk fluid and the initial boundary conditions (pressure, velocity) wherever the liquid is in contact with an open boundary of the geometric structure. At the closed boundaries, the so-called “no-slip” condition is usually employed which states that molecules in direct contact with the wall cannot move due to their strong interaction with the surface.

1.6.4. Laminar flow through a long cylinder

If one wants to calculate macroscopic parameters of liquid flow through long uniform channel geometries, the Navier-Stokes equation can be greatly simplified. We assume there is no convective flow due to the uniform channel geometry. Furthermore, there are no unsteady accelerations because the flow is assumed to be fully developed (steady-state flow). The pressure term can also be simplified into a term containing dependence only along the \( x \) axis. The velocity vector, on the other hand, shows dependence only in \( y \) and \( z \) direction. Equation (1.33) becomes
\[
\frac{\delta p}{\rho} = \frac{\delta^2 v_y}{\delta y^2} + \frac{\delta^2 v_z}{\delta z^2}
\] (1.34)

For a cylindrical channel, \(v_y\) and \(v_z\) are equal which allows us to introduce the velocity function \(v(r)\) which depends only on the radius \(r\):

\[
\frac{1}{\rho} \frac{\delta p}{\delta x} = \frac{1}{\eta} \frac{\delta^2 v(r)}{\delta r^2}
\] (1.35)

Double integration results in a second order polynomial with the two unknown parameters \(A\) and \(B\):

\[
v(r) = \frac{1}{2\eta} \frac{\delta p}{\delta x} r^2 + Ar + B
\] (1.36)

The exact solution for the cylindrical channel is found by knowing that the axial velocity at the walls equals zero (no-slip condition) and that the maximum velocity is found along the center axis (axisymmetry) (Figure 28):

\[
v(\pm R) = 0 \quad v(0) = v_{\text{max}}
\] (1.37)

Equation (1.36) thus simplifies to:

\[
v(r) = -\frac{R^2}{2\eta} \frac{\delta p}{\delta x} \left(1 - \frac{r^2}{R^2}\right) = v_{\text{max}} \left(1 - \frac{r^2}{R^2}\right)
\] (1.38)

To calculate the quantity for average volume flow velocity \(Q\), we integrate over the volume using

\[
\frac{\delta V}{\delta t} = Q = \int_0^{2\pi} d\phi \int_0^R v(r) r dr
\] (1.39)

to obtain the Hagen-Poiseuille relation

\[
Q = \frac{\pi R^4 \Delta p}{8\eta \Delta x}
\] (1.40)

The Hagen-Poiseuille equation describes the relation between the flow rate \(Q\) and pressure drop \(\Delta p\) for a defined length segment \(\Delta x\) inside a cylinder with radius \(R\). It is analogous to Ohm’s law for electrical conductivity. Therefore, we can introduce an extensive quantity which describes the hydrodynamic resistance \(R_h\) of a cylinder using

\[
R_h = \frac{\Delta p}{Q} = \frac{8\eta \Delta x}{\pi R^4}
\] (1.41)
For water at the standard temperature of 20 °C we obtain (in SI units)

\[ R_h^* = 2.55 \cdot 10^{-3} \frac{\Delta x}{R^4} [Pa \cdot s] \]  

(1.42)

Figure 28: Parabolic flow profile of fully developed liquid flow through a uniform, infinitely long channel in 2D space. The velocity of the elements directly at the wall is zero, where the no slip condition is true. The elements with the highest velocity are found along the center axis of the fluid pathway.

1.6.5. Laminar flow through a long rectangular duct

Since many microfluidic devices are fabricated using lithographic processes, their channel cross-sections are most often of rectangular shape. The exact solution for the hydrodynamic resistance of a rectangular duct is

\[ R_h = -\frac{3\eta}{4ba^3} \Delta x \left[ 1 - \frac{192a}{\pi^3 b} \sum_{i=1,3,5,...}^{\infty} \frac{\tanh \left( \frac{i\pi b}{2a} \right)}{i^2} \right]^{-1} \]  

(1.43)

with \(-a \leq y \leq a\) and \(-b \leq z \leq b\) [42]. Since the fraction in the sum series converges to zero with the fifth power, it is generally sufficient to approximate the equation considering the first term only:

\[ R_h \approx -\frac{3\eta}{4ba^3} \Delta x \left[ 1 - \frac{192a}{\pi^3 b} \tanh \left( \frac{\pi b}{2a} \right) \right]^{-1} \]  

(1.44)

For a rectangular duct where \(a\) and \(b\) have comparable length, we can use the hydraulic diameter derived from Equation (1.31) and insert it into the Poiseuille-Hagen Equation (1.41) to obtain

\[ R_h = \frac{8\eta\Delta x}{\pi} \left( \frac{w + h}{wh} \right)^4 \bigg|_{w=h} \]  

(1.45)

where \(w\) is the width and \(h\) is the height of the channel.
1.6.6. Global flow parameters in a microfluidic setup

In a typical setup of a microfluidic system, the main fluid components consist of a microfabricated device, external tubing, and reservoirs. Depending on the method used for driving the liquid through the microchannels, global flow parameters such as total hydrodynamic resistance, hydrostatic pressure or changes in total liquid volume need to be considered.

In pressure-driven systems, determination of the resulting flow rate requires knowledge of the total hydrodynamic resistance. Due to the analogy between electrical and fluidic circuits, Kirchhoff’s laws for calculating the total resistance of a network of individual resistances can be used. For fluidic pathways connected in series, the total resistance becomes

$$ R_{total} = \sum_{i=1}^{N} R_i $$

(1.46)

For fluidic pathways connected in parallel, the total resistance becomes

$$ R_{total} = \left( \sum_{i=1}^{N} \frac{1}{R_i} \right)^{-1} $$

(1.47)

In applications where the pressure drop resulting from the fluid driving source is in the range of millibars, the contribution of hydrostatic pressure may need to be taken into account. At equilibrium, each centimeter of height difference $\Delta h$ between the source and the sink of the system generates one millibar of hydrostatic pressure $\Delta p_h$ according to

$$ \Delta p_h = \rho g \Delta h $$

(1.48)

where $g$ is the standard gravity constant. Note that the compressibility of the fluid is neglected such that the mass density can be considered constant. If both, hydrostatic and externally applied pressure $p_{ext}$ is present, the total pressure drop across a fluidic system equates to

$$ \Delta p_{total} = \Delta p_{ext} + \Delta p_h $$

(1.49)

Hydrostatic pressure is the force per area exerted by a water column with an equal cross-sectional area. The potential energy stored by the water column can be converted into kinetic energy to create a flow through the system. The total energy of the system remains constant, according to Bernoulli’s equation for conservation of energy:

$$ \Delta p_{ext} + \rho g \Delta h + \frac{1}{2} \rho v^2 = const. $$

(1.50)

In volume-driven systems, such as syringe pumps, the hydrostatic pressure cannot be used for converting potential energy into kinetic energy. This is because the volume change per time unit is entirely controlled by the linear motor of the pump system.
1.6.7. Transport of solutes in a microchannel

On a macroscopic scale, free diffusion of a solute occurs upon the presence of a concentration gradient, as characterized by Fick’s first law:

\[ J = -D \nabla \phi \]  

(1.51)

where \( J \) is the resulting diffusion flux, \( D \) is the diffusion coefficient and \( \phi \) is the concentration of the solute.

To calculate the change in concentration over time, Fick’s second law is employed:

\[ \frac{\delta \phi}{\delta t} = D \nabla^2 \phi \]  

(1.52)

The diffusivity of a solute is dependent on the kinematic viscosity of the fluid and the hydrodynamic radius \( R_H \) of the molecules or particles of a solute. The drag force \( F_d \) of a spherical particle suspended in a laminar flow is found by solving the Navier-Stokes equation for a non-compressible, Newtonian liquid (Equation (1.33)):

\[ F_d = 6 \pi \mu R_H v_s \]  

(1.53)

which is known as Stokes’ law. The settling velocity \( v_s \) is the velocity reached when the drag force and the driving force are perfectly balanced. The driving force of diffusion originates from the thermal energy of the solute, put into relation with the macroscopic quantity temperature \( T \) by the Boltzmann constant \( k_B \). For the diffusion coefficient we therefore obtain:

\[ D = \frac{k_B T}{6 \pi \eta R_H} \]  

(1.54)

If there is fluid flow (advection) inside the microchannel, Fick’s second law gets expanded to obtain the formula for convection-diffusion

\[ \frac{\delta \phi}{\delta t} = D \nabla^2 \phi - \nu \nabla \phi \]  

(1.55)

As can be seen from the above equation the velocity field can be calculated separately. In a typical CFD problem involving convective solute transport (Figure 29), the user would first solve the velocity field using the Navier-Stokes equation and then insert it into the convection-diffusion equation to obtain the concentration field for a given point in time.
1.6.8. Proton mobility mechanism

Currently, the mechanism of proton mobility in water is not completely clear. Most will agree, however, that the net diffusion of a proton is not driven by free Brownian diffusion, as generally assumed for other ions. More likely, solvated protons are believed to interact heavily with the lone pairs of water molecules. As a consequence, net displacement of a proton is the result of association/dissociation events between structural water and solvated protons. These events are believed to be connected in a chain-like fashion, where an association/dissociation event at the start of the chain may propagate through the chain to ultimately induce an association/dissociation event at the end of the chain. Consequently, net proton displacement is an incoherent process where the originally displaced proton does not necessarily appear at the new position of appearance. Although somewhat misleading, this mechanism is often called proton hopping \[43\]. Nuclear magnetic resonance and \(^{17}\text{O}\)-resonance techniques revealed proton hopping times of around \(\tau_p \approx 1.5\) picoseconds \[44\]. These times are much faster than solvent reorganization times such that we consider the solvent’s structure to be static during a proton hopping process (Figure 30).

Figure 29: CFD simulation of convective transport of a solute in a fully developed flow in orthogonal 2D space. At the inlet, a solute with concentration \(c_0\) flows into the lower half of the channel. Diffusion into the upper half of the channel occurs until there is no concentration gradient across the channel cross-section (\(c=0.5c_0\)). The colored lines represent frontlines of equal concentration.

![Figure 30](image)

Figure 30: (a) Classical diffusion mechanism. The diffusion time depends on the mobility of the free ion and the travelled distance \(r\). (b) Proton hopping mechanism. The diffusion time depends on the hopping time \(\tau_p\) and the number of hopping events \(N\). In this figure, molecule orientation is not taken into account.
1.7. **Scope of the thesis**

During the course of my dissertation I developed two non-invasive methods for monitoring membrane transport activity in *Xenopus laevis* oocytes. The first method, the asymmetrical transoocyte voltage clamp (ATOVC), was developed specifically for use under extreme environmental conditions. Most electrophysiological recording systems are designed for use under standard laboratory conditions. The ATOVC, on the other, had to fulfill the following criteria:

i. Simple, non-invasive sample preparation
ii. High sample throughput per time unit
iii. Automatable operation
iv. Low susceptibility to electronic noise originating from external sources
v. Low power consumption
vi. Small form factor, portability
vii. Resistance to changes in gravitational acceleration
viii. Resistance to mechanical stress from vibration and shock

The ATOVC had to be operated during parabolic flight campaigns of the European Space Agency ESA. There are a number of limitations and constraints associated with parabolic flight that make the successful use of existing laboratory-based methods difficult or impossible.

The second method was based on a completely different methodology for monitoring transport activity at the cell membrane. Instead of measuring membrane conductance, a sensing method based on surface charge detection was employed. Implementation of the detection method was achieved using an ion-sensitive field-effect transistor (ISFET) integrated with a microfluidic perfusion system. The design criteria were similar to those mentioned above with the exception of points viii and ix due to the method’s restriction to standard laboratory conditions. Furthermore, the second method was designed to further facilitate parallelization through multiplexing capability, a feature which is easier to achieve in charge-based detection methods compared to conductance-based methods.

The applicability of ISFET sensor technology to mammalian cells was also determined during the dissertation. To achieve this, modification of the microsystem developed for *Xenopus laevis* oocytes was necessary.
2. Microfluidic platform for electrophysiological studies on *Xenopus laevis* oocytes under varying gravity levels

Voltage clamp measurements reveal important insights into the activity of membrane ion channels. While conventional voltage clamp systems are available for laboratory studies, these instruments are generally unsuitable for more rugged operating environments. In this study, a non-invasive microfluidic voltage clamp system developed for the use under varying gravity levels is presented. The core component is a multilayer microfluidic device that provides an immobilization site for *Xenopus laevis* oocytes on an intermediate layer, and fluid and electrical connections from either side of the cell. The configuration that is termed the asymmetrical transoocyte voltage clamp (ATOVC) also permits electrical access to the cytosol of the oocyte without physical introduction of electrodes by permeabilization of a large region of the oocyte membrane so that a defined membrane patch can be voltage clamped. The constant low level air pressure applied to the oocyte ensures stable immobilization, which is essential for keeping the leak resistance constant even under varying gravitational forces. The ease of oocyte mounting and immobilization combined with the robustness and complete enclosure of the fluidics system allow the use of the ATOVC under extreme environmental conditions, without the need for intervention by a human operator. Results for oocytes over-expressing the epithelial sodium channel (ENaC) obtained under laboratory conditions as well as under conditions of micro- and hypergravity demonstrate the high reproducibility and stability of the ATOVC system under distinct mechanical scenarios.
Chapter 2: Microfluidic platform for electrophysiological studies on Xenopus laevis oocytes under varying gravity levels

2.1. Introduction

The regulated movement of selected ion species through ion conducting channels in the plasma and intracellular membranes is essential for cell survival as well as cellular functions such as osmoregulation and intra- and intercellular signaling [45]. Disturbance to ion channel function undermines cell survival and may potentially manifest itself as a diseased state on the organismal level [46]. Ion channel function can be physiologically regulated by chemical, physical or mechanical gradients. The actions of chemical and pharmacological agents such as channel blockers and modulators of ion channel function have been well characterized and the underlying molecular mechanisms elucidated; in contrast, the role and influence of physical and mechanical stresses is less clear. Mechanical stimulation such as shear forces and mechanical strain is known to induce rearrangements of the cell’s mechanotransductive apparatus, including the cytoskeleton, as well as the expression pattern of cell-regulatory proteins that in turn can modulate ion channel activity [47, 48].

Previous studies have shown that gravitational force influences the activity of ion channels involved in cellular mechanotransduction [49, 50]. In this context, it has been shown that muscle cells are particularly vulnerable to reductions in gravitational force, ultimately leading to muscle atrophy in response to chronic exposure to a low gravity environment [51, 52]. Moreover, intracellular calcium, a ubiquitous cellular second messenger, is also responsive to gravitational force, which in turn, would modulate channel or pump activity [53]. Finally, some cation channels are both calcium conductive and mechanically-regulated, uniting both modalities of cellular response influenced by gravitational forces [54]. Given the importance of proper ion channel function for cell survival and metabolism, and the inherent difficulties of making electrophysiological recordings under conditions of altered gravitational force, a multidisciplinary collaboration was initiated with the aim of designing a stable and automated recording platform to reliably monitor the biophysical consequences of altered gravitational forces on ion channel activity.

Characterization of ion channels is usually performed by controlling the transmembrane potential and simultaneously measuring the resulting transmembrane current (voltage clamp). Laboratory-based voltage clamp systems commonly use glass microelectrodes (intracellular, or patch) to control the transmembrane voltage. These electrodes are fragile and require careful micromanipulation by the operator. Recently, the feasibility of planar electrodes manufactured from polydimethylsiloxane (PDMS) [25, 55] or glass [24] has been successfully demonstrated, thereby providing a reliable and robust alternative to glass microelectrodes and, importantly, obviating the need for micromanipulation typically required for patch clamping. Moreover, a number of microfluidic chips for performing planar patch clamping under laboratory conditions has been developed recently [26, 32, 33, 56]. Advances in automated electrophysiology have demonstrated that reproducible recordings can be obtained without significant operator intervention [57]. Nevertheless, these devices are generally designed for operation under standard laboratory conditions, and are not suitable for remote operation in a physically demanding environment. On the other hand, specialized microfluidic devices have been realized for use under extreme environmental conditions such as the sea [58] or the Martian atmosphere.
The implementation and optimization of a voltage-clamp device for studies under varying gravity conditions is particularly challenging, since changes in gravity are associated with alterations of the mechanical environment with profound physiological consequences, be it on an airplane (e.g. during parabolic maneuvers), sounding rocket, or in ground laboratory-based systems such as random position machines, 3D clinostats or centrifuges.

