SCUOLA DI DOTTORATO DI RICERCA IN INGEGNERIA DELL'INFORMAZIONE
INDIRIZZO DI BIOINGEGNERIA
XXV CICLO

# LAB on CHIP: capacitive stimulation of cells

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# Abstract

The main target of the LAB on CHIP phD project, funded by Fondazione Cariparo, was to develop a device to allow the handy production of CHO cell clones in order to built recombinant proteins for therapeutic targets. In particular, a major aim is to reduce time and costs associated with clones manufacturing.
Cultivated mammalian cells have become the dominant system for the production of recombinant proteins for clinical applications, because of their capacity for proper protein folding, assembly and post-translational modification. The quality and efficacy of a protein can be superior when expressed in mammalian cells versus other hosts such as bacteria, plants and yeast. Today more than 60 % of all recombinant protein pharmaceuticals are produced in mammalian cells. Expression vectors for recombinant cell line generation generally use a strong viral or cellular promoter/enhancer to drive the expression of the recombinant gene. But non-viral gene transfer remains the preferred approach to generate stable cell lines for manufacturing purposes: calcium phosphate transfection, electroporation, lipofection, [1].
Transfection is a complex process, and in order to be successful all steps involved must work efficiently. The device developed is based on the physical phenomenon called electroporation, that is the formation of temporary pores in the plasmatic membrane upon application of electric fields.
As the biological world is intrinsically variable, common approaches for improving electrotransfection rely on time consuming empirical attempts. It is therefore important to develop new methods enabling a fine control of all critical parameters involved to identify causes of failure and to improve efficiency. On-chip electroporation through capacitive currents can be such a method. To directly assess the formation of pores in the cell membrane, we performed patch-clamp experiments during on-chip electroporation. Thus, most promising protocols were selected and assessed for their electrotransfection efficiency. Moreover, patch clamp experiments allowed to study the dynamics of pores formation and resealing.
Regarding the develop of the device, the biocompatibility of titanium dioxide, selected as dielectric material, was tested. The inertness against cellular environment and the state of cell culture were considered. Cell cultures showed healthy state and normal development, good adhesion and normal replication time. No chemical reactions that can damage the culture were observed. The chemical inertness was considered in the reverse direction too. Metabolic products of cell culture did not lead to chemical corrosion of the dielectric surface. So, the patch-clamp on-chip electroporation recordings allowed to select the promising protocol that was tested on CHO cultures.
The prototype proposed demonstrated electrotransfection through capacitive coupling between cell and chip. The electroporation efficiency obtained is around 30%. Moreover, the selectivity of the device was demonstrated, and its applicability both in electrotransfection

and electroporation for staining application. Collateral results were obtained concerning the formation of pore on attached and free membrane and the possibility of study pore dynamics.

# Abstract

Scopo principale del progetto di Dottorato "LAB on CHIP" finanziato dalla Fondazione Cariparo è stato lo sviluppo di un dispositivo che agevoli la creazione di cloni di cellule CHO per la produzione di proteine a scopo terapeutico. In particolare il fine ultimo è quello di ridurne tempi e costi associati alla produzione. Le cellule di mammifero in coltura sono ormai il sistema pi diffuso per la produzione di proteine per applicazioni cliniche. La qualità e l'efficacia di una proteina possono essere superiore se essa è espressa in cellule di mammifero rispetto ad altri organismi, quali batteri, piante e lieviti. Ad oggi più del 60 % di tutte le proteine ricombinanti per applicazioni farmaceutiche è prodotto in cellule di mammifero. Vettori di espressione per la creazione di linee cellulari stabili da DNA ricombinante utilizzano vettori virali per indurre l'espressione del gene. Ma la transfezione senza l'ausilio di virus rimane l' approccio prediletto per la generazione di linee stabili per questi scopi.
La transfezione è un processo complesso e, affinchè avvenga con successo, tutti i sottoprocessi conivolti devono svolgersi efficientemente. Il dispositivo proposto si basa sul fenomeno fisico chiamato elettroporazione, che non è altro che la formazione di pori temporanei nella membrana plasmatica a seguito dell'applicazione di opportuni campi elettrici. I comuni approcci utilizzati per migliorare la transfezione tramite elettroporazione richiedono tempi lunghi e possono essere inefficaci.È importante poter sviluppare metodi nuovi che permettano un controllo di tutti i parametri critici coinvolti in modo da poterne identificare le cause in caso di fallimento e dunque migliorare l'efficienza. L'elettroporazione su chip utilizzzando correnti capacitive può essere un valido approccio. Per poter rilevare la formazione di pori, sono stati fatti esperimenti di patch-clamp su chip durante l'elettroporazione. In tal modo sono stati selezionati i protocolli più promettenti. Per quanto riguarda lo sviluppo del dispositivo, ne è stata verificata la biocompatibilit. Si valutato lo stato delle colture cellulari che hanno mostrato normali sviluppo, adesione e tempo di replicazione. Non sono state rilevate reazioni chimiche tra il mezzo di coltura e il diossido di titanio. Non si sono inoltre rilevati problemi di corrosione o danneggiamento dell'ossido a causa di prodotti metabolici della cellula.
Gli esperimenti di patch-clamp hanno permesso di selezionare un protocollo che stato poi testato sulle cellule in coltura. Il prototipo sviluppato ha dimostrato l'elettroporazione di cellule CHO in coltura, ottenendo un'efficienza media del 30 %. È stata inoltre dimostrata la selettività di tale dispositivo e la sua applicabilità sia per la transfezione che per l'introduzione nella cellula di marcatori. Risultati "collaterali" ottenuti riguardano la dimostrazione della formazione di pori temporanei sia sulla membrana adesa che su quella libera e la possibilit di studiare la dinamica dei pori.

# Contents

## Thesis structure

The project covered interdisciplinary topics, that go from cell biology for cell culture to physics for the device, to electrophysiology for the patch experiments, from basic molecular biology for transfection experiments to programming language for the data analysis. The experimental problems met during the work are numerous, some of them fundamental and tricky even if they can seem banal, as the proper chip prototype building that allowed the performing of a patch experiment at the same time of an electroporation one.
The thesis is organized as follow: after an Introduction in bioelectricity concerning cell membranes on chapter 1, Materials and Methods I deals with cell culture technique on CHO cells in Chapter 2. Then an overview on transfection most common techniques and lipofection experiments performed, constitute Chapter 3. Chapter 4 presents the basic concepts on electroporation. Chapter 5, in the first experimental part, deals with the device: its biocompatibility, the prototype production, the cell culture on it and the starting experiments for the introduction of nonpermeant dyes into cells. The most of the second part of Materials and Methods is reserved to the patch-clamp technique in Chapter 6. Then next chapter deal a model of cell in adhesion on a substrate in Chapter 7 and finally in Chapter 8 on the transfection performed in the experiments. The experimental data are collected in Experimental II and in the Results in chapter 11, 12 and 13. Chapter 14 on Conclusion and Outlooks ends the work.
All work described in this thesis was carried out by myself except only for the microelectronic process in the chip prototype.

# Introduction

# Chapter 1

# Bioelectricity

## 1.1 Electrical Potentials and Currents

A cell derives its electrical properties mostly from the electrical properties of its membrane. This membrane is called plasmatic membrane and consists physically of a double layer of molecular strands which are a chemical composition of phosphates and lipids. An electrical potential difference exists between the interior and exterior of cells. The potential difference across the plasmatic membrane is called transmembrane potential and is defined as the potential at the inner side of the membrane relative to the potential at the outer side of the membrane. It is generated by the "pump" proteins that harness chemical energy to move ions across the cell membrane. This separation of charges creates the potential difference. Because the lipid membrane is a good insulator, the transmembrane potential is maintained in the absence of open pores or channels that can conduct ions. The transmembrane potential at a steady-state condition with no net flow of electrical current across the membrane is called resting membrane potential.

Typical transmembrane potentials amount to less than 0.1 V, usually 30 to 90 mV in most animal cells, but can be as much as 200 mV in plant cells. Because the salt-rich solutions of the cytoplasm and extracellular milieu are fairly good conductors, there are usually very small differences at steady state (rarely more than a few millivolts) between any two points within a cell's cytoplasm or within the extracellular solution, [2].

Electrophysiological equipment enables to measure potential differences and currents in biological systems. Usually, currents measured by electrophysiological equipment range from picoamperes to microamperes. For instance, typically, $10^4$ $Na^+$ ions cross the membrane each millisecond that a single $Na^+$ channel is open. This current equals 1.6 pA (1.6 x $10^{-19}$ C/ion x $10^4$ ions/ms x $10^3$ ms/s).

In electrophysiology, it is convenient to discuss currents in terms of conductance instead of resistance, because parallel conductances simply sum. When several ion channels, or pores as in our topic, are open in a membrane simultaneously, the total conductance is simply the sum of the conductances of the individual open channels or pores.

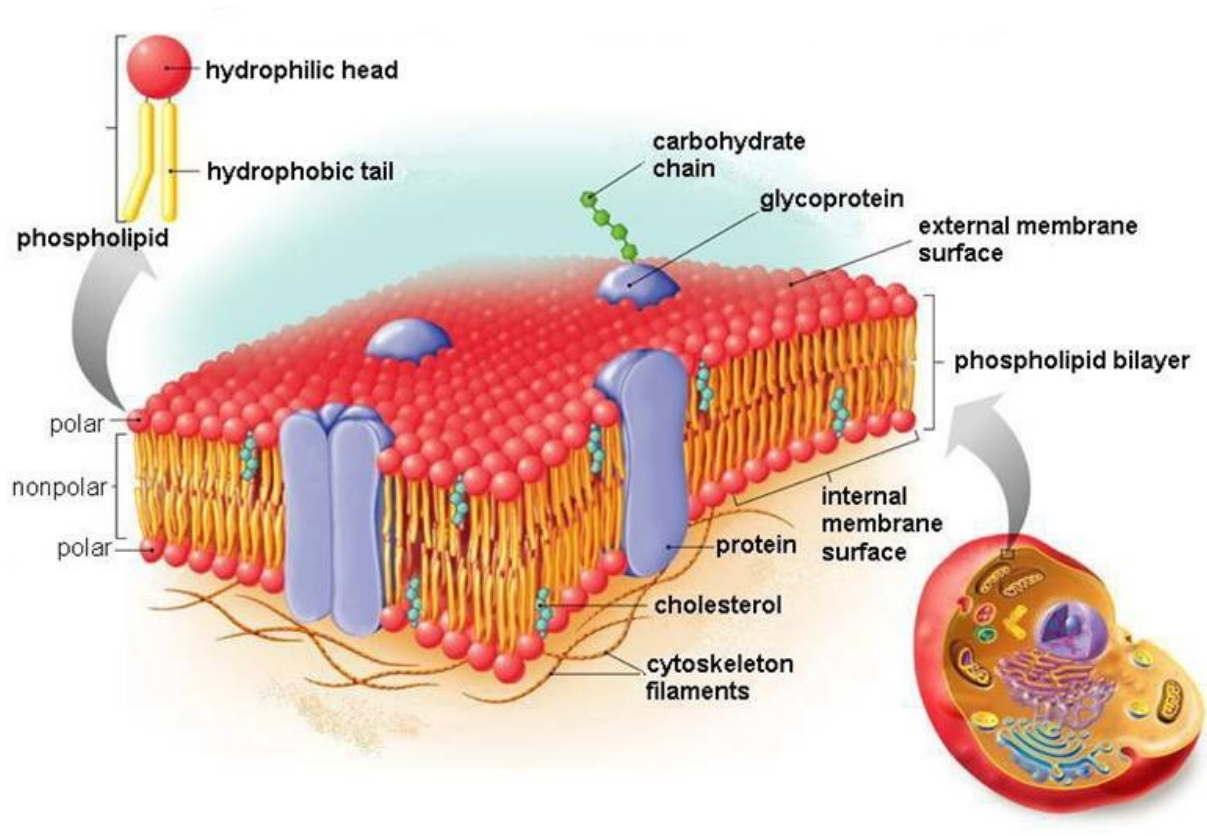


Figure 1.1: The cell is the basic unit of all living organisms. All cells, whether prokaryotic or eukaryotic, have a membrane that envelops the cell, separates its interior from its environment, regulates what moves in and out (selectively permeable), and maintains the electric potential of the cell. Inside the membrane, a salty cytoplasm takes up most of the cell volume. All cells possess DNA, the hereditary material of genes, and RNA, containing the information necessary to build various proteins such as enzymes, the cell's primary machinery.

## 1.2 Capacitors and Electric Fields

The concept of the electrical field is important for understanding membrane function. Biological membranes are typically less than 10 nm thick. Consequently, a transmembrane resting potential of about 100 mV produces a very sizable electrical field in the membrane of about $10^5$ V/cm. This is close to the value at which most insulators break down irreversibly because their atoms become ionized. Of course, typical electrophysiological equipment cannot measure these fields directly. However, changes in these fields are presumably sensed by the gating domains of voltage-sensitive ion channels, which determine the opening and closing of channels, and so the electrical fields underlie the electrical excitability of membranes.

Another consequence of the membrane's thinness is that it makes an excellent capacitor. Capacitance, C, represents the ability to store charge, Q, when a voltage drop, $\Delta$V, occurs across the two "ends", so that: $Q = C\,\Delta V$. A planar capacitor is an excellent approximation of the lipid bilayer. As plasmatic membrane is multiple capacitors connected in parallel,

it is electronically equivalent to a single large capacitor; that is, the total capacitance is the sum of their individual capacitance values. Thus, membrane capacitance increases with cell size. Membrane capacitance is usually expressed as value per unit area; nearly all lipid bilayer membranes of cells have a capacitance of 1 $\mu F/cm^2$ (0.01 $pF/\mu mm^2$), [2].
The stored charge on the membrane capacitance sets the resting potential, and any change in the voltage across the membrane is accompanied by a change in this stored charge. Indeed, if a current is applied to the membraneit first satisfies the requirement for charging the membrane capacitance, then it changes the membrane voltage. Formally, this can be shown by representing the membrane as a resistor of value R in parallel with capacitance C, pictured in fig. 1.2. if we apply a pulse of current to the circuit, the current first

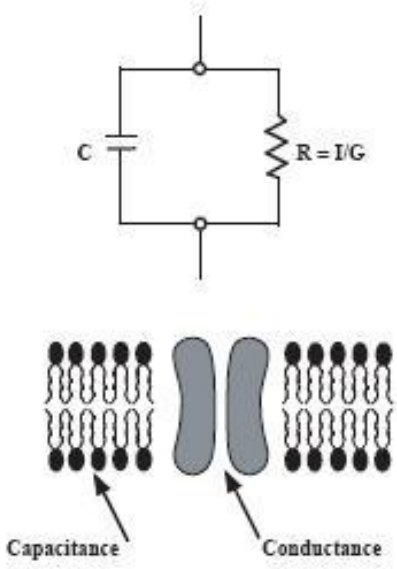


Figure 1.2: Equivalent circuit for a patch of the cell membrane

charges up the capacitance, then changes the voltage, that approaches steady state along an exponential time course:

$$V(t) = V_\infty(1 - e^{(t/\tau)})$$

The steady-state value V$\infty$ (also called the infinite-time or equilibrium value) does not depend on the capacitance; it is simply determined by the current I and the membrane resistance R: $V_\infty = IR$ and the voltage is approached with the time constant $\tau = RC$.

# Part I

# Materials and Methods I

# Chapter 2

# Cell biology

## 2.1 Cell culture of Chinese Hamster Ovary - K1

The Chinese Hamster Ovary(CHO)-K1 epithelial cell line was derived as a subclone from the parental CHO cell line, [3], initiated from a biopsy of an ovary of an adult Chinese hamster by T. T. Puck in 1957, [4]. This cell line is one of those in use in the Neurochip Lab in Padova. It was chosen for the project for its good adhesive properties on the common supports for cell biology and a good response to the common transfection methods in terms of transfection efficiency. The typical CHO heterogeneous morphology can be seen in figure 2.1.

### 2.1.1 Thawing of cells

When needed the cells stored at -80 $^o$C are thawed by a thermostated bath at 37 $^o$C. Culture medium, is added and the cells were precipitated through centrifuge in order to eliminate the cryopreservation agent, Dimethyl Sulfoxide (DMSO), toxic to cells. The culture medium is the Nutrient Mixture F-12 HAM (Sigma Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% with Fetal Bovin Serum (FBS) (Sigma Aldrich) and 0.1 % of the antibiotics penicillin and streptomycin. Then the cells are resuspended in fresh medium, seeded into flasks and kept in an incubator at 37 $^o$C and $CO_2$ concentration of 5% (v/v). The day after the thaw the medium will refreshed in order to remove any traces of DMSO and death cells that may affect the health of the culture.

