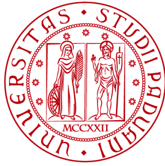

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di Padova

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CORSO DI LAUREA MAGISTRALE IN BIOINGEGNERIA

"CHEMO-MECHANICAL TRIGGERING OF MEMBRANE POTENTIAL AT THE SINGLE CELL LEVEL"

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Abstract

Rapid changes in cell membrane potential are associated with important physiological phenomena such as the release of neurotransmitters in neurons, and contraction in cardiomyocytes. These membrane potential variations are mediated by the activity and the dynamics of ion channels present on the cell membrane. However, these physiological phenomena can become pathological for instance when there is a mutation in the genes that encode the ion channels, causing the onset of serious diseases. Understanding these physiological and pathological processes is tremendously important and requires studying membrane potential variations as a consequence of the triggered activity of ion channels. Many technologies are available to archive this. In particular, two emerging technologies FluoVolt and FluidFM stand out against their competitors, thanks to their versatility. The research project, main topic of this thesis, aims to combine these two technologies, to deliver to the field of biophysics a new methodology for investigating electrophysiological processes that occur in cells.

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Introduction

Cells are the basic functional unit in living organisms. The task of cell biology is to study them from a morphological and functional point of view, in order to deepen human knowledge of the cellular and molecular mechanisms which are at the basis of all vital processes: microscopic and macroscopic. Understanding the physiological mechanisms that regulate the functioning of life is therefore tremendously important to understand also when and where pathological mechanisms born, leading to diseases at the expense of the entire organism.

Despite morphological and functional differences, all living cells have a surrounding cell membrane that separates the internal content of a cell from the external environment. The cell membrane performs multiple functions including regulating the exchange of matter that occurs between intracellular space and extracellular space. Therefore, the cell membrane is an important interface between the cell and the extracellular environment in which there is a multiplicity of fundamental physiological processes which, in turn, can become pathological processes in presence of "malfunctions".

For instance, thanks to the peculiar characteristic of the cell membrane (in particular due to the presence of ion channels) to be selectively permeable to different substances, including ion charges (e.g., Na^+, Ca^{2+}, K^+, Cl^-) the cell undergo a physiological process that generates a different distribution of electrical charges between intracellular and extracellular space, leading to the formation of the transmembrane electric potential and its electrical properties. Through a combination of many physiological mechanisms involving ion channels, the transmembrane potential can vary rapidly, causing important biological processes such as contraction/beating phenomena in heart cells, with obvious consequences on the functioning of the cardiovascular system. If malfunctions appear in this delicate set of processes, diseases such as, for instance, arrhythmias arise.

Among all the physiological processes that govern the functioning of the cell, those associated with the transmembrane potential and its variations are therefore fundamental. Malfunctions, which typically arise due to mutations in the genes encoding ion channels, have important implications in the cardiovascular system (as saw in the example), the nervous system and the muscular system. Ion channels are therefore the subject of important scientific studies, because they can become the target of new drugs development/treatments.

In order to study the pathophysiological processes associated with the transmembrane potential and its variations induced by the activity of ion channels, a method for measuring membrane potential over time and a method to stimulate single-cell (to trigger ion channels activities that can in turn trigger membrane potential responses) are required.
There are many methods to both measure the transmembrane potential and stimulate the single cell, but each of them suffers from several disadvantages. In the scientific research panorama, however, two new technologies have emerged. The first, called FluoVolt, is a method for monitoring membrane potential characterized by a considerable number of advantages over competitors. The second, called FluidFM, is an innovative technology, born in the Laboratory of Biosensors and Bioelectronics (LBB) of Swiss Federal Institute of Technology in Zürich (ETH Zürich) that allows the controlled perturbation, isolation and analysis of the single-cell.

In this broad context it is clear the necessity of studying deeply the electrical properties of the cell in order to understand its pathophysiology. The main topic of this master thesis is the research project that I carried out at LBB, which is is exactly positioned in this context. The project consists in the attempt to combine FluidFM technology (single-cell stimulation) with Fluovolt (membrane potential variations monitoring) to obtain a versatile, innovative and useful method in the field of electrophysiology studies, paving the way for new investigation opportunities in cellular processes involving transmembrane potential and ion channel studies.

In particular, the goal was to work on a proof-of-concept project that could demonstrate the feasibility, through ad-hoc designed experiments, of combining FluoVolt and FluidFM for the measurement of membrane potential variations, under chemical and/or mechanical stimulation of single cell, remembering that single cell studies have substantial impact on biology and medicine, providing more details on cell functions, cell-to-cell interactions and cell responses to exogenous stimuli.
During the course of the project many challenges were encountered. They have been described in the thesis together with the solutions adopted to tackle them.

The thesis is divided as follows.

In the first chapter the fundamentals of cellular electrophysiology are summarized, highlighting the most important points to understand how the electrical properties of the cell and their physiopathological implications arise.
The second chapter presents various methodologies available for monitoring membrane potential, their advantages and the disadvantages that lead FluoVolt to emerge among competitors, by representing a smart and convenient choice to measure membrane potential.
Similarly, in the third chapter some of the various methods available for the stimulation of single-cells are presented, with advantages and disadvantages that lead to the choice of using the versatility and capabilities offered by the FluidFM. Substantial part of this third chapter is precisely spent to describe in detail some technical sides of this technology, and some of its most important applications in biophysics.
The fourth chapter describes the first attempt to realize this proof of concept project, through the use of adult mouse primary cardiomyocytes during experiments.
Due to some technical and biological problems that have been described, experiments have also been conducted with another type of cells: HEK293 cells. The experiments carried out with the latter and the results obtained from them are described in the fifth (final) chapter.
The thesis ends with a brief recap of what has been done and the results obtained.

Chapter 1

Cell membrane and electrical properties

Despite differences in structure and function, all living cells in multicellular organisms have a surrounding cell membrane. Just as the outer layer of the skin separates the body from its environment, the cell membrane separates the inner contents of a cell from its exterior environment. This cell membrane provides a protective barrier around the cell and regulates which materials can pass in or out. By being able to select what passes through it, the cell is able to create an ion concentration difference between intracellular and extracellular space. This leads to the creation of an electrical potential difference between inside and outside of the cell, which has an important impact physiologically and pathologically. This chapter first discusses the basic characteristics of the cell membrane and how the cell is able to create and maintain an electrical potential difference between intracellular space and extracellular space. The consequences and importance of variations in the membrane electric potential are then emphasized. Finally it is given a brief description of what ion channels are, why they produces variations of the membrane potential, and why it is critical to learn more about their functions and how they work.

1.1 Structure and composition of the cell membrane

The cell membrane is an extremely pliable structure composed primarily of two layers of phospholipids, called bilayer. Cholesterol and various proteins are embedded within the membrane giving the membrane a variety of functions that will be described below.
A single phospholipid molecule has a phosphate group on one end, called the “head” and two side-by-side chains of fatty acids that make up the lipid “tails”. The lipid tails of one layer face the lipid tails of the other layer, meeting at the interface of the two layers. The phospholipid heads face outward: one layer exposed to the interior of the cell and one layer exposed to the exterior (Figure 1.1) The phosphate group is negatively charged, making the head polar and hydrophilic. The phosphate heads are thus attracted to the water molecules

of both the extracellular and intracellular environments. The lipid tails, on the other hand, are uncharged, or nonpolar, and are hydrophobic. A hydrophobic molecule (or region of a molecule) repels and is repelled by water. Phospholipids are thus amphipathic molecules. An amphipathic molecule is one that contains both a hydrophilic and a hydrophobic region.

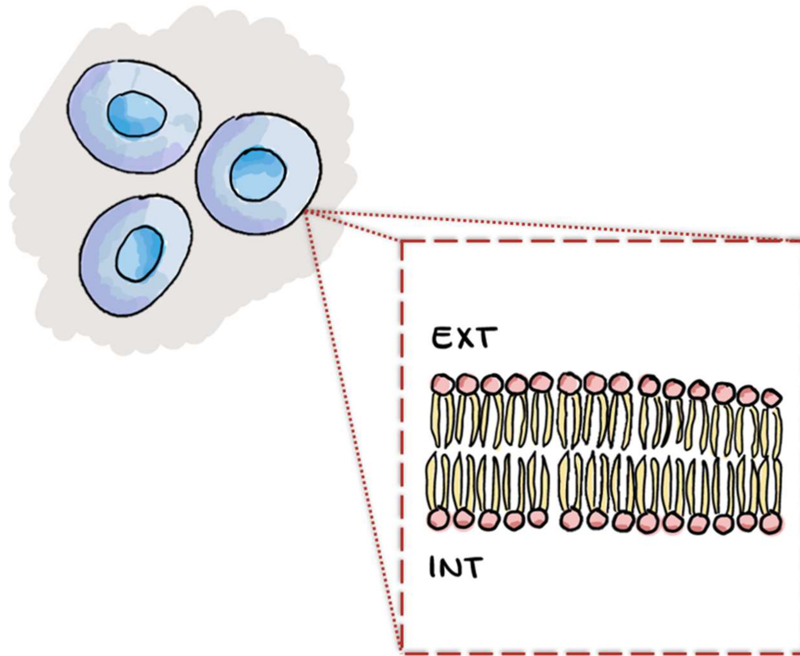

Figure 1.1. *The cell membrane consists of a phospholipid bilayer. The phospholipid heads face outward: one layer is exposed to the interior of the cell and one layer is exposed to the exterior.*

1.2 Membrane proteins

The lipid bilayer forms the basis of the cell membrane, but it is peppered throughout with various proteins. Two different types of proteins that are commonly associated with the cell membrane are the integral protein (Figure 1.2) and peripheral protein. An integral protein is a protein that is embedded in the membrane. Many different types of integral proteins exist, each with different functions. For example, an integral protein that extends an opening through the membrane for ions to enter or exit the cell is known as a channel protein. Peripheral proteins are typically found on the inner or outer surface of the lipid bilayer but can also be attached to the internal or external surface of an integral protein. All these types of membrane proteins perform many functions. Among all of them, one extremely important function carried on through integral protein is the transport of substances.

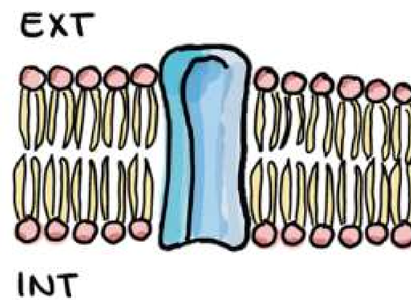

Figure 1.2. *Integral proteins connect intracellular and extracellular spaces together.*

1.3 Transport across the cell membrane

One of the great capabilities of the cell membrane is its ability to regulate the concentration of substances inside the cell. These substances include ions such as Ca^{++}, Na^{+}, K^{+}, and Cl^{-}, nutrients including sugars, fatty acids, and amino acids, and waste products, particularly carbon dioxide (CO_2), which must leave the cell.

The membrane's lipid bilayer structure provides the first level of control. The phospholipids are tightly packed together, and the membrane has a hydrophobic interior. This structure causes the membrane to be selectively permeable; this means that is allows only substances meeting certain criteria to pass through it unaided. In particular only relatively small, nonpolar materials can move through the lipid bilayer. Some examples of these are other lipids, oxygen, carbon dioxide gases, and alcohol. However, water-soluble materials like glucose, amino acids, and electrolytes need assistance to cross the membrane because they are repelled by the hydrophobic tails of the phospholipid bilayer. All substances that move through the membrane do so by one of two general methods, which are categorized based on whether or not energy is required. Passive transport is the movement of substances across the membrane without the expenditure of cellular energy. In contrast, active transport is the movement of substances across the membrane using energy from adenosine triphosphate (ATP), the energy of the cell.

1.3.1 Passive transport

Whenever a substance exists in greater concentration on one side of a semipermeable membrane, such as cell membranes, any substance that can move down its concentration gradient (from high concentration to low concentration) across the membrane will do so. A

concentration gradient is the difference in concentration of a substance across a space. Molecules (or ions) will spread/diffuse from where they are more concentrated to where they are less concentrated until they are equally distributed in that space. Diffusion is the movement of particles from an area of higher concentration to an area of lower concentration.