The core module of my system is a multilayer microfluidic chip that allows for stable positioning of a single cell (in this case, a *Xenopus laevis* oocyte) and accurate determination of the current passing across the entire cell membrane in response to an externally imposed transcellular electrical potential. This design, which I term the asymmetrical transoocyte voltage clamp (ATOVC) is akin to the previously described loose macropatch voltage clamp \[60, 61\] and transoocyte voltage clamp (TOVC) \[30\], however in my system only a small, well-defined patch of the oocyte membrane is exposed to test solutions. This has two consequences: (i) it facilitates the fast exchange of fluids exposed to the membrane patch through the microperfusion layer of the device, yet only requires low solution volumes; (ii) by permeabilizing the remainder of the cell membrane I can readily gain electrical access to the cell interior and achieve voltage clamping of the patch. The microfluidic device is made of PDMS and encapsulated in a polymethylmethacrylate (PMMA) assembly that comprises fluid, pressure and electronic connections. A similar design was previously described by Dahan et al. \[32\] but did not incorporate a fully enclosed cell recording chamber and fluidics. I now describe how this concept was specifically adapted and modified to satisfy the design criteria by incorporating a cell immobilization feature and, moreover, by having a liquid- and airtight system.

To demonstrate the feasibility of the system, measurements on *Xenopus laevis* oocytes that heterologously expressed the amiloride-sensitive epithelial sodium channel (ENaC) were performed. This channel was chosen because of its high specificity, fast kinetics and documented sensitivity to mechanical stimuli \[47, 62, 63\]. The advantages of the oocyte expression system in the context of gravity research is that these cells are extremely robust and they can be easily transported, maintained and handled without requiring special incubation conditions or stringent sterility procedures. Moreover, after removal from the frog, they can be kept healthy for typically up to a week, provided the incubation temperature remains below 20 °C. A further unique feature of *Xenopus laevis* oocytes is that their orientation with respect to the Earth’s gravitational field is well defined, with the easily identified animal pole (pigmented hemisphere) facing upwards for healthy cells.

In addition to extensive laboratory testing under 1 g conditions, the system has been successfully used in a parabolic flight campaign in Bordeaux (F) under the auspices of the European Space Agency (ESA) and in the Large Diameter Centrifuge (LDC) at the European Space Research and Technology Center ESTEC at Noordwijk (NL).

### 2.1.1. Principle of the ATOVC

Previously described electrophysiology techniques such as the TOVC \[30\] and the loose macropatch voltage clamp \[60, 61\] do not involve physical disruption of the membrane and therefore allow non-invasive electrophysiological measurements of membrane protein activity. For the TOVC, the oocyte is positioned in an orifice separating two fluid-filled compartments...
resembling an Ussing chamber and the electrical potential between the compartments is controlled to measure the transoocyte current. This arrangement has been used for transoocyte noise and impedance measurements [30]. In the loose macropatch [60, 61], the extracellular potential is operator-defined and current is measured from a small membrane patch by means of a glass pipette that lightly touches the cell membrane. For both methods, some of the current measured results from leakage because the membrane is not perfectly sealed to the orifice or patch pipette, respectively. Importantly, the transmembrane potential is poorly defined because the intracellular potential is unknown and in the case of large currents, errors in membrane voltage control may occur. One solution to this problem was recently described by Dahan et al. [32], in which a defined area of the oocyte membrane was permeabilized using an ionophore to allow a low resistance access to the cytosol.

As in the Dahan approach, I have used a conical structure with a small hole which separates the two fluid filled compartments. With the oocyte in place, this effectively divides the oocyte membrane surface into a lower “patched” region that is exposed to a microfluidic perfusion channel, and an upper “body” region that is exposed to the upper compartment. The surface area of the patch membrane is much smaller than the surface area of the entire oocyte (Figure 32, Figure 33) and in this context, the arrangement can be considered a variation of the loose macropatch [60, 61]. By defining the electrical potentials in the two compartments, the system offers the flexibility of allowing transoocyte impedance measurements as for the TOVC [30]; loose macropatch measurements [60, 61] and, by permeabilizing the body membrane, electrical access to the cytosol can be gained [32]. Due to the uneven surface topology of the oocyte membrane and associated vitelline layer [64], it is impossible to fully prevent ohmic leakage currents passing around the cell between the two compartments. However, these leak currents will remain constant, assuming the oocyte is stabilized. Moreover, they can be eliminated when control and test recordings are subtracted from one another.

2.1.2. Description of the ATOVC

The arrangement shown in Figure 31 permits voltage clamping of the patch membrane by controlling the cytosolic potential and assumes adequate electrical access to the cytosol from the upper compartment has been established. The same arrangement could be used for transimpedance measurements. Furthermore, by rearranging the connections to the core module so that the potential in the lower compartment is externally defined, and the upper compartment is held at 0 V, a loose macropatch configuration would be created.
2.1.3. Patch surface area considerations

The patch opening diameter was chosen on the basis of maintaining cell stability, considerations of the electrical properties of the cell and having rapid superfusion of the exposed area. Too large an opening would result in poor electrical access to the cytosol whereas too small an opening would compromise the signal-to-noise ratio for the patch current. The surface area $S$ of a sphere with radius $r$ is given by

$$S = 4\pi r^2$$  \hspace{1cm} (2.1)

The patch area $S_p$ (curved surface area of the cap) is calculated using

$$S_p = 2\pi rh$$  \hspace{1cm} (2.2)

where, $h$ is the height of the patch and $a$ the patch radius. For a sphere with $r=0.5$ mm (typical for *Xenopus laevis* oocytes) and $a=0.15$ mm (Figure 32) the surface area ratio is
The relationship between the impedance ratio of the two membrane parts and their respective surface areas can be expressed through

\[
\frac{S}{S_p} \approx 43
\]  

(2.3)

\[
\frac{R_B}{R_p} = \frac{C_p}{C_B} = \frac{4\pi r^2}{S_p} - 1
\]  

(2.4)

Here we take the apparent geometrical areas and ignore the increased surface area of the oocyte due to the microvilli and invaginations. This would simply scale the calculated areas equally. Assuming homogeneous distribution of membrane conductance the membrane impedances of the two regions will differ, according to the ratio of the membrane areas of each region. Consequently, changes in the transoocyte current will mainly result from changes in the impedance of the patch membrane due to its much higher impedance compared to the impedance of the body membrane.

Thus, even though the oocyte is effectively voltage clamped across two membranes, the largest fraction of applied AC voltage falls across the patched membrane, i.e. by having a large ratio between the non-patched area and the patched area, a voltage clamp from the cytosol across a single membrane is approximated (Figure 33b). In practice, the measured values will deviate somewhat from the calculated values due to the deformation of the immobilized oocyte. Nevertheless, this relation is useful for comparing membrane currents of the ATOVC with membrane currents measured with a conventional instrument (TEVC). Moreover, to simplify the analysis, this model ignores the effect of inhomogeneities of membrane access resistance close to the patch hole.

Figure 32: Left: Micrograph showing the oocyte immobilization site from above. The patch hole diameter is 0.3 mm. Right: Idealized cross-section of the oocyte showing geometrical parameters.
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Figure 33: The dependence of the surface ratio (a) and voltage ratio (b) plotted as function of the patch hole diameter. The values were obtained by simulation (Figure 34) for a typical oocyte diameter of 1 mm and an AC clamp voltage at 10 Hz. Actual values chosen for our design are indicated (dashed lines/red symbols).

Figure 34: Bode plot of the equivalent circuit of the ATOVC (made using LT SPICE IV by Linear Technologies) [63]. The absolute values for the resistances and capacitances are rough estimates. The DC resistance of the entire circuit is typically around 150 kΩ.
2.2. Materials and methods

2.2.1. Fabrication of the core microfluidic module

The design and realization of the microfluidic module is shown in Figure 35. The perfusion microchip was made of PDMS (Sylgard, Dow Corning) by molding from a structured silicon wafer [66]. The wafer was made from a high-viscosity positive resist (SU-8 2100, Microchem) employing standard lithography technology. Using a slow spinning step (500 RPM for 30 s), a final structure height of approximately 500 µm was obtained. This channel height was chosen to minimize the susceptibility to channel blockage should the oocyte become damaged and pass through the hole and to facilitate the integration of the Ag/AgCl electrodes. A precisely machined polytetrafluoroethylene (PTFE) frame was aligned with the master structures to obtain a PDMS microchip with exact dimensions on all axes (24 x 24 x 8 mm³) and a centered microfluidic structure. Well-defined curing conditions were used to keep the shrinkage of PDMS constant [67]. A ratio of 1:8 of curing agent to prepolymer was used for easier removal of the cured PDMS from its mold and for higher dimensional stability. Curing was done in an oven at 100°C for 1 hour. After removal of the PDMS microchip from its mold, 1 mm wide holes were punched at predefined positions using a biopsy puncher. These access holes were necessary for interfacing the fluid pathways and the sintered Ag/AgCl electrodes (Ag/AgCl 1 mm pellets, Warner Instruments, CT). The perfusion microchip was finally integrated with 3 CNC-machined PMMA parts that serve multiple functions such as interfacing, sealing and oocyte accommodation (Figure 35).

An important feature of the assembly is the clamping of the PDMS microchip between the PMMA parts. The thickness of the microchip is slightly larger than the cavity of the PMMA parts, resulting in compression of the microchip and perfect sealing of the microfluidic pathways. Moreover, as a result of the PDMS molding process, the oocyte patch hole in the PMMA is automatically aligned with the perfusion channel when the device is assembled.

In the present system I opted for a 300 µm diameter patch hole, which results in a sphere to patch surface ratio of 43 (Figure 33), giving a good compromise between electrical access to the cytosol, signal-to-noise ratio and mechanical stability.
Figure 35: Exploded view of rendered 3D CAD data used for the fabrication of the PMMA parts. The bottom electrodes are inserted into the PDMS microchip using 1/16 inch PFA tubing as a sleeve (tubing not shown for clarity).

Figure 36: (a) Cross-section view of rendered 3D CAD schematic of the assembled microfluidic module. (b) Photograph of the microfluidic module. (c) Design of the microfluidic channels in PDMS. The solution exchange volume is given approximately by the length of the main channel times its cross sectional area (in this case approx. 1 µl). The exact volume is smaller due to the protrusion of the pellet electrodes and part of the oocyte patch into the channel.
2.2.2. Fluid power systems

The scheme of the fully closed peripheral system is depicted in Figure 37 (see appendix, Figure 92 for technical schematic). To provide the pressure source a small air bottle was used (Sure/Pac 275 ml, Sigma-Aldrich). To control the pressure on the fluids, an electronic pressure regulator with feedback control was used (T3110, 1000 mbar, Marsh-Bellofram). The output pressure of the regulator pressurized glass vials filled with the perfusion fluids. Switching between the fluids was achieved by using solenoid valves (LFVA1220110H, Lee Co.). Teflon capillary tubes (0.18 mm inner diameter, 30 cm length)\(^3\) between the solution vials and the solenoid valves ensured a well-defined steady-state flow rate through the microfluidic module. To reduce the water hammer effect resulting from the switching action of the valves, air pocket elements between the valves and the microfluidic device were introduced. The outlet of the microfluidic device was connected to an expandable polypropylene reservoir \([68]\) acting as a sealed waste container. For the pressurization of the oocyte a second pressure regulator was used (T3110, 68 mbar, Marsh-Bellofram). To avoid large pressure transients and fluctuations a compensation volume between the regulator and the microfluidic device was introduced.

![Figure 37: Schematic of the peripheral system. Yellow denotes air, blue denotes liquid and grey denotes an electrical connection.](image)

\(^3\) Earlier prototypes used tubings with 0.25 mm inner diameter.
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Regulation and power requirements. Pressure-driven perfusion systems, as opposed to volume-driven perfusion systems, are characterized by a very steady and pulsation-free flow profile. The reason for this is that the driving force in pressure-driven systems can be generated in a quasi-static fashion. Usually, the pressure drop across the perfusion systems is generated using a pressure differential in air pressure acting on the liquid. In volume-driven systems, however, the liquid needs to be displaced as a function of time which requires the use of linear motors. Such motors use gears to convert rotational motion into linear motion which produces a periodical modulation of the linear motion. Pressure-driven systems, on the other hand, make use of air reservoirs that can virtually have limitless buffering capacity. This capacity depends on the elasticity and total volume of the involved gas or gas mixture. In microfluidic perfusion systems, the requirements towards buffering capacity of the gas are low, since the desired flowrates are usually very low in comparison with the available gas volume. The change in pressure drop across the perfusion system $\Delta p$ per time period $\Delta t$ can be found employing Boyle’s Law

$$p_0 V_0 = p_1 V_1 = (p_0 - \Delta p)(V_0 + \Delta V) \quad (2.5)$$

The change in volume of the gas is related to the volumetric flowrate $Q$ through

$$\Delta V = Q \cdot \Delta t \quad (2.6)$$

Substitution of Equation (2.6) into Equation (2.5) results in

$$\frac{p_1 - p_0}{p_0} = \frac{\Delta p}{p_0} = 1 - \frac{1}{1 + \frac{Q \cdot \Delta t}{V_0}} \quad (2.7)$$

It becomes evident that the relative decrease in pressure as a function of time depends on the ratio between the $Q$ and $V_0$. We can therefore introduce a scaling factor

$$K \equiv \frac{Q}{V_0} \quad (2.8)$$

In the case of the ATOVC, we require a flowrate of 1 µl/s. Since the perfusion system needs to be compact a pressurized air reservoir with a volume of 10 ml ($K=0.0001$) is assumed. For a time period of 1 second we obtain

$$\frac{\Delta p}{p_0} = 1 - \frac{1}{1 + \frac{1 \mu l / s \cdot s}{10000 \mu l}} = 0.01\% \quad (2.9)$$

At low $K$ values, Equation (2.7) becomes linear for a small time window (see Figure 38). The input power $P_{in}$ required to keep the pressure constant depends on the number of gas molecules $\Delta n$ that need to be added per time unit. Employing the molar volume $V_m$ for an ideal gas, we obtain
The energy required for adding molecules to a volume is described through the ideal gas law

\[ pV = nRT \]  
(2.11)

Where \( R \) is the ideal gas constant. Substitution of Equation (2.10) finally yields

\[ P_{in} = \frac{QRT}{V_m} \]  
(2.12)

For a flowrate of 1\( \mu \)l/s at 20 °C, we obtain

\[ P_{in} \approx 100 \mu W \bigg|_{Q=1\mu l/s} \]  
(2.13)

In conclusion, pressure-driven perfusion systems for microfluidic applications show a linear short-term decrease in pressure over time. This makes the use of low-speed proportional regulation sufficient. Furthermore, the input power required for proper regulation is very low. This enables realization of compact and portable (battery-operated) control systems.

**Flowrate-pressure drop relationship.** To calculate the volumetric flowrate for a given pressure drop across the perfusion system, the hydrodynamic resistances involved need to be known. Due to the absence of co-flowing streams, the total hydrodynamic resistance is composed of individual resistances connected in series. They include the resistance of the inlet and outlet tubings \( R_i \) and \( R_o \), capillaries \( R_c \), the solenoid valve (Figure 39) \( R_s \), and the microfluidic chip (Figure 40) \( R_m \). The total hydrodynamic resistance\(^4\) \( R_{tot} \) is therefore

\(^4\) Note that the back pressure at the outlet resulting from the elasticity of the expandable reservoir is neglected.
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\[ R_{\text{tot}} = R_c + R_s + R_l + R_m + R_o \]  \hspace{1cm} (2.14)

In the actual implementation (see Figure 39, Figure 40 and Figure 92), the values of the individual resistances, according to Equations (1.42) and (1.45), are (at 20 °C)

\[ R_c = 2.55 \cdot 10^{-3} \frac{30 \text{cm}}{(0.090 \text{mm})^4} Pa \cdot s = 1.17 \cdot 10^{13} Pa \cdot s \cdot m^{-3} \]

\[ R_s = 0.475 kPa \cdot ml^{-1} \cdot \text{min} = 2.85 \cdot 10^{10} Pa \cdot s \cdot m^{-3} \]

\[ R_l = 2.55 \cdot 10^{-3} \frac{10 \text{cm}}{(0.5 \text{mm})^4} Pa \cdot s = 4.08 \cdot 10^9 Pa \cdot s \cdot m^{-3} \]  \hspace{1cm} (2.15)

\[ R_m = 2.55 \cdot 10^{-3} \cdot 16 \text{mm} \left( \frac{0.5 \text{mm} + 0.5 \text{mm}}{0.5 \text{mm} \cdot 0.5 \text{mm}} \right)^4 Pa \cdot s = 1.04 \cdot 10^{10} Pa \cdot s \cdot m^{-3} \]

\[ R_o = 2.55 \cdot 10^{-3} \frac{10 \text{cm}}{(0.5 \text{mm})^4} Pa \cdot s = 4.08 \cdot 10^9 Pa \cdot s \cdot m^{-3} \]

As can be seen from the above calculations, the total hydrodynamic resistance of the perfusion system is dominated by \( R_c \). We can therefore assume that

\[ R_{\text{tot}} \approx R_c \]  \hspace{1cm} (2.16)

Which implies that we get a flowrate to pressure drop scaling of

\[ \frac{Q}{\Delta p} = 0.01 \frac{\mu l}{s \text{ mbar}} \]  \hspace{1cm} (2.17)

Figure 39: Typical flow characteristics of the LFVA series solenoid valve by The Lee Company [69].
Figure 40: 3D model of the microfabricated perfusion channel system as used in the ATOVC system. The arrows indicate the direction of the flow.