### 2.1.2 Trypsinization

Trypsinization is the process of cell dissociation using trypsin, that is an enzyme. When added to a cell culture, trypsin breaks down proteins which enable the cells to adhere to the vessel they are being cultured in. The trypsinization starts removing the culture medium. Then the culture is washed with Phosphate Buffer Saline (PBS) in order to remove any traces of serum that could inhibit the trypsin. The cells are detached from the support by enzymatic action of trypsin, at 37 $^o$C for 1 minute. Then trypsin is inactivated by the addition of complete medium that contains serum. The cells thus obtained are precipitated by centrifugation and reseeded after counts in a new flask at the desired density. This process as a whole is called “passage”. The cells are maintained up to the 25th passage,

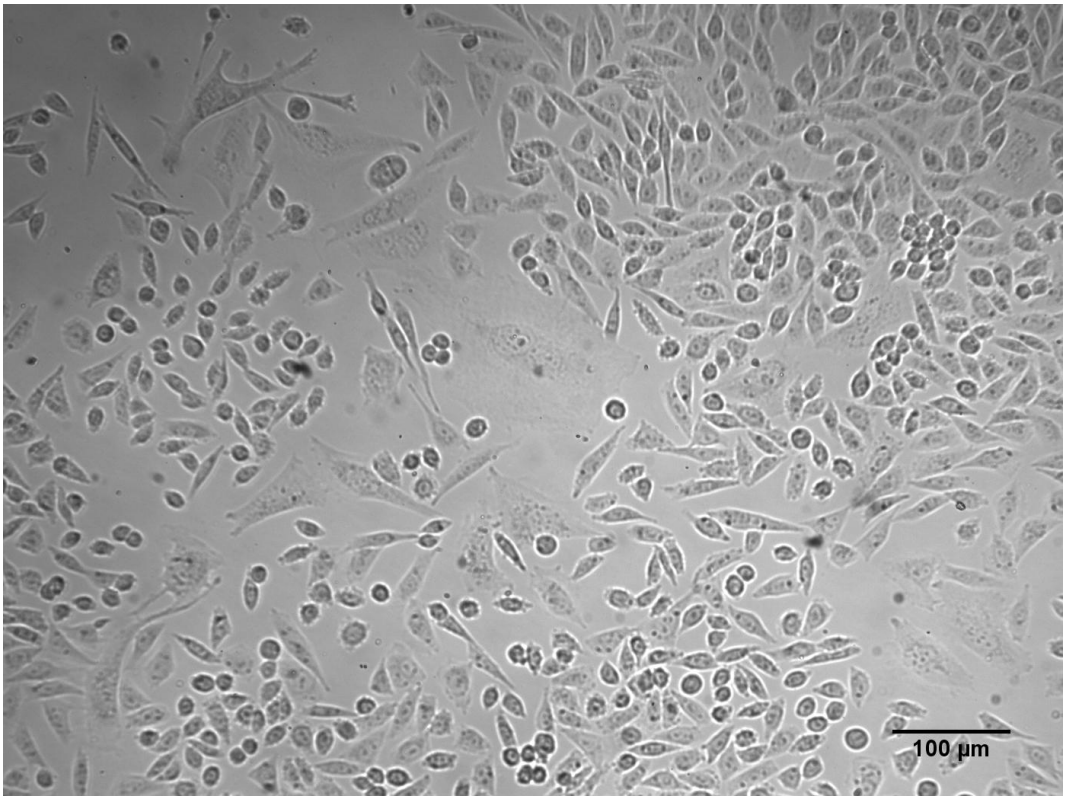


Figure 2.1: Chinese Hamster Ovary culture on a Petri dish (FALCON® 35 3001 Tissue Culture Treated by Vacuum Gas Plasma 35x10 mm polystirene Non-Pyrogenic BECTON DICKINSON). The mixed morphology is clear, from rounded cell to lengthened ones up to polygonal ones.

after which they are thrown away to avoid mutations that may arise with time and a new ones have to be thawed.
The cells are maintained in culture flasks with a surface area of 25 $cm^2$ in complete nutrient medium, in an incubator at a controlled temperature of 37 $^oC$ and $CO_2$ concentration of 5% (v/v) , and cooled to the achievement of confluence with trypsinization

### 2.1.3 Maintenance

Aliquots, containing approximately $10^6$ cells, are drawn and frozen to maintenance of the cell line. The freezing of the cells is performed only in the first steps when a crop is relatively "young" and few mutations could have grown. This allows for virtually cell clones in use always at the same level in terms of age. The freezing occurs in FBS at 5% DMSO through the use of "cryostep" which is a container that allows a gradual lowering of the temperature ($1^oC$/ minute), [5]. The cells can be stored up to one year at -80 $^oC$, while for a conservation of years it is necessary to use special tools that use liquid nitrogen to keep the cells in a state of hibernation .

# Chapter 3

# Transfection

## 3.1 Transient and stable transfection

The cell plasma membrane is a highly selective barrier for the diffusion of solutes between the intracellular and extracellular environment, in particular of ionic and polar substances. However, thanks to proper mechanisms of transport, communication is allowed, and the exchange of substances for the maintenance of homeostasis and cell metabolism. But sometimes it is important to introduce into cells particular molecules artificially, such as drugs, ions or other biological molecules of which one wants to study the influence on the cellular level, or nucleic acids used to inhibit or promote the expression of a particular gene. This process is called cell transfection. The exogenous DNA overcomes the plasmatic membrane, enters into the cytoplasm, enters into the nucleus, mRNA is transcripted, it moves out of the nucleus into the cytoplasm where finally the protein is expressed, sketched in figure 3.1. The transfection can be transient or stable. In the former the exogenous genetic material is not integrated in the DNA of the host cell. And so the cell eliminates it in the next replications. Otherwise, if and only if DNA is integrated in the chromosomal DNA of the host cell, and inserted in a "right" position in the host DNA, it will be duplicated in the subsequent cell replication and a clone is created. And the transfection is defined stable.
The dependance on the plasmid concentration is rather complex and it is observed that high levels of plasmids are toxic [6].
Transfection can be reached using different ways: biochemical, viral and physical methods briefly described in the next paragraph.

### 3.1.1 Biochemical methods

The methods take advantage of biochemical molecules that bind to DNA vectors creating aggregates that are endocytated by the cell. These substances exploit the negatively charged phosphate groups of DNA strands. These aggregates are able to overcome the barrier of the plasma membrane and thus allows the introduction of exogenous material into the cell. The method of "calcium phosphate"' exploits the reaction between the calcium chloride and the phosphate groups of DNA, with the formation of an insoluble molecule that adheres to the surface of the cells.It is then absorbed by endocytosis. This technique is widely used, because the necessary components are of low cost and easily available but the protocol,

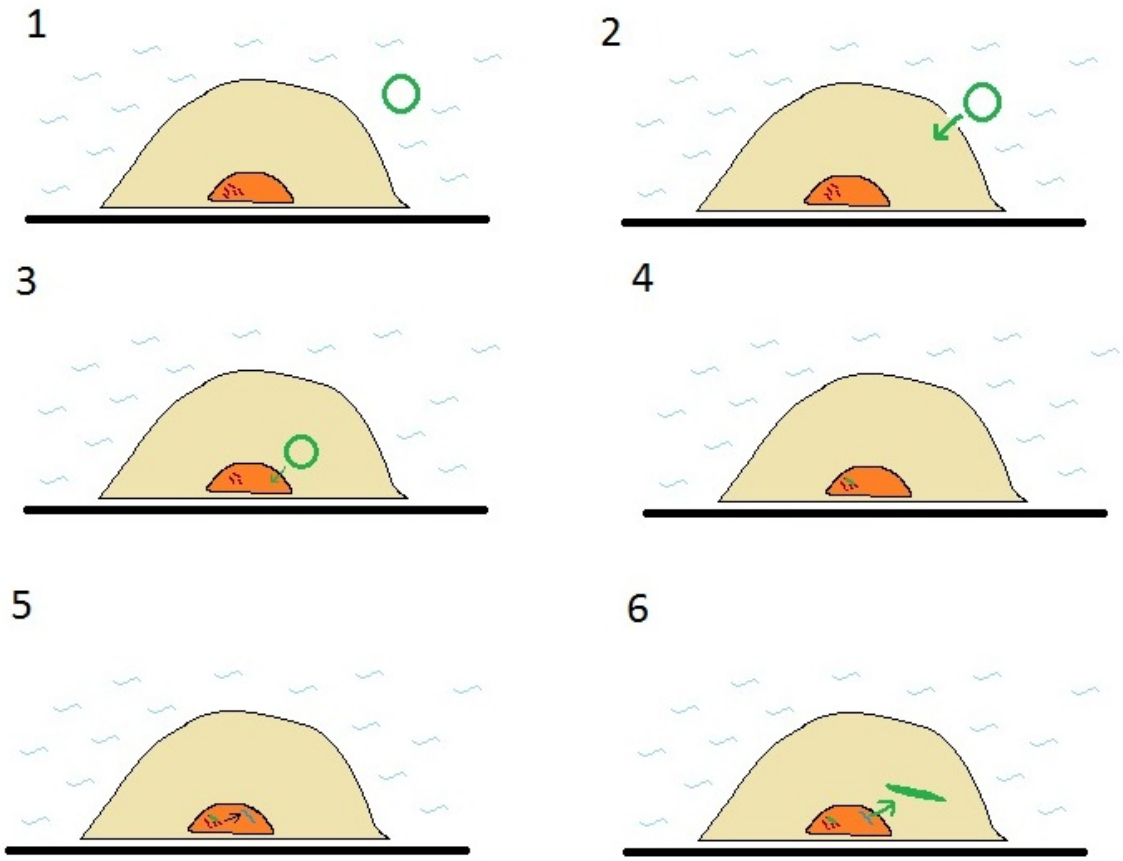


Figure 3.1: DNA transfection sequence: 1. Plasmidic DNA in solution. 2. DNA enters into the cytoplasm (through pores on the plasmatic membrane in our experiments, chapter 5). 3. From cytoplasm DNA enters into the nucleus 4. In case of stable transfection it is integrated in the DNA of the host cell. 5. In the nucleus mRNA is transcripted by DNA 6. mRNA comes out of the nucleus and enter into the cytoplasm, where the protein is produced by the cell. Picture not to scale.

though easy to use, is extremely delicate because small changes in pH can affect it making it ineffective [7].
Another protocol based on the creation of insoluble complexes and assimilable by the cell involved the DEAE-dextran. It is a cationic polymer which binds to the phosphate groups of DNA, forming compounds that are deposited on the membrane. The use of such a compound is toxic for cells, and requires careful optimization of the protocol in order to limit the time of exposure to the cells [8].
Among the methods with high transfection efficiency there is the use of liposomes. Such compounds are synthetic lipid vesicles positively charged, or neutral, which bind to the nucleic acid in solution. The liposome-DNA complex is internalized thanks to the fusion of the liposome with the plasma membrane, allowing the entry of exogenous material. The liposome-mediated transfection will be focused more in detail in section 3.2. It is advantageous for his efficiency of gene transfer and the possibility of finding different types

on the market suitable for different cell lines, [9].

### 3.1.2 Viral methods

The methods with the highest transfection efficiency use modified viruses. These methods consist in the insertion of the gene by transfecting into a virus-vector genetically modified so as not to be infectious for the operator, but able to infect the cell culture.

### 3.1.3 Physical methods

Physical methods of transfection are invasive techniques involving the direct introduction of the molecule within the cell: nucleic acids are conveyed in the cytoplasm, without going through the natural mechanisms of transport, overcoming obstacles due to electrical resistance. Some methods consist in the creation of pores through sound waves at different frequencies (Sonoporation), [10], or through the use of lasers which "pierce" the membrane due to the high energy that can reach [11]. The latter two methods are shown for cells particularly difficult to transfect although requiring expensive equipment. Finally electroporation is perhaps the most widely used physical method. This method uses short and intense electric pulses to induce the formation of pores in the membrane. It will be described in detail in chapter 5.

## 3.2 Lipofection

Lipofection (or liposome transfection) is a technique used to inject genetic material into a cell by means of liposomes, which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer. Lipofection generally uses a positively charged (cationic) lipid to form an aggregate with the negatively charged (anionic) genetic material. A net positive charge on this aggregate has been assumed to increase the effectiveness of transfection through the negatively charged phospholipid bilayer. This transfection technology performs the same tasks as other biochemical procedures utilizing polymers, DEAE dextran, calcium phosphate, and electroporation. The main advantages of lipofection are its ability to transfect all types of nucleic acids in a wide range of cell types, its ease of use, reproducibility, and low toxicity. In addition, this method is suitable for all transfection applications (transient, stable, co-transfection, reverse, sequential or multiple transfections). And last but not least its main advantage is its efficiency, surely extolled by the producers of the molecule. The transfection efficiency on the Chinese Hamster Ovary line declared by the Lipofectamine's producers should be 51-79 % for the plasmid DNA transfection and co-transfection using Lipofectamine® 3000 [12], the last one available, and Lipofectamine® 2000 ($life$technologies) available in our laboratory, widely diffuse and cheaper than the previous. Just to have an idea on the costs, Lipofectamine® 2000 Transfection Reagent 0.75 ml costs about 430 euro and the same amount of Lipofectamine® 3000 about 470 euro [12]. To transfects a cell culture on a substrate of a petri dish of $\simeq$ 9.6 $cm^2$ you need 2 $\mu$l of Lipofectamine® 2000/ 1 $\mu$g of DNA , that means 4 $\mu$l as 2 $\mu$g of DNA has to be used.

But the cost is not the only aspect that could be improved using the electroporation technology on adherent cells, as we will underline in the following. Actually lipofection can not be selective, the cells are randomly transfected so the method is not space resolved. As consequence it is not time-resolved too.

## 3.3 Lipofection protocol and experimental efficiency on CHO

The lipofection protocol I follow, states to transfect the CHO cells two days after the plating. The lipofection process is a stress for the cell culture and in order to avoid the cell detachment in order to preserve the culture, we need good cell adhesion. The solution of plasmidic DNA and lipofectamine is prepared in a small volume of medium, and left 30 minute to incubate. Then it is distributed with drips on the culture, put in the incubator three hours and then the medium is changed by a fresh one. The cells takes at least 6-8 hours for the protein expression so usually the results are checked the day after. The microscope is Olympus (BX51WI) equipped with a video-camera Leica CCD DFC350FX 12 bit gray scale. For the visualization of the fluorescence signal produced by the different molecules (ECFP,EYFP,Alexa 568 and autofluorescence of Trypan Blue) is equipped with dichroic filters (Chroma). The software used for the image analysis is ImageJ.

When lipofection is performed the number of cell in the culture is less than the one of the day after, when the efficiency is estimated. In order to avoid an undervaluation of the lipofectamine efficiency, we consider the total population half than the one of the checking day, that is an undervaluation. The efficiency is a mean of the efficiency determined in different fields of the culture, simply by the radio # transfected cells/ # total cells: we have 9 % $\pm$ 2%, that is not undervalued for sure. And it is far away from the 50% expected by the manufacturer.

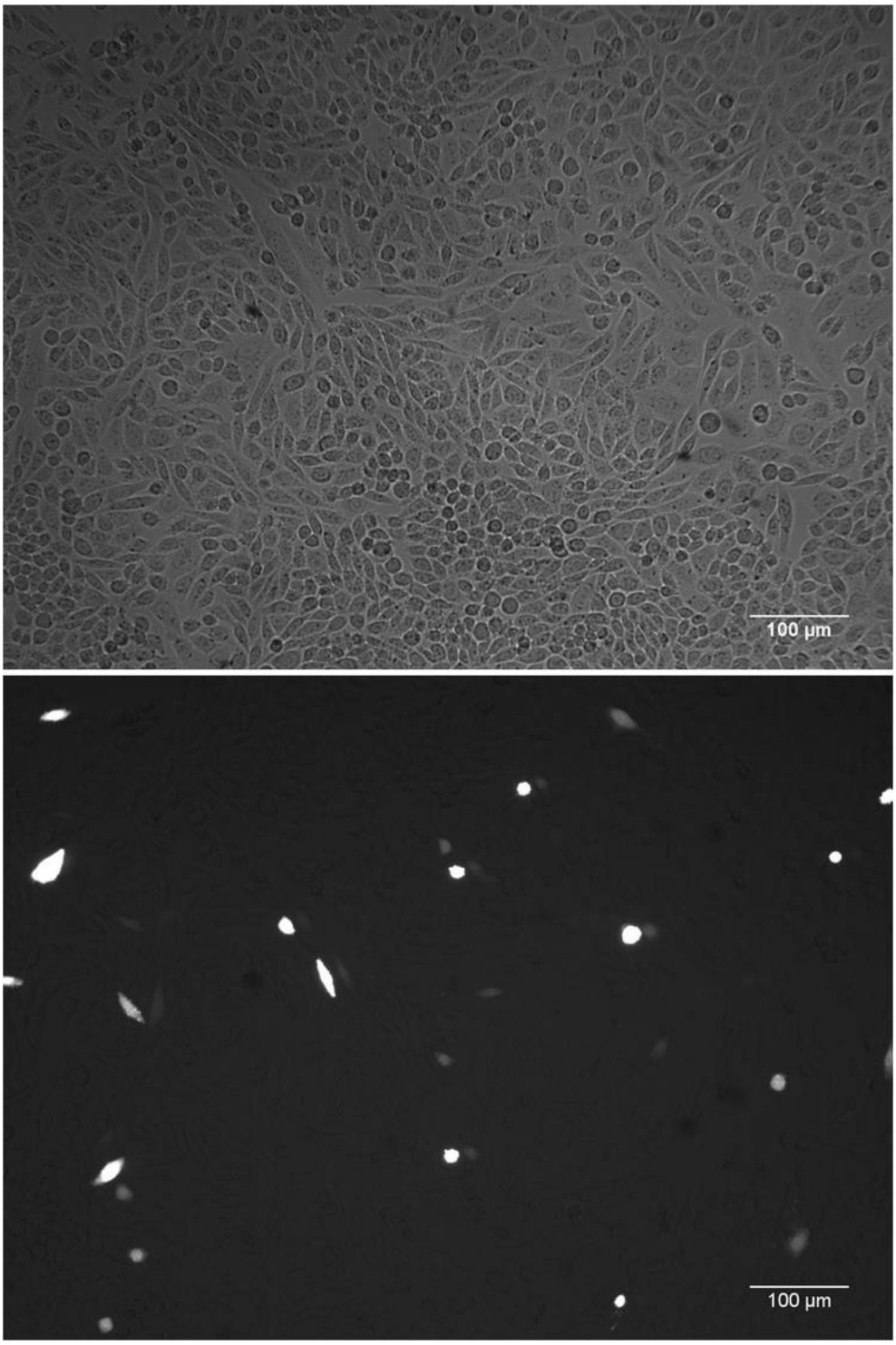


Figure 3.2: On top, CHO culture plated at the density of $157000/ml$ in order to arrive at confluence three days after the seeding. On bottom, UV light image of the same field of the culture in figure on the top. Transfected cells are easily visible as they are fluorescent, different intensity of fluorescence are present because any cell express the protein with different quantities.

# Chapter 4

# Electroporation

## 4.1 Introduction

The permeability of a cell membrane can be transiently increased when an external electric field pulse is applied. Under suitable conditions depending mainly on the pulse parameters, field strength, pulse duration and number of pulses, the viability of the cell can be preserved. This process is called electroporation or electropermeabilization and it is therefore an elegant way to gain access to the cytoplasm and to introduce chosen foreign molecules. Electropermeabilization allows the free diffusion of small molecules and ions whatever their chemical nature such as ions, drugs, markers, dyes, antibodies. Polar compounds, as DNA is, can cross the membrane though the pores too [13]. There is a general agreement that very little is known about what is really occurring in the cell and its membranes at the molecular level.

Many effects are induced by an electric field when applied on a cell. When a voltage is applied between two electrodes an electric field is obtained. Scalar as vectorial effects result from this perturbation of the interelectrode space:

- Electromechanical effects. A cell is submitted to an electrophoretic drag and can move during the pulse. If we consider that charged proteins that belong to the plasmatic membrane can move in the lipid matrix, a new topology of the cell surface results. This process may be very important when the pulsed cells cannot move under the field effect. This is indeed the case in plated cells growing on dishes or substrates.

- Membrane potential difference modulation. This is the most important effect. It is due to the dielectric character of the membrane.

- Joule effect. As an electrical current is flowing, Joule heating is taking place. The temperature of the sample is going to increase. We minimize this problem using an EOS system, as the one will showed, that avoids the use of conventional metal electrodes for the stimulation of cells on adhesion.

Literature underlines that the cell metabolism plays indeed a critical role in the recovery. It should be taken into account the fact that the transient permeability can be influenced by the stadium in the cell cycle, if it is at mitosis for example [6].