When the substances can move across the cell membrane without the cell expending energy, the movement of molecules is called passive transport. Substances that can easily diffuse through the lipid bilayer of the cell membrane, such as small, fat soluble gasses and other small lipid soluble molecules like gases oxygen (O_2) and carbon dioxide (CO_2) can dissolve in the membrane and enter or exit the cell following their concentration gradient. This mechanism of molecules moving across a cell membrane from the side where they are more concentrated to the side where they are less concentrated is a form of passive transport called *simple diffusion*. O_2 generally diffuses into cells because it is more concentrated outside of them, and CO_2 typically diffuses out of cells because it is more concentrated inside of them. The concentration gradients for oxygen and carbon dioxide will always exist across a living cell and never reach equal distribution. This is because cells rapidly use up oxygen during metabolism and so, there is typically a lower concentration of O2 inside the cell than outside. As a result, oxygen will diffuse from outside the cell directly through the lipid bilayer of the membrane and into the cytoplasm within the cell. On the other hand, because cells produce CO2 as a byproduct of metabolism, CO2 concentrations rise within the cytoplasm; therefore, CO2 will move from the cell through the lipid bilayer and into the extracellular fluid, where its concentration is lower.
Large polar or ionic molecules, which are hydrophilic, cannot easily cross the phospholipid bilayer. Charged atoms or molecules of any size cannot cross the cell membrane via simple diffusion as the charges are repelled by the hydrophobic tails in the interior of the phospholipid bilayer. Solutes dissolved in water on either side of the cell membrane will tend to diffuse down their concentration gradients, but because most substances cannot pass freely through the lipid bilayer of the cell membrane, their movement is restricted to protein channels and specialized transport mechanisms in the membrane. Facilitated diffusion is the diffusion process used for those substances that cannot cross the lipid bilayer due to their size, charge, and/or polarity but do so down their concentration gradients. For instance, even though sodium ions (Na^+) are highly concentrated outside of cells, these electrolytes are charged and cannot pass through the nonpolar lipid bilayer of the membrane. Their diffusion is facilitated by membrane proteins (Figure 1.3) that form sodium channels (or “pores”), so that Na^+ ions can move down their concentration gradient from outside the cells to inside the cells.

A common example of facilitated diffusion using a carrier protein is the movement of glucose into the cell, where it is used to make ATP. Although glucose can be more concentrated outside of a cell, it cannot cross the lipid bilayer via simple diffusion because it is both large and polar, and therefore, repelled by the phospholipid membrane. To resolve this, a specialized carrier protein called the glucose transporter will transfer glucose molecules into the cell to facilitate its inward diffusion. There are many other solutes that must undergo facilitated diffusion to move into a cell, such as amino acids, or to move out of a cell, such as wastes. Lastly, a specialized example of facilitated transport is water moving across the cell membrane of all cells, through protein channels known as aquaporins. This process, called osmosis, is the diffusion of water through a semipermeable membrane.

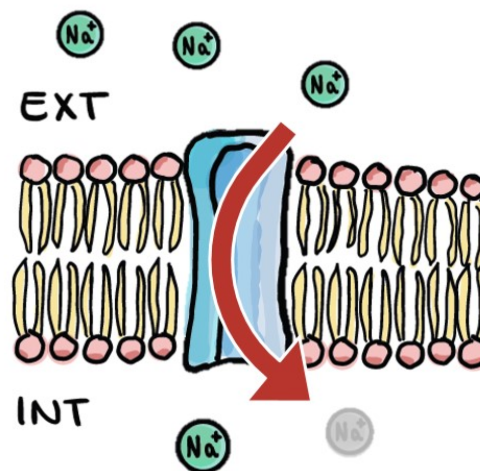

Figure 1.3. *Ion channels allow specific ions to pass through the cell membrane. For passive transport, ions move down their concentration gradients.*

1.3.2 Active transport

For all of the transport methods described above, the cell expends no energy. Membrane proteins that aid in the passive transport of substances do so without the use of ATP. During active transport ATP is required to move a substance across the membrane, with the help of membrane protein, and against its concentration gradient. One of the most common types of active transport involves proteins that serve as pumps. Energy from ATP is required for these membrane proteins to transport substances (molecules or ions) across the membrane, against their concentration gradients: from an area of low concentration to an area of high concentration. The sodium-potassium pump, which is also called Na^+/K^+ ATPase,

transports sodium out of a cell while moving potassium into the cell. The Na^+/K^+ pump is an important ion pump found in the membranes of all cells.

1.3.3 Other forms of membrane transport

Other forms of active transport do not involve membrane carriers. Endocytosis, for example, is the process of a cell ingesting material by enveloping it in a portion of its cell membrane, and then pinching off that portion of membrane. Endocytosis often brings materials into the cell that must to be broken down or digested. In contrast with endocytosis, exocytosis is the process of a cell exporting material using vesicular transport. Many cells manufacture substances that must be secreted. These substances are typically packaged into membrane-bound vesicles within the cell. When the vesicle membrane fuses with the cell membrane, the vesicle releases its contents into the interstitial fluid.

1.4 Resting membrane potential

The resting membrane potential is the result of the movement of several different ion species (mainly potassium, sodium, calcium, and chloride) through various ion channels and transporters (uniporters, cotransporters, and pumps) in the plasma membrane, with sodium (Na^+) and potassium (K^+) providing a dominant influence (Figure 1.4). Various negatively charged intracellular proteins and organic phosphates that cannot cross the cell membrane are also contributory. These movements result in different electrostatic charges across the cell membrane and they exist due to the differences in membrane permeability to those ions, which in turn result from functional activity of various ion channels, ion transporters, and exchangers. It is important to notice that sodium, calcium and chloride ion concentrations are lower inside the cell than outside, and the potassium concentration is greater inside the cell. Neurons and muscle cells are excitable such that these cell types can transition from a resting state to an excited state. The resting membrane potential of a cell is defined as the electrical potential difference across the plasma membrane when the cell is in a non-excited state. The resting membrane potential is a relatively stable, ground value of transmembrane voltage in cells. Traditionally, the electrical potential difference across a cell membrane is expressed by its value inside the cell relative to the extracellular environment and it is around -70mV

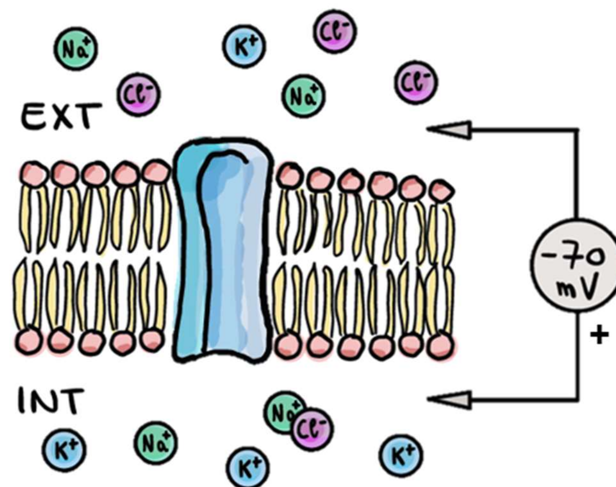

Figure 1.4. *The resting membrane potential is the result of the movement of several different ion species through various ion channels and transporters. Typically, it is around -70mV.*

1.5 Membrane potential variations

The membrane potential may change over time. In particular, it is known that the activation of different ion channels, in different moments of time but in an orderly manner, can provoke large variations in membrane potential, in a process called depolarization of the cell membrane. This process can even lead to a more positive intracellular space than extracellular space.

Typically, these large variations occur in the so-called excitable cells. In general, the term excitable cell refers to the ability of some cells to be electrically excited, resulting in the generation large variations in membrane potential. Neurons, muscle cells (skeletal, cardiac and smooth) and some endocrine cells (e.g., pancreatic β cells that release insulin) are excitable cells. In these cases, variations occur in the order of milliseconds and are called action potentials. An action potential is generated by the rapid inflow of Na^+ ions followed by a slightly slower outpouring of K^+ ions. These rapid changes in membrane potential are very important from a physiological point of view. For example, in neurons action potentials allow the release of neurotransmitters in the post-synaptic region allowing communication between neurons. At the same time, in cardiac muscle cells, called cardiomyocytes, the action potentials depolarize the cell allowing it to contract. This microscopic mechanism of cell contraction underlies the macroscopic contraction of the heart.

Then there are a whole other kind of variations of the membrane potential (ΔV_{mem}), which happen on different timescales compared to the one of action potentials: seconds, hours, days (Figure 1.5).

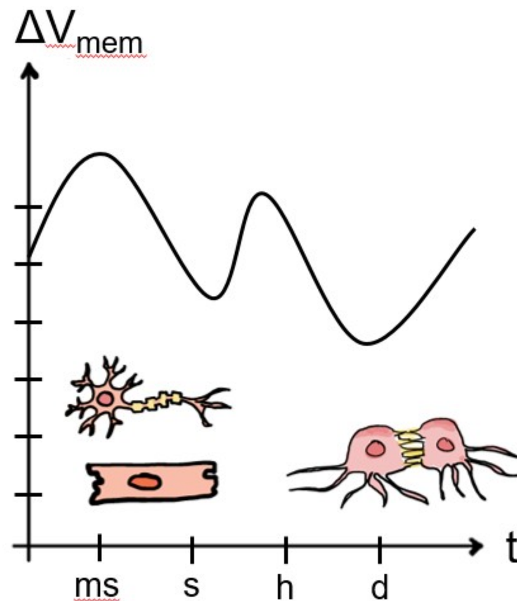

Figure 1.5. *Variations of the membrane potential can happen at different timescale: rapid and large variations are associated with action potentials, having important physiological consequences like neurotrasmitters release from neurons and contraction in cardiomyocytes. Variations in larger timescales have other consequences, like cell proliferation*

For instance, in healthy cells, the cell cycle is a key parameter of cellular behavior that must be tightly regulated and coordinated during morphogenesis associated with development and regeneration. In addition to this normal turnover, changes in membrane potential have been linked to the proliferation of cells during wound healing. In cultured cells, modulation of V_{mem} using K^+ channel-inhibiting drugs increases wound healing in cell monolayers. This modulation of the voltage gradient across an epithelium is a crucial component of wound healing and normal development. Furthermore, in human endothelial cells, modulation of V_{mem} through applied electric fields revealed that hyper-polarizing currents arrest cell division.

Changes in membrane potential are not only important physiologically, but also pathologically. Neoplasia, the uncontrolled and uncoordinated growth of a group of cells, to the detriment of tissue homeostasis, has long been associated with aberrant changes in cell cycle. Alterations in membrane potential and ion channel expression/function have been observed in a wide array of cancers.

It is therefore clear that it is important to study changes in membrane potential, on different timescales, to better understand the functioning of both physiological and pathological cellular processes.

1.6 Ion channels

Ion channels are essential for life since they play a fundamental role in physiological processes, such as neuronal signaling, muscle contraction, and nutrient transport. Ion channels are pore-forming proteins, located in the plasma membrane of virtually all living cells. Ion channels also exist in membranes of intracellular organelles, such as the endoplasmic reticulum, endosomes, lysosomes, and mitochondria. They form aqueous pores across the lipid bilayer and they are often highly selectively. This allow the flow of particular inorganic ions, primarily sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), or chloride (Cl^-). These flows are involved and play critical roles in very important physiological and pathological processes, such as neuronal signaling and cardiac excitability, therefore, they serve as therapeutic drug targets.
A multitude of diseases result from the disruption of normal ion channel function. Disorders resulting from a mutation in the gene encoding an ion channel or its regulatory proteins are called channelopathies. Common channelopathies include cystic fibrosis and long QT syndrome. Among the inherited mutations of ion channels are neurological disorders, e.g. neuromuscular and movement, migraine, pain, depression, and epilepsy, as well as, cardiovascular diseases and other human disorders.
Gating of ion channels involves the process of opening and closing of the pore. This is determined by various factors, including external or internal ligands, mechanosensitivity, voltage, thermosensitivity, etc., permitting ions either to pass through or block the ion flow. In one single second, an open channel can pass up to ten million ions in and out of a cell.

1.6.1 Voltage-gated ion channels

Voltage-gated ion channels are a class of transmembrane proteins that form ion channels that are activated by changes in the electrical membrane potential near the channel. Voltage-gated ion channels are generally composed of several subunits arranged in such a way that there is a central pore through which ions can travel down their electrochemical gradients. The functionality of voltage-gated ion channels is attributed to its three main discrete units: the voltage sensor, the pore or conducting pathway, and the gate. The

membrane potential alters the conformation of the channel proteins, regulating their opening and closing. They have a crucial role especially in excitable cells such as neuronal and muscle tissues, allowing a rapid and co-ordinated depolarization in response to triggering voltage change. Found along the axon and at the synapse, voltage-gated ion channels directionally propagate electrical signals. In particular, voltage-gated sodium channels (Na_vs) are responsible for generating the Na^+ currents underlying the initiation and propagation of action potentials in nerves and muscle fibers.

In primary sensory neurons, for example, the depolarization of an axon by a noxious stimulus leads to the transmission of sensory information through the nervous system to the brain which may be perceived as pain. In skeletal and cardiac muscle cells, received action potentials produce muscle contraction enabling body movements and blood flow. Often, Na_v channels are the molecular targets for a broad range of natural neurotoxins. Voltage-gated ion-channels are usually ion-specific, and channels specific to sodium, potassium, calcium, and chloride ions have been identified. The opening and closing of the channels are triggered by changing ion concentration, and hence charge gradient, between the sides of the cell membrane.