2.2.3. **Electronic hardware and system control**

The general arrangement of the voltage clamping hardware configuration is detailed in Figure 31. It was designed to be easily adapted to perform different electrophysiological measurements such as transimpedance and loose macropatch voltage clamping. For the experiments described in the present study, aimed at voltage clamping the patch membrane itself, the electrical potential in the compartment facing the oocyte patch (microfluidics channel) was defined as signal ground by means of a virtual ground amplifier connected to the two electrodes in the microfluidics channel. The electrical potential in the upper compartment was defined by the input command potential using a conventional TEVC arrangement connected to the two upper compartment electrodes.

Two prototypes were developed, an earlier version used during the 51st, 52nd and 53rd ESA Parabolic Flight and hypergravity campaigns (Figure 56) and the latest version offering higher flexibility and better performance. It was used during the 55th ESA Parabolic Flight Campaign (see appendix, Figure 96).

**System version used during the 51st, 52nd and 53rd ESA Parabolic Flight and hypergravity campaigns.** The analogue command potential, defined according to the proprietary control software, was generated by a 16 bit digital-to-analog converter (DAC) in the microcontroller unit. Two analogue signals were measured: the potential of the upper compartment and net current passing between the two compartments. They were acquired by 16 bit analog-to-digital converters (ADC) in the microcontroller unit with timing and sampling rates defined by the proprietary software user interface. Data were written automatically to a removable solid-state memory device. The pressure controllers and fluid solenoid valves were driven from the microcontroller unit by pulse width modulation and transistor-transistor level (TTL) signals, respectively. The microcontroller unit was a fully customized design (Interstate University for Technology Buchs NTB, Switzerland) based on the MPC555 microcontroller with a number of connected peripherals. The unit could be connected to a computer for direct human control or
operated fully automatically with data continuously written to removable media via a universal serial bus (USB) interface.

**System version used during the 55th ESA Parabolic Flight Campaign.** For the new ATOVC setup, a modular design concept around the commercially available LabJack U6-Pro data acquisition and control unit (DAQ) was opted for (Figure 41). The main components connected to the DAQ were a personal computer for user interaction, a programmable signal generator module, the voltage clamp circuit (Section 2.2.6) and power electronics. In time for the parabolic flight campaign, four units of the system were manufactured externally (Spacetek GmbH, Switzerland), three of which were used for the campaign.

![Figure 41: Schematic of the integration concept for the ATOVC system for microgravity experiments.](image)

The DAQ unit incorporates various programmable peripherals for communication and control of other devices. Moreover, it features high-accuracy multi-channel data acquisition functionality. The DAQ also provides a USB port for communication with a personal computer. Together with the drivers and software libraries available from LabJack Corporation, the DAQ can be controlled via software run on a personal computer. Details and technical specifications on the LabJack U6-Pro are available on the corporate’s website [70].

The graphical user interface (GUI) provides monitoring and control of the ATOVC for the experimenter. It was written in Visual Basic .NET language using the Visual Studio 2010 integrated development environment (IDE) commercially available from the Microsoft Corporation [71]. Employing multi-threaded operation, the GUI provides numerical and
graphical representation of relevant input variables (voltage, current, etc.) in realtime. Control of the perfusion system, pressurization system and voltage clamp circuit is accessible on the main panel of the GUI. An integrated scripting function allows experimental procedures to be predefined such that experiments can be run automatically. In addition, triggering of the script can be done via a user-defined condition from the accelerometer output variable. See Figure 42 for more details on the interface.

Figure 42: Graphical user-interface for control of the ATOVC system in parabolic flight configuration. (I.) Realtime input signal graph; (II.) controls for perfusion system; (III.) controls for the top pressurization system; (IV.) controls for the voltage clamp system; (V.) indicators for the accelerometer; (VI.) controls for the script macro feature; (VII.) logging subsystem; (VIII.) general system info; (IX.) ATOVC output variables for voltage, current and resistance; (X.) controls for data recording.

The signal generator unit used for creating the command voltage in the ATOVC was based on a programmable microcontroller unit (MCU). The STM32 by STMicroelectronics was chosen due to its high-quality DAC peripheral, ease of programming and low cost. The STM32 uses the Cortex M3 architecture developed by ARM Holdings [72, 73].

The DAC peripheral has good DC because the output voltage is referenced to the integrated band-gap voltage reference. To generate the control voltage for the ATOVC, the output voltage of the DAC is sent to a gain stage with offset control. Control of the DAC is achieved through digital communication using the serial periphery interface (SPI) bus which is
supported by both the DAQ and the STM32. SPI communication is managed by the user software.

To power and control the electrofluidic components of the liquid handling system, it was necessary to implement electronic stages between the DAQ and the electrofluidic components. For the electronic pressure regulator (Figure 43a), an input voltage between 0 and 10 V was required, realized through an instrumentation amplifier (INA118, Burr Brown/Texas Instruments) with offset compensation. The normally-closed, isolation solenoid valve (Figure 43b) used for solution switching required a continuous voltage to stay open. A bipolar transistor (2N3904) was used for current amplification of the TTL signal coming from the DAQ. 12 Volts were used for powering the valve, as specified in the datasheet. Figure 93 in the appendix shows all the electronic circuits that were implemented in the final prototype of the ATOVC system.

2.2.4. Reagents and solutions

Modified Barth’s solution for storing oocytes contained (in mM): 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.82 MgSO₄, 2.5 NaHCO₃, 2 Ca(NO₃)₂, 7.5 HEPES. The solution was adjusted to pH 7.5 with Tris and supplemented with 5 mg/l doxycycline and 5 mg/l gentamicine. For storage of oocytes after the injection, a low Na⁺ Barth’s solution was used with the Na⁺ concentration reduced to 10 mM and replaced with 78 mM methyl-d-glucamine. Standard extracellular solution (100Na) contained (in mM): 100 NaCl, 2 KCl, 1.8 CaCl₂, 10 HEPES, pH 7.4 adjusted with TRIS. The 10 mM Na⁺ test solution (10Na) had the same composition as 100Na except that 90 mM of the NaCl was replaced with choline chloride. For test solutions containing amiloride, this was added from 100 mM stock (in DMSO) immediately prior to priming the solution reservoirs to give a final concentration of 10 μM. All standard reagents were obtained from either Sigma-Aldrich or Fluka.
2.2.5. Oocyte preparation

Female *Xenopus laevis* frogs were purchased from *Xenopus* Express (France) or African *Xenopus* Facility (R. South Africa). Portions of ovaries were surgically removed from frogs anesthetized in MS222 (tricaine methanesulphonate) and cut in small pieces. Oocytes were treated for 45 min with collagenase (crude type 1A) 1 mg/ml in 100Na solution (without Ca$^{2+}$) in presence of 0.1 mg/ml trypsin inhibitor type III-O. Healthy stage V-VI oocytes were selected, maintained in modified Barth’s solution at 16 °C and injected with 5 ng total of cRNA of the α, β, γ sub-units of the *Xenopus* isoform of ENaC according to previously described procedures [75]. After injection, the oocytes were incubated for 3 days at 16 °C in the low Na$^+$ Barth’s solution (10 mM) to maintain a low intracellular Na$^+$ concentration and ensure cell survival. Non-injected oocytes of the same batch were used as a negative control as the amiloride-sensitive inhibition of endogenous mechanically sensitive channels is only detected under tight seal patch conditions and the microvilli and invaginations present in the healthy stage VI oocyte membrane are thought to buffer direct mechanical activation of these channels in the intact oocyte [76, 77].

2.2.6. Design of the Voltage Clamp circuit

In general, the circuit comprises six stages - four being responsible for clamp voltage regulation and two being used for conversion and conditioning of the current (Figure 44). Implementation of the circuit is achieved entirely using operational amplifiers (opamps) and instrumentation amplifiers (see appendix, Figure 94). The first realization of the circuit is shown in Figure 95 of the appendix. The implementation followed conventional design practice, whereas two stages required special consideration: the adjustable phase shifter and the current-to-voltage converter.

Figure 44: Design concept for the voltage clamp circuit comprising six stages connected to the device under test (DUT). Input ($V_c$: command voltage) and output ($A_V m$: amplified transoocyte voltage, $V_{out}$: converted transoocyte current) variables are denoted via small circles. Squares with numbers denote a stage. 1: voltage preamplifier, 2: phase shifter, 3: feedback amplifier, 4: feedback time constant, 5: current-to-voltage converter, 6: inverter.
Adjustable phase shifter. There are a number of signal phase shifts introduced into the voltage clamp by the various stages. Since the signal at the output of the current output stage (stage 4 on schematic) is indirectly fed back into the input of the voltage preamplifier (stage 1 on schematic), the phase shift can cause unstable operation of the voltage clamp. Therefore, it becomes necessary to integrate a stage allowing the signal’s phase at the input of the voltage clamp to be shifted relative to the signal’s phase at the output to find the optimal stability condition. An externally adjustable implementation is desirable due to the fact that the device under test (DUT) itself causes a phase shift which may vary from DUT to DUT due to manufacturing tolerances.

I opted for an opamp-based active phase shifter, also called all-pass filter, which allowed the phase to be shifted from 0° to -180° [78]. The transfer function of the circuit (Figure 45) is

\[
\frac{V_{\text{out}}}{V_{\text{in}}} = \frac{RCs - 1}{RCs + 1} \quad (2.18)
\]

with the phase response function

\[
\varphi(f) = \pi - 2 \arctan\left(2\pi fRC\right) \quad (2.19)
\]

Since the opamp is configured as an inverting amplifier, the signal is inverted at the output. This is taken into account for the configuration of the feedback amplifier.

The phase shifter requires an opamp with stable operation at unity-gain. The general purpose, precision OPA277 was thus chosen [79].

![Figure 45: Schematic of the phase-shifter used in the ATOVC.](image)

Current-to-voltage converter. This stage converts the current flowing from the microperfusion channel into the external current electrode into an output voltage \(V_{\text{out}}\) according to

\[
V_{\text{out}} = -I_{\text{ext}} \left(\frac{1}{R_f} + 2\pi fC_f\right)^{-1} = -I_{\text{ext}} \frac{R_f}{2\pi fR_fC_f + 1} \quad (2.20)
\]
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As can be seen from the above equation, $V_{out}$ is frequency dependent. The capacitor in parallel to the resistor in the feedback loop generates a first-order low-pass filter (-6 dB per octave). The use of 330 pF for the capacitor and 1 MΩ for the resistor results in a corner frequency of 482 Hz. This frequency limits the bandwidth of events that can be detected in the current measurement. The chosen value allows the detection of events as fast as a few milliseconds.

At DC, the resistor creates a current-to-voltage scaling of 1 µA/V. The selection criterion of the feedback resistor is based on the assumption that the expected current from the ATOVC is below 10 µA and that the full-scale range of the data acquisition is 10 V.

As seen in Figure 46, the inverting input of the opamp is not shorted to the feedback network. This arrangement allows sensing of the voltage close to the DUT, essentially factoring out the series resistances of the electrodes. What does need to be taken into account, however, is the resistance $R_{ext}$ between the two electrodes. The accuracy of the measured current $I_{meas}$ is calculated from

$$
\frac{I_{meas}}{I_{real}} = 1 - \frac{R_{ext}}{R_f}
$$

(2.21)

It is thus important to keep the electrodes close to each other. $R_{ext}$ can be estimated using the volume resistivity $\kappa$ of the solution and the distance $d$ between the electrodes. For the solution, we assume a standard 100 mM NaCl solution at 20 °C [20]. The distance between the electrodes is approximately 1 mm. We therefore obtain

$$
R_{ext} = \frac{1}{\kappa \cdot d} \approx 1.2k\Omega_{\kappa=0.82S/m,d=1mm}
$$

(2.22)

For the actual implementation, the opamp model OPA140 (Burr Brown/Texas Instruments) was chosen due to its very low offset voltage (30 µV) and input bias current (0.5 pA) [80]. This makes the use of external offset compensation unnecessary and allows the accurate recording of current in the sub-nA range. The accuracy of the current measurement is thus limited only by the feedback resistor $R_f$ and $R_{ext}$. The design criterion was defined such that the initial total error would be well below 1 %. Using a feedback resistor with a tolerance of 0.1 % and keeping the electrodes close together (~1 mm) fulfilled the criterion.
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Figure 46: Schematic of the current-to-voltage converter used in the ATOVC. The resistor network enclosed in the grey box is the equivalent circuit of the pellet electrodes immersed into the solution inside the microperfusion channel. Only the resistive components are taken into account.

2.2.7. Transfer function of the ATOVC

The schematic used for calculating the transfer function of the ATOVC is shown in Figure 47. In this model, the phase shifter, current-to-voltage converter and inverter are not taken into consideration. The simplification can be justified via the fact that these stages do not contribute to the circuit’s response as they operate at unity gain within a bandwidth that is much larger than that of the other stages.

Figure 47: (a) Simplified logic circuit of the ATOVC. (b) Equivalent schematic of the impedance of the oocyte immobilized in the core module.

To determine the membrane voltage \( V_m \) as a function of the command voltage \( V_c \), we can start by applying Ohm’s law on the load. Since no current flows into the sense voltage

---

5 Since the current-to-voltage and inverter stages both invert the signal, no net inversion results at the output of the circuit.
preamplifier, the sensed voltage corresponds to the voltage drop across the impedance of the oocyte $Z_m$ and the series resistance $R_s$. $V_m$ thus becomes

$$V_m = I(Z_m + R_s)$$

To eliminate $I$, we can use

$$V_i = I(Z_m + R_s + R_c)$$

which is the voltage drop across the load including the series resistor $R_c$. The transfer function becomes

$$\frac{V_m}{V_i} = \frac{Z_m + R_s}{Z_m + R_s + R_c}$$

$V_i$ is also given by the amplification factors involved and the comparison with $V_c$:

$$V_i = A_f A_l (A_v V_m - V_c)$$

where $A_f$ is the gain of the feedback amplifier, $A_l$ is the gain of the loop time constant and $A_v$ is the gain of the sense voltage preamplifier. Combination of Equations (2.25) and (2.26) results in the transfer function

$$\frac{V_m}{V_c} = \frac{A_f A_l}{Z_m + R_s + R_c} - A_f A_l A_v = \left(1 + \frac{Z_m + R_s + R_c}{Z_m + R_s} - A_v\right)^{-1}$$

We now assume that the bandwidths of the sense voltage preamplifier and the differential feedback amplifier are much higher than that of the feedback control time constant. The frequency dependence of the feedback loop thus adopts a first-order low-pass characteristic

$$\frac{V_m}{V_c} = \left(1 + s\tau_i Z_m + R_s + R_c - A_v\right)^{-1}$$

The impedance of the immobilized oocyte, as depicted in Figure 47b, is given by

$$Z_m = \left\{ \frac{1}{R_s} + \left(\frac{1}{R_b} + sC_p + \frac{1}{R_p}\right)^{-1} \right\}^{-1}$$

This can also be expressed by replacing $R_b C_b$ and $R_p C_p$ with time constants as
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\[
Z_m = \left\{ \frac{1}{R_l} + \left[ R_p \frac{1}{1+s\tau_b} + R_c + R_p \frac{1}{1+s\tau_p} \right] \right\}^{-1} \tag{2.30}
\]

Factoring out \( R_p \) of the innermost term yields

\[
Z_m = \left\{ \frac{1}{R_l} + \left[ \frac{R_b}{R_p} \frac{1}{1+s\tau_b} + \frac{R_c}{R_p} + \frac{1}{1+s\tau_p} \right] \right\}^{-1} \tag{2.31}
\]

If we now assume that the surface area of the body membrane is much larger than that of the patch membrane, the resistance of the patch membrane will dominate. Furthermore, the resistance of the cytosol is assumed to be very low compared to the resistance of the patch membrane. We can therefore simplify the equation using

\[
R_p \gg R_b \quad R_p \gg R_c
\]  

which simplifies Equation (2.31) to

\[
Z_m \approx \left\{ \frac{1}{R_l} + \frac{1}{R_p} \left( 1+s\tau_p \right) \right\}^{-1} = \frac{R_l}{1 + \frac{R_l}{R_p} \left( 1+s\tau_p \right)} \tag{2.33}
\]

It is evident that \( Z_m \) becomes purely resistive if \( R_p \) is much larger than \( R_l \). If \( R_l \) is much larger than \( R_p \), then \( Z_m \) inherits the algebraic form seen in the TEVC configuration, only with different values for the resistance and capacitance.