## 4.2 A membrane permeabilization model

When the transmembrane potential exceeds the breakdown voltage of the membrane, 200-250 mV [14], there is a rearrangement of the lipid chains, with the temporary formation of pores.We underline that this valuation and the models proposed deal with electroporation of cells in suspension and not in adhesion on a substrate as it is our case. So we will not entered in details but just give a look at the principal model. Although the real molecular mechanism by which this occurs is not jet really understood, it is generally believed that a rapid structural rearrangement of the membrane occurs, that induce the formation of aqueous pathways ("pores") that are hydrophylic, as pictured in figure 4.1.

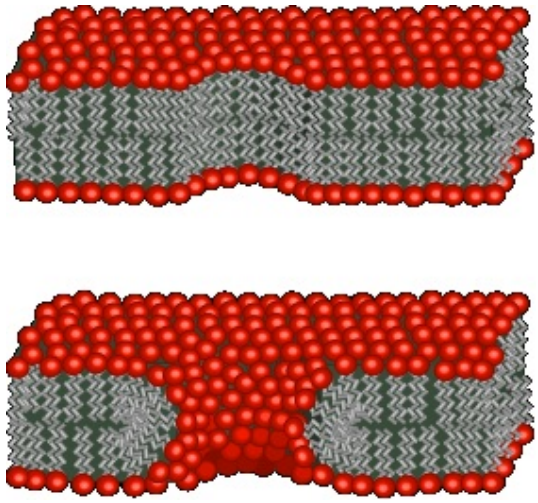

Figure 4.1: Schematic picture of two possible conformations of the lipids at the edge of a pore. In the bottom image some of the lipid heads have bent over, so the pore wall is hydrophilic and the one proposed in the Neumann model. In the top image the lipids have not rearranged, so the pore wall would be hydrophobic.

The most popular model that describe the interaction of an external electric field with a cell is the one by Neumann. Following the review of Somiari, in the Neumann model 4.2, a poration process is initiated by elongation of the cell along the axis of the field, termed Maxwell or electroporative deformation. This elongation is associated with a slight (0.01% to 0.03%) increase in membrane surface area while maintaining constant internal volume. The resulting ellipsoid deformation also decreases the radius of curvature of the membrane at the surfaces perpendicular to the field. The combination of these effects is proposed to exacerbate the lipid packing difference between the adjacent membrane leaflets at the apices of the ellipse. The second step in the Neumann model involves redistribution of intracellular ions under the influence of the electrical field, resulting in an increase in the transmembrane electrical potential. Discharge of the potential across regions of more pronounced lipid packing differences is proposed to then result in the formation of conical hydrophobic pores. Under the continuing influence of external electrical field forces, the pores are proposed to coalesce, restructure and enlarge to yield inverted hydrophilic pores, which then facilitate the passage of large, hydrated, polynucleotide anions. The porated

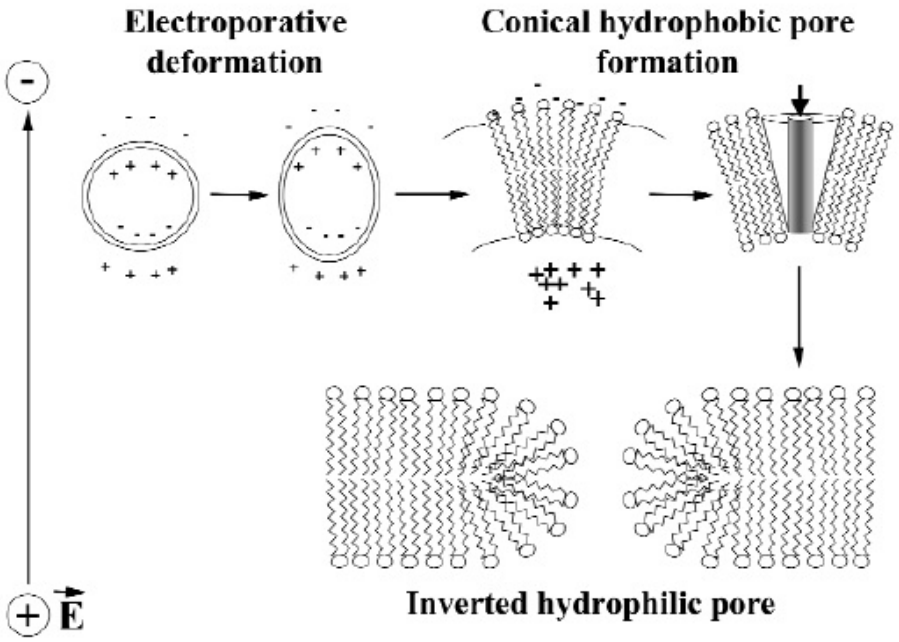


Figure 4.2: Neumann model for electroporative channel development. Under the influence of an electrical field, the distribution of ions adjacent to the inner surface of cell membranes is proposed to be altered, resulting in a series of membrane alterations that predispose to pore formation. See text for a description of the model. [15] from [16].

state is transient, with cells remaining in a destabilized state for minutes after the electrical treatment, after which the membrane defects are sealed and the cells revert to baseline status. The general mechanism of transportation induced by electroporation clearly starts with an increase in membrane permeability following treatment with electrical pulses [17] and is then followed by passive diffusion through the permeabilized membrane defect. In addition to passive diffusion, electrophoretic and electroosmotic transport under the influence of electric fields may also facilitate transport of charged molecules and ions across membranes. To vary the type of cells and electronic devices used for the transmission of stimuli, different are the parameters to be optimized to get a good outcome of transfection, such as the waveform, the amplitude and duration of the pulses, and solutions in which to perform electroporation. Despite the biophysical modalities with which occurs electroporation have not yet been fully characterized, it is a technique applicable both in vivo and in vitro, for animal cells, plant, human. Once the pore is formed it will remain even for several minutes as a function of the electrical parameters used in the protocol and temperature. The creation of the pore is not irrelevant to the state of health of the cell, this fact allows both the incoming and the outgoing molecules, solutes which if prolonged can lead to cell death. It is therefore necessary to reach a critical level but below the threshold through which molecules can be introduced into the cell but without destroying their vitality. We underline that these consideration don't refer to cell in adhesion, as the constraints given by the adhesion to a substrate could be reasonably do their part.

# Part II

# Experimental I

# Chapter 5

# The device

## 5.1 The working principle

The stimulation is performed via a capacitive current using an electrolyte/oxide /silicon (EOS) structure. It is a capacitor in which one plate is the aluminum back and the second one is the electrolyte solution. An Ag/AgCl reference electrode in the bath is the reference for the potential, sketched in figure 5.1 The bath is an ionic conductive solution. The capacitive current is induced applying a potential difference at the ends of the device by a function generator manually programmed (Agilent 33220A, 20 MHz Function/Arbitrary Waveform generator).

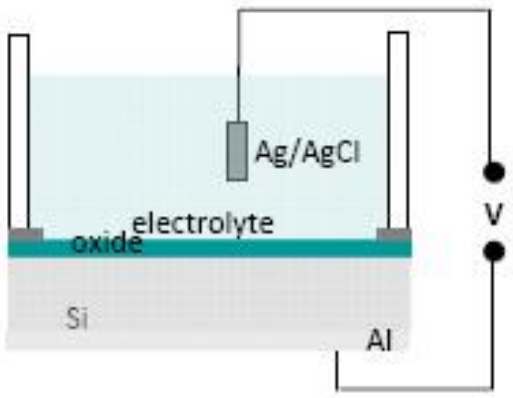


Figure 5.1: EOS configuation

## 5.2 The dielectric

The dielectric layer is the core of the device, its characteristics and quality determine the real feature of the capacitor.
In order to have a high capacitive coupling between cell and oxide, the dielectric layer needs to be an high-K material. Intensive research is underway to develop these oxides into new high quality electronic materials and many advances have occurred in the last

years. [19] Among the materials with high dielectric constant titanium oxide is the best candidate. There are two crystalline phases in which titanium dioxide exists, known as rutile and anatase. Titanium dioxide dielectric constant go from 30 for anatase phase up to 80 for the pure rutile. Although rutile one has a double permittivity, it is rather difficult to produce thin films. Anatase phase is the most common one of thin film samples. So we are talking about dielectric constants of around 30-35. It is therefore a factor of about 10 compared to that of the silicon oxide, 3.9, that was used in previous device in our lab, that can be enough for dye entering but not for efficient transfection. The transition to an high-K dielectric layer, therefore, promises to increase significantly the performance of the device reaching higher fields intensity and allowing, inter alia, to have the proper electric field between the cell and chips using lower applied voltages.

## 5.3 Biocompatibility

Biocompatibility is, by definition, a measurement of how compatible a device is with a biological system. The biocompatibility of the titanium dioxide was tested on two different cell lines, the CHO-K1 chosen for the project and the Human Astrocytes H4. The inertness against cellular environment and the state of cell culture were considered.

### 5.3.1 Surface cleaning

The test surface of titanium dioxide and the future chips are cleaned with a 1% solution of Tickopur detergent (DR H Stamm), bubbled to maximize its action on cells eventually still attached to the oxide. Very gently, using a cotton swab the surface is cleaned in a mechanical way, strictly in the presence of the solution, with circular and delicate movements. Then they have to be washed thoroughly with deionized water in order to eliminate any traces of the detergent and dried under the hood air flow. The sterilization is then carried through exposition to UV light for 30 minutes in the hood.

### 5.3.2 Cell culture on titanium dioxide substrate

Cell cultures show healthy state and normal development, good adhesion and normal replication time. No chemical reactions between cell culture medium and the titanium, that can damage the culture, were observed.
The chemical inertness has to be considered in the reverse direction too. Metabolic products of cell culture did not lead to chemical corrosion of the dielectric surface. These results were obtain on samples of titanium dioxide on silicon substrate, deposited through the same Atomic Layer Deposition Process and in the same reactor of the single chip I describe in section 5.5. The detailed results on this work, that was carried on in parallel during the first part of the project, are collected in the paper : E. Cianci, S. Lattanzio, G. Seguini, S. Vassanelli, M. Fanciulli, Atomic layer deposited TiO2 for implantable brain-chip interfacing devices, Thin Solid Films 520(14): 4745-4748.
Titanium dioxide thin film is a successful candidate, which combines the biocompatibility, inertness against cellular environment and high capacitive coupling.

## 5.4 Single spot chip

Each chip has square shape, with an edge lenght of 4 mm, the circular capacitor in the middle is the stimulation area. The chips were fabricated in the laboratory of professor Peter Fromherz in Munich, [20]. The substrate is p+doped silicon (0.01 Ω) with 1 $\mu$m $SiO_2$. Circular capacitors are etched. A dielectric layer of 10 nm of $TiO_2$ is deposed by atomic layer deposition at the Laboratorio MDM-INFM, Milan. An aluminum layer (200 nm) is evaporated on the back. The structure of a single stimulation chip is shown in figure 5.2. The chip were properly assembled in a petri dish in order to allow the cell culture and the possibility of connection on the back side as described in the next section.

## 5.5 Prototype production

A wafer contains approximately 100 chips with capacitor diameters of 0.100 mm, 0.25 mm, 0.5 mm, 1 mm and 2 mm. A picture of the wafer, is shown in figure 5.3.

The round black areas correspond to the capacitor elements. I isolated along the black lines every single 4x4 $mm^2$ chip with the help of a proper plastic tweezers. Using a drill with a proper tip I made a hole in 35 mm polistirene culture petri dishes, in decentralized position (as required from the patch clamp experiment, see chapter 6).
I glued the chip on an aperture using a silicon paste (Silicones Elastosil E43, WACKER)

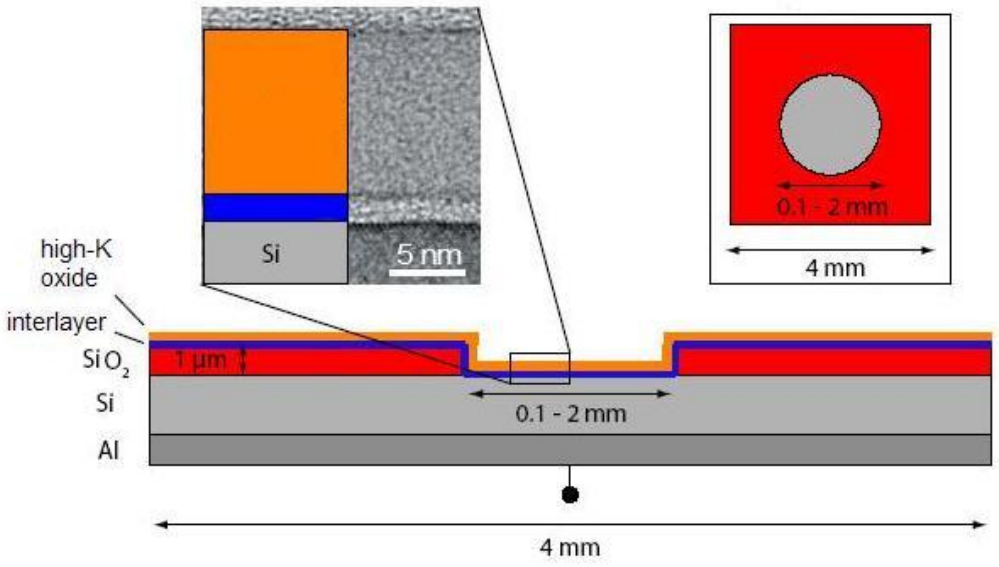


Figure 5.2: Sketch of a single stimulation spot chip. The starting material was highly p+doped - 4 ” wafer with a specific resistance of 0.006-0.01 cm (p + -Si). This corresponds to a doping concentration of about $10^{19}$ cm $^{-3}$ [21]. After a standard RCA cleaning (Radio Corporation of America), a 1 $\mu$m thick $SiO_2$ field oxide is grown by wet oxidation. Circular capacitors are etched. A thin buffer layer of silicon nitride was reported to suppress the formation of an interfacial layer of $SiO_2$ on silicon during the deposition of the thin layer of $TiO_2$, 9.5±0.5 nm, specific capacitance $\geq 1.44\mu$ F/$cm^2$ [20].

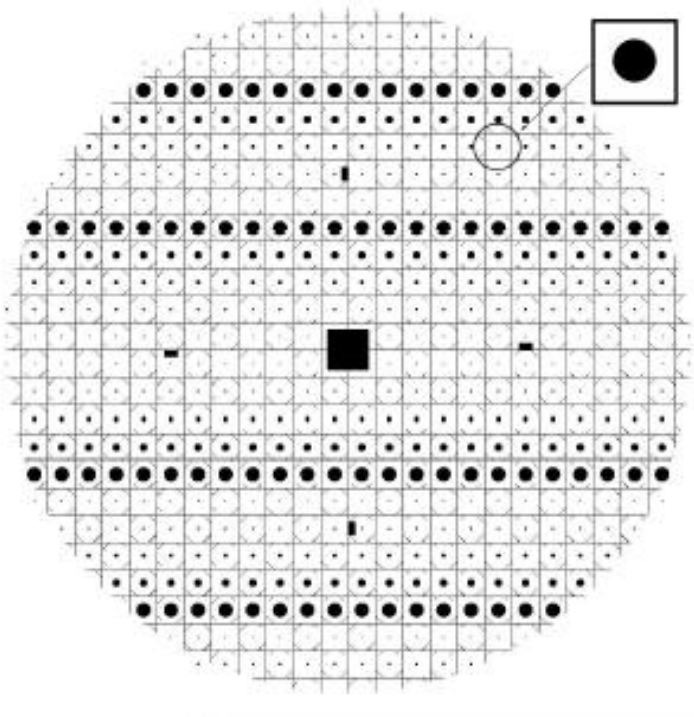

Figure 5.3: Scheme of the photolithography mask. The entire wafer has a diameter of 10 cm and is divided into 4  4 $mm^2$ chips. The round capacitor is in the middle of each chip. There are a diameter of 0.1 mm, 0.25mm, 0.5 mm 1 mm and 2 mm.

after the proper gluing with conductive paste (Silver Conductive Paste, RS) of the pin for the metal contact on the back. The assembled chip are of two kind. The ones for the patch experiments, shown in figure 5.4 with the chip on the upper surface of the petri, in order to allowed the micropipette to reach the culture. The ones for the transfection experiments, with the chip on the lower surface in the bottom of the petri, in order to create a sort of well in which it is easy to maintain the electroporation solution for transfection and insert the reference electrode.

## 5.6 Cell culture on the prototype

### 5.6.1 Chip surface cleaning and sterilization

The photoresist film on the chip's upper surface is removed with ethanol and a very gently help of a cotton swab. Then the chip are washed with deionized water and then sterilized through the exposition to UV light for 30 minutes in the hood. After any experiment's section, the patch solution or the electroporation one are removed. Chips are cleaned with a 1% solution of Tickopur detergent (DR H Stamm), bubbled to maximize its action on cells eventually still attached to the oxide. Very gently, using a cotton swab the surface is cleaned in a mechanical way, strictly in the presence of the solution, with circular and delicate movements in order to remove any biological residual. Then they have to be washed thoroughly with deionized water in order to eliminate any traces of the detergent and dried

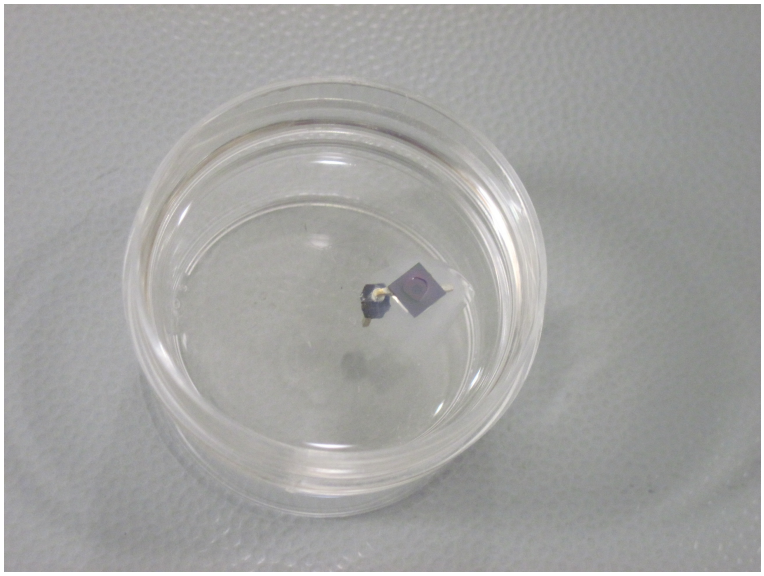

Figure 5.4: Prototypes for patch-clamp-electroporation experiments

under the hood air flow. The sterilization is then carried out through exposition to UV light for 30 minutes in the hood.