1.6.2 Mechanosensitive ion channels

Mechanosensitive ion channels (MSCs) are multimeric integral membrane proteins that translate mechanical signals into a biochemical response. They respond to increased lipid bilayer tension (e.g., lipid packing, bilayer thickness, curvature, and/or lateral pressure profile) by opening their pores to release solutes and relieve increased cytoplasmic pressure. In particular they alter their conformation between an open state and a closed state as a response to mechanical stimulus, transducing mechanical forces into ion fluxes (Figure 1.6). MSCs are found in all types of cells ranging from bacteria to animal and human cells. Different types of channels vary in selectivity for the permeating ions: from nonselective between anions and cations in bacteria, to cation selective allowing passage Ca^{2+}, K^+ and Na^+ in eukaryotes.

One type of mechanically sensitive ion channel activates specialized sensory cells, such as cochlear hair cells and some touch sensory neurons, in response to forces applied to proteins. Therefore, they are the sensors for a number of systems including the senses of touch, hearing and balance, as well as participating in cardiovascular regulation and osmotic homeostasis (e.g. thirst).

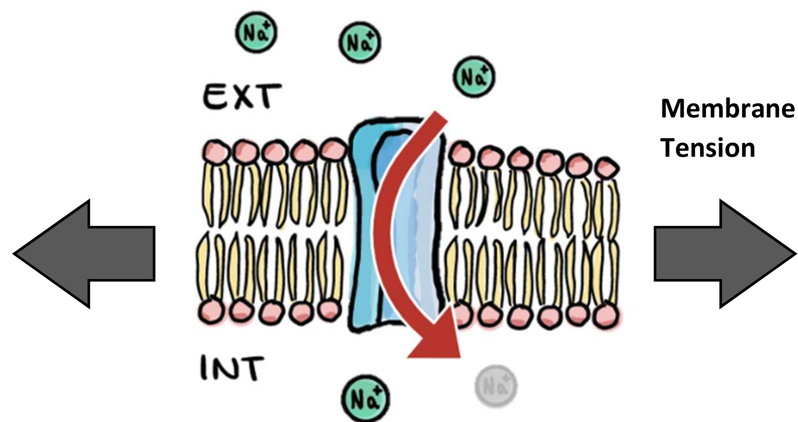

Figure 1.6. *Gating Mechanism of MSCs stretch activated model: tension in the lipid bilayer triggers conformational changes which open the channel.*

Apart from mechanosensation, MSCs have been implicated in several basic cellular functions, including the regulation of cell volume, cell shape, motility, growth, and cell death. Because abnormalities in MSCs may also contribute to major human diseases, including muscular dystrophy, kidney disease, cardiac arrhythmias, hypertension, and tumor cell invasion ("mechanochannelopathies"), there is great interest in identifying the molecules that form MSCs and discovering agents that can selectively block their activity and/or expression.

Chapter 2

Recording membrane potential

Membrane potential (V_{mem}) is a fundamental biophysical signal present in all cells. V_{mem} signals range in time from milliseconds to days, and they span lengths from microns to centimeters. V_{mem} affects many cellular processes, ranging from neurotransmitter release to cell cycle control to tissue patterning. For this reasons it is very interesting to measure the membrane potential and its variations. There are many techniques to do this, and these can be classified into two major categories: Electrode-based and optical-based. In this chapter we describe some of these techniques, analyzing their advantages and disadvantages. Finally, an innovative compound, called FluoVolt, belonging to optical based techniques, is described, along with advantages and disadvantages that led to its selection as the method of measurement for membrane potential variations in the research project of this thesis, during mechano-chemical stimulation of single cells.

2.1 Electrode-based approaches

Electrode-based techniques are the gold standard for recording V_{mem}, ion channel properties, and electrical currents in cells. Three primary configurations are used in cellular Vmem recordings: whole-cell patch clamp, cell attached, and perforated patch (Figure 2.1). All three can quantify absolute V_{mem} with excellent temporal resolution, but they suffer from high invasiveness, instability over time, low throughput, and poor spatial resolution.
In intracellular recording, V_{mem} is determined from the difference between a recording electrode in the cytosol and a reference electrode in the extracellular solution. Two main types of intracellular recordings exist: *sharp electrode* and *whole-cell recordings*.

Due to very fine tips and high tip resistances, *sharp microelectrodes* produce a variable tip potential and a V_{mem} artifact on the order of tens of millivolts. Furthermore, they create a leak in the cell of interest, producing artificially depolarized apparent V_{mem} and altered

input resistance. Sharp electrode recordings have largely been replaced by intracellular recordings in the whole-cell configuration.

Whole-cell intracellular recordings use much larger patch electrodes with lower tip potentials; such electrodes can be sealed onto the cell with less damage than sharp electrodes. Whole-cell recordings can also provide higher-resolution information about the particular ions and types of channels involved in setting V_{mem}. They provide a direct, in situ measurement of V_{mem} with sub-mV precision and excellent temporal resolution, but this measurement suffers from a variety of drawbacks. First, the wholecell patch is highly invasive and can disrupt the very processes under investigation. By dialyzing the cytosol with electrode internal solution, whole-cell patch clamp washes out soluble signaling factors. In addition, physical contact with the electrode can activate mechanosensitive channels and signal transduction pathways introducing further artifacts. Errors in recorded V_{mem} can occur also due to leaks in the seal between the recording electrode and the membrane. Lastly, an electrode has poor spatial resolution: reporting V_{mem} at a point of contact may or may not reflect other parts of the cell or tissue. Furthermore, intracellular recordings are challenging to execute. Attempting each whole-cell recording takes an expert researcher or specialized robot 5–10 minutes, with a 10–30% success rate for these efforts.

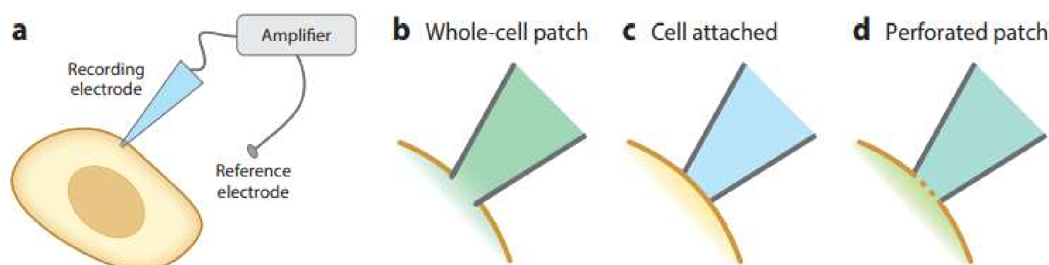

Figure 2.1. *Electrophysiological configurations for Vmem recording. (a) General schematic for electrode-based Vmem recordings, in which a cell comes into direct contact with an electrode. (b–d) Voltage is measured as the difference between the recording electrode and a reference electrode in the bath solution. (b) In whole-cell patch-clamp electrophysiology, the plasma membrane is ruptured, and the cytosol mixes with the recording electrode solution. (c) In the cell-attached configuration, the plasma membrane is left intact. (d) In perforated patch, the membrane is not ruptured, but ionophores introduced into the recording solution allow ionic exchange across the membrane.*

To mitigate the washout of soluble factors associated with whole-cell recording, V_{mem} must be measured without disrupting the plasma membrane. This is possible in the cell-attached

configuration, in which an extracellular patch electrode is sealed onto the cell without rupturing the membrane.
Perforated patch recordings offer a more general solution to reduce invasiveness of whole-cell recordings. In perforated patch, an ionophore placed in the pipette internal solution allows ions to cross the membrane without completely disrupting it, thereby electrically connecting the cytosol with the pipette solution. An ionophore is a chemical species that reversibly binds ions. Many ionophores are lipid-soluble entities that transport ions across the cell membrane. They catalyze ion transport across lipid bilayers like cell membranes. Structurally, an ionophore contains a hydrophilic center and a hydrophobic portion that interacts with the membrane. The two broad classifications of ionophores are: *carrier ionophores* that bind to a particular ion and shield its charge from the surrounding environment. This makes it easier for the ion to pass through the hydrophobic interior of the lipid membrane. The other types are *channel formers.* They introduce a hydrophilic pore into the membrane, allowing ions to pass through without coming into contact with the membrane's hydrophobic interior. Channel forming ionophores are usually large proteins. Ionophores (and thus perforated patch recordings) carry some drawbacks: they modify the permeability of biological membranes toward certain ions to which they show affinity and selectivity, disrupting the membrane potential. Therefore, these substances could exhibit cytotoxic properties. Because the membrane remains impermeable to the diffusion of larger molecules, perforated patch recording can last over an hour without run-down of relevant cytosolic factors.

Developments in the design of electrodes have reduced invasiveness and improved throughput of electrode-based recordings. Planar patch clamp and nanoscale electrodes represent two examples.

Planar patch clamp uses low-noise arrays of patch electrodes to perform simultaneous, automated electrophysiological experiments in multi-well arrayed formats. These systems are increasingly used to screen compound libraries against ion channel targets, expediting the drug discovery process. However, the system requires a suspension of dissociated cells, which precludes recording in situ and over extended time courses. As a result, planar patch clamp is more suited for high throughput screening of ion channel pharmacology than for analysis of cellular or tissue-level voltage signaling.

The development of *nanopipettes and nanopillar* electrodes has enabled electrode-based access to broader length scales. Nanopipettes are a smaller version of sharp microelectrodes; their reduced size brings flexibility and access to small cellular compartments. In addition, the flexibility of the nanopipette allow for in vivo recordings

during animal behavior. As with sharp electrodes, the high tip resistances of the nanopipettes filter the signal and create a shunt that changes the observed V_{mem}, so the output signal must be processed to obtain accurate results. Nanopillar electrode arrays enable simultaneous intracellular recordings from many cells in a culture via a grid of nanoscale electrodes. These platforms facilitate mapping of electrical activity across neuronal circuits or beating cardiomyocytes. However, the cells of interest must be cultured directly onto the electrode grid.

2.2 Optical-based approaches

A variety of optical V_{mem} measurement strategies use fluorescence as a proxy for V_{mem}, employing small-molecule or protein-based sensors engineered to display V_{mem}-sensitive fluorescence. Optical platforms generally enjoy excellent spatial resolution, low invasiveness, and medium throughput. Disadvantages include difficulties in achieving the requisite temporal resolution, voltage resolution, and signal-to-noise ratio. Optical strategies fall into four categories: single-color intensity (Figure 2.2), ratio based, pump probe, and single-color lifetime. In all cases, rigorous calibration should be performed to relate the optical signal to the underlying absolute V_{mem}, which is most effectively achieved by performing the optical recording while V_{mem} is tuned across a range of known values. If calibration is not performed, only changes in the potential can be measured. A key difference among the various fluorescence-based strategies is voltage resolution, which is a result of the reproducibility of this calibration over time and between cells. In the next paragraph only single-color intensity probes are discussed.

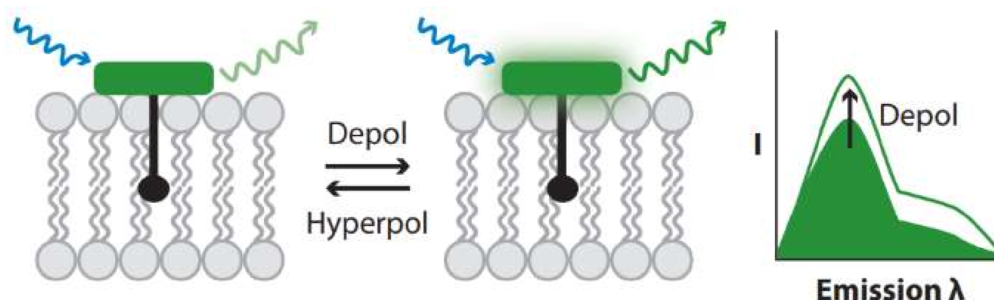

Figure 2.2. *Single-color fluorescence intensity recordings detect changes in V_{mem} by changes in a sensor's fluorescence quantum yield (shown), extinction coefficient, or cellular concentration. This technique generally cannot report absolute V_{mem} optically, although estimates can be made if calibrations are performed for every cell of interest.*

In single-color fluorescence intensity–based recordings, V_{mem} affects the quantum yield or absorption coefficient of a sensor, producing changes in its fluorescence emission. Therefore, to capture the optical signal from cells loaded with these substances, cells have to be shined by a laser at the excitation wavelength of the used substance, and the emitted light at the correct wavelength have to be captured.
Fluorescence intensity of a voltage sensor in a membrane is recorded over seconds or minutes, enabling detection of V_{mem} changes and characterization of V_{mem} waveforms. These sensors are typically small-molecule dyes which display sufficiently fast temporal resolution to follow millisecond-time action potentials. Single-color fluorescence intensity is best suited for V_{mem} event detection and generally cannot report absolute V_{mem}. Methods that rely solely on fluorescence intensity to monitor V_{mem} display large differences in baseline signal between cells and over minutes to hours. These variations are V_{mem} independent, arising from variable sensor loading, cell morphology, sensor photo bleaching, fluorescence quenching, and illumination intensity. As a result, calibrations made on one cell at a given time cannot be extended to other cells or even to the same cell hours later. Electrophysiological calibration of each individual cell is often challenging or impractical, so fluorescence intensity calibrations are generally performed with less accurate pharmacological strategies, which are prone to artifacts, or they are not calibrated at all if just variations of V_{mem} are considered. When calibrating from a single pharmacological set point, rather than a range of V_{mem} set by an electrode, the researcher must assume that the fractional change in fluorescence (%$\Delta F/F$) per mV of the indicator is the same for all samples. In reality, the $\Delta F/F$ response to a given ΔV_{mem} will depend on the ratio of productively engaged sensor to background sensor, which may vary in space with trafficking or loading of the sensor. Furthermore, even with electrode-based calibration, many voltage sensors display sensitivity to membrane composition which varies not only between cells but also within cells with complex membrane structures. Calibration for these types of sensors, being complex and difficult to interpret, is often avoided. They are therefore used for the study of relative changes in membrane potential.