To calculate the natural frequencies of the ATOVC, Equation (2.33) needs to be substituted into Equation (2.28) and the Eigenvalues of \( s \) determined. This can be achieved using the factorization function of a computer algebra system. In this case, Maple was used which yielded

\[
\frac{V_m}{V_c} = \frac{a_0 s + a_1}{b_0 s^2 + b_1 s + b_2}
\]

\[
a_0 = A_f R_l \tau_p
\]

\[
a_1 = A_f \left( R_l R_p + R_l R_p + R_l R_l \right)
\]

\[
b_0 = -R_l \tau_p \tau_l \left( R_l + R_l \right)
\]

\[
b_1 = -R_l R_p \tau_l - R_l R_p \tau_l - R_l R_l \tau_l - R_l R_l \tau_l - R_l R_l \tau_l - R_l R_l \tau_l + A_f A_f A_f \left( R_l R_p + R_l R_p + R_l R_l \right)
\]

\[
b_2 = -R_l R_p - R_l R_p - R_l R_l - R_l R_l + A_f A_f \left( R_l R_p + R_l R_p + R_l R_l \right)
\]

\[
(2.34)
\]
The corresponding Eigenvalues are

\[ s_{1,2} = \frac{-b_1 \pm \sqrt{b_1^2 - 4b_0b_2}}{2b_0} \]  

(2.35)

The resonant frequency \( \omega_0 \) and its time constant \( \tau_0 \) are extracted using

\[ \omega_0 = \begin{cases} s_1 & |s_1| > 0 \\ s_2 & |s_2| > 0 \end{cases} \]

(2.36)

2.2.8. Parameterization of the voltage clamp and comparison with numerical analysis

To tune and evaluate the voltage clamp circuit, a small printed circuit board (PCB) incorporating a network of resistors and capacitors was used. The network was designed to emulate the impedance of the oocyte immobilized in the core module. Moreover, numerical analysis on the voltage clamp was done using the SPICE-based circuit analysis software TINA V9 (Texas Instruments) [81, 82]. All calculations are based on the schematic shown in Figure 48.

Figure 48: Schematic used for the numerical analysis of the ATOVC.
Adjustment of the voltage clamp. Tuning of the voltage clamp is a balancing act between accuracy, response and stability of the regulation. From the behavior of feedback control systems we know that in order to increase accuracy, the gain must be increased. This, in turn, lowers the feedback loop’s stability and may induce oscillations. To reduce these oscillations, the time constant of the feedback loop can be increased, which in turn lowers the response. In case of the ATOVC, it makes sense to adjust the time constant to match the fastest biophysical event expected during the experiment. This is done with the gain set at the lowest setting (Figure 49). Subsequently, the gain may then be increased up to the point just before the feedback loop starts becoming unstable. The phase shift between the command voltage and the sensed membrane voltage is caused by the various time constants in the feedback loop. They depend on the bandwidth of the integrated circuits (ICs), the feedback control time constant, the reactive elements of the load and of course the setting on the adjustable phase shifter. In principle, the phase shifter’s setting is chosen such that the signals at the inputs of the differential feedback amplifier are in phase. Oscillation of the ATOVC occurs at phase differences equal or greater than 180°. The step responses of the voltage and current after final adjustment are in very good agreement with the predicted response as shown in Figure 50.

Figure 49: (a) Oscilloscope screenshot of I_{out} and V_{meas} at minimum gain after adjusting the phase and time constant. (b) Simulated response of V_{meas} using comparable settings. The step response has the characteristic of an overdamped system.
Figure 50: (a) Oscilloscope screenshot of $I_{out}$ and $V_{meas}$ after final adjustment of the voltage clamp. (b) The simulated response of $I_{out}$ and $V_{meas}$ deviates approximately 5% from the measurement, which is lower than the tolerance of the capacitors used in the physical model (10%).

**Prediction of the voltage step response.** After final adjustment, we obtain the following parameters:

$$R_i = R_s = 2.2 \cdot 10^3 \Omega$$

$$R_l = 10^5 \Omega$$

$$R_p = 2 \cdot 10^6 \Omega$$

$$\tau_p = 4 \cdot 10^{-2} \text{s}$$

$$\tau_l = 1.34 \cdot 10^{-4} \text{s}$$

$$A_v = 10$$

$$A_f = 4.9$$

Substitution of these values into Equation (2.34) yields the resonant frequency $\omega_0$ and its time constant $\tau_0$: 

\[ (2.37) \]
We find that the calculated value for the time constant corresponds very well with that determined via the SPICE model (Figure 51). Moreover, the SPICE model confirms that the response of the voltage clamp does not change significantly with changing resistance of the body membrane (Figure 51a,b).

\[
\frac{\omega_0}{\frac{2\pi}{\omega_0}} \approx 34 \mu s 
\]

\[ |s_0| = \omega_0 \approx 1.86 \cdot 10^5 \text{s}^{-1} \approx 29602.8 \text{Hz} \]

\[ \tau_0 = \frac{2\pi}{\omega_0} \approx 34 \mu s \] (2.38)

Figure 51: Step response of the sensed membrane voltage with a membrane body resistance of (a) 100 kΩ and (b) 9.52 kΩ. The influence on the time constant is negligible as the deviation between the two cases is only about 1%.
2.3. **Results and discussion**

2.3.1. **System evaluation under standard laboratory conditions**

I first optimized the entire system in the laboratory environment to verify its robustness and ease of operation. The best conditions for cell immobilization were evaluated. To ensure the cell remained correctly and stably positioned on the patch hole, a constant and well-defined air pressure to the upper compartment was applied. This pressure served two purposes: (i) it increased the leak resistance between the two compartments around the oocyte by counteracting forces acting on the oocyte resulting from the flow inside the perfusion channel - if the air pressure in the bath is high compared to the pressure resulting from the perfusion flow, the leak resistance will become independent of the flow rate inside the perfusion channel; (ii) when in operation it served to ensure that any effects of gravitational forces on the leak resistance were negligible and therefore allowed the system to operate under microgravity conditions. Tests for determining the influence of variations in the pressure differentials on the leak resistance were performed to demonstrate its low susceptibility to changing environmental conditions (Figure 54).

To demonstrate the viability of the system for monitoring the membrane conductance change of the patch under voltage clamp conditions, oocytes that overexpressed a channel with known properties were expressed, namely the epithelial sodium channel (ENaC). The large macroscopic conductance associated with ENaC expression meant that the conductance of the cell membrane exposed to the upper compartment established a low resistance electrical access to the cytosol and the cytosolic side of the patch. Moreover, as ENaC can be reversibly blocked by application of the inhibitor amiloride, the leak current between the two compartments could be eliminated by subtraction of recordings made in the presence of amiloride from control recordings. For these experiments, the perfusion system was filled with the 100Na, 100Na + amiloride and 10Na solutions, and the oocyte was placed in the chamber together with approximately 200 µl of 10Na solution. The amiloride concentration (10 µM) was used to ensure the complete inhibition of all ENaCs exposed to the microperfusion channel [83]. The top PMMA plate was positioned and fixed with screws and the oocyte compartment was pressurized at 20 mbar. Various leak evaluation tests had shown that this value was the best compromise between maximizing the leak resistance (typically in the range of 150 to 200 kΩ for 10Na) and minimizing stress to the oocyte membrane. It was found that increasing the pressure beyond 20 mbar did not yield a substantial increase in leak resistance, most probably because the area of the contact between the oocyte and funnel remained unchanged and the oocyte would simply be pushed through the hole further. Furthermore, oocytes pressurized at 50 mbar and beyond tended to burst after a few minutes.

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6 Permeabilization of the body membrane could also be achieved using ionophores as demonstrated Dahan et. al. In the present design, a perfusion pathway was already incorporated (Figure 36a) to allow changes in upper compartment composition and this could be adapted for the permeabilization.
After top pressurization, the perfusion system was started and the oocyte constantly perfused with 10Na solution to replicate the standard incubation conditions and avoid loading the cells with sodium. It was found that the oocyte typically required 3 to 5 minutes to reach a stable condition after starting pressurization. The perfusion sequence was then initiated (Figure 53a), which consisted of switching from 10Na solution to perfusion with 100Na solution for 10-20 s followed by 100Na solution containing 10 μM amiloride for 10-20 s. This was followed by a washout in 10Na solution to return to the initial conditions. The individual steps of the perfusion procedure as well as the measurement parameters were configured by the software. Two electrophysiological characterizations were performed using the system: time-dependent current measurements at a constant holding potential and current-voltage (I-V) characterizations using a voltage ramp or voltage step sequence.

Figure 53a shows a representative steady-state recording at a transoocyte potential of -50 mV (upper compartment relative to lower compartment) for an ENaC overexpressing oocyte subjected to the perfusion sequence indicated. Assuming a solution exchange volume of approximately 1 µl, the solution exchange time is around 1 s for the flow rate used (1 µl/s, Figure 53a), giving reasonable response time of the microfluidic exchange. The rapid response to the application of amiloride within a few seconds for the ATOVC confirms the good performance of this system. The I-V relation of the amiloride-sensitive current (Figure 53b) for repeated applications and wash-out on the same oocyte at the time intervals indicated shows that the system responded reproducibly with little change in the currents between successive measurements. For this cell, the reversal potential remained constant and indicated that the internal [Na⁺] did not change significantly between successive measurements. Moreover, comparison of I-V traces agreed well with the TEVC recording (Figure 52b).

The curvilinear nature of the I-V reflects the typical amiloride-sensitive macroscopic current carried by ENaC as previously reported [84]. This characteristic shape was found for oocytes overexpressing different levels of ENaC. Figure 53c shows the I-V traces obtained from 5 different oocytes with amiloride-sensitive currents varying from -28 nA to -80 nA (at -50 mV). The data were normalized to the current at -100 mV and superimposed to show that the voltage-dependence was unchanged, independent of expression level. The reversal potential of (+20 ± 10) mV is lower than predicted (+59 mV) from the assumed 10:1 Na concentration gradient across the patch membrane. As this did not change significantly with expression level it is unlikely that it resulted from inadequate voltage control of the patch membrane, but rather suggests that the internal [Na⁺] was higher than assumed.

Finally, I tested the system on two different laboratory shakers providing either horizontal or wave-like movements (see electronic supplementary information, movie 1 and 2)⁷. The output signals were not affected by the movement, which confirmed the high stability and robustness of the system.

⁷ Available on the website of RSC Publishing.
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Figure 52: (a) Real-time TEVC recording of membrane current from a representative oocyte expressing ENaC and voltage clamped to -50 mV. (b) I-V data from ENaC expressing oocytes (n = 7). Amiloride-sensitive currents were normalized to the current at -100 mV and data pooled (mean ± sem).

Figure 53: (a) Real-time current measurement on an ENaC expressing oocyte. The transoocyte voltage was kept constant at -50 mV except immediately before switching solutions where voltage sweeps where applied as indicated. The blue line represents the initial current demonstrating the steady-state stability before and after the solution switching procedure. The flow rate was kept constant at 1 µl/s. (b) Four I-V curves from one selected oocyte measured consecutively at the times indicated. (c) I-V curve representing the average of 5 oocytes and normalized to the current at -100 mV. The mean reversal potential is approx. +20 mV. For the data in plots b and c a voltage ramp from -200 mV to +100 mV with a period of 20 s was applied.
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2.3.2. Influence of pressure variations on the leak conductance

To study the influence of air pressure variations on the leak conductance, a series of experiments were conducted where either the air pressure (Figure 54a) or the perfusion pressure (Figure 54c) was varied. These tests demonstrate the system’s susceptibility to variations in the immobilization pressure (top pressure) and tolerance to the hydrodynamic resistance of the individual solution pathways. All tests were conducted on native oocytes which have negligible membrane conductance compared to the leak. Therefore, the current resulting from an applied transooocyte voltage is predominantly leak current.

![Figure 54](image)

Figure 54: (a) Time-dependent measurement of leak current varying the air pressure acting on the native oocyte. The transooocyte voltage and the perfusion flow rate were kept constant at -50 mV and 2 μl/s (200 mbar), respectively. (b) Statistical evaluation of 5 oocytes subjected to air pressure variation of 1 mbar in both directions (base pressure 20 mbar). (c) Time-dependent measurement of the leak current varying the perfusion pressure in the microchannel. (d) Scheme showing the direction of the forces (arrows) involved in the experiment.

2.3.3. Measurements under hypergravity

To study if extended periods of hypergravity affected the macroscopic conductance of ENaC overexpressing oocytes, tests on a large diameter centrifuge (LDC) were conducted, provided at the European Space Research and Technology Center ESTEC at Noordwijk (NL). The entire system was mounted in a centrifugation chamber and exposed to 1.8 g for about 20 minutes.
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After an initial time of about 2 minutes to allow cell settlement on the hole, the complete experimental protocol, including solution exchange and current recording, was remotely triggered at defined time intervals varying from 90 seconds to 5 minutes.

In Figure 55 the relative changes of total and amiloride-sensitive currents are shown for an oocyte overexpressing ENaC that was exposed to hypergravity and compared to the results obtained for another oocyte exposed to the same experimental protocol at 1 g but with an immobilization pressure of 20.5 mbar to account for the increased hydrostatic pressure under hypergravity. Analysis of the data revealed that under these conditions, the amiloride-sensitive current increased to 150% of the initial value, whereas the total current decreased slightly (Figure 55). In contrast, for the oocyte on the ground (1 g), an insignificant change in amiloride-sensitive current was observed, and a slight decrease in the total current (leak plus oocyte current), which was comparable to the 1.8 g experiment. Taken together, these findings indicated that the increase in amiloride-sensitive current under hypergravity could not be attributed to either changes in patch size (i.e. an increase in the number of channels exposed to amiloride) or leak conductance. These findings strongly suggested that exposure to hypergravity appeared to influence the sodium conductance mediated by ENaC.

Figure 55: Results of ATOVC measurements from two representative ENaC overexpressing oocytes at 1 g and 1.8 g. Hypergravity was achieved using a Large Diameter Centrifuge. After a short stabilization time of about 2 min, the relative changes of the total current at 100Na and the relative changes of the amiloride-sensitive current over a time course of 18 minutes were recorded. The transoocyte potential was held constant at -100 mV.

2.3.4. Measurements during a parabolic flight

Two ATOVC systems were fixed on a stable baseplate, which was mounted within a metal enclosure in the airplane used for parabolic maneuvers (Figure 56). It was mandatory that no access to the enclosure was possible during each set of parabolic maneuvers and therefore the entire measurement protocol was fully automated, with controls and real-time monitoring via a customized touch-screen interface. Each maneuver comprised two hyper-g (ca. 1.8 g) phases
that bracketed the nominal micro-g phase. A typical flight involved a total of 31 such maneuvers. Before the flights, oocytes were freshly prepared and stored on site. They were introduced into the core module prior to each set of 5 parabolas.

Figure 56: Photograph of two identical ATOVC setups. I. ATOVC core module (air pressure tubing not connected). II. Holder for the three solution vials, compensation volume and expandable waste reservoir. III. Voltage clamp hardware and system controller. IV. Preampifier headstage. V. Electronic pressure regulators. VI. Solenoid valves for solution switching. VII. Control module for manual valve switching. VIII. Air supply bottle.