### 5.6.2 Density protocols

Depending on the experiment you want to do it is necessary to prepare the device with a suitable density of cells. The cells are deposited on the chip surface under a biological hood. They recline and adhere to the surface. Then they develop proper proteins that attach very good to the substrate. Actually to knock off them is necessary the trypsinization process described in section 2.1.2. You can not use the cells the same day they are plated but better at least two or three days after plating, in order to have a strong adhesion to the substrate. Time flowing, cells replicate occupying all the available area. In this case the culture reaches the confluence and can no longer be used because the cells begin to grow out layers.
The experimental density protocols, I determined experimentally, suggest the density of cell in small amount of culture medium, 300 $\mu$l, in order to handy plate small quantities of cell and have the desired density at the desired time as summarized in the tabular 5.1. A first quantity of medium and cells is deposit on the chip, usually from 4 to 10 $\mu$l taken from the dilution as soon as mentioned. Then the chip has to be put in the incubator for twenty minute, in order to let the cells deposit on the surface and start to attach to it. Then other culture medium is added, up to 2 ml, in order to completely cover the chip surface and the petri dish support. Some examples of CHO-K1 cultures on the single-spot prototypes are shown in figure 5.5.

| to reach the confluence n$^o$ days after the plating | number of cell in 300 $\mu$l | corresponding density cellml |
|---|---|---|
| 2 | 66000 | 220000 |
| 3 | 47000 | 157000 |
| 4 | 28300 | 94200 |
| 5 | 9400 | 31200 |
| 6 | 5100 | 15700 |

Table 5.1: Plating's protocol on single spot chips. In the first column there is the day at which the confluence should be reached. In the second one the number of cell that have to put in the 300 $\mu$l medium in order to have the density reported in the third column, with the usual unit of measurements of the cell density, as used in the trypsinization process. The optimal quantity of liquid on the open area of the chip, varies from 4 $\mu$l up to 10 $\mu$l. This variability derives from the different area exposed after the silicon gluing that I did by hand on every single chip and so is subject to varibility.

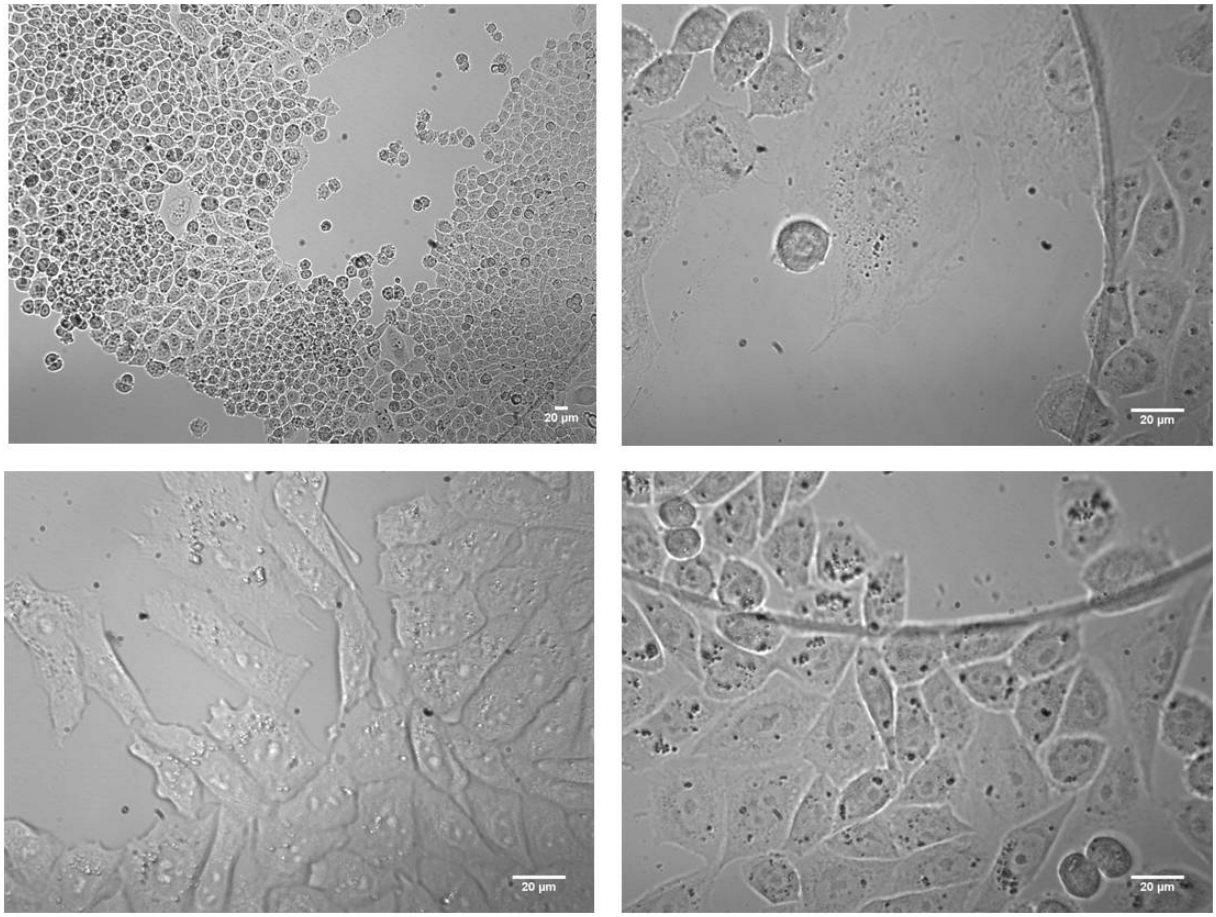


Figure 5.5: And this is an image of the culture of epithelial CHO-K1 cells on some prototypes in which is visible the rim of the spot of stimulation. It is possible to observe the mixed morphology of this cell line, already mentioned introducing the cell line: cells are more or less elongated, more or less flat or rounded. The bars are 20 $\mu$m.

## 5.7 Direct detection of the permeabilization by mean of dyes

From experiment on planar bilayer membranes, it is know that lipid bilayers are not able to withstand an increase in the applied voltage above a threshold value. A conductive state followed by a rupture is observed for values of the order of 200-250 mV, as mentioned in chapter 5. This apparently depends on the nature of the lipids and on the pulse duration [6]. But value of potential are linked to a different experimental configuration respect to the one we use. These values refer to cell cultured in suspension instead of in adhesion. In this configuration you directly control the electric field applied in a space region in which the suspension is.
Instead, we are not able to measure directly the voltage drop above the plasma membrane, as we don't control it directly in our experiments, and we are not interest in it directly. We control the voltage signal applied to the chip. And it can stimulate the plasmatic membrane only after, by mean of the capacitive coupling of cell and the dielectric substrate, as it will be presented in next chapters.
The work started on the confirmation of the electroporation protocols previously developed in the laboratory, that allowed the penetration of nonpermeant dye into the cytoplasm.

### 5.7.1 Trypan Blue

Electropermeabilization of cells can be quantified by penetration of nonpermeant dyes, such as Trypan Blue (TB, MW 891.8 Da)(Sigma-Aldrich). Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. Live cells or tissues with intact cell membranes are not coloured. If trypan blue is absorbed by an alive cell it means that pores opened in the plasmatic membrane. Cells are stained blue and can be detected without specific excitation. TB also excites and absorbs over a broad range extending from the blue to the orange. It has intrinsic fluorescence in the far-red region of the spectra (600-720 nm) where in our case the electroporation results can be checked.

# Part III

# Materials and Methods II

# Chapter 6

# Patch-clamp technique

## 6.1 Why Patch clamp

The efficiency of the electromediated cell transfection is strongly influenced by biological constrains connected to the cell variability that influence the efficiency of the process.
Electro-mediated gene transfer and expression can be described as a complex process with different successive steps, that cover the plasma membrane permeabilization, the electrophoretic migration of DNA toward the membrane, the DNA/membrane complex formation, the conversion of the metastable form of the DNA/membrane complex to a stable one, the translocation/diffusion across the membrane, the migration toward the nucleus and finally the gene expression, [24], as explained in chapter 3.
In order to understand the fail point in the electroporation process really, we perform on-chip electroporation patch-clamp experiment.

## 6.2 Introduction

The patch-clamp technique is an extremely powerful and versatile method for studying electrophysiological properties of biological membranes. It was developed by Erwin Neher and Bert Sakmann, who were awarded the highest scientific recognition for their discoveries concerning "The Function of Single Ion Channels in Cells". It caused a revolutionary advancement of many research areas in both cellular and molecular biology. "Each living cell is surrounded by a membrane which separates the world within the cell from its exterior. In this membrane there are channels, through which the cell communicates with its surroundings. These channels consist of single molecules or complexes of molecules and have the ability to allow passage of charged atoms, that is, ions. The regulation of ion channels influences the life of the cell and its functions under normal and pathological conditions. (...) Patch-clamp allows the registration of the incredibly small electrical currents (amounting to a picoampere) that passes through a single ion channel. The technique is unique in that it records how a single channel molecule alters its shape and in that way controls the flow of current within a time frame of a few millionths of a second. Neher and Sakmann conclusively established with their technique that ion channels do exist and how they function.", [30].

## 6.3 Patch-clamp configurations

There exist different patch-clamp configurations. Each of them has its peculiarities, advantages, and disadvantages. Which patch-clamp configuration is chosen as the experimental paradigm depends on the type of question to be addressed. We underline that we focused on the application of patch-clamp technique in an unusual contest, as we were interest in studying electroporation, that is pores and not channels. We will focus on the two main configurations that are also the ones used in this work: whole-cell recording and cell-attached recording, sketched in figure 6.1.

The so-called "cell-attached" configuration is the precursor to all other variants of the patch-clamp technique. Tightly sealing a glass microelectrode on to the plasma membrane of an intact cell, thereby isolating a small patch, is possible to measure by means of a connected patch-clamp amplifier, the currents flowing through ion channels enclosed by the pipette tip within that patch. The resistance between pipette and plasma membrane is critical for determining the electrical background noise from which the channel or pore currents need to be separated. The seal resistance should typically be in excess of $10^9$ $\Omega$ "gigaseal". The cell-attached configuration may be used (as such) to record single-channel activity and in our case single or more probably few pores currents.
Breaking the patch by applying a pulse of suction through the patch pipette, thereby we create a hole in the plasma membrane and we gain a direct access to the cell interior. We obtain a direct communication between the micropipette solution and cell cytoplasm. Amazingly, this maneuver does not compromise the gigaseal between pipette and plasma membrane. The tightness of the gigaseal both prevents leak currents flowing between the pipette and the reference electrode and prevents flooding of the cell with the constituents of the bath solution.

## 6.4 Patch-clamp "approach": Voltage and Current clamp

Properly the term "patch clamp" stands for a special voltage clamp that allows one to resolve currents flowing through single ion channels. But we will regard a more wide use of this term, dealing with all kinds of configuration available, and we will specify each time the kind of clamp and the configuration used.
In a patch clamp experiment one variable is "clamped" to a fix value. We can clamp voltage and measure current, or we can clamp current and measure voltage. So typically in a current-clamp experiment, one applies a known constant or time-varying current and measures the change in membrane potential caused by the applied current.
In a voltage clamp experiment one controls the membrane voltage and measures the transmembrane current required to maintain that voltage. Despite the fact that voltage clamp does not mimic a process found in nature, there are three reasons to do such an experiment:

- Clamping the voltage eliminates the capacitive current, except for a brief time following a step to a new voltage.

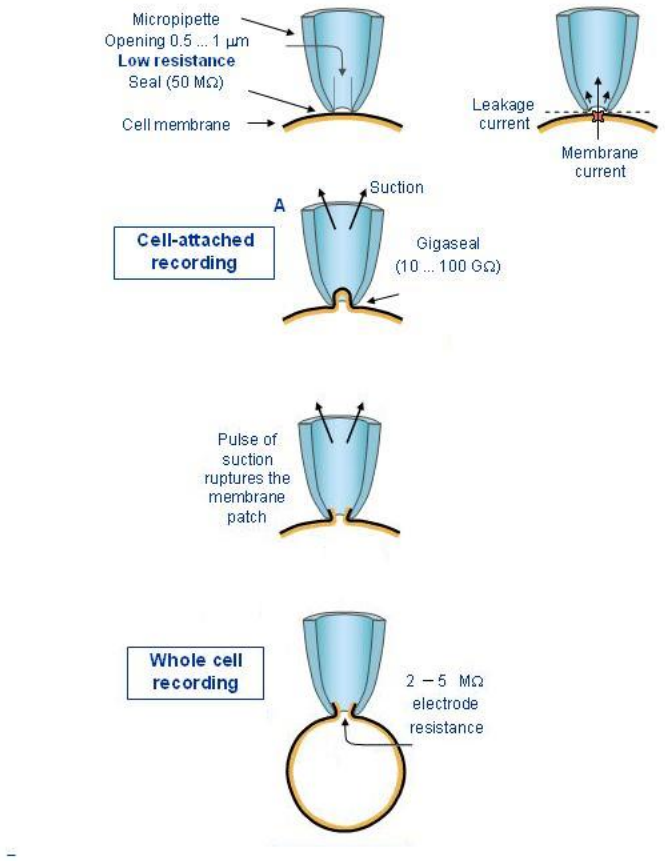


Figure 6.1: Sketch of the main patch-clamp configurations. Seal creation on the top. Cell-attached and Whole cell configuration

- Except for the brief charging time, the currents that flow are proportional only to the membrane conductance, i.e., to the number of open channels, and in our case, to the number of pores

- If channel gating is determined by the transmembrane voltage alone (and it is insensitive to other parameters such as the current and the history of the voltage), voltage clamp offers control over the key variable that determines the opening and closing of ion channels. We assume that similar consideration works for pores too, even if some more detailed observation will be done on the 'history'.

## 6.5 Patch-clamp setup

In its simplest form, a patch-clamp setup may consist of a microscope (for cell visualization) placed on a vibration isolation table within a Faraday cage, a patch-clamp amplifier and pulse generator for voltage-clamping the cells, a micromanipulator and data-recording devices (e.g., ocilloscope, computer, chart recorder). In addition, some instruments for pipette fabrication are required (i.e., a pipette puller and a microforge).

### 6.5.1 Vibration isolation table

In most patch-clamp experiments (particularly whole-cell or cell-attached recordings from small cells), mechanical stability of all setup components must be considered crucial, as even the slightest vibrations or relative movements of the pipette/cell assembly are detrimental to stable recordings and pipette tip integrity. We use a table by Newport, Model LW 3048 B-OPT.
Microscopic movements and vibrations are present to different degrees in all buildings and must be damped out by an appropriate vibration isolation table.

### 6.5.2 Faraday cage

A Faraday cage surrounds the table. Its main purpose is to shield the sensitive patch-clamp preamplifier from electrical noise. Although this is not an essential requirement (if proper electrical shielding can be accomplished otherwise).

### 6.5.3 Microscope, Video Camera and light source

Microscopic observation of the cells during the measurement and, even more importantly, the approach to the cell by the patch pipette for seal formation require a good optical visualization of the cell culture. We use a microscope Olympus (BX51WI) equipped with a video-camera Leica CCD DFC350FX 12 bit gray scale. The light source is a mercury burner lamp Olympus U-RFT-T.
Video cameras is attached to the microscope. The advantages of having a video camera to monitor the preparation during seal formation and throughout the experiment are numerous. One can form seals much more easily, as one can simultaneously observe the approach of the patch pipette and the change in pipette resistance on the computer screen when the pipette touches the cell membrane. During an experiment one can monitor any morphological changes of the cell under investigation (e.g., swelling or shrinking, blebbing, contraction). Considering the particular case of patch-clamping during electroporation, it can be important to have photos of the patched cell in order to establish on the offline analysis a link between the cell surface and shape and the recorded signals. And it also can be use to detect the morphological changes induced by electroporation.

### 6.5.4 Micromanipulator and pipette holder

In order to place patch pipettes on cells as small as a few micrometers, it is essential to be able to precisely control the movement of the patch pipette in the submicrometer range. Another important requirement is that the position of the pipette be free of drift after seal formation to maintain stable recordings for several minutes. Micromanipulator accomplishs this target. It is the tool that allow to establish a real physical connection between a macroscopic object, as the patch pipette and a single cell. The one available in our lab is manual one and has a piezoelectric control. It is *PatchStar Micromanipulator* $\Psi$ Scientifica. The amplifier probe is mounted directly on the micromanipulator. The headstage is fixed tightly to the manipulator. The arrangement of the manipulator and the attached headstage should also allow for easy access to the pipette holder for exchange

of pipettes. To accomplish this, the manipulator in mounted on a rotatable platform that makes it possible to swing out the headstage for pipette exchange and return it to its fixed position for experiments.

### 6.5.5 Patch-clamp amplifier

We use a Axopatch200B Amplifier (Axon, USA). Patch-clamp amplifier is capable of recording single channels as well as whole-cell currents and operating in voltage- or current-clamp mode. Then it is capable of applying a steady command voltage to the pipette, in order to fix the voltage at a desired value, as in our configuration.

### 6.5.6 Data Acquisition

Patch-clamp recordings requires equipment for data acquisition: an analog to digital conversion card and a board for the data acquisition and storage and the software to perform this. We have the card National Instrument card BNC 2110.

### 6.5.7 Grounding

Because of the extreme sensitivity of the headstage, special care must be taken in grounding all surfaces that will be near the probe input in order to minimize line-frequency interference. Even 1 mV of AC on a nearby surface, which can easily arise from a ground loop, can result in significant 50- or 60-Hz noise. A high-quality ground is available at the terminal of the probe; this is internally connected through the probe's cable directly to the signal ground in the main amplifier unit. The ground terminal on the probe is best used for the bath electrode. But an aluminum sheet is put on the headstage to reduces the noise too.