Single-color intensity probes are generally characterized as slow- or fast-response probes (Figure 2.3).

Fast-response probes are molecules that change their structure in response to the surrounding electric field: they can detect transient (millisecond) potential changes, but they suffer from low sensitivity, typically 2–10% $\Delta F/F$ per 100 mV. Fast-response probes are commonly used to image electrical activity from intact heart tissues or measure membrane potential changes in response to pharmacological stimuli.

Slow-response dyes function by entering depolarized cells and binding to proteins or membranes. They are characterized by high sensitivity, typically 1% ΔF/F per 1 mV. Increased depolarization results in additional dye influx and an increase in fluorescence, while hyperpolarization is indicated by a decrease in fluorescence. Slow-responding probes are often used to explore mitochondrial function and cell viability.

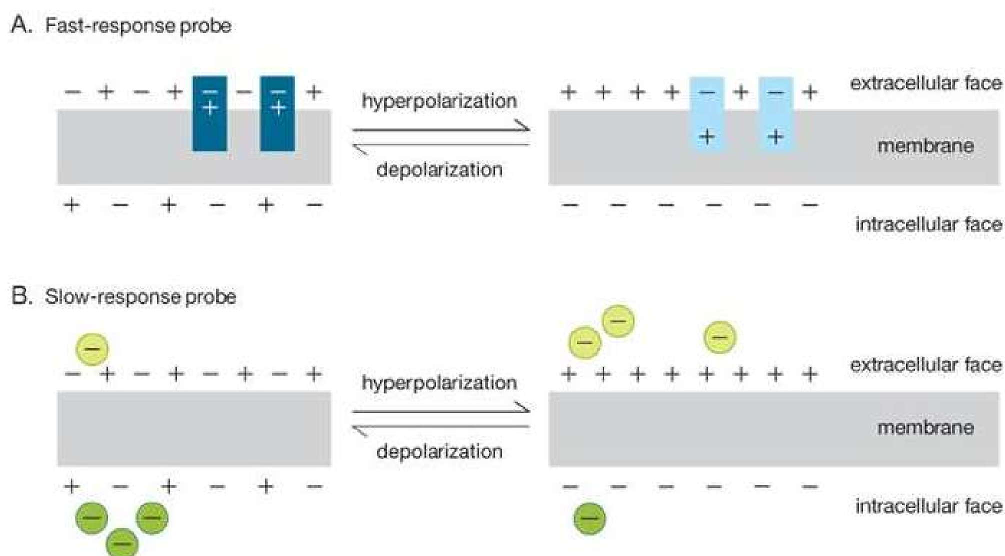

Figure 2.3. *Response mechanisms of membrane potential–sensitive probes. (A) Fast-response probes undergo electric field–driven changes of intramolecular charge distribution that produce corresponding changes in the spectral profile or intensity of their fluorescence (represented by color changes in the illustration). (B) Slow-response probes are lipophilic anions (in this illustration) or cations that are translocated across membranes by an electrophoretic mechanism. Fluorescence changes associated with transmembrane redistribution (represented by color changes in the illustration) result from sensitivity of the probe to intracellular and extracellular environments. Thus, potentiometric response speeds directly reflect the time constants of the underlying processes—fast intramolecular redistribution of electrons versus relatively slow transmembrane movement of entire molecules.*

2.3 Fluovolt

It is possible to summarize the advantages and disadvantages of techniques for measuring membrane potential with a summary table (Figure 2.4). It is clear, therefore, that electrode-based methods hold a great advantage in terms of voltage-resolution and temporal-resolution. But they suffer from invasiveness, low spatial resolution and a complicated

procedure to implement, highly labor dependent skills. These disadvantages make electrode-based techniques low throughput. Optical techniques, on the other hand, solve almost all the disadvantages introduced by electrode-based techniques, but require highly sophisticated cameras to achieve high FPS values (frames per second), especially when it is necessary to capture rapid changes in intensity in fast-response probes. Instrumentation, in optical-based techniques, is therefore a limiting factor, typically in terms of costs. The biggest disadvantage of optical-based techniques is the necessity to choose between a fast-response probe and a slow-response probe. This corresponds to a tradeoff between a sensor that allows to measure rapid changes in membrane potential but characterized by low sensitivity, and a sensor with opposite characteristics. This makes optical-based techniques disadvantageous because they are not versatile in being able to measure both slow and rapid changes in membrane potential at the same time.

Electrode-based	Optical-based (single-color intensity)
Voltage resolution (1mv) Temporal resolution (µs-ms)	Tradeoff - low sensitivity, fast * (µs-ms) - high sensitivity, slow (min)
Invasive	Non invasive
Low spatial resolution	High spatial resolution*
Low throughput	Medium throughput
Complicated procedure	Easy procedure (- Image processing; - Signal interpretation; - Just variations)

*Camera dependent

Figure 2.4. *Comparison of the advantages and disadvantages of electrode-based and optical-based techniques for measuring membrane potential variations.*

In the panorama of single-intensity fluorescence optical probes, a probe called FluoVolt (FV) was released in 2017 by ThermoFisher Scientific (Figure 2.5). FluoVolt represents the next generation in voltage sensitive fluorescent probe-based kits. The FluoVolt probe brings together the best characteristics of the fast and slow response membrane potential fluorescent probes. It responds to changes in membrane potential in sub-milliseconds because the molecule, which stain the cell membrane, changes its structure in response to the surrounding electric field.

Furthermore it displays a high magnitude of response: the high sensitivity is characterized by a response range which is typically 25% per 100 mV. Emission/excitation works with standard FITC settings. Specific settings for FV are 470nm for excitation and 540nm for emission.

These high performances of FluoVolt both in sensitivity and response-speed make this substance extremely versatile for the measurement of membrane potential variations over different timescales: from milliseconds, up to seconds, minutes and hours, allowing to study a wide variety of biological phenomena and biological processes affecting the electrical properties of the cell. At the same time, all the advantages that brings the usage of an optical method for ΔV_{mem} measurement make FluoVolt an extremely convenient option such that it has been implemented as a solution for membrane potential measurement in the research project that is part of this thesis.

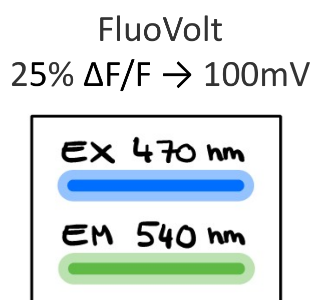

Figure 2.5. *FluoVolt main characteristics: sensitivity and laser excitation/emission fluorescence settings.*

Being an optical probe, Fluovolt is certainly easier to use than electrode-based techniques: it is only needed to load the substance into the buffer in which the cells are maintained during imaging. However, what becomes more difficult, at the expense of having a simplified procedure for data acquisition, is the image processing required to extrapolate a representative ΔV_{mem} signal, the necessity to interpret the signal to check if artifacts have been introduced, and the possibility of measuring only relative potential changes and not absolute values. This will become clear in next chapters. Nevertheless, FluoVolt remains an extremely convenient option because of the advantages described above.

Chapter 3

Single-cell stimulation

Variations in membrane potential are associated with important physiological and pathological cellular processes. These variations depend, ultimately, on the activity of ion channels present in the cell membrane. Monitoring changes in membrane potential is therefore a method for studying the behavior and the dynamics of ion channels. Deep understanding of ionic channels is of fundamental biological importance, with implications in the field of medicine.

Mutations, in fact, are often associated with serious pathologies (e.g., mutation in genes encoding voltage dependent sodium channels in heart cells are associated with pathologies such as cardiac arrhythmias) and understanding ion channels functioning in a physiological and pathological state paves the way to the discovery of new drugs for the treatment of diseases related to them. If monitoring membrane potential allows the study of ion channels, it is also necessary to remember that the latter are activated in the presence of stimuli including electrical, mechanical and chemical stimuli. For this reason, studying their dynamics requires not only measuring the membrane potential (*consequence*) but also requires stimulating their activity by stimulating the cell with chemical, mechanical or electrical methods (*cause*).

There are various methodologies to apply these types of stimuli and some of them (those related to chemical and mechanical stimuli) will be presented in the first part of this chapter. However, there is a technology called FluidFM invented at ETH Zürich's Bio-Sensors and Bioelectronics Laboratory (LBB) which combines many of these stimulation techniques in one instrument, which is extremely versatile and more capable with respect to classical stimulation method. The FluidFM is therefore a convenient and innovative method to stimulate the activity of ion channels through the stimulation of the single cell. The second part of this chapter is dedicated to FluidFM and its applications in single cell biophysics.

3.1 Chemical stimulation

Chemical stimulation at the cellular level is, in the most general definition, the perturbation of cellular homeostasis through the use of a chemicals: organic or inorganic compounds. The method that allows this stimulation is not specified, but the type of compounds that can be used for chemical stimulation also depends on it. In fact, a trivial example of chemical stimulation is represented by the loading of a drug in the medium of culture in the cell plate. This drug, if permeable to the cell membrane, will diffuse from the extracellular solution in which it is dissolved to the intracellular fluid, causing or not causing a cell response. But this method does not allow to study the effect of compounds that are impermeable to the cell membrane. For this reason there are other methods, such as injection via micropipettes. Some advantages and disadvantages of these techniques are listed below.

3.1.1 Diffusion-based drug loading

As mentioned above, the simplest and most direct method of studying the effect of a drug candidate or a chemical compound is to dissolve the drug directly in the culture medium in which cells are cultured. This process typically requires exposing the cells to the compound of interest by leaving them in the presence of the chemical compound for a certain amount of time. During this period of time, the chemical that must necessarily be permeable to the membrane, passively diffuse from the extracellular fluid to the cytoplasmic fluid, reaching the interior of the cell where it can eventually trigger a cell response. This simple and effective method has obvious limitations.

First of all, it does not allow to study the effect of potential drugs which can not to be transported in the intracellular fluid by passive diffusion, because of the impermeability of the cell membrane against them.
Secondly, for all those drugs that can still be studied with this method, the amount of drug that is actually causing an effect on each cell is unknown. It is known, in fact, that cells uptake of a compound present in the culture medium varies from cell to cell due to cell-cell variability.
Last but not least, with this methodology, you lose information about the initial instant in which the drug begins to take effect. It is not known, in fact, the exact moment in which the uptake of the drug by each cell takes place.

It is clear, therefore, that with this method it is not possible to stimulate single cells in a precise way, losing information on the initial instant of the perturbation, on how much drug is acting on a particular cell and losing the possibility to study drugs which are impermeable to the cell membrane.

3.1.2 Microinjection

Microinjection techniques solve the problems described allowing to stimulate single cells in a more precise way, opening new scenarios for the study of the biological effect of chemical compounds.

Microinjection is the use of a glass micropipette to inject a liquid substance at a microscopic level. Using these very small bore glass needles (outer diameter of usually less than 0.2 μm), DNA or other material is injected into the cell for subsequent integration and/or expression. Unlike many other approaches, the direct nature of the approach ensures delivery in almost every cell treated. Further, the exact copy number or number of DNA molecules delivered into each cell can be precisely controlled. Current applications include the delivery of genes (and other molecule) into isolated cells for research purposes, generation of transgenic mice and other animals, and forms of human in vitro fertilization (Figure 3.1).