Figure 57 shows first data obtained from an ENaC overexpressing oocyte that was exposed to several hyper- and microgravity phases. Here I compared for a single oocyte the change in total current (leak plus transoocyte current) of each phase with respect to the normal gravity condition before the maneuver. By subtracting the I-V data obtained during each phase from the I-V curve before the maneuver, the difference in transoocyte conductance is obtained. The resulting I-V curves can be fitted with a linear function from which the transoocyte conductance can be estimated. In this case, changes in transoocyte conductance were seen during hyper- and zero gravity conditions (Figure 57b, c). Comparison with the conductance before and after the parabolic maneuvers showed no significant change, which suggested that the oocyte reverted to initial conditions after the maneuver. The origin of these phenomena has yet to be elucidated, and it appears unlikely that they result from systematic errors due to

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8 Photograph was taken during the 53rd ESA parabolic flight campaign. For a photograph showing the latest setup, please refer to Figure 96 in the appendix.
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alternating changes in leak resistance during micro- and hypergravity, which was found to be minimal. In particular, the contribution of the gravitational force to the total force acting on the entire oocyte can easily be estimated. For a typical oocyte diameter of 1 mm and a stabilizing air pressure of 20 mbar, the gravitational force is approximately 1000-fold smaller than the force applied to the oocyte via positive pressure. The contribution of the change in hydrostatic pressure (approx. 0.5 mbar per g) to the leak conductance is less than 2 nA at -50 mV transoocyte voltage (Figure 54b). Therefore, the changes can indeed be assigned to the response of the oocyte to alteration in the gravity level. However, further studies are required to confirm the results and reveal the underlying mechanisms.

Figure 57: (a) Differential current of an ENaC overexpressing oocyte recorded during a parabolic flight maneuver. The I-V curve for each acceleration level was subtracted from that recorded prior to the maneuver. The 1 g trace was obtained by subtracting the I-V curves taken directly before and after the maneuver. Linear regression was applied to the datasets yielding the change in conductance for the slope (Pearson’s coefficients are shown on the bottom right of the graph) (b) Plot of the relative differential conductance resulting from the I-V data shown in a. (c) Readout of the on-board accelerometer during a parabolic maneuver. The sense of the acceleration vector was from the upper compartment of the core module to the microperfusion channel.

9 For this calculation the values for the force resulting from the air pressure and the oocyte weight are assumed to be $4\pi rp$ and $4/3\pi r^3\rho g$, respectively. For $\rho$ we chose a value of 1.2 g cm$^{-3}$ due to the oocyte’s slightly higher mass density compared to water.
2.3.5. Measurements on the electrogenic sodium/phosphate cotransporter NaPi-IIb

This section describes ATOVC measurements conducted on *Xenopus laevis* oocytes heterologously expressing the flounder sodium/phosphate cotransporter NaPi-IIb. Aside from characterizing the current-voltage relationship of phosphate-dependent transport, the influence of treatment of the oocyte with ionophores is described. The main purpose of these studies was to determine whether permeabilization of the body membrane with ionophores could improve the electrical access to the cytosol and thus improve the voltage control across the patch membrane. Furthermore, demonstration of the ATOVC’s applicability to carrier proteins was an important aspect of this work.

**Experimental procedures.** The procedures for general handling of oocytes and preparation of buffer solutions are identical to those described in Section 2.2. Stocks of ionophore solution were prepared at concentrations of 1 mM for nystatin and 100 mM for amphotericin B in millipure water (Figure 58). These solutions were then added individually to the oocyte bath solution and allowed to reach a steady-state. For all experiments, the negative control was performed (no ionophore) on the same oocyte before exposure to the ionophore.

![Molecular formulae of (a) nystatin and (b) amphotericin B.](image)

**Current-voltage relationship of the phosphate-dependent transport.** The voltage-dependence and dose response of phosphate-sensitive currents in NaPi-IIb are well known [85, 86]. It has been found that phosphate concentrations above 0.3 mM were required to fully activate the phosphate-sensitive current mediated by NaPi-IIb. This current shows a sigmoidal voltage-dependence and no reversal potential. These two features form the basis for comparison with the I-V traces obtained through the ATOVC.

Figure 59 shows averaged I-V plots of phosphate-sensitive current in oocytes expressing NaPi-IIb and in non-injected oocytes. For oocytes expressing NaPi-IIb, the shape of the I-V curve is comparable to that obtained with the TEVC [86]. The ATOVC results confirm the rectifying nature of phosphate-sensitive transport via NaPi-IIb (Figure 59a). Sigmoidicity of the I-V curve is present for the ATOVC measurement, though less pronounced than obtained using the TEVC. Especially at lower voltages the I-V curve of the ATOVC deviates from that

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10 Approximately 10 times the apparent affinity for phosphate.
obtained by TEVC. The I-V plot of phosphate-sensitive current in the non-injected oocyte (negative control) is close to zero (Figure 59b). These results suggest that the ATOVC is indeed able to measure phosphate-sensitive currents in oocytes expressing NaPi-IIb.

Influence of ionophore treatment on the voltage-dependence of phosphate-related currents. Nystatin and amphotericin B were tested for suitability as permeabilizing agents in ATOVC measurements. Nystatin, for example, is a well-known ionophore and has been used in patch clamping for studying intracellular mechanisms [87]. The perforating properties of nystatin and amphotericin make them also applicable as fungicides because they can kill fungi as a consequence of leakage of monovalent ions out of the cytosol [88, 89]. In the case of the Xenopus laevis oocyte, ionophore concentration ranges that did not compromise the cell’s health had to be found. At the same time, the concentration had to be high enough to have an effect on ion permeability of the membrane. Typical literature values are around the order of $10^{-5}$ M for nystatin and $10^{-6}$ M for amphotericin B [90-92].

For nystatin, the oocytes maintained reasonable stability at a concentration of 10 μM. The phosphate-dependent currents increased significantly (Figure 60). Treatment with 10 μM nystatin increased the conductance by 20 % ($\Delta$: 20 nA) at -100 mV and 200 % ($\Delta$: 15 nA) at 0 mV. The voltage-dependence increased below -50 mV, whereas it decreased above 20 mV. The overall shape of the I-V curve improved with regard to sigmoidicity, which suggests that the voltage control of the patch membrane was improved. A sigmoidal voltage-dependence is expected from previously conducted TEVC experiments [93]. Results for nystatin concentrations other than 10 μM are not shown as often the I-V traces before and after treatment did not seem to relate to each other.
Amphotericin B significantly increased phosphate-dependent conductance already at a concentration of 0.1 μM (Figure 61). The increase in conductance was approximately 120 % (Δ: 20 nA) at -100 mV and 100 % (Δ: 10 nA) at 0 mV. The voltage-dependence increased after treatment, but the overall shape of the I-V curve remained unaltered. At an amphotericin B concentration of 1 μM an increase in conductance of 160 % (Δ: 40 nA) at -100 mV and 500 % (Δ: 12 nA) at 0 mV was observed (Figure 62). An increase of amphotericin B concentration to 10 μM did not yield a higher phosphate-induced current below 40 mV (Figure 63). What could be observed, however, was a smoothing of the I-V curve resulting in a higher sigmoidicity. This indicates an improvement in voltage control across the patch membrane. At voltages above 40 mV, phosphate-current increased, as seen also at 1 μM concentration. This increase in phosphate-induced current may be explained by an increase in phosphate leak through the pores formed by the ionophores.
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Figure 61: I-V curves of phosphate-sensitive current with (red) and without amphotericin B treatment (black). Amphotericin B concentration was 0.1 μM.

Figure 62: I-V curves of phosphate-sensitive current with (red) and without amphotericin B treatment (black). Amphotericin B concentration was 1 μM.
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Figure 63: I-V curves of phosphate-sensitive current with (red) and without amphotericin B treatment (black). Amphotericin B concentration was 10 μM.

Influence of ionophore treatment on the transient behavior of phosphate-related currents.
In order to study the influence of ionophore-treatment on the phosphate-sensitive current over time, repeated application and washout of phosphate on oocytes expressing NaPi-IIb was done. First, a phosphate perfusion step was conducted without ionophore-treatment. Then, ionophore was added to the bath solution and the phosphate perfusion step repeated three times in intervals of 5, 15 and 30 minutes. The transoocyte voltage was kept constant at -50 mV for all experiments. To verify that the phosphate-sensitive current was attributed to the expression of NaPi-IIb, negative control was performed using a non-injected oocyte. The change in steady-state current was typically less than 1 nA (Figure 64).

Figure 64: Change in phosphate-sensitive current in a non-injected oocyte. 0.5 mM phosphate with subsequent washout. In this oocyte, stability of the steady-state current was slightly compromised with non-perfect behavior regarding repeatability. This is most likely attributed to the oocyte’s health condition (see Section 2.3.2 for stability tests).
Figure 65a shows the change in phosphate-sensitive current before and after treatment with 10 μM nystatin at subsequent time intervals (5, 15 and 30 minutes). The ionophore treatment increased the phosphate-sensitive current throughout the sequence. The amplitude of the current within the first 20 seconds after exposure to phosphate remained constant (Figure 65b). After 15 and 30 minutes of the start of the experiment there was an increased rundown of the current, but their pre-washout levels were still higher than those before treatment. Especially at the beginning the treatment also improved reversibility of the phosphate exposure, demonstrated by a smaller offset between the initial and final baseline.

In contrast to nystatin, the phosphate-sensitive current following amphotericin B treatment increased only during the first cycle (5 minutes after start of treatment) (Figure 66). The subsequent cycles showed a decrease in phosphate-sensitive current relative to the current obtained for the untreated oocyte. Furthermore, reversibility of the phosphate exposure decreased after treatment with amphotericin B. The transient behavior of the phosphate-sensitive current during each exposure cycle was different from that observed for nystatin. In comparison with nystatin, amphotericin B showed a slower response with no sign of rundown before washout. A number of factors could be responsible for the observed difference, such as differences in membrane conductance and overall cell viability.
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2.3.6. Measurements of calcium-dependent currents

The results presented in this section were produced within the scope of the 55th Parabolic Flight Campaign in Bordeaux under the auspices of the European Space Agency ESA. The goal of the campaign was the investigation of calcium-associated membrane transport under varying gravitational levels. Calcium levels are thought to be important in regulating muscle and bone development [94, 95]. The physiological pathways related to calcium are therefore of vital interest with regard to muscle and bone atrophy associated with lack of gravity [96].

Experimental procedures. The assay used for this study involved the use of thapsigargin as an activating agent for calcium activated currents. Thapsigargin is an enzymatic inhibitor of the Ca²⁺ ATPase in the sarcoplasmic and endoplasmic reticulum (SR, ER) of the cell. It causes the depletion of calcium storage within the ER releasing calcium into the cytosol. The increase of cytosolic calcium then activates voltage-dependent calcium channels in the membrane (Figure 68) [97, 98]. To exclude extracellular calcium and to block endogenous potassium currents, the calcium in the buffer medium is replaced with barium [99]. This way, experiments with external perfusion can be performed that depend only on intracellular calcium. To extract currents that are specific to calcium, 2-aminoethoxydiphenyl borate (2-APB) can be used. 2-APB is an inhibitor of the non-specific cation channel family TRP and has also been demonstrated to be a general blocker of calcium channels [100-102].

In this study, the procedures for general handling of oocytes and preparation of buffer solutions are identical to those described in Section 2.2, except for the Ca²⁺, which was replaced by 1.8 mM Ba²⁺. For preparation of the blocker solution, 100 μM 2-APB was added from a 100 mM stock solution (in millipure water) to the buffer. Prior to the experiment, Xenopus laevis oocytes were incubated for 30 to 45 minutes in buffer solution additionally containing 1 μM thapsigargin.

Figure 66: (a) Change in phosphate-sensitive current in a NaPi-IIb oocyte repeatedly exposed to 0.5 mM phosphate and washed out. 10 μM amphotericin B was added after 5 minutes of the start of the experiment. (b) Phosphate-sensitive current for each exposure cycle after 20 seconds (indicated by arrow) of the start of exposure to phosphate.
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Figure 67: Molecular structures of (a) thapsigargin and (b) 2-aminoethoxydiphenyl borate (2-APB).


**Current-voltage relationship of 2-APB-sensitive currents in thapsigargin-treated oocytes.** Figure 69 shows the I-V plots of the 2-APB-sensitive current for a treated and non-treated oocyte. It is evident that for the treated oocyte there is an increasing outward current with increasing transoocyte voltage at depolarizing potentials. This is in agreement with the expected influx of chloride into the cytosol. These channels are known to be activated by increasing levels of cytosolic calcium [98]. The non-treated oocyte showed almost no voltage-dependence, suggesting no change in cytosolic calcium had occurred.
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Figure 69: I-V plots of the 2-APB-sensitive current in oocytes with (black trace, square symbols) and without (red trace, round symbols) thapsigargin treatment. There was a number of technical problems at the time when the I-V curves were recorded. First, there was coupling of the 50 Hz signal into the measurement apparatus. Second, the current signal of the voltage clamp was not properly zeroed, so that the traces are offset by about -20 nA. Third, there was a problem with the acquired signal for transoocyte voltages above 100 mV due to a configuration problem in the multiplexer. All of the problems were fixed in time for the 55\textsuperscript{th} ESA parabolic flight campaign.

2-APB-sensitive currents under varying gravitational levels. For the measurements during the parabolic flights, the experimental protocol was modified to allow the investigation of changes in steady-state as well as transient currents under zero gravity. This was achieved via the use of a pulse protocol with pulse lengths of one second. First, the oocyte was kept at holding potential of -50 mV. Then, the oocyte was hyperpolarized using a transoocyte voltage of -200 mV. Subsequent depolarization to +200 mV was used to evoke the influx of calcium-activated chloride into the cytosol. The immediate transition from a highly negative to a highly positive voltage would allow maximal activation of transient currents. The pulsing sequence was repeated after switching to the blocker solution to enable evaluation of 2-APB-sensitive currents through subtraction of the signals.

Figure 70 shows the transoocyte current as a function of time for the complete experiment as conducted during the parabolic flight. The capacitive transient upon depolarization of the membrane is clearly developed. Moreover, reduction of steady-state transoocyte current can be directly observed in both polarization steps. After return to the holding potential (-50 mV), the transoocyte current returned to the initial holding current with reasonable accuracy, demonstrating the assay’s reversibility. However, artifacts did appear after solution switching. The origin of these artifacts are probably to be found in the release of a bubble during the actuation of the solenoid valve. Vibration levels on the aircraft were probably high enough to promote bubble formation in regions of dead volume within the perfusion system of the ATOVC.
To investigate the influence of change in gravity on the 2-APB-sensitive current, the transoocyte currents recorded during the zero gravity phase were compared with the currents recorded at normal gravity. To minimize the contribution of time-related effects, control measurements were made directly before and after the zero gravity phase. Figure 71 shows exemplary data of the 2-APB-sensitive current after depolarization. The plots clearly show that the 2-APB-sensitive current at steady-state decreased during the zero g phase, suggesting an inactivation of calcium-dependent chloride currents. Figure 71b, however, not only shows a reduction in 2-APB-sensitive current, but also its inversion. This may be attributed to the lack of specific effects of 2-APB [101]. The evaluation of the transient current was not possible because the transients were too fast to be recorded accurately at the set sampling rate. The two examples were chosen due to the reversibility of 2-APB sensitive current. Experiments where the 2-APB-sensitive current did not return to its initial baseline were not deemed reliable.
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Figure 71: 2-APB-sensitive current after membrane depolarization. The black trace (square symbols) was taken during the 1 g phase preceding the 0 g phase, the red trace (circular symbols) was taken during the 0 g phase and the blue trace (triangular symbols) was taken during the 1 g phase following the 0 g phase. (a) shows data of an oocyte measured during parabola number 20 and (b) shows data of another oocyte measured during parabola number 7.
2.4. Conclusions

A system for performing non-invasive electrophysiological measurements on *Xenopus laevis* oocytes under varying gravity levels was described. Benchmark experiments conducted in the laboratory demonstrated that the system is able to faithfully reproduce the results typically obtained by the TEVC method. Furthermore, due to the high stability of the leak current, changes in current with nanoampere resolution could be measured. Initial results on ENaC overexpressing oocytes exposed to micro- and hypergravity were presented that suggest that ENaC activity is indeed sensitive to gravitational forces. Future analysis of other ion channel classes under micro- or hypergravity will certainly provide additional biologically relevant insights, into which physiological pathways are affected by gravitational change.