## 6.6 Pipette fabrication

An optimal adjustment of the size, shape, glass type, and coating of the patch pipette is required. The main steps in pipette fabrication involve pulling of appropriately shaped pipettes from glass capillary tubes, coating the pipette with a suitable insulation to reduce the background noise (that we avoid, as we can record without coating too). Patch pipettes are made in the laboratory too, no more than one day before the experiment. They are pulled from borosilicate glass capillary tubes. A patch-pipette horizontal puller passes large currents through a metal filament made of platinum and applies elastic force to pull the glass in multiple steps (typically 3 in our case). Pipettes should be used within 5-8 hour after fabrication, even if stored in a covered container; small dust particles from the air stick readily to the glass and can prevent sealing. However experience has been that pipettes may even be used the next day. It is very important to filter the filling solutions, we use a $0.2\mu$m syringe filter Minisart® sartorium stedim biotech. Pipettes can be filled by sucking up a small amount of solution through the tip. This can be done by applying negative pressure to the back of the pipette (e.g., using a 5-ml syringe). Thereafter, the pipette is back-filled, and any bubbles left in the pipette can be removed by tapping the side of the pipette. Overfilling the pipette has disastrous consequences for background noise because

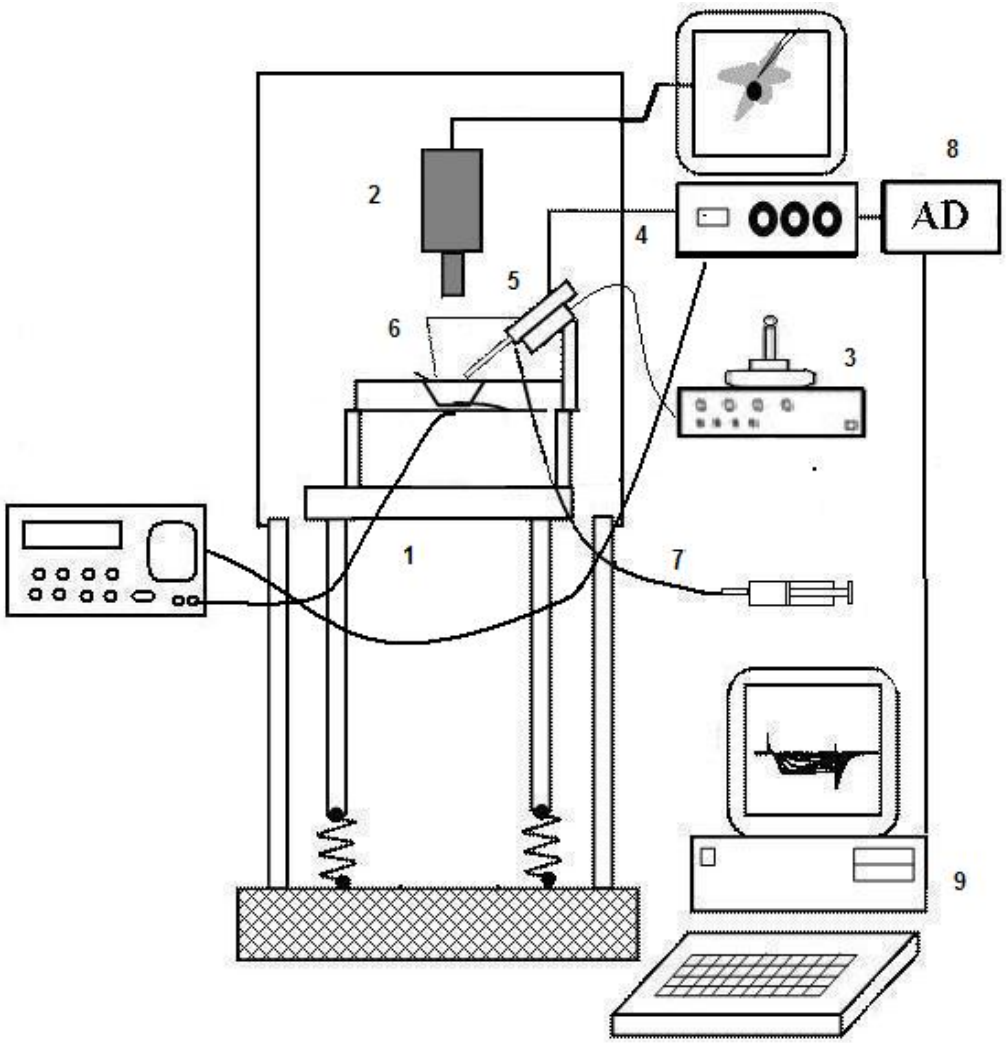


Figure 6.2: Patch-clamp set up used: 1. antivibrating table, 2. microscope and light source, 3. micromanipulator, 4.patch-clamp amplifier, 5. pipette holder, 6. reference electrode, 7. suction line and valve, 8. AD converter, 9. acquisition board and the function generator

the solution can spill into the holder, sketched in figure 6.5, wetting its internal surfaces with films that introduce thermal noise. Therefore, the pipette should only be partially filled, just far enough to make reasonable contact with the electrode wire (the pipette holder is not filled with solution but is left dry). Patch-clamp pipette are fabricated from borosilicate capillars cylinders:
ex.∅1.5 in.∅1.050 l 100 mm (SCIENCE PRODUCTS GmbH, GB150T-10 1.050x1.5x100 mm). The term micropipette usually refers to a pulled capillary glass into which the Ag/AgCl electrode is inserted. The patch-clamp pipette fabrication protocol has to be built by an experimental optimization of the parameters: heat, pull, velocity, time, pressure and the number of steps, usually 3 or 4. The resistance range is 2-3.5 MΩ and it is checked at the patch-clamp amplifier filling the pipette with intracellular solution and measuring in the extracellular one. The tip opening diameter is $\simeq 1$ $\mu$m.


Figure 6.3: Horizontal puller for glass patch-clamp microcapillaries (SUTTER INSTRUMENT CO. MODEL P-97 FLAMING/BROWN MICROPIPETTE PULLER).

## 6.7 A note on ions in solution and electrodes

Ohm's law describes the linear relation between potential difference and current flow and it applies to aqueous ionic solutions too. Complications are introducedby two factors, [2]:

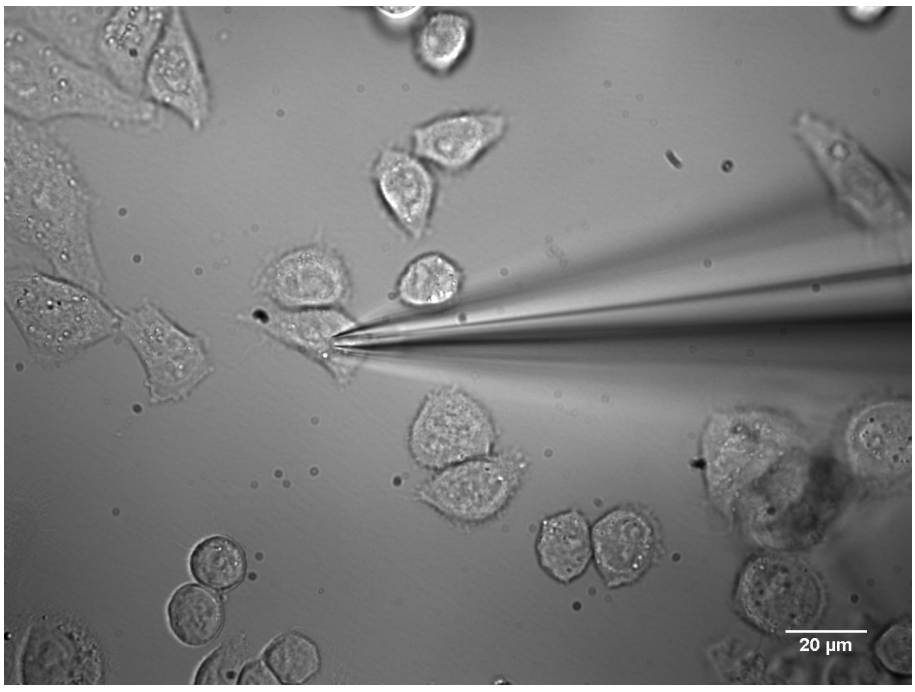


Figure 6.4: Patch clamp-pipette attached to a cell. It has the typical conical shape and an access resistance 2-3.5 MΩ, filled with intracellular solution and measured in extracellular one in a standard patch-clamp experiment. Seal is formed, the pipette is in cell-attached configuration to a CHO-K1 cell in adhesion on a titanium dioxide single-spot chip.

1. The current is carried by at least two types of ions (one anion and one cation) and often by many more. For each ion, current flow in the bulk solution is proportional to the potential difference. For a first approximation, the conductance of the whole solution is simply the sum of the conductances contributed by each ionic species. When the current flows through ion channels, it is carried selectively by only a subset of the ions in the solution. Instead, when the current flows through pores it is carried by all the type of ions in the solution.

2. At the electrodes, current must be transformed smoothly from a flow of electrons in the copper wire to a flow of ions in solution. Many sources of errors (artifacts) are possible. Several types of electrodes are used in electrophysiological measurements; we use the most common, a silver/silver chloride (Ag/AgCl) interface, which is a silver wire coated with silver chloride. If electrons flow from the copper wire through the silver wire to the electrode AgCl pellet, they convert the AgCl to Ag atoms and the Cl- ions become hydrated and enter the solution. If electrons flow in the reverse direction, Ag atoms in the silver wire that is coated with AgCl give up their electrons (one electron per atom) and combine with $Cl^-$ ions that are in the solution to make insoluble AgCl. This is, therefore, a reversible electrode, i.e., current can flow in both directions.

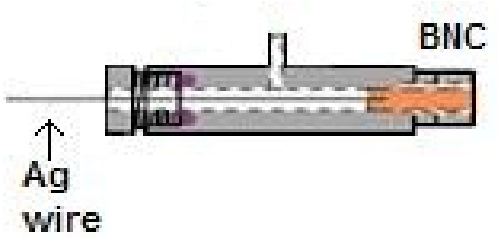


Figure 6.5: Pipette holder picture. The BNC connector to the headstage of the patch amplifier and the connection to the microcapillare for the communication between the pipette and the suction line are visible. Several types of electrodes are used in electrophysiological measurements; a silver/silver chloride (Ag/AgCl) interface, which is a silver wire coated with silver chloride. If electrons flow from the copper wire through the silver wire to the electrode AgCl pellet, they convert the AgCl to Ag atoms and the Cl- ions become hydrated and enter the solution. If electrons flow in the reverse direction, Ag atoms in the silver wire that is coated with AgCl give up their electrons (one electron per atom) and combine with Cl- ions that are in the solution to make insoluble AgCl. This is, therefore, a reversible electrode, i.e., current can flow in both directions. The Ag wire that has to be clorurated before experiments, we use a 1 M KCl solution.

## 6.8 Patch-clamp experiment: a summary

A patch clamp experiment needs the communication between different instruments in order to record the biological signal that come from cells: headstage and patch amplifier, A/D board and computer. Figure 6.6 is a diagram of a patch clamp experiment with "plot and actors" summarized. Moreover, we there is a function generator that is not represented in the diagram.

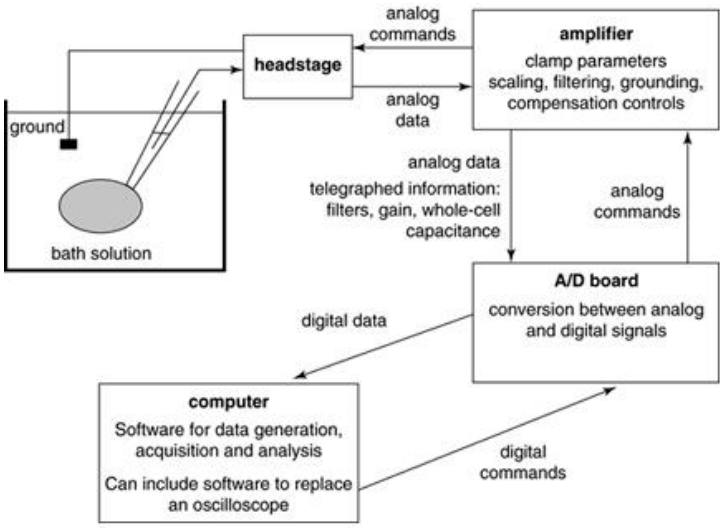


Figure 6.6: Patch-clamp experimental "plot and actors" chart.

# Chapter 7

# Cell on a substrate

## Two-domain-stimulation model

The adhesion of a cell on a substrate is mediated by protein molecules that protrude from the cell membrane and by CAM molecules and extracellular matrix molecules secreted from the cell and deposited on the surface of growth. These protein interactions lead to the formation of a thin layer that separates the cell membrane from the surface of adhesion, called "cleft". Following the interpretation given by Peter Fromhertz and his collaborators, [31], we underline some basic concept that stand behind the interpretation of electrophisioloigical experiments. Usually the interpretation of electrophysiological experiments relies on three concepts:

1. The electrical potential is constant across the cytoplasm and across the bath.
2. The difference of the two electrical potentials-the membrane voltage-is given by the voltage between two Ag/AgCl electrodes in the cytoplasm and in the bath.
3. The current through all domains of the membrane is driven by the same membrane voltage.

In a situation of extracellular stimulation these assumptions are no longer valid. Current from the stimulation electrode to the bath electrode gives rise to a gradient of the electrical potential in the extracellular electrolyte that depends on the geometry of cell and stimulation electrode as well as on the resistivity of the bath. Even though the cytoplasm may still be isopotential, the membrane voltage depends on the position of the membrane domain in the field of the extracellular potential. Thus the current through different membrane domains is controlled by different voltages. In the special case of cell adhesion on a planar electrode, we may distinguish

1. The region of adhesion with a large drop of the electrical potential due to the high electrical resistance of the narrow cleft between cell and electrode
2. The region in the surround of the cell with a minor drop of the electrical potential in the surrounding bath.

As a basis for the planning of the experiments and for the discussion of the results, we introduce a two-domain-stimulation (TDS) model that relies on two approximations:

1. The minor drop of the electrical potential in the surround of the cell above the uncovered capacitor is neglected. The extracellular electrical potential near the free membrane is assumed to be probed by the bath electrode.

2. The potential profile in the area of adhesion is replaced by a mean extracellular potential. There exists a potential difference-an extracellular voltage-between the attached membrane and the bath. Hence the current through the attached membrane is driven by a different voltage as compared to the current through the free membrane.

Deviations between the TDS model and the experimental data may occur if these assumptions are not perfectly valid.

Consider a cell on a dielectric layer. The attached plasma membrane is separated from the substrate by a thin film of electrolyte as illustrated in figure 7.1 A. The contact area with the insulating layers of membrane and oxide forms a planar core-coat conductor, [26] [27],. When a changing voltage $V_S$ is applied to the substrate with an area-specific capacitance $c_S$, current flows along the cell-chip junction with a sheet resistance $r_J$ and across the membrane with an area-specific capacitance $c_M$. A profile of extracellular voltage $V_J$ arises in the junction as well as a change of the intracellular voltage $V_M$ with respect to the bath at ground potential. A drop of extracellular voltage in the surround of the cell with respect to the bath is neglected. To account for crucial features of capacitive stimulation, we use a model that describes the core-coat conductor as a single equipotential compartment with a representative extracellular voltage $V_J$ as illustrated in Fig. 1 B. We distinguish two domains of the membrane with a total area $A_M$: the free membrane with an area $A_M A_J$ is controlled by the voltage $V_M$, whereas the attached membrane with an area $A_J$ and a fraction $\alpha_{JM} = A_J/A_M$ is controlled by the voltage $V_M V_J$. The two-compartment stimulation (TDS) model is determined by the capacitance $(A_M A_J)c_M$ and the ionic conductances $(A_M - A_J)g^i_{FM}$ of the free membrane and the capacitance $A_J c_M$ and ionic conductances $A_J g^i_{JM}$ of the attached membrane (area-specific conductances $g_i\ F_M$ and $g_i\ J_M$), as well as by the chip capacitance $A_J c_S$ in the junction and the conductance $A_J g_J$ from the junction to the bath. The areaspecific conductance $g_J = \eta/r_J A_J$ is defined in terms of the sheet resistance, the contact area, and a geometry factor that is $\eta_J = 8\pi$ under stationary conditions, [31].

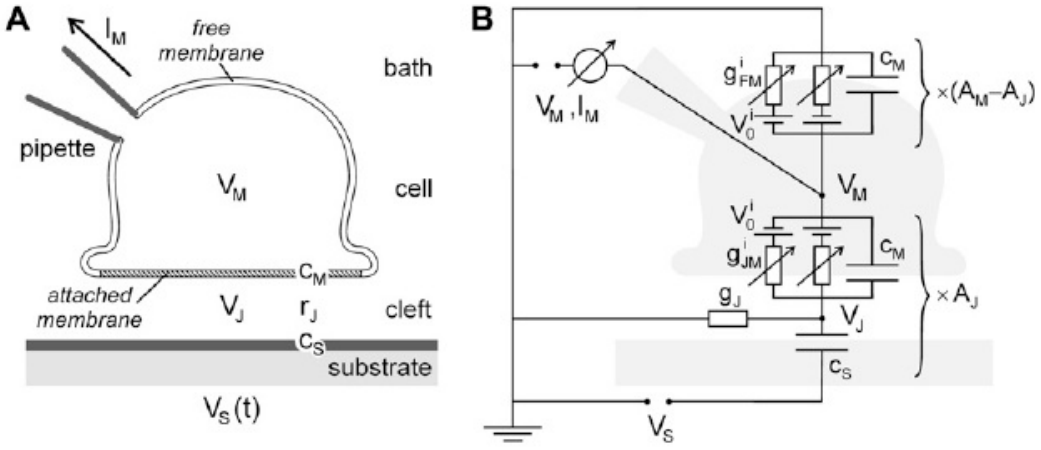


Figure 7.1: Capacitive stimulation of a cell in the geometry of cell adhesion , adapted from [31]. On the left. Schematic view, (not to scale). A cell (diameter of a small CHO rounded cell $\simeq$20 $\mu$m) is attached to an insulated substrate from which it is separated by a narrow cleft ($\simeq$ 30 nm as order of magnitude [31] and [28], not directly measured in case of CHO cells) filled with electrolyte. The attached and free areas of the membrane can be considered. For the measurements, the cell is contacted by a patch pipette under voltage clamp. The voltages with respect to bulk electrolyte are$V_J$ in the cell-capacitor junction, and $V_S$ in the substrate. A voltage protocol applied to the substrate evokes a capacitive current that creates temporary pores in the membrane. Two domains of the plasma membrane can be distinguished, an attached domain with area $A_J$ (shaded) in contact to the capacitor and a free domain with area $A_M$ in contact to the bath. A voltage ramp $V_S(t)$ applied to the substrate induces an extracellular voltage $V_J$ in the cell-capacitor junction. The resulting change of the membrane current$I_M$ are measured with a pipette. On the right Equivalent circuit of TDS model. Substrate, cell-chip contact, membrane in the junction ($J_M$) and free membrane ($FM$) are characterized by the area-specific parameters of substrate capacitance $c_S$ ( $c_{TiO_2} \simeq= 1.44\,\mu F/cm^2$ [20]), of membrane capacitance $c_M$ ($\simeq$0.01 $pF/\mu mm^2$ as order of magnitude), [2], of seal conductance $g_J$, and of ionic conductances $g^i_{JM}$ and $g^i_{FM}$ (reversal voltages $V^i_0$). The parameters of the junction are weighted by the area $A_J$ and the parameters of the free membrane by the area $A_M - A_J$ as indicated.