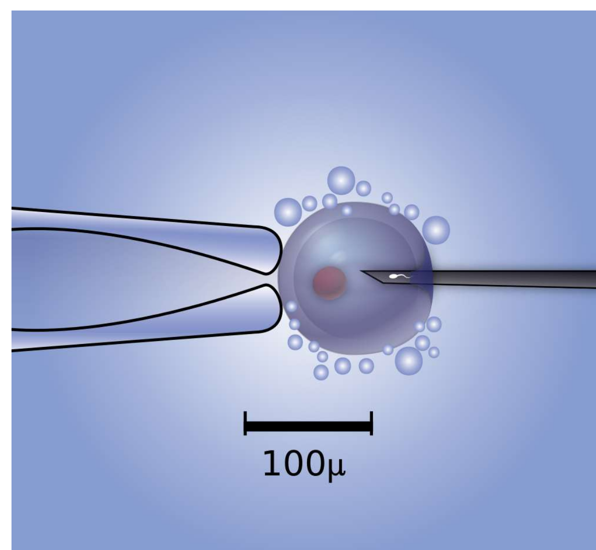

Figure 3.1. *Diagram of the intracytoplasmic sperm injection of a human egg. Micromanipulator on the left holds egg in position while microinjector on the right delivers a single sperm cell.*

Any microinjection system typically comprises three basic functions: the imaging of the target cell and the micropipette, the positioning of the micropipette, and control of the pressure inside the micropipette.

- An inverted microscope (one whose objective lens turret is located below a fixed stage, with the transmitted light source located above) is typically used for imaging in microinjection systems.
- The micropositioning device (the micromanipulator) is used to position and move the micropipette in proximity to the tissue to be manipulated or injected and it is mounted to the microscope by means of an appropriate adapter.
- The microinjector is used to control the pressure level in the micropipette. Depending upon the application being employed, different pressures are required. For instance, direct-pressure microinjection of fluids such as nucleic acid solutions, dyes and drug compounds into cells uses a sharp hollow capillary-glass micropipette with a tip diameter typically less than one micron. Considerable pressure (in the order of a few hundred hPa) is required to force the solution out of the tip of such a micropipette and into the cell.

The micropipette is thereby slowly approached to the cell by using micro manipulators and visual control through an optical microscope. During this process, however, the cell is often mechanically injured. This leads to cell death and failure of the experiment. To overcome these challenges and limitations of conventional microinjection method the FluidFM technology has been developed at LBB (ETH Zürich, Switzerland). This extremely versatile technology, described in more detail in a later paragraph, can be regarded as a multifunctional micropipette with force feedback working in liquid environment. It is precisely due to the presence of the force feedback that FluidFM is able to solve challenges associated with conventional microinjections.

3.2 Mechanical stimulation

Mechanical stimulation of the cell is necessary in studies of mechanobiology. Mechanobiology emerges at the crossroads of medicine, biology, biophysics and engineering and describes how the responses of proteins, cells, tissues and organs to mechanical cues contribute to development, differentiation, physiology and disease. The grand challenge in mechanobiology is to quantify how biological systems sense, transduce, respond and apply mechanical signals. In mechanobiology, living systems are described by

cycles of mechanosensation, mechanotransduction and mechanoresponse. In addition to its state, the functional response of a living system depends on the nature of the mechanical signal, whether it is applied at the nanometre or micrometre scale, for a short or long time, with low or high magnitude, and on whether it is scalar or vectorial. Nanotechnological and microtechnological approaches have enabled progress in quantifying the mechanical properties of biological systems. The most widely used approaches to structurally map the mechanical properties and responses of biologicalsystems, ranging from millimetre to sub-nanometre resolution and from micronewton to piconewton sensitivity, are based on atomic force microscopy (AFM). Of particular importance for the characterization of biological systems, atomic force microscopes can operate in aqueous environments and at physiological temperatures. AFM in its most standard configuration features a pyramidal tip at the extremity of a flexible cantilever that acts as a spring (Figure 3.2). The cantilever is fixed to an x-y-z piezo, and the tip is scanned on a surface while the deflection of the cantilever in response to the tip–surface interaction is monitored with a laser beam reflected on a four-quadrant position sensitive detector.

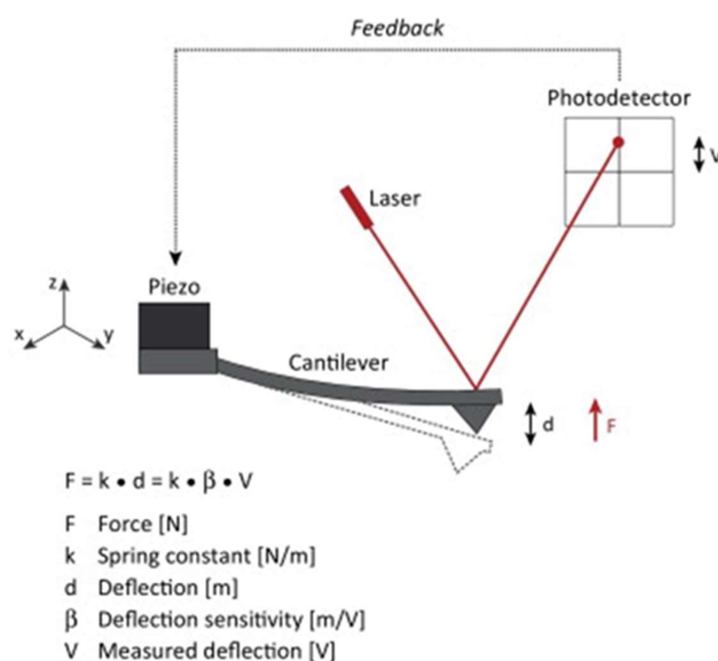

Figure 3.2. *Schematic representation of an atomic force microscope. Forces affecting the cantilever tip cause bending of the cantilever, which is measured via the reflection of a laser beam onto a photodetector. An x-y piezoelectric scanner allows for scanning the tip over a sample, whereas a z-piezo and a feedback system enable vertical tip position control. The force F exerted on the tip is related to cantilever's deflection d and spring constant k; the deflection is measured in volt V, and the deflection sensitivity b converts it from volt to meters.*

Thus, mechanical stimulation of the single cells by AFM allows to measure mechanical properties of the cell and at the same time stimulate mechano-gated ion channels. The easiest way to archive this is to indent the probe into the sample and to record the applied force, which is proportional to the cantilever deflection, and the distance travelled by the probe in a force–distance (FD) curve (Figure 3.3). These AFM single point measurements in which the cantilever approaches and "pokes into" the sample, and then withdraws is called "force spectroscopy mode". Recorded upon approaching and retracting the probe, FD curves measure the mechanical deformation and response of the sample under load. Force can also be plotted against time in force–time (FT) curves, which are particularly useful if the force applied by the indenting probe or the indentation depth of the probe is to be held constant. These mechanical readouts are particularly useful when the sample changes mechanical properties with time, when viscoelastic properties need to be determined or to know what kind of stimuli the ion channels are experiencing.

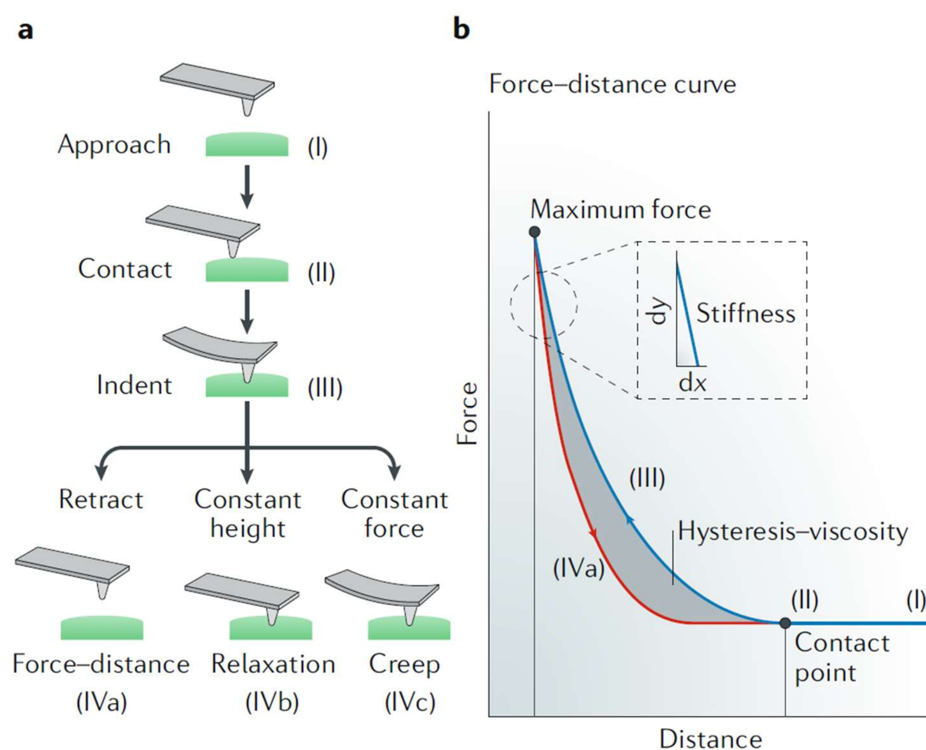

Figure 3.3. (*a) Schematic illustration of the methods used for quantifying the mechanical responses of a biological system to the indentation of an atomic force microscope probe. (b) Example of force–distance (FD) curve: the probe indents the sample until a defined force is reached (blue approach curve) and the cantilever is retracted (red retraction curve). From the approach FD curve, the contact point between the probe and sample and the sample stiffness can be estimated. From the difference between the approach and retraction curves, the sample viscosity can be estimated.*

A recent extension of AFM is represented by the invention of fluidic force microscopy (FluidFM) which combines a conventional atomic force microscope mounted on top of an optical microscope with microchanneled cantilevers that are connected to a pressure controller for operation in liquid. Together with complementary AFM applications, FluidFM is tremendously capable in helping to address some of the challenges of single-cell biology. The next section describes in detail the FluidFM and present how it can help overcome several shortcomings of conventional AFM, especially for single-cell biology.

3.3 FluidFM

A different solution to work at the single cell level that integrates the advantages of AFM such as the force feedback for gently touching the substrate with the advantages of glass micropipettes was born in late 2006, at the Biosensors and Bioelectronics Laboratory at ETH Zürich. This new tool, called "fluidic force microscopy" (FluidFM) is based on AFM (accurate force-controlled positioning) and AFM microchanneled cantilevers (versatility of fluidics) is described below. The result is a tool that is as versatile as glass micropipettes, but capable of improving the control over when, where and how the substrate (e.g., cell) is touched.

3.3.1 Instrument technology

In the FluidFM a microsized channel is integrated in an AFM cantilever and connected via channels in the AFM chip holder to a delivery system, thus creating a continuous and closed fluidic channel that can be filled with an arbitrary chosen solution and can be immersed in a liquid environment (Figure 3.4). An aperture in the AFM tip at the end of the cantilever allows liquids with any viscosity (ranging from 1 to 10’000 cP) to be dispensed locally, with femtoliters precision. Force feedback is ensured by a standard AFM laser detection system that measures the deflection of the cantilever and thus the force applied by the tip to the sample during approach and dispensing.

It is due to the various tip geometries (at the extremity of hollow cantilevers) that the FluidFM can be such a versatile instrument. Thanks to different cantilever tips (Figure 3.5e, Figure 3.5f, Figure 3.5g) it can easily perform precise three dimensional manipulations on individual living cells (e.g. adsorbing, extracting, transporting, placing, delivering, and injecting) by controlling the pressure of the fluidic channel in the AFM cantilever.