The experiments on NaPi-IIb for studying the effect of ionophores showed that in comparison with nystatin, amphotericin B appeared to be more effective in permeabilizing the membrane of *Xenopus laevis* oocytes. The resulting improvement in voltage control of the patch membrane was demonstrated via the increase in phosphate-sensitive current and smoothing of the I-V curve. On the other hand, nystatin showed better stability over time of phosphate-sensitive current. Even after 30 minutes, nystatin showed a positive effect on the current while oocyte health did not seem to be compromised. Amphotericin B, however, induced an increase in phosphate-sensitive current only during the first 5 minutes. Reproducibility for both ionophores was low, which made a statistical evaluation of the data unpractical. As such, the presented results are preliminary and are thus considered proof-of-concept only.

The results obtained for the calcium experiments demonstrate that the use of thapsigargin and 2-APB for studying calcium-dependent chloride currents in native *Xenopus laevis* oocytes is feasible. Furthermore, a reduction in steady-state 2-APB-sensitive current could be observed during selected zero gravity phases of parabolic flight. The evaluation of transient currents was not possible due to the sampling rate being too low. However, reconfiguration of the ATOVC system would enable the study of transient 2-APB-sensitive currents in future parabolic flight campaigns.

The key advantages of this system, in accordance with our stringent design criteria, are: (i) the very short time needed for setup and measurement, (ii) capability of fully automatic operation (after mounting the oocyte) and (iii) high reliability. Although the system was developed specifically for electrophysiological studies under varying gravity conditions, due to its operational simplicity it could also be conveniently used for electrophysiological investigations in ground-based laboratories.
3. **An integrated field-effect microdevice for monitoring membrane transport in living cells via lateral proton diffusion**

An integrated microdevice for measuring proton-dependent membrane activity at the surface of *Xenopus laevis* oocytes is presented. By establishing a stable contact between the oocyte vitelline membrane and an ion-sensitive field-effect (ISFET) sensor inside a microperfusion channel, changes in surface pH that are hypothesized to result from facilitated proton lateral diffusion along the membrane were detected. The solute diffusion barrier created between the sensor and the active membrane area allowed detection of surface proton concentration free from interference of solutes in bulk solution. The proposed sensor mechanism was verified by heterologously expressing membrane transport proteins and recording changes in surface pH during application of the specific substrates. Experiments conducted on two families of sodium-phosphate cotransporters (SLC20 & SLC34) demonstrated that it is possible to detect changes in phosphate-induced transport for both electrogenic and electroneutral isoforms and distinguish between transport of different phosphate species. Furthermore, the transport activity of the proton/amino acid cotransporter PAT1 assayed using conventional whole cell electrophysiology correlated well with changes in surface pH, confirming the ability of the system to detect activity proportional to expression level.
3.1. Introduction

For many decades, electrophysiological methods have been at the forefront of investigations of membrane conduction and excitation in living cells [103]. To measure membrane conductance, control of either the transmembrane voltage or current is required. Therefore, a key experimental challenge is to establish adequate electrical access to the cytosol. For example, in the commonly used two electrode voltage clamp (TEVC) applied to large cells such as *Xenopus laevis* oocytes, two microelectrodes impale the oocyte to sense and control the membrane potential. This procedure requires delicate glass microelectrodes and a high degree of micromanipulation, either by a human operator or precision robotics [37, 104]. In the whole cell patch clamp, electrical access to the cytosol is gained by sealing the tip of a glass microelectrode to the membrane and applying suction to eventually rupture the cell membrane within the electrode [105]. Recently, several non-invasive voltage clamp techniques for *Xenopus laevis* oocytes have been developed that leave the membrane intact. These methods rely on the physical compartmentalization of the membrane into two areas and measurement of trans-cellular currents across the entire oocyte. In the transoocyte voltage clamp (TOVC) an AC voltage is applied across symmetrically distributed membrane impedances [106]. Asymmetric variants of the TOVC have been realized to better define the transmembrane potential across a smaller region of membrane [31, 107] and are akin to the loose macropatch [61].

Even though voltage clamping has established itself as a reliable tool for performing electrophysiological experiments, its application in large-scale industrial screening has not been fully realized. Apart from the aforementioned cell manipulation requirements, parallelization of the voltage clamp requires duplication of many hardware components - including the electrodes, voltage clamp electronics and the fluidic pathways. Electronic detection methods based on field-effect devices, on the other hand, are attractive alternatives for large-scale integration. As the sensors detect local surface charge accumulation or depletion as opposed to passing current, from the cell medium, liquid domains can be shared without significant crosstalk occurring between channels. Furthermore, as the sensing field is limited by the shielding properties of ionic solutions, this would allow the realization of high density sensor arrays. The detection distance can be calculated using Debye’s relation for low electrolyte concentrations [1]. Recent work on the theory of electrostatic fields in ionic solutions allows for more accurate predictions on high electrolyte concentrations, as present in physiological solutions [108]. The most appropriate field-effect sensor for use in ionic solutions, the ion-sensitive field-effect transistor (ISFET), has first been described by Bergveld [109]. The ISFET, which is derived from the metal oxide semiconductor field-effect transistor (MOSFET), replaces the metal gate electrode on the insulator with the bulk solution, the potential of which is defined using a reference electrode. Charge separation at the insulator-liquid interface then results in a detectable potential difference due to the double-layer capacitance between the reference electrode and the semiconducting channel.

ISFETs have mostly been used to measure local changes in pH, such as acidification in cell cultures [110, 111]. There has also been interest in using ISFETs for studying membrane transport. The first successful transport assays were reported on the human anionic transporting
peptide C (OATP-C) heterologously expressed in *Xenopus laevis* oocytes [34]. In these experiments, the uptake of estrone-3-sulfate and estradiol 17β-D-glucuronide was detected with good sensitivity, and differences in uptake rates between the wild-type and mutant of the OATP-C transporter were resolved.

*Xenopus laevis* oocytes, like other cells, are believed to have endogenous proton-regulating mechanisms at the cell membrane that are responsible for a pH gradient across the cell membrane. In particular, there is evidence of the existence of a Na+/H+ exchanger that is at least partially responsible for the proton countertransport across the cell membrane [112, 113]. Furthermore, overexpression of proton-dependent membrane proteins modulates local proton binding affinity upon transport activation. Transport activity mediated by heterologously expressed transport systems by pH detection using a glass microelectrode placed in close proximity to the membrane has been demonstrated [114, 115]. Considering the extremely fast bulk diffusion rate of protons in water [43, 116], it may at first seem surprising that pH values different from the bulk solution can be detected at all. However, proton sinks and sources at the cell membrane change the local association/dissociation rate constants that results in lateral diffusion rates different from the bulk diffusion rate. Recently, a mathematical model for lateral diffusion kinetics at the cell membrane for protons was described [117], which provides numerical solutions for the dwell time of a proton in buffered solutions commonly used in physiology. The results support previously conducted experiments that show that a proton can migrate laterally hundreds of micrometers along the cell membrane before diffusing back into the bulk solution [118].

Based on the lateral diffusion model, I developed a method for sensing proton-dependent membrane transport in *Xenopus laevis* oocytes by utilizing a novel arrangement of ISFET technology. A proton-selective diffusion barrier is established that is created by direct contact of part of the membrane with the sensor. The membrane is stabilized against the sensor surface using previously reported immobilization technology (see Section 2.2.2) [107]. Due to the much higher lateral diffusion rate of the protons compared to other solutes, the detected potential change at the sensor is exclusively proton-dependent (Figure 72). Once equilibrated, the proton concentration at the detection site [H+]D is equal to the proton concentration at the membrane surface [H+]S, assuming ideal coupling between the sensor surface and the membrane surface. The surface potential of the sensor then reflects the surface pH for steady state membrane transport. It is predicted that in contrast to protons, substrate molecules S that interact with transport proteins at the cell membrane exposed to the bulk solution, will diffuse poorly across the diffusion barrier for two reasons. First, their lateral mobility relative to the protons is greatly reduced and second, they are effectively removed from the local medium by the transport proteins themselves before reaching the detection surface. As a consequence, modulation of substrate concentration in proximity to the detection surface will be minimal for large migration distances, as present in the system.
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3.2. Materials and methods

3.2.1. Microdevice

The device comprises a sensing element, a microperfusion system and a circular orifice for immobilization of the oocyte and alignment with the sensing surface. The sensor is an n-channel field-effect transistor (FET) without metal gate and with tantalum pentoxide ($\text{Ta}_2\text{O}_5$) as the insulator material (ISFETCOM Co. Ltd., Saitama, Japan). $\text{Ta}_2\text{O}_5$ exhibits superior proton buffering capacity compared to other metal oxides while providing a good diffusion barrier [17]. The sensor was inserted into the cavity of a precisely machined support made of aluminum, creating a flush fit. A rectangular (5 mm x 10 mm) piece of perfluoroethylene (PTFE) with a thickness of 0.2 mm was then placed over the sensor to serve as a gasket and spacer for forming the microperfusion channel. A CNC-machined block made from polymethylmethacrylate (PMMA) was screwed onto the aluminum support. It integrated 6 liquid inlet channels and 1 outlet channel as well as the immobilization orifice. A hole (1.6 mm dia.) intersecting with the outlet channel was drilled for insertion of the reference electrode. Due to the low dimensional tolerances of all parts involved, the oocyte orifice is self-aligned with the sensor surface upon assembly of the device.

The FET was driven with a source-drain follower circuit that fixed the source-drain voltage and drain current to constant values. For all experiments, the source-drain voltage and current were set to 500 mV and 500 µA, respectively. The reference electrode was connected to the circuit’s signal ground which defined the reference potential, against which the output signal $V_{GS}$ was measured at the source connection. In this configuration, changes in $V_{GS}$ are directly

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Figure 72: Simplified cartoon of the proton-selective lateral diffusion model where the oocyte interfaces to the sensor element (not to scale). The vitelline layer of the (defolliculated) oocyte comprises a network of fibrous filaments that surround the cytoplasmic membrane, which itself is not smooth due to numerous microvilli protrusions (not shown). The total thickness of the vitelline layer is estimated to be 2-3 µm for stage VI oocytes [6]. ([S]: concentration of substrate interacting with the membrane transport proteins. $[\text{H}^+]_D$, $[\text{H}^+]_S$, $[\text{H}^+]_B$: Proton concentration at the detection site, membrane surface and in buffer, respectively.) The diffusion barrier is assumed to be narrow compared with the sensor area so that “edge effects” can be neglected and transporters within the sensor region do not “see” the substrate $S$. 

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...proportional to changes in the Nernst potential. The technical implementation of the drain-source follower was realized using constant current drivers [119]. Acquisition of the gate-source voltage was achieved using a high-resolution data acquisition and control (DAQ) unit (LabJack U6-Pro, LabJack Corp./USA). The DAQ unit also served as a controller for the air pressure system used for cell immobilization and solution exchange. User control of all systems was provided by a proprietary PC-based graphical user interface (GUI), which employs the Windows.NET application programming interface (Microsoft Corp./USA). An expanded version of an air pressure system, which provided solution exchange and perfusion, as well as oocyte immobilization was used as described earlier [107].

Figure 73: a) Schematic view of the cross-section of the device (not drawn to scale). The height of the microperfusion channel is around 200 µm. A typical Xenopus oocyte would be 1000-1200 µm in diameter. b) Micrograph of the sensor as seen through the hole (ca. 800 µm in diameter) of the oocyte immobilization compartment. The oocyte membrane completely covers the active area of the sensor due to its deformability.

Figure 74: a) Exploded view of the device using the original 3D CAD engineering data. b) Photograph of the assembled, but unconnected device.
3.2.2. ISFET biasing and readout circuit

The complete circuit for the ISFET sensor system used for the experiments on *Xenopus laevis* oocytes is shown in Figure 97 of the appendix. It integrates a source-drain follower, a Sallen-Key low-pass filter (40 Hz corner frequency) and voltage generators for setting the biasing parameters of the ISFET [78]. Power is provided through a linear-type regulated power supply with +15 V and -15 V output.

**Source-drain follower.** The purpose of the source-drain follower circuit is to maintain the source-drain voltage $V_{DS}$ and the drain current $I_D$ constant. According to Ohm's law, the resistance between source and drain is thus kept constant. This ensures that the output voltage $V_{GS}$ depends solely on the change in Nernst voltage at the insulator/liquid interface (see Section 1.4.2 for theoretical background). It is also important to ensure that the potential at the insulator, and thus the entire FET and electronic circuit, is able to float relative to the reference potential. In other words, currents generated within the source-drain follower circuit are not allowed to flow into the reference potential.

Figure 75 shows a simplified schematic of the source-drain follower as implemented in the ISFET sensor system for monitoring membrane transport in *Xenopus laevis* oocytes. It utilizes two floating current drivers to define the source-drain voltage $V_{DS}$ and the drain current $I_D$ [119]. The source-drain follower can be realized in other ways [120], but this version was chosen due to its simplicity and the possibility of defining $V_{DS}$ and $I_D$ independently. Setting $V_{DS}$ is achieved by sourcing a user-defined current through resistor $R_{src}$. Opamp 1 ensures that the potential at node 3 (drain) is equivalent to node 1 and that no current originating from $I_{src}$ is allowed to flow into node 3. Opamp 2 regulates the potential at node 2 to match the potential at node 4 (source). At the same time the output of opamp 2 provides a return path for $I_{src}$. $I_D$ originates from the output of opamp 1 and flows into the current sink. The voltage between node 2 and ground is equal to the source-gate voltage and is therefore called $V_{SG}$.

![Figure 75: Simplified electronic schematic of the source drain follower connected to the ISFET. The voltage difference between node 1 and 2 is equivalent to the voltage difference between node 3 and 4.](image-url)
**Current source/sink.** The current source or sink generates a current $I_s$ flowing through a scaling resistor $R_i$ as described by the relation

$$I_{src} = \frac{V_+}{R_i} \quad (3.1)$$

As shown in Figure 76, this is achieved by connecting node 1 of the scaling resistor to the output of the instrumentation amplifier and connecting node 2 to a voltage follower driving the reference voltage input of the instrumentation amplifier. Since the potential at node 2 is equal to the potential at the reference voltage pin of the instrumentation amplifier (node 3), the voltage drop across the resistor is equal to $V_+$. Since $I_{src}$ cannot flow into the voltage follower, it has to flow through the load connected at node 2 in its entirety. The distinction between the current source and the current sink is given by the direction of the current. If the conventional current flows out of the instrumentation amplifier’s output into the load, the arrangement becomes a current source. If the current flows from the load into the instrumentation amplifier’s output, the arrangement becomes a current sink. In both cases, the load needs to be terminated with a current return path for proper operation. Since the input voltage at the instrumentation amplifier can be both positive and negative, the circuits for the current source and current sink are identical (Equation (3.1)).

For the actual implementation, the INA105 instrumentation amplifier (Burr Brown/Texas Instruments) was chosen due to its very low gain error (0.05 %) [121]. Since the source-drain follower requires very stable DC operation, an adjustable band-gap voltage reference was used as the input voltage. The model REF102 (Texas Instruments) was chosen due to its high long-term stability (5 ppm per 1000 hours) and low output noise (5 µV) [122].

![Figure 76: Schematic of the current source as implemented in the source-drain follower circuit.](image)
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3.2.3. Solutions and reagents

For storage of oocytes, modified Barth’s solution contained (in mM): 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.82 MgSO₄, 2.5 NaHCO₃, 2 Ca(NO₃)₂, 7.5 HEPES, pH was adjusted to 7.5 with Tris and supplemented with 5 mg/l doxycycline and 5 mg/l gentamicine. Standard buffered saline solutions (100Na) contained (in mM): 100 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, adjusted to pH 7.5, pH 7.4 or pH 6.5 with TRIS. For test solutions containing activating or inhibiting substances the following stock solutions were used: 1M (in H₂O) inorganic phosphate (Pi) at pH 7.4, 100 mM (in DMSO) amiloride, 100 mM (in H₂O) L-Proline, 100 mM (in H₂O) γ-aminobutyric acid (GABA) and 100 mM (in H₂O) sodium phosphonoformate tribasic hexahydrate (PFA). After addition of the reagents, all solutions were filtered using a 0.22 µm filter, prior to filling the solution vials. All standard reagents were purchased from either Sigma-Aldrich or Fluka.