# Chapter 8

# Transfection experimental procedure

The genetic material used to perform the transfection is a plasmidic DNA that encodes the Enhanced Cyan Fluorescent Protein (ECFP) and allows the expression in mammalian cells. ECFP is a versatile biological marker for monitoring physiological processes, visualizing protein localization, and detecting transgenic expression in vivo. CFP can be excited by the 405 nm laser line and is optimally detected at 485 nm [32].
The plasmid is added to the electroporation solution and incubated for 10 minutes at room temperature. The culture medium is removed and the culture is covered with the electroporation solution with the DNA. The reference electrode is carefully inserted in the electroporation solution and the pin on the bottom of the chip is connected to the function generator.
The culture on the stimulation spot is electroporated with the plasmid in the solution, in order to obtain transfection. Then the protein expression is checked the day after, even if 6 our later would be sufficient. The electroporation process should be done in a sterile environment but with the proper care it can work even out of a hood, but for sure the probability of contamination grows. After the electroporation the chips with the culture and the electroporation solution are put in the incubator for 20 minute, than the electroporation solution is removed and the usual culture medium is added on the chip.

# Part IV

# Experimental II

# Chapter 9

# Electroporation experiments

## 9.1 Electoporation protocols

The target of the project is the development of a device and the relative experimental protocols to allow the handy CHO clone production. The experimental electro-transfection protocols will not made explicit because not already published and possibly (hopefully) patent. A complete protocol consists of:

- stimulus
    1. shape of the signal
    2. eventually offset
    3. amplitude
    4. frequency
    5. number of pulses
- electroporation solution
    1. chemical composition
    2. DNA adding time and quantities

I developed a new electroporation solution respect to the one used before in the laboratory. I did not do any particular experiments or analysis on this topics, for time reasoning, but it could be a point of interest in future. Regarding the DNA, instead, an important observation will be in the section 13.3.

## 9.2 Data acquisition

In order to see the current traces at screen in real time and record them, we use WIN-WCP program, an open source program for electrophysiology. The most of the traces are recorded with a sampling interval of 0.026 ms. The voltage recording window is $\pm$10V. The signal is filtered by the patch amplifier at 10 kHz by a lowpass filter.
In order to obtain the seal, a proper protocol is created, that applies voltage square pulses

at fixed time interval. A convenient pulse amplitude is 20 mV and a duration of 20-25 ms. As soon as the seal is formed and stabilized, after the proper capacitance and resistance compensation, the attached configuration is reached. If the desired configuration is the whole-cell one, after the gigaseal is formed, the access to the cytosol has to be gained. Then the electroporation experiment can start.
A trigger signal is sent to the function generator that applies the proper voltage selected electroporation stimulation protocol to the device that was properly connected through the pin on his bottom and the reference electrode in the extracellular medium. This allows to have synchronization between stimulation and recording.
An example of typical recording is shown in figure 9.1.

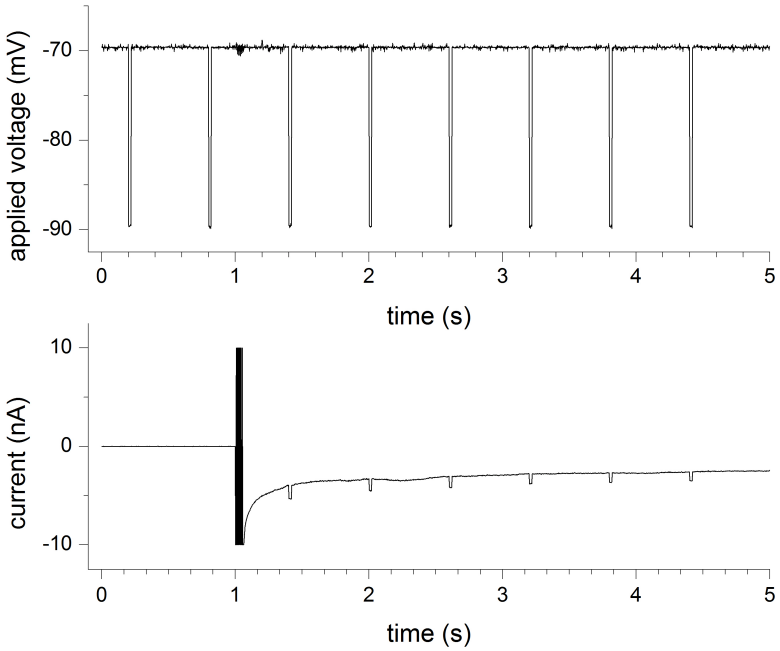


Figure 9.1: Voltage clamp experiment: example of recording. Top, voltage square wave applied to monitor the conductance changes, square wave of amplitude -20 mV to measure the conductance of the membrane, making the ratio between the current increase and the voltage applied. Bottom, current signal recorded through the micropipette. A trigger signal is sent through WIN-WCP to the function generator in order to apply the proper stimulation protocol created and stored on the function generator. The trigger signal occurs 1 second after the start of the recording. The characteristic jump in current is indicative of pore's opening, as ions are allowed to pass. In order to give a quantitative result and to avoid uncontrolled offset oscillations that can affect the interpretation of the results, conductance increase can be obtain from the increased current signal in correspondence of the square waves. This is a case of very good seal, so that the current signal at the square wave before the electroporation signal is practically indistinguishable from noise. It means that there is not significant flow of current through the plasmatic membrane before the electroporation.

Square wave of voltage are applied by the patch-clamp amplifier controlled by the WIN-

WCP protocol in order to monitor the membrane conductance's changes. Furthermore this strategy ensures that undesired and uncontrolled offset oscillations don't affect the interpretation of the results.
A little bit different recording protocols were used, the target is to monitor the conductance without perturbing too much the measurements: 20 mV amplitude, 20 ms duration and usually 600 ms of distance between the pulses is the most common choice in my measurements, but different interval and duration were used too. We interpreted the changes of membrane conductance (following an increase and decrease) as a result of the opening and closing of pores in membrane structures.

## 9.3 Experimental strategy

The experiments are performed in voltage clamp mode, as anticipated in section 6.4, clamping the voltage and measuring the current in whole-cell and in cell-attached configuration. Some consideration has to be done: in order to find the efficient electroporation protocol, different protocols are compared.
The recording more influenced by the cell dimension is the one in whole-cell configuration because the current recorded comes from the pores on the attached membrane, as depicted in figure 9.2 on the left and explained in the caption. The recording in attached configuration, instead, is less influenced by the cell dimension variability. This happens because the area from which the current is recorded, is always the one included in the pipette tip. An so it has less variability than the one that could be recording on different cells in whole-cell configuration. In order to establish a hierarchy in the protocols I compared the conductances after the electroporation stimulus. The amount of conductance has to be balance with the health of cell culture. Indeed, very efficient stimuli produce high conductance increasing but that can induce cell death.
We would like to electroporate as gentle as possible for improve resealing. So we have to balance this against the issue of having the pores large enough such that heavy molecules, as DNA is, can enter into the cell, and/or keeping the pores open long enough such that impermeable exogenous compounds can diffuse into the cytoplasm. Preserving the cell viability, more efficient stimulation protocols determine higher conductance's increases and longer times for reseal.
The best information should be obtained by applying different protocols on the same cell but these procedure can not be used. Indeed we realized that the same protocol applied to the same cell in sequence is less efficient - if applied in the same stationary condition, that is waiting the need resealing time between one electroporation and the subsequent. Indeed to a less effective capacitive coupling, probably due not only to a "stress"' of the membrane but also to a progressive detachment from the substrate due to a real rearrangement of the membrane. The approach at the beginning was to test some protocols to fix the first two parameters: shape of the signal and the eventually offset, in order to correlate previous preliminary results of electroporation experiments with a conductance increase. So a shape and an offset value were chosen. Then, I focused on the three other parameters: amplitude, frequency and number of cycles.
I considered cells of similar area, typically I compared the conductance values after electroporation, see section 10.2.

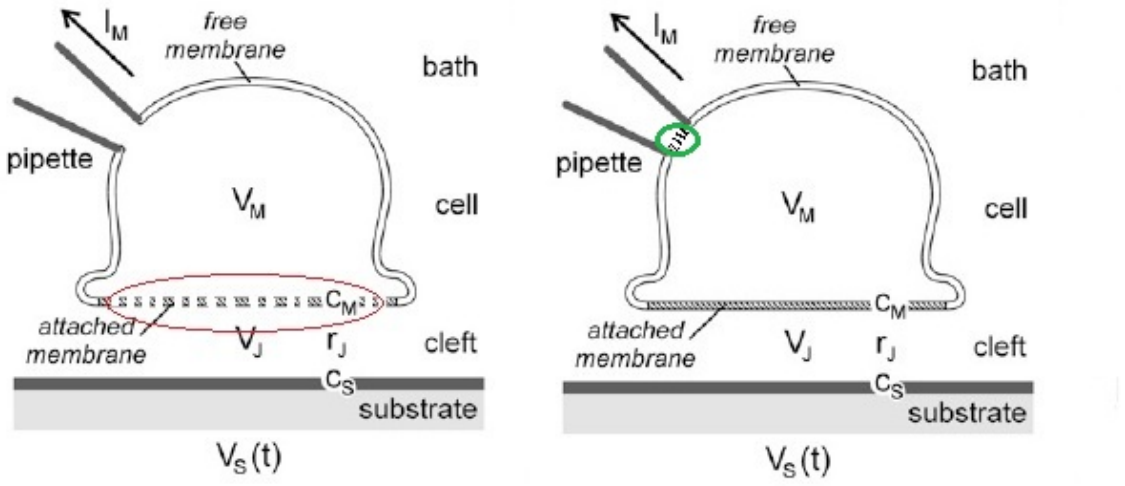


Figure 9.2: On the left, whole cell configuration is depicted. This is a case of a perfect seal where no leakage current sums to the signal. The current increase comes only from the pores that arise on the attached membrane. Indeed, after an electroporation stimulus, $V_S$ changes, $V_J$ changes and as consequence $V_M$ should change. But the patch amplifier in voltage clamp configuration works exactly in order to "clamp" the voltage $V_M$ fixed, at -70 mV, so the voltage drop across the plasmatic membrane is fixed. The current recorded is the current that the amplifier applies to maintain the voltage clamped to the chosen value. The current flow through the pores is a response of the attached membrane only, as the free membrane is in contact with the pipette and so no voltage drop can occur on it. On the right, cell attached configuration is sketched. In this configuration the potential in the cytosol can change, as the one clamped is the external surface of the membrane. So a potential drop rises across the "patch" of the membrane in the area of the pipette tip.

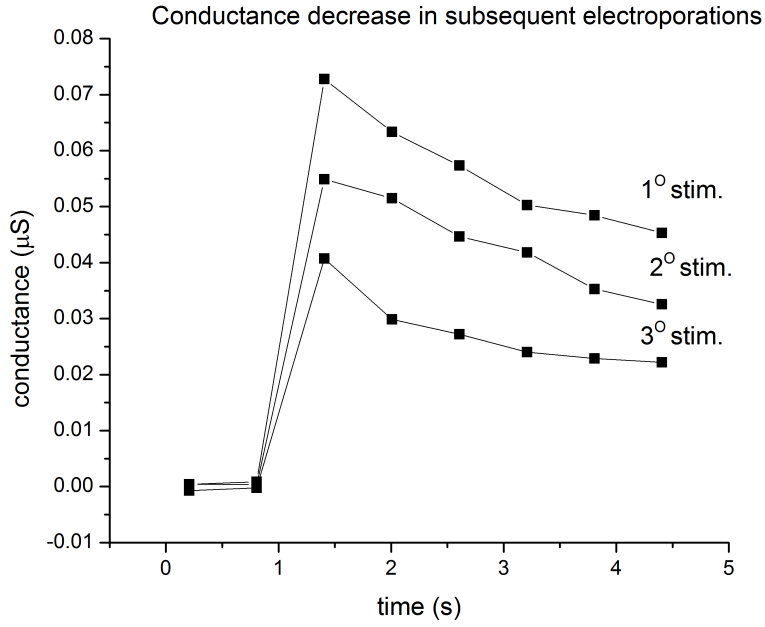


Figure 9.3: Comparison of conductance increase after electroporation stimulations in sequence performed waiting the pore resealing after any stimulation. Error bars not shown are less than 5%. Errors computed as standard deviation on the conductance value determined as the mean conductance during each square wave applied.

# Chapter 10

# Data

## 10.1 Data Analysis

The data were treated by a program written in C language. The tasks of the program are the following:

- creating a file of data that can be plotted and easily understood, with time flowing from zero to the end of the recording (as WIN WCP is built for standard patch clamp experiment and in order to have a sufficient sampling it works with short time sweeps.
- collecting information on the acquisition, duration of the recording, offset values
- calculating the membrane conductance before and after the electroporation, calculating the contribution due to the pores, in order to determine if the pore resealing is complete or not
- creating a file of data for the study of the decay of the current recorded, for possible future analysis that goes beyond the target of the thesis

## 10.2 Recorded segnals

The patch-clamp recordings during electroporation are around eighty. Due to the high time consuming connected to this kind of measurements the statistic can seem not very high. It is true but the way followed converged to the target. Our goal was not the study of the pore dynamics, but use it for tranfection purpose. The thing is that as a negative result can have many reasons, a positive one means that it works. An increase in conductivity, recorded as described, can not have other reasons but pore rising. In the figure 10.1 is reported the first sample of data obtained applying square wave of 10 mV amplitude instead of 20 mV. Only the data that have current increase are shown in the following graphs. The conductance values are calculated as $G_{pores} = \Delta I_{pores}/\Delta V_{applied}$. Current and conductance data shown in the same pages in figures 10.1, 10.2 and 10.4,10.3.

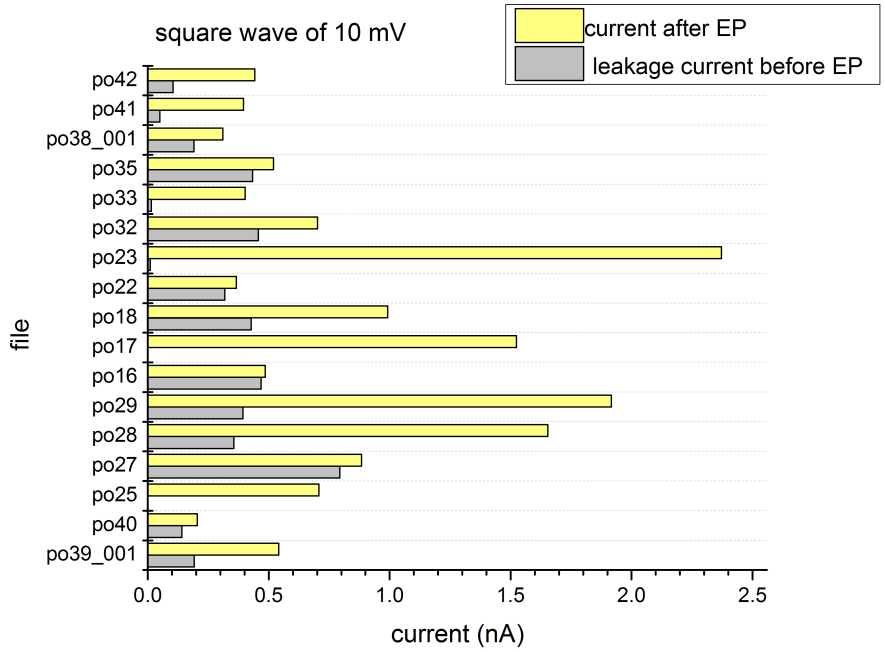


Figure 10.1: A sample of the recorded current values, before and after the electroporation. The data that gave null increase in current are not shown. Error bars are not shown, they are less than 2%.

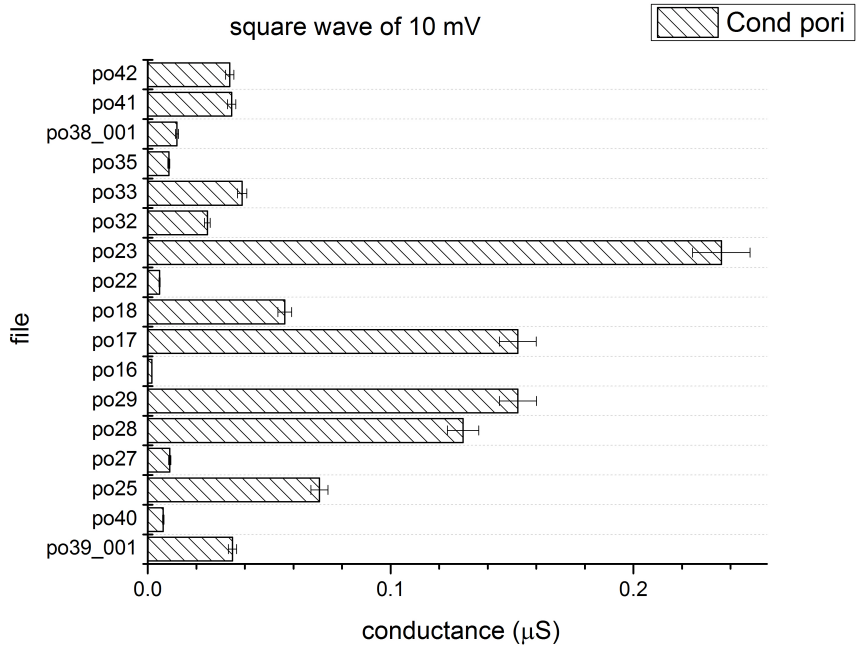


Figure 10.2: Conductance values of the first group of data, applying square wave of 10 mV amplitude. Errors computed as standard deviation on the conductance value determined as the mean conductance during each square wave.

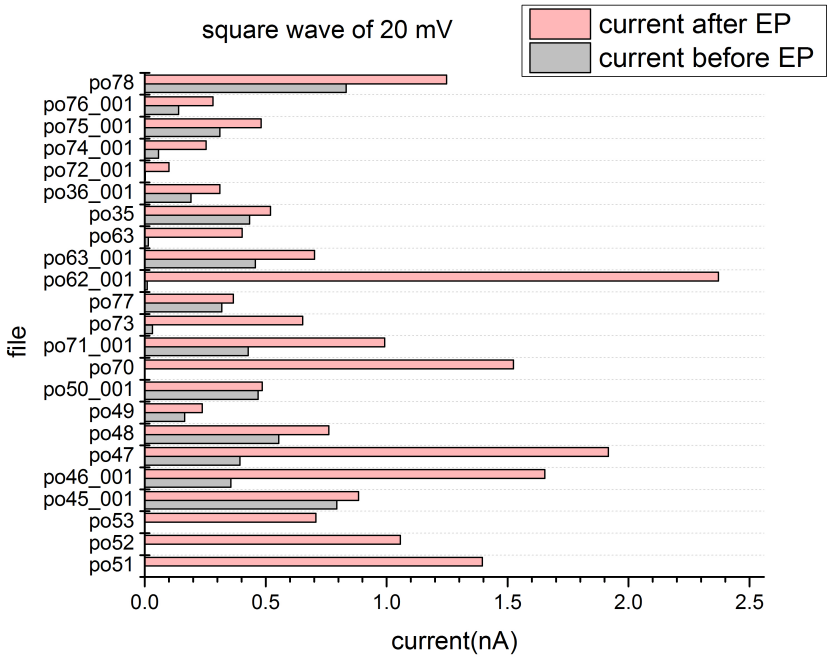


Figure 10.3: A sample of the recorded current values, before and after the electroporation. The data that gave null increase in current are not shown. Error bars are not shown, they are less than 2%.