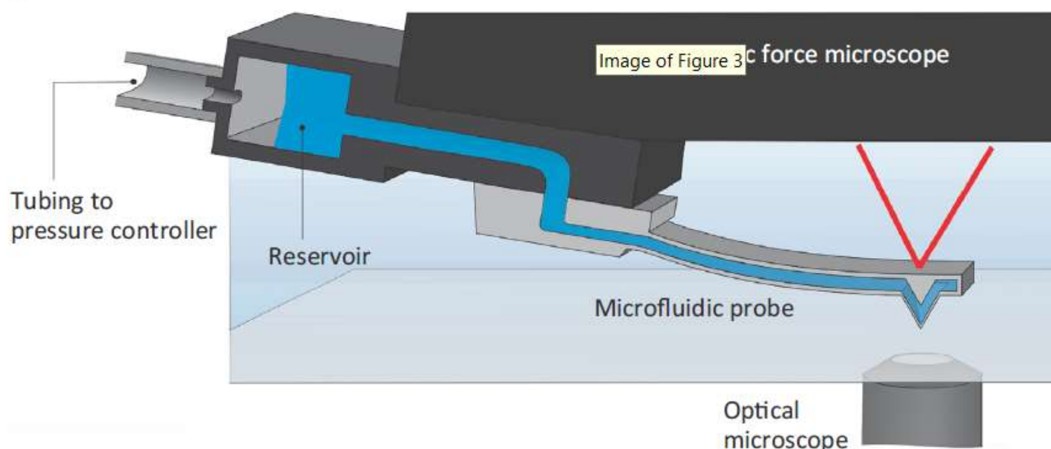

Figure 3.4. *Diagram showing a microchanneled cantilever chip fixed to a AFM probeholder. The FluidFM can be operated in air or with the whole system (probeholder and chip) immersed in a liquid. The external liquid or bath and the liquid in the microchannel may be the same or may be different. The substrate (e.g., cells) can be observed with an optical microscope through the glass holder (e.g., glass dish) on which it is mounted*

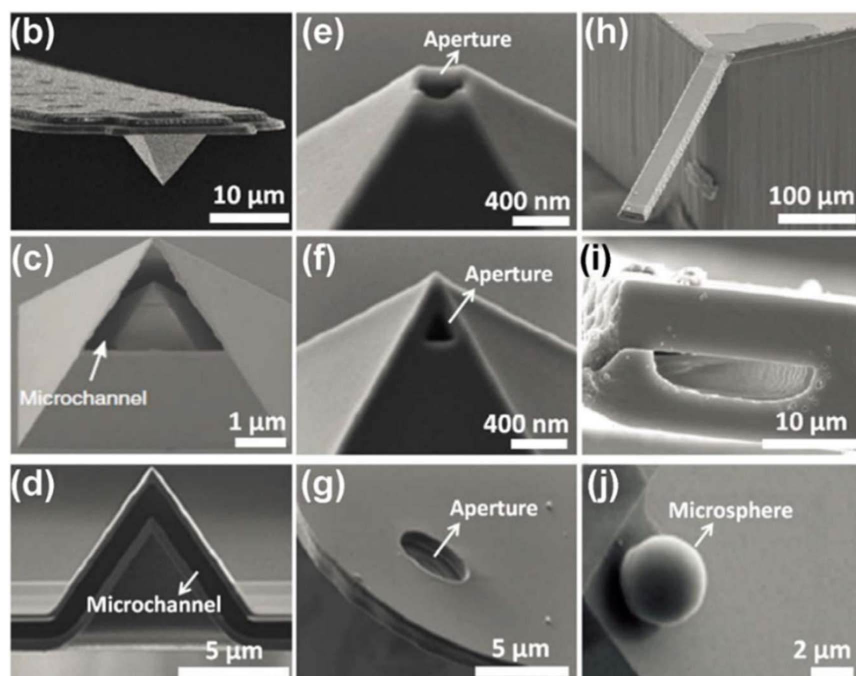

Figure 3.5. *(b)–(j) SEM images of the different types of microchanneled probes (b) The whole probe (c) Cutaway view of the pyramidal tip with embedded microchannel (d) Hollow pyramid after sectioning the probe with FIB (e) and (f) Aperture at the apex (e)/side (f) of the pyramidal AFM tip. (g) Aperture formed in tipless cantilever. (h) Whole image and (i) magnified image of the hollow probe fabricated by SU-8. (j) Microsphere tip prepared based on the microchanneld cantilever.*

There are also various types of hollow cantilevers, and they are divided mainly by the material they are composed of. The two main classes are: silicon-based hollow cantilevers and polymer-based hollow cantilevers (Figure 3.5b, Figure 3.5i).

Silicon-based hollow cantilevers

Because of its good elastic properties, silicon nitride, along with silicon and silicon oxide, is the most popular material for cantilevers. (Figure 3.6) Silicon nitride is often used as an insulator and chemical barrier in manufacturing integrated circuits, to electrically isolate different structures or as an etch mask in bulk micromachining. Silicon nitride is a chemical compound of the elements silicon and nitrogen. Si_3N_4 is the most thermodynamically stable and commercially important of the silicon nitrides and the term "silicon nitride" commonly refers to this specific composition. It is a white, high-melting-point (1,900 °C) solid that is relatively chemically inert, being attacked by dilute HF and hot H_3PO_4. It is very hard (8.5 on the mohs scale). Insoluble in water.

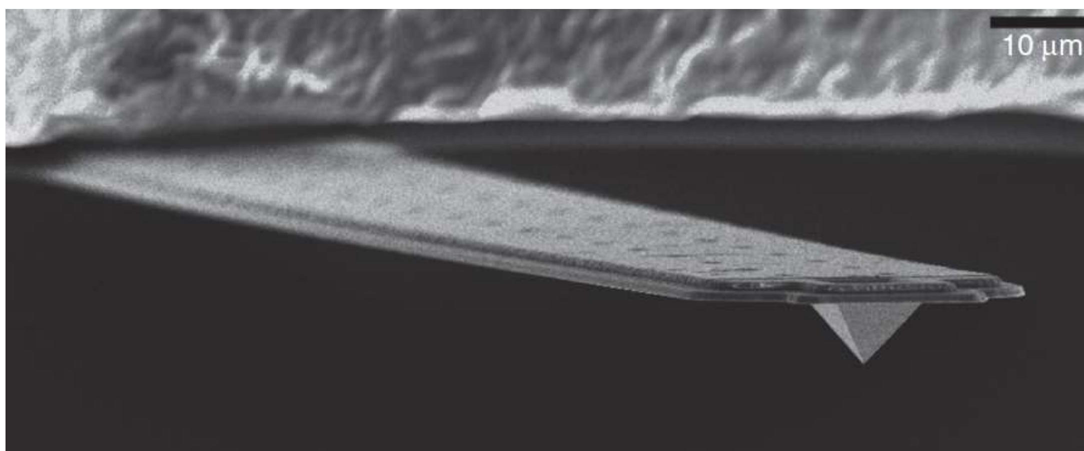

Figure 3.6. *SEM image of silicon nitride FluidFM probe: large view of a pyramidal cantilever. For mechanical stability the upper and lower layers of the probes are connected by two lines of pillars having a diameter of 3um each. Cantilever dimensions: 200 x 36 x 1.7 µm (LxWxH). Channel height: 1500nm. Spring constant (nominal): 2.2N/m but cantilevers with spring constant in different ranges are available.*

Nowadays the main process used to fabricate FluidFM probes is based on a “sacrificial layer process” (Figure 3.7). The apertures are milled by focused ion beam (FIB) with a diameter ranging from 1 µm down to 100 nm. Thin Si_3N_4 walls are compulsory for milling such nanosized holes. Before the FIB milling, a metallic layer (gold, chromium or platinum) is deposited on both sides of the cantilever to avoid charging effects during milling and increase the reflectivity for the AFM laser. The FluidFM technology and a wide

variety of different probes are currently commercialized by CytoCurge (LBB spin-off co-founded by some of the FluidFM co-inventors) and Nanosurf AG, a partner company established to develop AFM-based technologies.

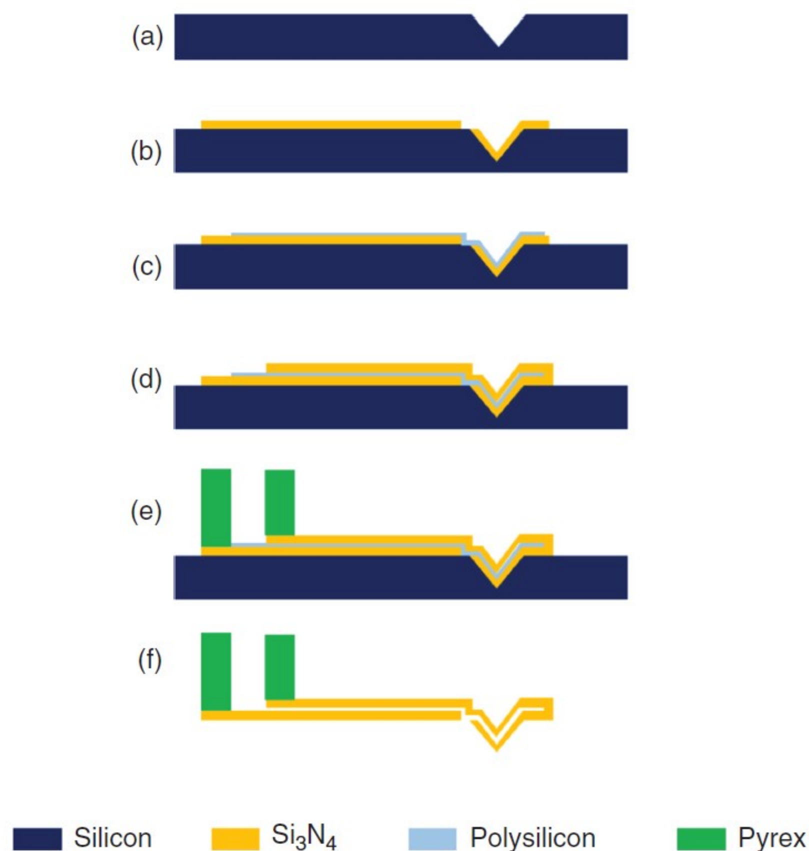

Figure 3.7. *Simplified microfabrication process for producing microchanneled silicon-based cantilevers with sacrificial layer. (a) KOH etching a single-crystal <100> silicon wafer to obtain the molds for the tips by anisotropic attack (54.7° angle of sidewalls with the surface) (b) Deposition and patterning of a first Si3N4 layer with an outlet side hole for the cantilevers (c) Deposition and patterning of a sacrificial polycrystalline silicon layer (d) Deposition and patterning of a second Si3N4 layer for the cantilevers (e) Patterning/dicing an additional Pyrex wafer and bonding of the two wafers for the reservoir (f) Etching of the polycrystalline silicon for the channels and final release of the probes.*

Polymer-based hollow cantilevers

An example of polymer-based hollow cantilevers are the ones fabricated with SU-8 polymer (Figure 3.8). Functional cantilevers integrating for instance 22 um thick channels can be fabricated. Such thick hollow cantilevers can benefit from novel applications such as single-cell deposition.

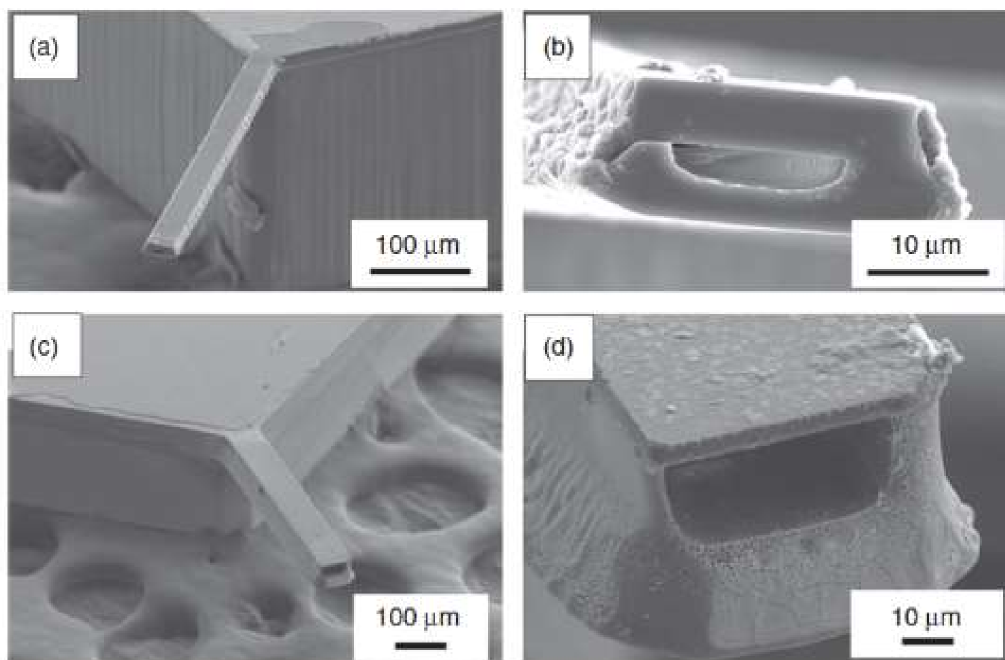

Figure 3.8. *SEM micrographs of SU-8 hollow cantilevers with an aperture deliberately defined in the front plane to inspect the cross section. (a) 12 um thick cantilever integrating a 3 um thick and 20 um wide channel with the corresponding closeup view in (b). (c) 50 um thick cantilever integrating a 22 um thick and 60 um wide channel with the corresponding closeup view in (d).*

3.3.2 Applications in single-cell biophysics

The two special features of FluidFM, namely the ability to deposit or extract liquid levels in the order of femtoliters, and force feedback enable the instrument to be extremely versatile, allowing it to operate in the fields of physics biology, chemistry and materials science. Below there is briefly description on how FluidFM addresses biomechanical and biophysical issues in the life sciences, particularly single-cell manipulations, single-cell force spectroscopy and single-cell electrophysiology, present in the scientific literature. In contrast, other applications of FluidFM such as local surface modification and three-dimensional microprinting are not discussed.