3.2.4. Oocyte preparation

Female *Xenopus laevis* frogs were purchased from Xenopus Express (France) or African Xenopus Facility (R. South Africa). The frogs were anesthetized in MS222 (tricaine methanesulphonate) after which portions of ovaries were surgically removed and cut in small pieces. Collagenase treatment on oocytes was done for 45 min with 1 mg/ml of crude type 1A in 100Na solution (without Ca²⁺) in presence of 0.1 mg/ml trypsin inhibitor type III-O. Healthy stage V-VI oocytes were selected, maintained in modified Barth’s solution at 16 °C and injected with typically 5 ng total of cRNA of the α, β, γ sub-units of the *Xenopus* isoform of ENaC [75], sodium/chloride-dependent GABA cotransporter GAT1 [123], flounder NaPi-IIb [124], mouse NaPi-IIc [125], mouse PAT1 [126], or PiT-2 [127], according to procedures previously conducted in our laboratory. After injection, the oocytes were incubated for 3 days at 16 °C in Barth’s solution. Non-injected oocytes of the same batch were used as negative controls. All animal handling procedures were approved by Swiss Cantonal and Federal veterinary authorities.

3.2.5. Procedure for oocyte experiments

Before the experiment, the oocytes were thoroughly washed in 100Na solution at pH 7.4 to prevent the diffusion of protons between the oocyte chamber and the microperfusion channel. After priming of the system with 100Na pH 7.4 solution, the cell was pipetted into the oocyte chamber. The air port plug was then inserted into the oocyte chamber and a constant air pressure of 5 mbar was applied. The perfusion system was then pressurized at 100 mbar which, in combination with the inlet capillaries (30 cm length, 180 µm inner diameter), resulted in a flow rate of approx. 1 µl/s when one of the solenoid valves was open. The oocyte was perfused with 100Na solution at pH 6.5 (for PAT1) or pH 7.4 (all others) for 5 min to create stable steady-state conditions before initiating a transport activation protocol. The perfusion sequence was programmed beforehand using a built-in macro feature of the GUI. This feature provided high
timing accuracy of the solution switching and guaranteed the repeatability of the perfusion sequence.

3.3. Results and discussion

3.3.1. pH-sensing characterization of the ISFET

![Graphs showing pH-sensing characterization of the ISFET](image)

Figure 77: a) Time-dependent measurement of phosphate reference buffers at pH 4.01, pH 7.00 and pH 9.21 and b) linear fit of the data points obtained from the baselines of each buffer. c) Time-dependent measurement of 100Na solutions buffered at pH 7.40 and pH 7.50 for two different perfusion pressures (100 mbar and 200 mbar). d) Expanded view of data in panel c during the exchange of solutions from pH 7.40 to pH 7.50. Valves were switched at t = 0 s. All experiments were conducted at room temperature.

To determine the response of the sensor to changes in bulk proton concentration and correlate the changes in output voltage to the pH change, the sensor was superfused with buffered solutions at varying pH (Figure 77a). A slope of \(-58.0 \pm 1.5 \text{ mV/pH} \ (R^2_{adj} = 0.99862)\) was found using a three-point extrapolation with standard phosphate buffer solutions at pH 4.01, pH 7.00 and pH 9.21 (Figure 77b). This value was close to the prediction from the Nernstian equation \((-59 \text{ mV/pH})\), which demonstrated the excellent proton buffering capacity of Ta_2O_5. The detection limit of \(\Delta pH\) was determined to be approx. 0.003 units (0.2 mV peak-to-peak noise). The drift slope depended on a number of factors, such as ambient light intensity, but was typically less than 0.1 mV/min. The sensor-to-sensor variation was insignificant, which resulted from the large sensor structure and precise CMOS manufacturing processes. In a second experiment, the sensor was superfused with 100Na solutions at pH 7.50 and pH 7.40 (Figure
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The experiment showed an expected decrease of 5.4 mV (i.e. $\Delta pH = -0.1$) with a rapid transition from the first steady-state signal to the next one. Even though the flow inside the microperfusion channel was laminar, the signal response was not perfect due to cross-diffusion at the junction where the inlet channels meet. Also, there was some dead time between the switching of the valves and the signal response onset due to the relatively large solution exchange volume (approx. 4 µl) in relation to the flow rate (1 or 2 µl/s). In total, it took approx. 8 s at 100 mbar and approx. 5 s at 200 mbar to reach steady-state conditions after valve actuation (Figure 77d). Nevertheless, for the uptake experiments on oocytes these values are fully acceptable due to the comparatively slow proton diffusion between the membrane surface and the sensor surface (see below). With regards to selectivity, the sensitivity of the Ta$_2$O$_5$-based sensor towards Na$^+$ and K$^+$ is less than 1 mV/M, as has been described previously [128].

3.3.2. Transport experiments on oocytes heterologously expressing various membrane transport proteins

Figure 78: Experiments conducted on oocytes heterologously expressing various membrane transport proteins indicated with their respective controls on non-injected (NI) oocytes showing sensor readout ($V_{SC}$) as a function of time. Only part of the initial stabilizing baseline region that preceded substrate application is shown (see Materials and methods): a) PAT1, b) NaPi IIb, c) NaPi-IIc, d) PiT-2, e) Proline control, f) P$_i$ control, g) GAT1, h) ENaC. In each case either the same or representative oocytes from the same batch were pretested using a two-electrode voltage clamp to confirm functional expression. The bars indicate the duration of application of the respective activating and blocking agents. Arrows indicate flux direction of substrate according to the assumed driving force conditions.
Proline transport mediated by PAT1. Assays conducted on oocytes heterologously expressing the proton-driven amino acid transporter (PAT1) showed a significant decrease of $V_{GS}$ upon exposure of the cell to a 1 mM proline solution (Figure 78a). The signal reached a steady state after 5 min and returned to the initial baseline after washout of proline. When switching back to the proline-free buffer solution, the signal returned to the initial baseline. PAT1 is known to reversibly bind amino acids and cotransport them stoichiometrically with one proton/amino acid per cycle \cite{129}. Even though PAT1 works bidirectionally, the exposure time of 5 min was apparently not sufficient to significantly change the proton gradient across the cell membrane. The downward deflection of $V_{GS}$ upon exposure to proline translates into an increase in surface pH. This is to be expected since the translocation of free protons by PAT1 results in their depletion at the extracellular membrane surface and a consequent lateral movement of protons away from the sensor region.

Phosphate transport mediated by NaPi-IIb, NaPi-IIc and PiT-2. In contrast to PAT1, for oocytes heterologously expressing the electrogenic sodium-coupled phosphate cotransporter (NaPi-IIb) the signal deflection was reversed, which indicated that a decrease in surface pH occurred (Figure 78b). This can be understood when we consider that Pi is present at the membrane surface as both divalent ($\text{HPO}_4^{2-}$) and monovalent ($\text{H}_2\text{PO}_4^-$) phosphate species with an assumed $\text{pK}_a=6.8$ under physiological conditions and distributed according to the following equilibrium,

\[
\text{HPO}_4^{2-} + \text{H}^+ \rightleftharpoons \text{H}_2\text{PO}_4^-
\]

The electrogenic NaPi-IIb translocates 3 Na\(^+\) together with one divalent Pi per transport cycle \cite{130}, which results in the increase of $[\text{H}^+]_S$ due to the depletion of the divalent species. For the electroneutral isoform NaPi-IIc, a detectable pH decrease at the membrane surface is also induced upon exposure to Pi (Figure 78c). This was expected given that NaPi-IIc also prefers divalent Pi \cite{131}, and translocates two Na\(^+\) per cycle with no net charge movement \cite{132}. This result also establishes that the sensor (i) is capable of sensing electroneutral transport processes and (ii) does not simply respond to a change in the cell membrane potential as might be predicted for the electrogenic transporters that mediate net charge translocation.

In contrast to the SLC34 family of sodium coupled Pi cotransporters, members of the SLC20 family prefer monovalent Pi ($\text{H}_2\text{PO}_4^-$), and translocate two Na\(^+\) per transport cycle together with one positive net charge. The resulting shift of the equilibrium to the right (see equation above) would therefore result in a decrease in $[\text{H}^+]_S$, which explains the downward deflection (increase in pH) of $V_{GS}$ in PiT-2 upon exposure to Pi (Figure 78d). The amplitude of the change in $V_{GS}$ at steady state was significantly lower compared to NaPi-IIb and NaPi-IIc and most likely resulted from the lower surface expression of protein compared to NaPi-IIb,c. The changes in surface pH registered by the ISFET sensor for NaPi-IIb and PiT-2 were in qualitative agreement with TEVC measurements conducted in combination with surface pH measurements using a pH-sensitive glass microelectrode \cite{115}. 

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Control experiments confirm the specificity of the sensor for detecting local $\Delta p\text{H}$. Control experiments on non-injected oocytes showed minimal deflections of $V_{SG}$, both for P$_i$ and proline (Figure 78e,f). These may be due to endogenous membrane proteins that could interact with proline and P$_i$. In particular, the presence of amino acid-modulated membrane activity has been determined in uptake studies [133]. Nonetheless, considering that relatively high concentrations of proline and P$_i$ were used in our experiments, the contribution of the endogenous activity can be neglected when the level of exogenous expression is sufficiently high. To further demonstrate that modulation of proton-independent transport proteins did not induce a potential change at the sensor surface, experiments on oocytes overexpressing the γ-aminobutyric acid (GABA) transporter (GAT1) and the epithelial sodium channel (ENaC) were conducted. GAT1 is a sodium-chloride-dependent cotransporter highly specific for GABA [134]. ENaC is a sodium channel whose conductance can be blocked using amiloride ($K_i < 1 \, \mu\text{M}$) [135, 136]. For oocytes expressing either of these membrane proteins, a significant change in $V_{SG}$ could not be resolved. This finding allowed the exclusion of the presence of detection artifacts at the sensor surface and confirmed the specificity of the sensor to changes in local proton concentration.

Correlating the ISFET response to transport activity. Oocytes overexpressing PAT1 were used to correlate the change in membrane surface pH with PAT1 activity. For the oocyte on the ISFET sensor, application of proline will result in a net intracellular translocation of protons into the oocyte, and a concomitant membrane depolarization. A new steady-state membrane potential will be reached that is a function of both the PAT1 protein expression level and transport rate, as well as the endogenous leak conductance of the oocyte. The latter effectively shunts the secondary active transport process mediated by PAT1. As the endogenous leak may vary from cell to cell, for a given expression level, the change in membrane potential will also vary and is therefore not a good quantitative measure of transport activity. Moreover, the proline transport rate will itself be a function of membrane potential due to voltage-dependent partial reactions in the transport cycle [126]. Therefore, to correlate transport activity with $\Delta p\text{H}$ detected by the sensor for individual oocytes, the steady-state membrane potential reached during transport under the same conditions as for the ISFET assay was first determined (inset, Figure 79b). For that case, the net membrane current is zero and comprises the inward transporter-related flux that is balanced by an equal, but opposite current mediated by endogenous channels and pumps. The oocyte was then voltage clamped to determine the transporter-related current by subtracting the current in the absence of proline from that obtained with proline to eliminate proline-independent endogenous currents. From this I-V data the transport flux corresponding to the steady-state potential for the ISFET assay could be estimated (Figure 79b). To ensure minimal substrate accumulation, the TEVC response was tested first to avoid long substrate exposure incurred with the ISFET system.

A reasonable correlation between changes in $V_{SG}$ and the substrate-dependent current for six oocytes expressing PAT1 was observed (Figure 80). This finding supports the assumption that the change in surface proton concentration is directly proportional to the total substrate current, which for a given turnover rate and driving force, is a measure of the number of active transporters in the membrane. Indeed, this is to be expected if the dependence of protons on the
translocation of a substrate-specific substance is stoichiometric. The substrate turnover rate is then proportional to \([\text{H}^+]_s\), which allows quantitative substrate activation and inhibition studies. This implies that a high coupling strength between \([\text{H}^+]_s\) and \([\text{H}^+]_d\) is maintained to minimize secondary effects compromising the linearity of concentration change. It is assumed that optimizing the geometry of the structures involved in the immobilization of oocytes (hole diameter, sensor-to-hole distance) will lead to improved coupling strength between the membrane transport proteins and the sensor surface, without compromising the effectiveness of the proton-selective diffusion barrier. This may also be the reason for the deviation of data points in Figure 80.

Figure 79: Correlating sensor response with transport activity. a) Sensor response to proline superfusion of a representative oocyte (designated #4 in c) heterologously expressing PAT1. b) TEVC I-V data of the proline-dependent current of oocyte #4 in response to the addition of 3 mM proline solution to the 100Na buffer. Inset shows the change in membrane potential induced by proline application for the same oocyte as in a.

Figure 80: Correlation of \(\Delta V_{GS}\) and the substrate-dependent current. Each point represents data from a single oocyte. Arrow marks the data point of oocyte #4 (-23 mV, -140 nA).
3.3.3. Extension of the ISFET sensor for monitoring activity in mammalian cell cultures

This section describes the first developments of a microchip-based platform intended for studying membrane transport in mammalian cell lines. The key technical differences from the previously described ISFET-sensor devices can be summarized as:

(1) **Sensor size.** The characteristic length of the sensors is in the order of 10 μm. Since mammalian cells are typically in the same range, analysis at single cell level is possible.

(2) **Sensor parallelization.** A number of sensors are arranged in a rectangular array. The sensors share a common source connection.

Due to these differences, the ISFET-sensor array allows multiplexed analysis on mammalian cells cultured on the sensor array. Via the control of cell density (confluency), the system can perform measurements on either isolated single cells (low confluency) or a conjugated cell layer (full confluency).

**Materials and methods.** The basic integration concept of the microdevice involved the utilization of a printed circuit board (PCB) with a card edge connection feature. This allowed rapid connection to and disconnection from the biasing and readout electronics. The sensor dies were purchased in the form of 4-inch wafers from the Tyndall research institute (Cork, Ireland). The dies, fabricated using a 1.5 μm silicon-on-insulator (SOI) process, featured arrayed p-channel ISFET devices with varying pitch and gate size\(^{11}\). The exposed insulator material consisted of silicon nitride (Si\(_3\)N\(_4\)). A 1.2 μm thick polyimide coating served as a passivation layer. It covered the entire die except the gate insulator and the bond pads. After dicing, the sensor dies were glued onto PCBs and wire-bonded using a manual wire-bonding station at the cleanroom facility (Figure 81a). Subsequent encapsulation of the wires ensured that no liquid could diffuse to the contacts and damage the die (Figure 81b).

Figure 81: (a) Photograph of the ISFET die after wire-bonding to the PCB. Shown is a 32-channel version. The size of the die is approximately 4.5 mm times 5 mm. (b) Die after encapsulation using an industrial-grade epoxy.

\(^{11}\) Tyndall National Institute proprietary information.
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The next step involved gluing a hollow glass cylinder on top of the die. This created a well with an internal volume of approximately 200 μl. Last, the wire of an Ag/AgCl sintered pellet electrode was soldered to the card. Images of the fully assembled device are shown in Figure 82 and Figure 83.

![Fully assembled and connected ISFET card. This image shows an early prototype where a transparent die encapsulant was used.](image1)

![Top view of the ISFET sensor area after completion of assembly. The dark area at the border of the image is the encapsulant.](image2)
Biasing of the ISFET array was achieved using two voltage sources and a multiplexer (Keithley 3700). In contrast to the oocyte-based system, reliable operation of the source-drain follower was not possible for the ISFET array. Thus, I opted for readout of the drain current at constant gate and source-drain voltage as the output variable of the system (Figure 84). Control of the device was achieved using an adapted version of the control system used in the ATOVC. The software interface was modified (see Section 2.2.3) to allow programming of the bias voltages, sending trigger commands to the multiplexer and readout of the drain current.

Sensor characterization. To determine the proper function of the ISFET devices, I-V sweeps at varying gate bias voltages were conducted. A phosphate buffer solution at pH7.01 was used for these measurements. Figure 85 shows the I-V traces obtained for one ISFET sensor at gate bias voltages of -1 V, 0 V and +1 V. In general, it can be seen that modulation of the gate bias voltage changes the conductivity between source and drain. The curves, however, do not show symmetric behavior. At drain voltages below zero, the voltage-dependence and thus conductivity is decreased compared to drain voltages above zero. On the other hand, changes in drain current are higher at voltages below zero. Furthermore, the dependence of the drain current on the gate bias voltage at constant drain voltage is not linear. This behavior can be explained by the fact that the drain current also depends on the threshold voltage (see Section 1.4.2 for theoretical background). In contrast to the source-drain follower arrangement, the threshold voltage itself depends not only on the surface potential at the insulator/liquid interface, but also on the change in junction potential at the n-p transition as a consequence of changes in channel conductance.