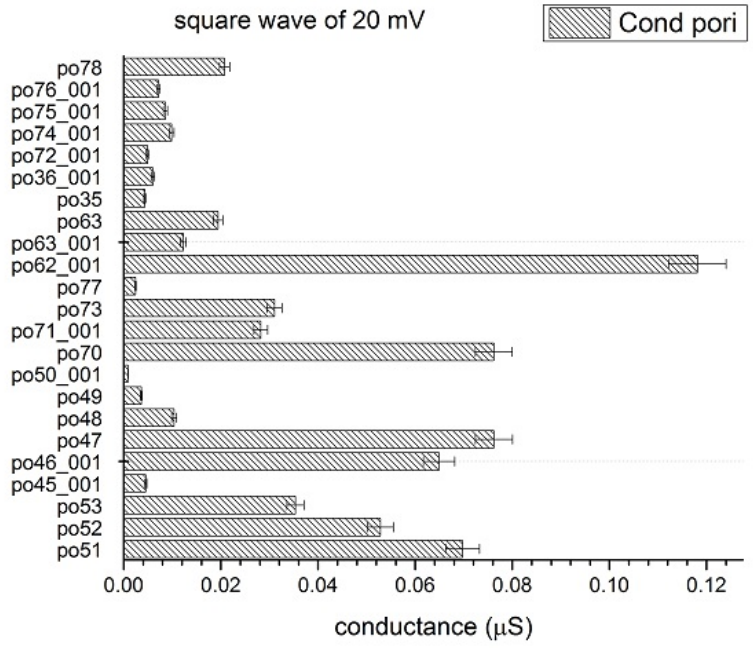


Figure 10.4: Conductance values of the second group of data, applying square wave of 20 mV amplitude. Errors computed as standard deviation on the conductance value determined as the mean conductance during each square wave.

# Part V

# Results

# Chapter 11

# On pore detection

## 11.1 Prove of the rising of pores on the attached membrane

Patch-clamp records in whole-cell configuration allow to record current that flows through the pore opening in the membrane attached to the dielectric substrate.

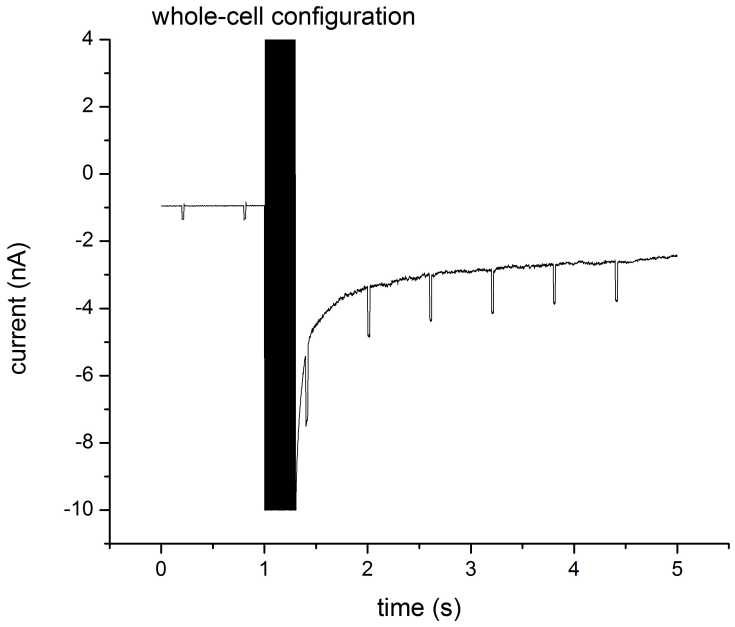


Figure 11.1: Current trace recorded in whole-cell configuration. Leakage current is present. At time 1 second the stimulus is applied. The stimulation protocol applied is not the one selected for the electrotransfection.

## 11.2 Prove of the rising of pores on the free membrane

Patch-clamp records in cell-attached configuration allow to record current that flows through the patch of membrane in the tip diameter and so it demonstrates the pore opening in the free membrane.

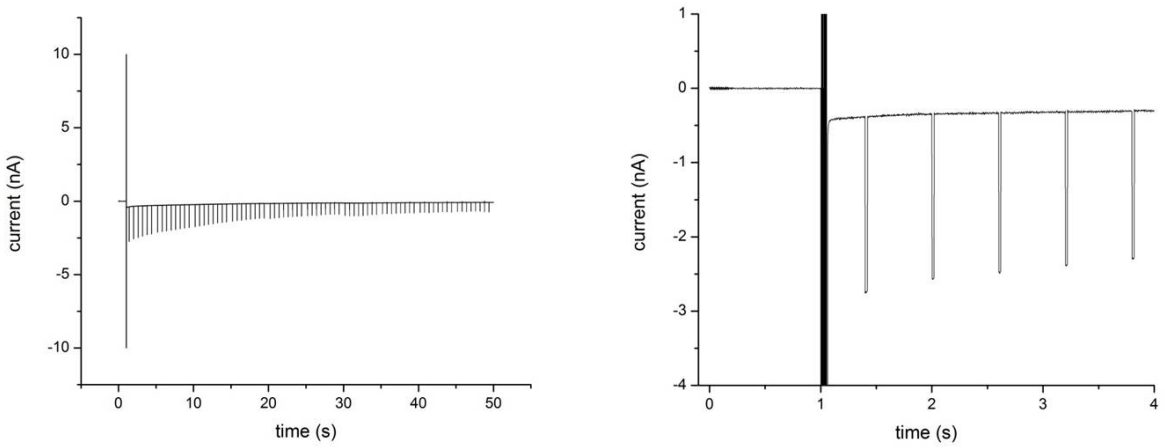


Figure 11.2: Current trace recorded in attached configuration, only the firts 50 seconds are shown for clarity. At time 1 second the stimulus is applied. The perturbation is very high respect to the scale of current of a patch stimulation and it takes the whole recording window. On the right, magnification of the first seconds of the record. It is clearly visible that the seal is a very good one, there is no significant leakage current.

An increase in the membrane conductance is clearly visible from the square waves that "go down" the current trace but to make it clearer, the pore conductance - that in this case coincides with the membrane one, as no leakage current is present - is calculated and represented as a function of time, 11.3. Pore opening determine an increase of pore conductivity that jumps at positive values after the capacitive stimulation. Then it declines as a sign of pore resealing.

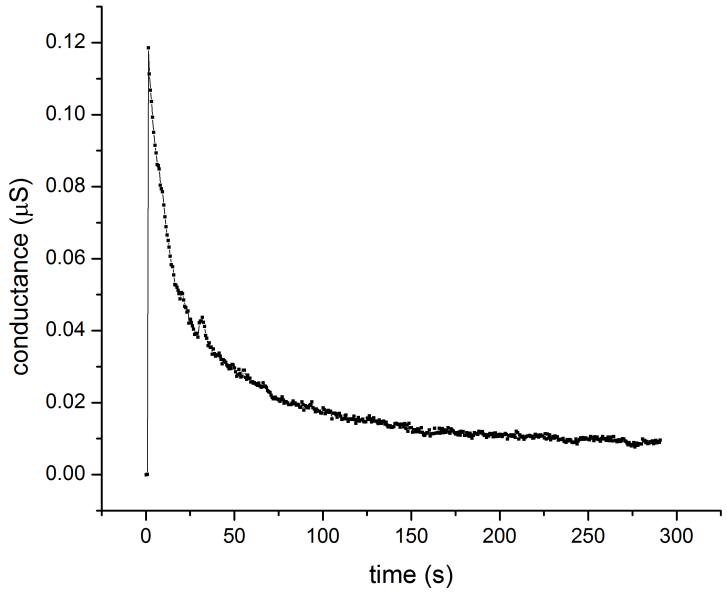


Figure 11.3: Conductance values calculated from the trace reported in figure 11.2. The peak is clearly visible after stimulation at time t=1 sec. The error bars, not shown, are less than the 5%, calculated as the standard deviation on the sample of points for each square wave applied.

## 11.3 Confirmation of the rise of pores with Lucyfer Yellow dye

The current traces recorded in cell attached configuration demonstrate that pores rise on the free membrane. But in order to dissolve any doubts on the real pore opening we decided to perform a new on -chip electroporation patch-clamp experiment. We added a fluorescent dye to the intracellular solution used to fill the pipette. Lucifer Yellow (LY) ( $life$technologies) is a fluorescent dye used in cell biology, [29]. The key property of Lucifer Yellow is that it can be readily visualized in both living and fixed cells using a fluorescence microscope.
The experiment was performed in cell-attached configuration with the voltage clamped at -70 mV as described in section 6.3. The electroporation stimulus was the best one selected, but applied with a smaller amplitude respect the one for the transfection in order to less stress the culture. The first seconds of recorded current are shown in figure 11.4.

The clear demonstration of the opening of pores in the "patch" of the free membrane is that the Lucyfer Yellow dye enters into the cytosol, and probably in the nucleus too indeed, as is shown in the image at the bottom right corner in figure 11.6.

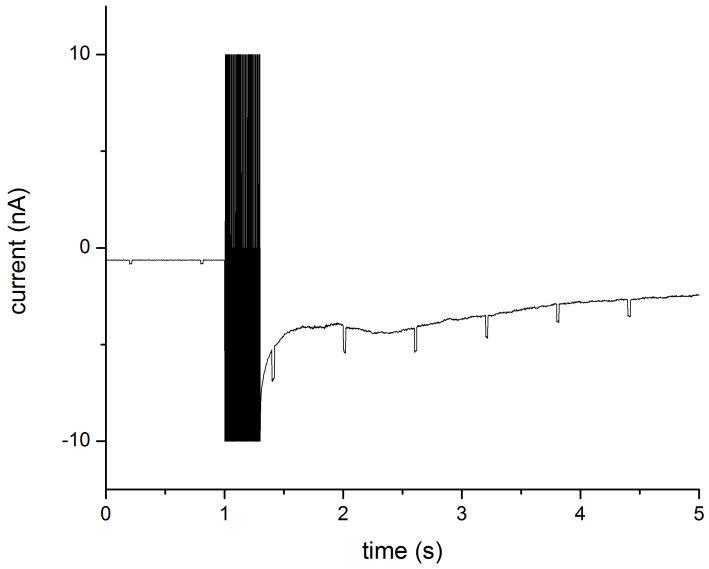


Figure 11.4: A zoom on the first seconds of the record. An offset oscillation is also present that is an example of how it could influence the evaluation of electroporation occurrence, and that we avoid checking the conductance as explained in section 9.2.

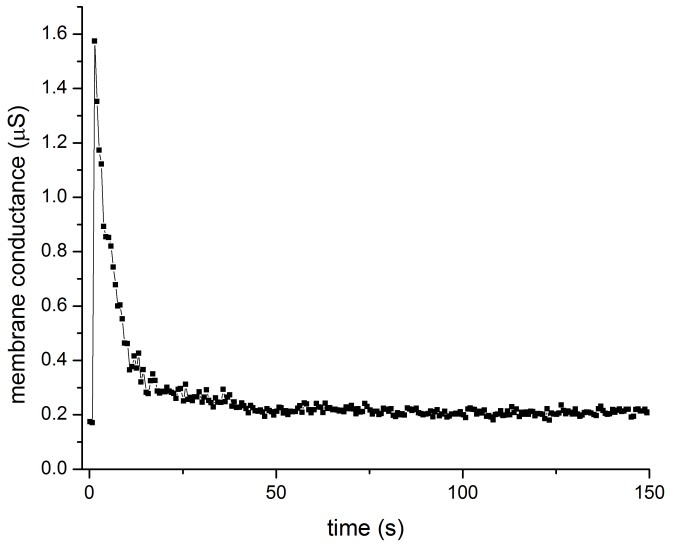


Figure 11.5: Membrane conductance time dependence. Electroporation stimulation protocol starts at time 1 second. Error bars not shown are less than 5%. Errors computed as standard deviation on the conductance value determined as the mean conductance during each square wave applied.

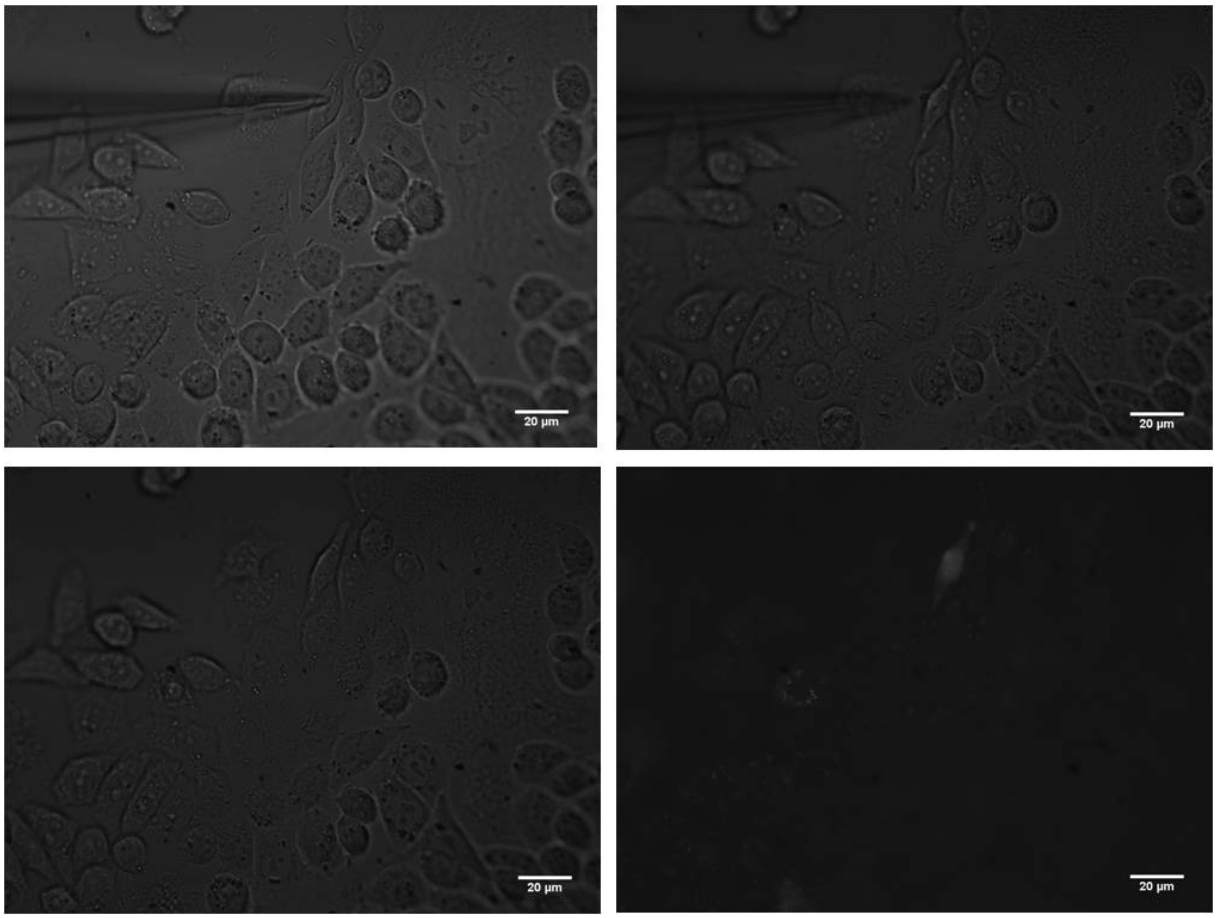


Figure 11.6: Starting from the left corner on top, clockwise: pipette on a CHO cell in attached configuration, pipette kindly removed, culture in visible light, culture in UV light where the cytosol uptaken the dye passed through the pores created in the free membrane. The images refer exactly to the traces shown

# Chapter 12

# On eletroporation to convey markers

## Phalloidin $A_{568}$

Electroporation protocols can be used to insert into cells not only genetic material, but also other kinds of molecules like markers that allow to visualize endogenous structures. This way electroporation allows to study cells when they are still alive, avoiding any fixation protocol that leads to cell death.
Phalloidin is a marker that can be used to visualize intracellular proteins. It is a toxin that binds to F-actin (filamentous actin) of the cytoskeleton of cells. The F-actin is a protein which intervenes in adherence of cells to the substrate and their movements, forming filamentous structures called *stress fibers*.
Phalloidin $A_{568}$ was added to the electroporation solution and incubated 10 minute at room temperature before the experiment. Then the complete electroporation solution was added on the culture a few moments before the stimulation.

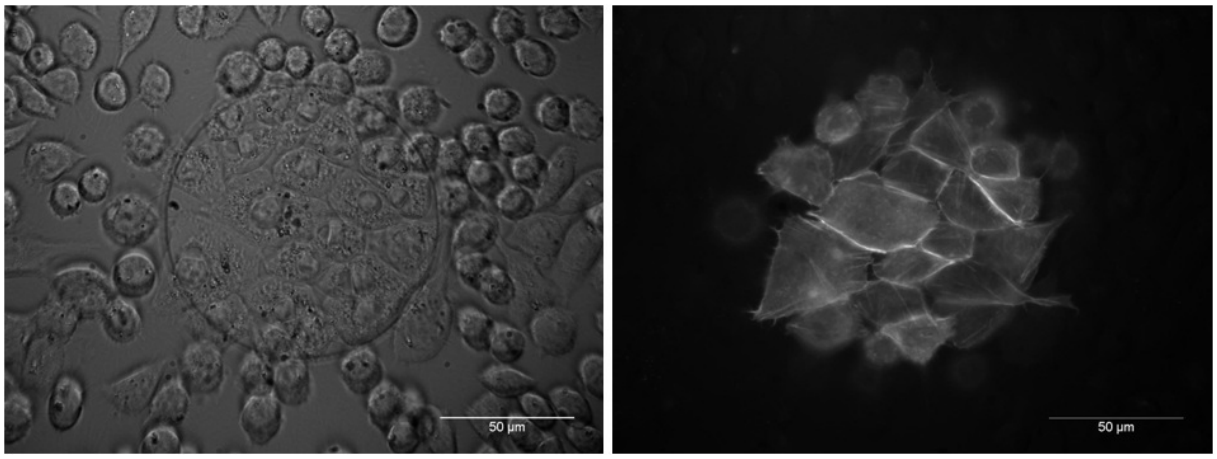


Figure 12.1: On the left, CHO culture on a single spot chip of 100 $\mu$m diameter after electroporation in visible light. On the right, the same culture but in UV light visualizing Phalloidin that entered into the cytosol thanks to the pores induced by electoporation. It is clear the selectivity of the system. Only the cells that were on the stimulation spot have been electroporated and have taken the marker.