Picking and placing single cells: by approaching the FluidFM probe onto a cell in contact mode and applying a underpressure in the fluidic channel, the cell can be tightly attracted to the aperture of the cantilever in the vertical direction and therefore can be picked up detaching from the surface. By reversing the pressure, the cell can be released onto a desired

spot. Taking advantage of the FluidFM multifunctionality, trypsin solution can also be loaded in the channel and locally delivered to individual adherent which activates the selective detachment (rounding) of the targeted cell while neighboring cells remain adherent. The detached cell can then be easily lifted up and manipulated by the FluidFM, providing an efficient way for precise isolation of chosen cells in a cell population. In this way, it is possible to transfer cells to targeted areas to observe cell behaviors.

Sucking colloidal beads as indenters: FluidFM opens the way to exchangeable colloidal AFM for single-cell mechanical assays. To probe the mechanical properties of cells, spherical AFM tips are commonly used, since the conventional conical tips only detect the local mechanics of the cell. In the case of FluidFM, a single sphere can easily be aspirated to the aperture by applying a negative pressure. Both tipless or pyramidal FluidFM can be used to prepare spherical tip, and specifically pyramidal probes are suited for preparing spherical tips with smaller sizes. Under the guidance of optical microscopy, the FluidFM probe is moved to touch individual spheres deposited on the substrate. Subsequently, applying adequate underpressure to the fluidic channel of the probe allows the adsorption of the sphere to the aperture of the probe. The spherical probes prepared by FluidFM can then be used for experiments such as probing the mechanics of single cells. A notable point is that the sphere sucked to the FluidFM cantilever can be exchanged in the working medium when probing cells. Applying positive pressure yields the release of the sucked sphere, and then moving the probe to the region of the substrate where the colloidal beads had been previously deposited and applying negative pressure will adsorb a new clean one. This is particularly useful for probing the specific molecular interactions which require the functionalization of the tip. For example, it is possible to firstly approach a cell with a bare sphere as control, and then detect specific molecular interactions on the cell surface with an ad-hoc chemically functionalized sphere.

Intracellular injection/extraction of single cells: using a pyramidal tip with a side-hole preserving the sharpness of the apex, FluidFM is able to puncture the cell membrane for intracellular delivery. Membrane perforation is indeed achieved by exerting a higher indentation force on the cell surface, which is visually confirmed by the abrupt peak in the recorded force curves. Subsequently, by applying overpressure to the fluidic channel of the probe, the solution inside the microchannel can be directly injected to the interior of the cell. Instead, applying adequate negative pressure enables extraction of intracellular structures.

Adhesion of eukaryotic cells: the adhesion forces between single living cells and substrates can be quantified by FluidFM. Traditional AFM-based SCFS (single cell force

spectroscopy) is based on attaching a living cell to the AFM cantilever via complex chemical functionalization. Firstly, the cantilever is coated with multiple substances, which is labor intensive process and requires specific expertise. With the use of FluidFM, single living cells can easily be physically immobilized onto the AFM cantilever by applying and maintaining adequate negative pressure of the fluidic channel in the AFM probe, allowing the SCFS measurements in native conditions (without chemical treatment on cells). Besides, regular functionalized probes can be used only once, whilst cells are prone to detach from the cantilever during SCFS experiments while the FluidFM probe can be reused (after completing the measurements of one cell, the FluidFM probe can be controlled to adsorb another cell for further measurements) and the adsorption force for immobilizing cell can be conveniently adjusted, therefore FluidFM facilitates increasing the throughput and efficiency of SCFS assays.

3.3.3 Combining FluoVolt with FluidFM

It is clear, therefore, that the FluidFM represents an extremely versatile tool in the field of single-cell biophysics thanks to its ability to gently interact with cells and at the same time to stimulate them in many different ways. These different types of stimuli also trigger the activity of ion channels (e.g., mechano-gated, voltage-gated, ligand-gated ion channels) which in turn can contribute to membrane potential variations.

Also FluoVolt, as described in the previous chapter, represents an innovative and versatile monitoring technique for measuring membrane potential variations. For this reason, combining FluoVolt and FluidFM to simultaneously study membrane potential variations and ion channels activity, would represent a new and versatile methodology for the study of physiological and pathological cellular processes. The possibility and feasibility of combining FluoVolt and FluidFM for membrane potential variations monitoring under chemical and mechanical stimulation has been studied and demonstrated in the proof of concept project part of this thesis, through ad-hoc designed experiments.

Chapter 4

Cardiomyocytes-based project

Cardiomyocytes are excitable cells. Their ability to fire action potentials is associated with the ability to contract. These microscopic contractions are the base of the heart macroscopic contraction, a fundamental vital process. Studying in depth the electrical activity of these cells, and how it depends on ion channels is therefore extremely important from the physiological and pathological point of view to get new insights into the underlying mechanisms of diseases such as arrhythmias and other heart complications that affect changes in membrane potential. The aim of this research project is to combine the measurement of membrane potential through FluoVolt with a simultaneous mechanical, chemical, or mechanochemical stimulation of the cell via FluidFM. In this chapter, the reasons behind the choice of adult mouse primary cardiomyocytes as cells to stimulate along with some details about their physiology and structure are first discussed. Subsequently, the process used to measure and extract the representative signal of changes in membrane potential using FluoVolt is described. It is then illustrated a methodology for the extraction of important parameters that are representative of the cell electrical activity measured with an optical probe. Finally, the reasons for not achieving the goal of chemical and mechanical stimulation of cardiomyocytes are explained.

4.1 Adult mouse primary cardiomyocytes

Cardiovascular diseases remain the primary cause of death in the world. To heal some of these pathologies, drugs are developed. To develop appropriate drugs it is increasingly necessary to know the true functioning of the heart at a microscopic level, and this requires the study of the building blocks that compose the heart: cardiac cells. Cardiac cells, called cardiomyocytes, therefore become targets for the development of drugs capable of attacking cardiovascular diseases. Evaluating the toxicity of a potential drug candidate is essential in the development process of a new treatment. The toxicity of a drug that targets

heart cells, called cardiotoxicity, can affect both cardiomyocytes structure and electrophysiology; the first is the case of most cytotoxic drugs, such as chemotherapeutic agents, that determine an increase in oxidative stress, mitochondrial damage and apoptosis; the second form of cardiotoxicity, instead, impairs cardiomyocytes electrical homeostasis by affecting heart frequency and/or the action potential shape.

The focus of the research in the development of treatment for cardiovascular diseases thus shift from macroscopic observations to more detailed functional examinations: the whole organ and isolated muscle preparations revealed several limitations (e.g., proper control of membrane potential during electrophysiological assessments is difficult with large sample sizes) and the necessity of having models based on isolated cardiomyocytes and cell lines to better understand the cellular and subcellular physiology and pathophysiology is increasing.

To study cardiomyocytes physiology and pathophysiology and the effect that drugs have on them, cardiovascular research has long relied on animal models as they recapitulate human cardiac physiology to a good approximation and primary cardiomyocytes obtained from small rodents represent an invaluable model to study cellular and subcellular physiology through electrophysiology, calcium imaging, cell mechanics, immunohistochemistry and protein biochemistry. Isolation of human cardiomyocytes from surgical waste is also a practicable procedure, however, the low availability of samples, the low yields of obtained cardiomyocytes and the risk of de-differentiation in extended cultures, together with ethical and logistic problems related to human tissue usage, makes impractical to employ human primary cardiomyocytes in most of the cases.

Freshly isolated adult mouse primary CMs are considered physiologically relevant in terms of both structure and function and they have been used in this work where the goal is to combine the measurement of membrane potential via FluoVolt with simultaneous chemical/mechanical stimulation of the cell via FluidFM.

Cardiomyocytes (CMs) are striated self-beating and cylindrical rod-shaped muscle cells that fundamentally govern the function of myocardium. The size of an adult mouse primary cardiomyocytes is 100–150μm by 20–35 μm. CMs are mainly obtained by the Langendorff method, performing perfusion of enzyme solutions through the aorta of an isolated heart.

CMs are excitable cells, capable of firing and conducting cardiac action potentials. The cardiac action potential is a recording of cell's membrane potential (V_{mem}), versus time. During each cardiac cycle, ions move back and forth across the cardiomyocyte cell

membrane, thereby changing V_{mem}. The cardiac action potential, which reflects the integrated behavior of numerous individual ionic currents, is largely dominated by the movement of Na^{+}, Ca^{2+}, and K^{+} ions. These ions traverse the cell membrane through ion-selective pores formed typically by integral membrane-spanning proteins. The behavior of these ionic pathways is highly regulated, and permeation of specific ions is influenced by multiple factors, the most prominent of which are changes in membrane potential (i.e., voltage gating). During the cardiac action potential there is a sustained increase in Ca^{2+} conductance, and all Ca^{2+} ions moving into the cell at that time contribute to mechanical activation of sarcomeric structures, the contractile apparatus of the cell.

To assess the viability of cardiomyocytes (necessary step before conducting an experiment with them), it is necessary to check their morphology under a bright-field microscope. Freshly isolated cardiomyocytes maintain a roughly rod-shaped morphology with sharp, distinct edges under brightfield illumination (Figure 4.1). Rapidly promoting cell attachment through serum-free medium and coating of the culturing substrate, result in better preservation of morphology compared to prolonged suspension cultures. Cardiomyocytes can be identified from non-cardiomyocytes by their characteristic morphology (rod-shaped) and size (100 - 200 μm in length).

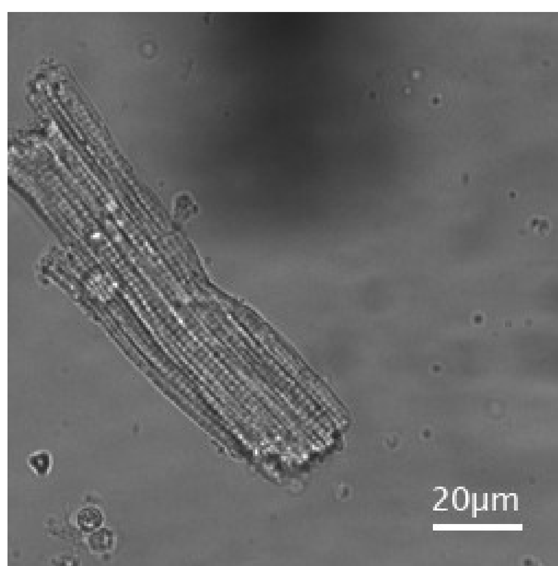

Figure 4.1. *Cardiomyocytes viability can be assessed from their characteristic morphology (rod-shaped) and size (approximately 100 - 200 μm in length) under a bright-field microscope.*

The reasons that lead to the use of cardiomyocytes in order to achieve the goal set for the thesis are many. The choice of electrically active cells such as cardiomyocytes is advantageous because capturing large variations in membrane potential, (e.g., cardiac

action potentials fired by heart cells) allows easier interpretation of the signal obtained via FluoVolt. It is safer to have a signal that, even in the presence of artifacts, is representative, at least from the qualitative point of view. At the same time, choosing to use primary adult mouse cardiomyocytes which are extremely fragile cells, and managing to realize a stimulation of the cell (e.g. injection) could prove how gentle is the FluidFM in interacting with cells. The versatility and ability of the FluidFM to gently stimulate the cell is demonstrated through the exploitation of the precise force-feedback that the FluidFM inherits from the AFM.

4.2 Seeding freshly isolated cardiomyocytes

Once the cardiomyocytes are isolated, it is necessary to conduct the experiment/imaging in the hours just after isolation, as cardiomyocytes are too fragile to be kept in culture. Thus, after receiving isolated CMs it is necessary to immediately proceed to the seeding of the cells and prepare them for the experiment. The cells were received suspended in a buffer, whose concentration of calcium ions is 100uM. To proceed with seeding it is firstly necessary to transfer the cells in a specific buffer with a calcium concentration of 800uM, which has been proven to be optimal for the maintenance of cardiomyocytes. This transfer must be gradual and take place through different buffers having intermediate calcium concentration steps: 100uM, 300uM, 500uM, 800uM. At each step the cells are gently resuspended by shaking the falcon tube. It takes 3-5 minutes for healthy cells to settle at the bottom of the falcon tube. In each step the supernatant is removed after 3-5 minutes to eliminate dead cells as much as possible which tend to float in the supernatent. The cells are then resuspended in the buffer with the highest Ca^{2+} concentration.

In order to measure the membrane potential through the use of FluoVolt it is necessary to load this substance into the cells. FluoVolt is thus dissolved in the buffer where the cells are kept: in this case is the one used in the last step of increasing Ca^{2+} concentration (800um). Therefore, having resuspended the cells in the specific buffer containing a calcium concentration of 800uM in which FluoVolt is also dissolved, it is possible to seed the cardiomyocytes on a coated glass dishes. Glass dishes are used for optimal imaging, compared to plastic dishes. The coating instead ensures that the cells adhere better. Coated dishes are prepared the day before the seeding with the following process: 2 minutes of plasma cleaning, then the dishes are covered with a solution containing laminin mice, and thy are incubated overnight at 37 ºC.