To test the leak resistance of the gate insulator, the current flowing from gate to source was measured while performing a voltage sweep. Drain-source was biased at 0 and -0.5 V (Figure 86). The results show that the gate current decreased with decreasing voltage, reaching zero current below -2.5 V. At this voltage, the gate resistance was approximately 150 MΩ for a
drain voltage of 0V, which is a reasonable value for an intact insulator. Therefore, the Si$_3$N$_4$ layer appears to provide sufficient protection from ion leak.

Figure 85: Representative I-V traces of an ISFET sensor with varying gate bias voltages.

Figure 86: I-V plot of gate-source current. Drain-source bias voltages of 0 V (black squares) and -0.5 V (red circles) were applied during tests. The disturbance between -1.5 V and 0 V is likely to have been caused by electromagnetic interference.
To test the sensor performance and sensitivity to pH, standard phosphate buffer solutions at pH values of 4.01, 7.00 and 9.21 were used. The bias voltages of the gate and the drain were set to -2.5 V and -0.5 V, respectively. Figure 87 shows the drain current as a function of time during an experiment with repeated solution exchange. Even though the plot shows a clear dependence of the drain current on pH, severe drift of the signal can be seen. The drift is strongest at pH 4.0 and weakest at pH 7.0. At present the reason for the drift is not entirely clear as there are a number of factors that could be responsible for it, such as limited buffering capacity of the Si₃N₄ [17]. However, the signal appears to be fairly stable at pH 7.0, which is very close to physiological pH values.

![Figure 87: Drain current as a function of time. Different pH buffers were exchanged to test the pH sensitivity of the sensor.](image)

**Experiments on C2C12 cells.** C2C12 is a myoblast cell line from mouse. The cells, capable of differentiation, were originally obtained from thigh muscle [137]. C2C12 cells provide good capabilities for expression of proteins and are useful for studying physiological pathways [138]. The main objective of this study was to evaluate the viability of an adherent cell line on the microchip. Of particular interest was the determination of the cells’ preference between the gate area and the rest of the chip surface. The general protocol for culturing C2C12 cells on the microchip was as follows:

1. Cleaning of chip in 2% sodium dodecyl sulfate (SDS) solution in an ultrasonic bath.
2. Chip sterilization in 70% ethanol.
3. Drying of chip under the clean bench hood.
4. Coating of the chip surface with 50 μl of 100 μg/ml fibronectin solution. Incubation at 37 °C and 7% CO₂ for 2 hours.
(5) Washing of the chip (three times) with phosphate buffered saline solution (PBS).

(6) Trypsination of C2C12 cells in the culture flask. Resuspension into fresh standard growth medium (Dulbecco’s modified eagle medium (DMEM) with high glucose + 20 % fetal bovine serum).

(7) Filling of chip with cell suspension containing 6250 cells. Filling up with medium for a total volume of 200 μl.

(8) Incubation at 37 °C and 7 % CO₂ for 24 hours.

(9) For experiments outside the incubator, replacement of growth medium with CO₂-independent medium + 2 mM L-glutamine.

As shown in Figure 88, the C2C12 cells cultured on the microchip appeared to be fully viable, despite their high confluency (80-90 %). Close-up images of the sensor array area revealed that the cells did not avoid the gate areas. The drain current of six selected ISFETs showed good stability of the signal whereas addition of 5 mM ethylene glycol tetraacetic (EGTA) had little effect on the drain current (Figure 89). EGTA is a chelating agent with strong preferential affinity to calcium and was used in this preliminary experiment to evaluate the detectability of changes in extracellular calcium concentration [139].

Figure 88: Adherent C2C12 cells on chip at 80-90 % confluency. (a) Shows the entire exposed area of the die. (b) Zoomed photograph showing twenty ISFET sensors. The C2C12 cells did not appear to discriminate between the sensor area and the rest of the substrate.
Chapter 3: An integrated field-effect microdevice for monitoring membrane transport in living cells via lateral proton diffusion

Figure 89: Time-dependent drain current in a microchip with cultured C2C12 cells (100 % confluency). The drain current for six individual ISFET sensors is shown. 5 mM EGTA was added after 3 minutes and removed approximately 4 minutes later. The offset between the signals stems from differences in gate area geometry and manufacture-related differences in the materials.

**Experiments on MDCK cells.** Madine-Darby canine kidney (MDCK) cells are epithelial cells originating from the dog kidney [140]. They are primarily used for studying epithelial cell functions. The cell line was chosen to evaluate the applicability of ISFET sensor technology to tight cell layers. MDCK cells are polarized, having an apical pole and a basolateral pole, and form a polarized monolayer at 100 % confluency. The individual cells are connected to each other via tight junctions that creates a separation of the medium into a large domain electrically connected to the reference electrode (apical domain) and an interfacial layer between the basolateral side and the sensing surface. The ISFETs are thus expected to primarily detect changes in basolateral membrane transport. A primary concern was a possible electrical separation of the reference electrode from the charge separation layer at the sensor. Therefore, this study’s main objectives not only included establishment of cell viability on chip, but also evaluation of the sensor signal for fully connected MDCK layers. The general protocol for culturing MDCK on the microchip was as follows:

1. Cleaning of chip in 2 % SDS solution in an ultrasonic bath.
2. Chip sterilization in 70 % ethanol.
3. Drying of chip under the clean bench hood.
4. Coating of the chip surface with 20 μl of 1 mg/ml fibronectin solution. Incubation at 37 °C and 7 % CO2 for 30 minutes.
5. Washing of the chip (three times) with PBS. Filling with standard medium and subsequent incubation for 1 hour.
(6) Trypsination of MDCK cells in the culture flask. Resuspension into fresh standard growth medium (DMEM, high glucose, +2 mM L-glutamine, +1 % non-essential amino acid solution, +10 % fetal bovine serum).

(7) Filling of chip with cell suspension containing 50000 cells. Filling up with medium for a total volume of 200 μl.

(8) Incubation at 37 °C and 7 % CO₂ for 3 days.

Viability of the cell layer was confirmed by optical microscopy (Figure 90). The sensor signal of six selected ISFETs remained intact despite the presence of the cell layer (Figure 91). A drift of approximately 30 nA/min was observed during the first 10 minutes after a solution exchange procedure where the standard growth medium was renewed. Otherwise, the signal appeared to be devoid of any disturbances other than the contribution from thermal noise.

Figure 90: Fully confluent layer of MDCK cells on chip. (a) Shows the entire exposed area of the die. (b) Zoomed photograph showing six ISFET sensors completely covered by the cell layer.

Figure 91: Time-dependent drain current of six ISFET sensors covered with an MDCK cell layer. The measurement was started directly after performing a solution exchange with fresh standard growth medium.
3.4. Conclusions

An integrated microdevice based on ISFET technology for monitoring proton-dependent membrane transport was presented. Based on the ability of protons to migrate along the cell surface without diffusing back into the bulk solution, the method can sense surface pH free from detection artifacts stemming from other solutes. To verify the hypothesis, experiments on *Xenopus laevis* oocytes heterologously expressing various membrane transport proteins were conducted. Only transport systems that resulted directly (e.g. PAT1) and indirectly (e.g. NaPi-IIb,c and PiT-2) in a change in local pH gave significant responses. The results show that surface pH can be monitored with high precision and reliability. Moreover, control experiments demonstrate that solutes involved in the transport cycle do not diffuse along the cell membrane sufficiently to be detected by the sensor. Furthermore, studies correlating the change in surface pH with the population density of the amino acid cotransporter PAT1 have been done, revealing the feasibility of quantitative experiments, such as dose-response screenings. Optimization of the coupling between the transporter surface and the detection site will then lead to devices suitable for large-scale integration with potential for efficient and cost-effective high-throughput screenings. Furthermore, application to other cell types may be feasible by further miniaturization and modification of the cell immobilization site.

First studies on a multi-channel ISFET microdevice demonstrated the compatibility between ISFET-sensing technology and mammalian cell lines. Experiments on C2C12 cells showed that it is possible to culture these cells on chip without a noticeable impact on their viability, as verified by optical microscopy images. Furthermore, the cells did not seem to have a preference for attaching to the inactive area (passivation layer) over the active area (gate area) of the ISFET die. This allows for detection of activity inside the microenvironment of C2C12 cells. Experiments on MDCK cells cultured on chip showed that it is possible to form a viable layer covering the entire surface of the ISFET die. In addition, the experiments verified that a stable ISFET signal could still be obtained, despite separation of the reference electrode from the insulator surface of the ISFET by the tight cell layer.
4. Summary and outlook

In this thesis, two novel microsystems for non-invasive monitoring of membrane transport in living cells are presented. Combining the benefits of microtechnology, mechanical engineering and automation technology, these micro total analysis systems (μTAS) enabled reliable analysis on Xenopus laevis oocytes with a minimum of operator intervention. In addition to the development of the μTAS, peripheral systems such as a pressure-driven perfusion system and custom-engineered electronics were developed specifically for the microdevices. This allowed the potential of the μTAS to be exploited and demonstrated the possibility for combining compactness, automation and high performance into a single analytical system.

The first method was based on a microchip-based voltage clamp system recently described by Dahan et. al., which enabled non-invasive current-voltage (I-V) characterization of the cell membrane [31]. In contrast to the traditional two electrode voltage clamp (TEVC), the new method did not require the insertion of electrodes into the cytosol. Instead, the cell was immobilized in a compartment resembling an Ussing chamber using an air pressure overhead with externally placed electrodes. A small orifice at the bottom of the compartment resulted in separation of the membrane into a small fraction (patch membrane) and a large fraction (body membrane). The highly asymmetric division of the membrane, and consequently impedance, was the central aspect of the new voltage clamp method. This and the fact that the system was used on oocytes were the source of inspiration for calling the new method asymmetrical transoocyte voltage clamp (ATOVC). The implementation of the ATOVC featured a microperfusion channel to which the patch membrane was exposed. By driving solutions with activating and deactivating agents through the microchannel, electrogenic transport processes across the patch membrane could be modulated, which were detected as a change in total transoocyte current.

The ATOVC’s function was verified via a number of experiments involving various membrane transport proteins whose transport mode had already been characterized on the TEVC. The first protein was the epithelial sodium channel ENaC, which was heterologously expressed in the cell membrane. I-V curves as well as time-dependent current traces were obtained with the ATOVC, exhibiting comparability with the TEVC and high signal fidelity. Measurements on the sodium/phosphate cotransporter NaPi-IIb proved to be more challenging, but successful recordings of phosphate-sensitive currents were finally obtained. The last series of experiments involved the study of calcium-dependent currents in native oocytes whose endogenous calcium-channels had been activated using thapsigargin. Exposure with the calcium channel-specific blocker 2-aminoethocydiphenyl borate (2-APB) resulted in inhibition of the transoocyte steady-state current at highly positive membrane potentials.

To further demonstrate the capabilities of the ATOVC system, experiments on a specially modified aircraft performing parabolic flight maneuvers were conducted. Successful measurements of sodium (ENaC) and 2-APB-sensitive (thapsigargin treated oocytes) currents during the zero gravity phase lasting only 20 seconds were made. These measurements
demonstrated the system’s usability in an extreme environment, the benefits of rapid solution exchange in a microfluidic system and the capability of fully automated operation.

So far, the focus of the system has been on proof-of-concept establishment and migration of the technology to a platform capable of reliably performing electrophysiological experiments under zero and hypergravity. Future special gravity experiments could focus on the physiological aspects of membrane transport, especially in space muscle atrophy [141]. Even though preliminary experiments on calcium-dependent transport have already been performed as part of this thesis, heterologous expression of proteins relevant to muscle physiology, such as the family of transient receptor potential cation channels (TRPC), could provide a promising basis for further exploitation [142]. Also on ground, the ATOVC could prove significantly beneficial as the simplicity of the preparation is an important step towards higher sample throughput. However, due to the nature of the voltage clamp, fully multiplexed operation of the ATOVC is not possible which reduces its potential for parallelization. In order to achieve true multiplexing capability, a different sensing method is required.

To address the aforementioned issue, another μTAS relying on a completely different detection method for monitoring membrane transport was developed. Employing an ion-sensitive field-effect transistor (ISFET), the method was able to detect changes in proton concentration in close proximity to the cell membrane. The proton kinetics at the cell membrane had been shown to be different from those in bulk solution. In particular, the existence of proton migration along the cell membrane had been shown both in theory and experiment [117, 118]. The distinction between membrane surface pH and bulk solution pH, as well as facilitated lateral diffusion of protons enabled monitoring of proton-dependent membrane transport. This was realized in a μTAS, combining an ISFET sensor, an integrated microperfusion channel and cell membrane immobilization on the sensor.

To validate the new method, a series of experiments on *Xenopus laevis* oocytes expressing various membrane transport proteins was conducted. By prediction of the expected result, the model for lateral diffusion of protons and decentralized detection of membrane transport events was verified. Experimentally, this was realized via chemical activation and deactivation of transport proteins with known proton-dependence. These included the amino acid transporter PAT1, the electrogenic sodium-coupled phosphate transporters NaPi-IIb and PiT-2, and the electroneutral sodium/phosphate cotransporter NaPi-IIc. The detection of a response in NaPi-IIc was a significant success, since its measurement is not possible with conductance-based electrophysiological methods, such as the TEVC. To further exploit the capabilities of the new detection method, studies were done to correlate the output signal of the ISFET-based system with the expression level of the membrane proteins via comparison with the TEVC measurement.

The full potential of ISFET-based membrane transport monitoring has not yet been exploited. Optimization of the sensor/geometry arrangement will probably improve the correlation between chemical activity of the substrate and the resulting sensor signal. This would enable dose response experiments that could be particularly useful with regard to determination of pharmacodynamic parameters in living cell assays. A number of novel assays could also be realized on the ISFET-based μTAS, as it does not rely on the electrogenicity of a
transport process. Important examples include the serotonin transporter, a target for antidepressant drugs, and the Na⁺/H⁺-exchanger, a target for treatment of inflammatory bowel disease [143, 144].

This thesis also described a μTAS extending the ISFET technology to measure membrane transport in mammalian cell lines. The differences between this μTAS and the one used for *Xenopus laevis* oocytes were mainly based on the sensor configuration. Due to the much smaller size of the sensors (10 μm versus hundreds of μm) and their arrangement as an array on a single μTAS, parallelized analysis on mammalian cells at single-cell level was targeted. By multiplexing between the individual ISFET sensors, quasi-parallel readout of the output signal was achieved with high reliability. On-chip-viability of C2C12 and MDCK cell lines was achieved, laying the foundation for physiologically relevant studies. An interesting application of the system could be the study of basolateral transport in epithelial cell layers. With traditional electrophysiological techniques it has so far only been possible to study apical and transepithelial transport [145]. The ISFET-based system could therefore bring valuable insights into the epithelial transport processes that serve many important physiological functions such as absorption, secretion and sensor signal transduction.
Figure 92: Fluid power schematic of the pressure-driven perfusion system.
Figure 93: Schematic of the power electronics implemented in the latest ATOVC system.
Figure 94: Complete voltage clamp circuit as implemented in the latest ATOVC system. The six individual stages are numbered and marked by the red square boxes: 1. Sense voltage preamplifier; 2. Adjustable phase shifter; 3. Differential feedback amplifier; 4. Feedback control time constant; 5. Current-to-voltage converter; 6. Inverter with offset compensation. The power supply (not shown) is a linear type with bipolar output voltages of +/-15 V. The power supply lines of all integrated circuits (IC) are bypassed using 100 nF capacitors close to the package to ensure stable operation.
Figure 95: Prototype of the latest ATOVC circuit, integrated into a die-cast aluminum case.

Figure 96: Three full ATOVC setups as used during the 55th ESA parabolic flight campaign. The electronic hardware was manufactured by Spacetek GmbH (Switzerland) according to the latest development design.
Appendix

Figure 97: Electronic schematic of the ISFET driving and readout circuit. 1. Voltage-controlled current source for setting the source-drain voltage; 2. Voltage-controlled current sink for setting the drain current; 3. Low-pass filter; 4. Band-gap voltage reference for generating the control voltages.
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