The phalloidin used is labeled with the fluorophore Alexa 568 that emits in red, and it is thus possible to evaluate the results of the experiments in fluorescence. Unlike the Trypan Blue, transfection with phalloidin has a biological interest, since it allows to view *stress fibers*.
It is clear one of the advantage of this kind of device, that is the selectivity. Only cells that were on the stimulation spot uptook the fluorophore. All the others are not influenced by the stimulation and maintained their state. Electroporation is a stress for cells as it can be seen from the different appearance of the cells on the spot than the ones out of it. But they are able to recover, as shown in this experiments and the ones on transfection in section 13.1.

# Chapter 13

# On transfection experiments

## 13.1 Transfection results

The selected electroporation protocol was tested on cell cultures. The transfection experiments performed through the selected protocol are not so many for time reasoning, but the results obtained are clearly positive. A sample of cells transfected on the single spot chip is shown in figure 13.1.
A vision of the whole chip is shown in figure 13.2.
The transfection experiments demonstrate that the opening and closing of pores can be

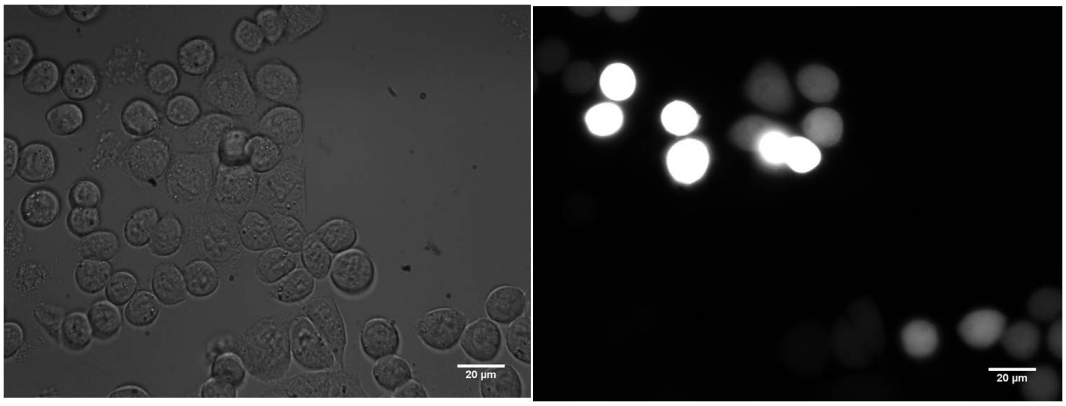


Figure 13.1: A magnification of group of electrotransfected cells . It can be visible a cell that has been transfected but is no more adherent to the substrate and is floating in the medium. It is probably due also to the stress imposed by the electroporation stimulation that can induce detachment.

obtained preserving cells alive and in an good health state, otherwise they could not express the protein codified by the plasmid. Another example of electrotransfection experiment is shown in figure 13.3

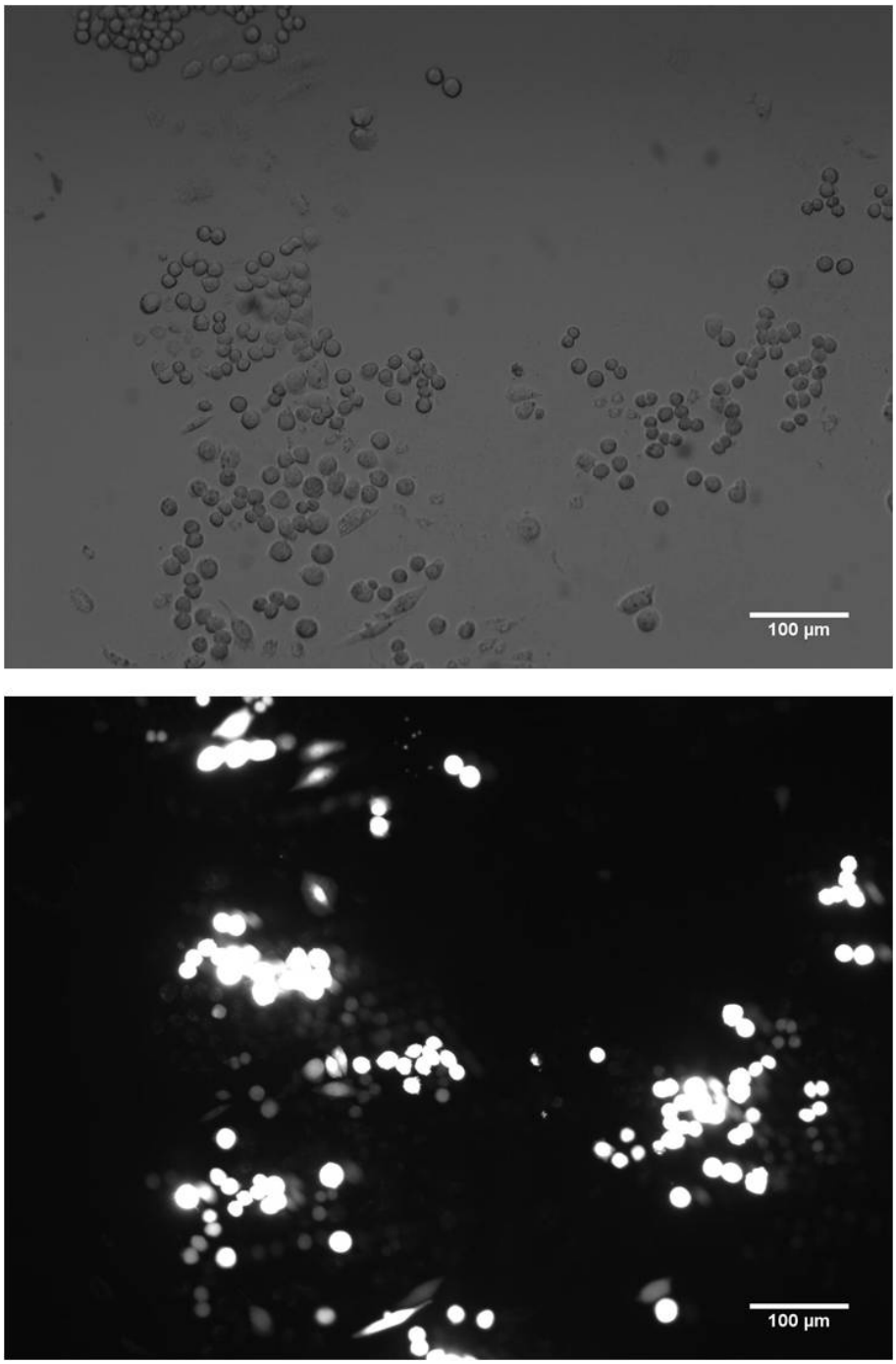


Figure 13.2: Cell culture in visible light the day after the electrotransfection. More cell than usual are rounded, due to electroporation stimulus, but the high protein expression, demonstrates their good health conditions.

## 13.2 Transfection efficiency

The evaluation of transfection efficiency is also influenced by the real detection of the fluorescence. The total number of cells is determined on photographs in visible light. The total

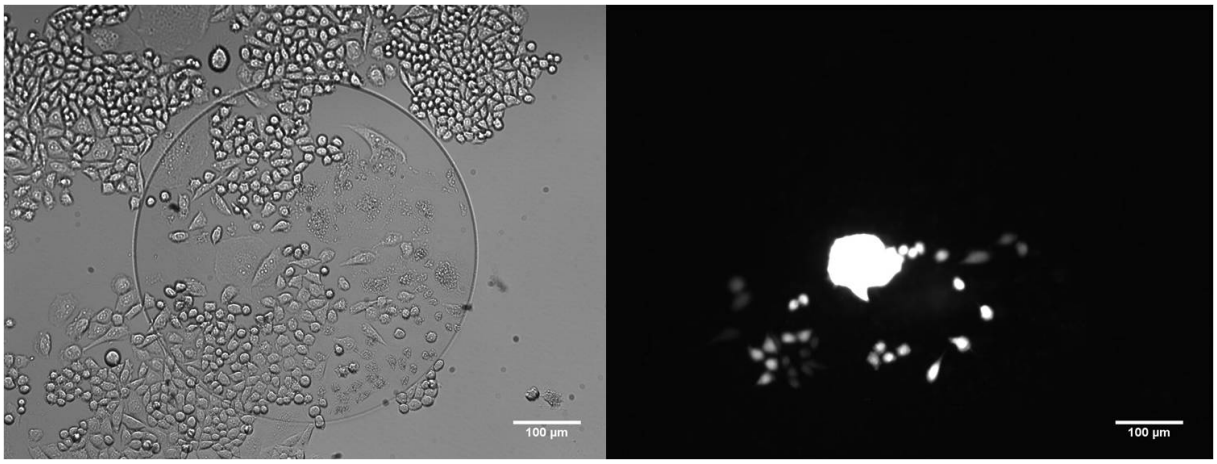


Figure 13.3: On the right, culture after electrotransfection. The round active region is visible in the whole, 500 $\mu$m of diameter, in visible light. A very big cell is in the middle of the chip, not easy to be seen in visible light, it is very bright under UV light. At the same stimulation conditions larger cells appear to be more sensitive, as their adherent surface is higher. Many cells that seem to had big area died.

number of transfected cells is determined on photographs in UV light. The cell counting should be done on images of high exposition time in order to take into account all the transfected cells, even those that produce the protein less than others, as is shown in figure 13.5, otherwise the transfection efficiency can be underestimated. In the following table the results are reported.

The mean value of efficiency is 29.5 $\pm$ 3.9. This is just a preliminary result, new experiments need to be performed, in order to increase the statistical sample.

| n$^o$ transfected cells determined on photos | total n$^o$ of cells determined on photos | efficiency trans.n$^o$/tot $^o$ |
|---|---|---|
| 95 | 321 | 31 % |
| 34 | 184 | 18% |
| 74 | 254 | 29% |
| 60 | 170 | 40% |

## 13.3 A note on DNA adding

The DNA is mixed in the electroporation solution and it is incubated at room temperature 10 minutes. It is then added on the chip few seconds before the stimulation. This choice comes from the patch clamp experiment results that show an increment of membrane conductance starting less than one second after the pulse.

But if the DNA is not mixed with the electroporation solution an incubated, and it is added directly on the electroporation solution on the chip. The electroporation efficiency drops down drammatically.

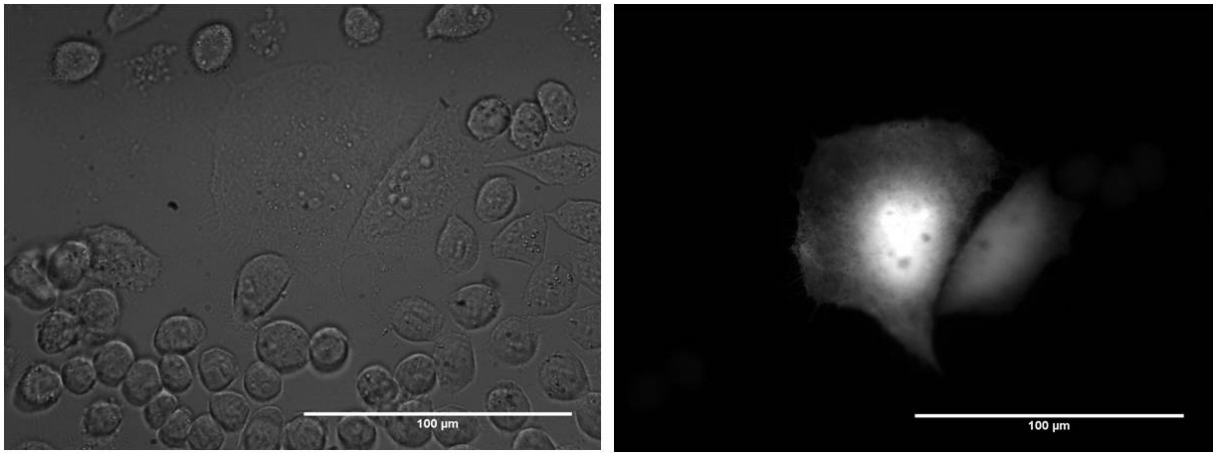


Figure 13.4: The magnification of image 13.3 in UV light lets see that the cells are two, very thin and very adherent to the substrate.

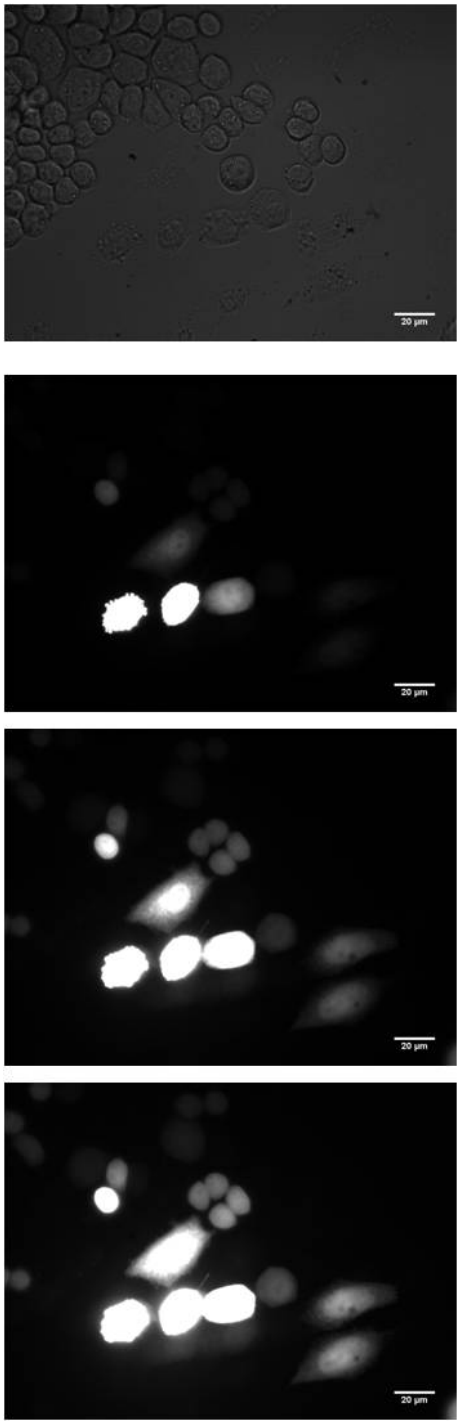


Figure 13.5: Starting on top, cell culture in visible light, cell culture in UV light photographed at different shutter speeds, increasing from top to bottom. In the first UV image cells that produced less protein are almost completely invisible

# Chapter 14

# Conclusions and Outlooks

I demonstrated the biocompatibility of titanium dioxide thin films for cell culture grown in adhesion and the effective operation of the prototype proposed.
I demonstrated the use of patch-clamp technique to effectively perform membrane conductance measurements before, during, and after electroporation events. This allows real-time detection of electroporation events, and monitoring of closing kinetics.
We interpreted the changes of membrane conductance (following an increase and decrease) as a result of the opening and closing of pores in membrane structures. Using this approach, different pulse parameters were evaluated in order to select an effective electrotransfection protocol. I found an effective electrotransfection protocol for the production of CHO-K1 clones, that was the target of the thesis.
Regarding the parameters of the electrotransfection protocol, without making explicit the numbers, as explainded in section 9.1:

- Effect of signal shape. A smooth signal shape is ineffective.
- Effect of stimulation signal amplitude (maximum voltage). There is a voltage threshold for pore opening
- Effect of stimulation duration. Pulse duration affects the pore formation and in particular the resealing. Too short stimulation induces fast opening and closing of pores, even less than a second. On the contrary, too long and high stimulation kills the cells.
- Effect of stimulation repetition: an efficient stimulation can be less efficient if performed again after the complete pore closing.

Some results come as "collateral" ones, on applied purposes and on researching ones:

- On pore dynamics, opening and "life time".
  - I demonstrated that it is possible to record current traces in cell-attached and whole-cell configuration. This paves the way to the study of pores dynamics in principle up to a single pore in attached configuration.
  - I demonstrated that pores form on the free and attached membrane.
  - A conductance peak occurs after the stimulation, than the conductance reduces.

  - The pore opening is fast. I recorded that pore conductance increases less than 500 ms after the starting of stimulation.
  - The decrease in membrane potential to fixed potential did not cause the pore to close immediately. The pore could be maintained opened up to some hundreds of seconds.

- On the selectivity. The selectivity of the device is an important surplus value. Quantitative data from a large number of individual cells provide a wealth of information and insight typically obscured by bulk measurements. Bulk population experiments output the mean value of a parameter of interest, whereas single-cell experiments allow for investigating the distribution of that parameter. This is an important distinction because even cells that are identical genetically exhibit marked variations in gene expression and behavior. Information on a cell's time- or stimulus-dependent gene expression is lost when the sample's specific signal is diluted with those from surrounding cells, which do no exhibit the same expression. The possibility to develop a device with a small stimulation spot can allow experiments on single cell.

- The possibility of a device with a small active stimulation area is also important for the selection of stable clones. It allows to select different clones, with the use of antibodies, avoiding very time consuming procedures.

- Concerning the production on large scale of protein for therapeutics targets take advantages from the possibility of using bigger surface of stimulation. The prototype proposed has no limitation about it and the active stimulation area can be increased.

Future developments are the attempt on neuron cultures concerning the electrotransfection and the study of pores dynamics and dimensions.

# Appendix A

# Chemicals and Solutions

Concentration expressed in mM

**Phosphate Buffer Saline**
$[NaCl]$= 137 , $[KCl]$= 2.7 , $[Na_2HPO_4]$= 10, $[KH_2PO_4]$= 2
pH 7.4, in millipore water,
sterilized by filtration

**CHO-K1 culture medium**
Nutrient Mixture F-12 HAM (Sigma Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% with Fetal Bovin Serum (FBS) (Sigma Aldrich) HAM-F12 with glutamine, 10 % inactivated FBS, 0.1 % penicillin and streptomycin sulphate (PEN/STREP)
pH = 7.4

**VERSENE**
NaCl 9 g/l, EGTA 0.2 g/l
pH 7.2, in millipore water,
sterilized by filtration

**DMSO**
cryopreservation medium
$life$technologies

**Extracellular Solution**
$[NaCl]$= 135, $[KCl]$=5.40 ,$[MgCl_2]$=1.00, $[CaCl_2]$=1.80, $[Glucose]$=10.00, $[HEPES]$=5
pH = 7.4, in millipore water,

**Intracellular Solution**
$[KCl]$=5.40 ,$[MgCl_2]$=1.00, $[EGTA]$=5, $[HEPES]$=5
pH 7.3, in millipore water,
sterilized by filtration

**Electroporation solution** confidential, potentially patent

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