Once the cardiomyocytes are obtained on the coated dishes and are covered by the buffer with known 800uM Ca^{2+} concentration and Fluovolt dissolved in it, the cells are incubated 30 minutes at room temperature (Figure 4.2).

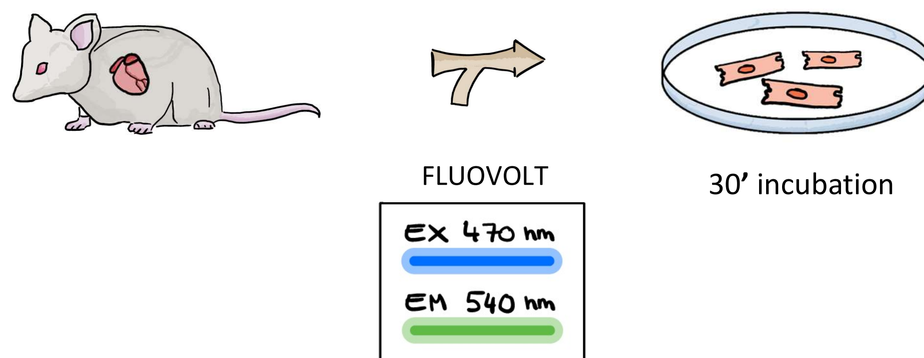

Figure 4.2. *Cardiomyocytes are fragile cells. As soon as they are isolated, they must be immediately prepared for the experiment. In order to measure the membrane potential, FluoVolt is loaded into the buffer where the cells are kept. They are incubated 30 minutes and then washed with the same buffer without FluoVolt.*

After this window of time, FluoVolt becomes toxic, for this reason it is necessary to wash the cells (which in the meantime have adhered to the coated dish) with the same buffer containing Ca^{2+} 800uM but without FluoVolt. The cells kept in this buffer are therefore ready for experiment/imaging.

4.3 Automatic signal extraction from FluoVolt data

After loading FluoVolt, cells can be imaged using the correct emission and excitation settings. The acquired images you are grayscale images. The greater the depolarization of the cell membrane, the greater the intensity of fluorescence, which will tend to lighter colors (Figure 4.3). To extract a one-dimensional signal, representative of the variations in the membrane potential over time, it is first necessary to identify the region of the image occupied by the cell, or the set of pixels representing the cell, for instance through a manual selection of the cell boundary. Summing the value of all pixels in this region gives a point in the FluoVolt fluorescence intensity vs time graph (Figure 4.4)

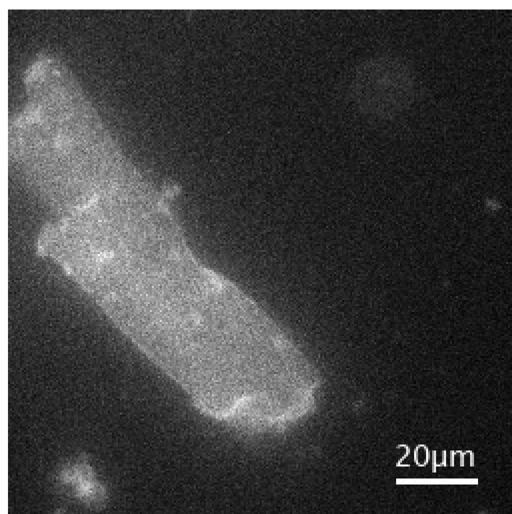

Figure 4.3. *Same cardiomyocyte as in Figure 4.2 but in the laser and filter configuration to display the Fluovolt signal. The greater the depolarization of the membrane, the greater the intensity of the fluorescence, resulting in a whiter color in the image.*

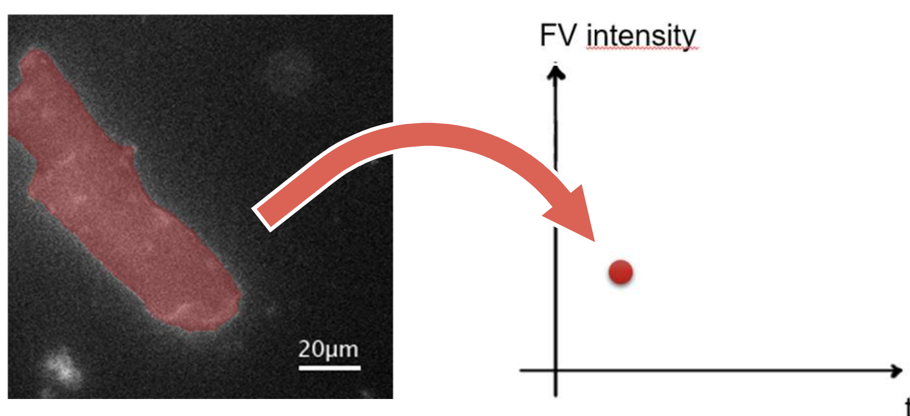

Figure 4.4. *From a FluoVolt image a point of the fluorescence intensity vs time curve is extracted, summing the values of the image pixels belonging to the region of the image occupied by the cell.*

Therefore, to obtain a curve that varies over time, it is necessary to acquire images in various moments of time and for each one repeat the manual procedure of cell boundary selection to archive the extraction of the total value of intensity in belonging to that region (Figure 4.5). To extract quantitative information through the use of Fluovolt it is therefore necessary to acquire video: a two-dimensional signal composed of a sequence of frames/images acquired in the GFP/FITC laser and filters fluorescence miscroscopy configuration, compatible with FluoVolt fluorescence characteristics. From the acquired video, a one-dimensional signal is extracted: the FluoVolt fluorescence intensity vs time

curve. Each frame requires a cell boundary selection step, since cardiomyocytes have spontaneous contractions that make the occupied cell region in the image to vary over time. Often acquiring video corresponds to capturing hundreds/thousands of frames. Thereby, finding automatically the contour of the cell becomes a key step in the pipeline to extract the fluorescence intensity signal from a video of a cardiomyocyte contraction since manually segmenting the cell boundary in hundreds/thousands of frame is too laborious and time consuming.

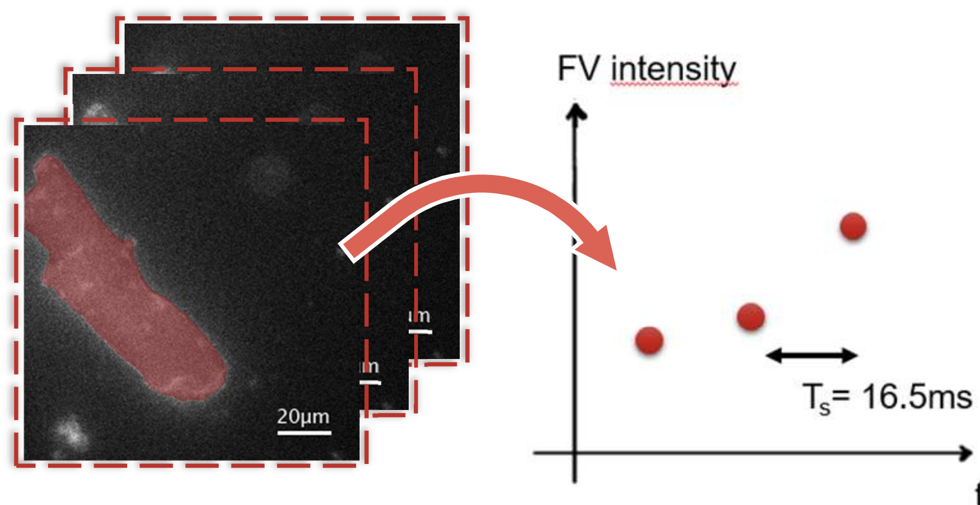

Figure 4.5. Multiple points of the fluorescence intensity vs time curve are extracted from various images captured in different time frames. Typically, images are captured at a fixed time interval and on each one you need to identify the region of the image occupied by the cell, in order to sum the pixel values.

For this reason, an automatic cell segmentation algorithm has been developed based on "Active Contour" model, which extracts the contour of the cell in each frame of the video (Figure 4.6). Active contour model, also called snakes, is a framework in computer vision for delineating an object outline from a possibly Noisy 2D image. A snake is an energy minimizing, deformable spline influenced by constraint and image forces that pull it towards object contours and internal forces that resist deformation.

By applying this algorithm to the video of a cardiomyocyte which was beating repeatedly, it is therefore possible to extract the fluorescence signal variation over time (Figure 4.7). This signal cannot be directly compared to the absolute values of the membrane potential because no calibration has been carried out, as the goal of the project is to measure variations in membrane potential in response to a mechanical/chemical stimulation of the

cell. These beatings are visible in the extracted signal showing the dynamic behavior of the cardiomyocyte, which by firing action potentials, depolarizes the membrane (the intensity of fluorescence increases rapidly), causing the cell to contract.

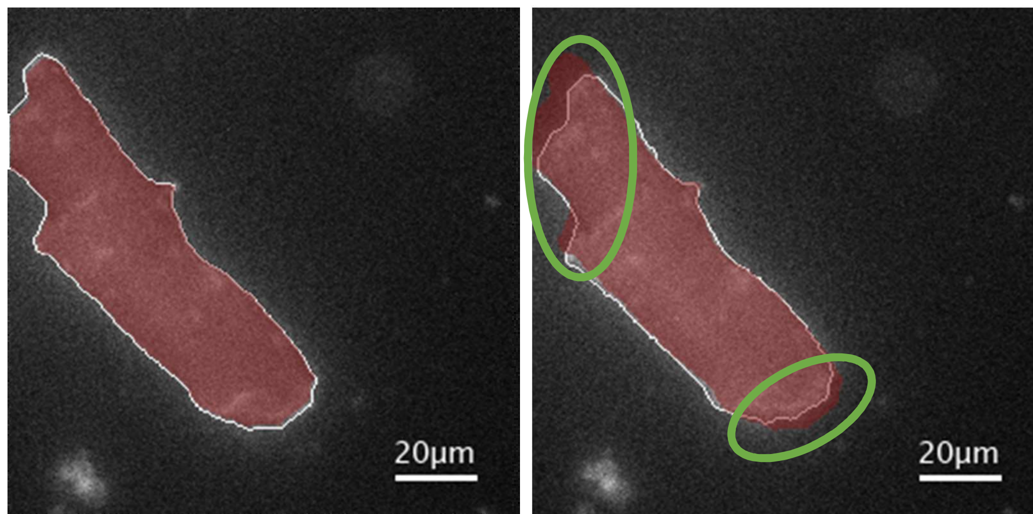

Figure 4.6. *Two frames of the video of a beating cardiomyocyte are visible. The active contour technique identifies the edge of the cell (white pixels), precisely, despite the contractions of the cell that are made visible by over-imposing in the second frame (b) a red mask that indicates the region of the space occupied by the cell in the first frame(a). Circled in green are the highlighted areas representing the error that would be committed by using mask extracted on frames different from the frame under analysis.*

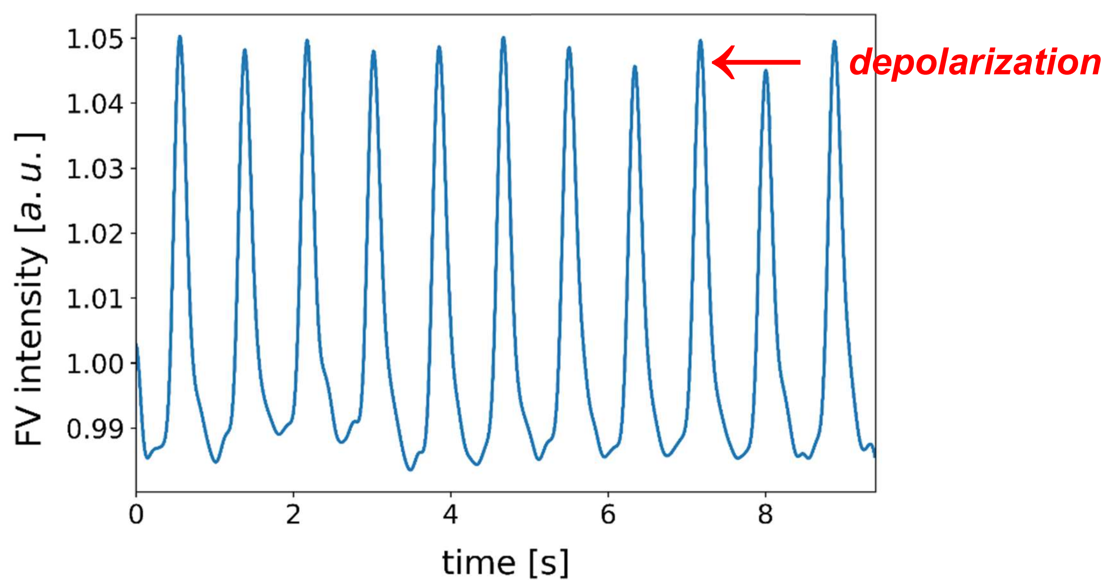

Figure 4.7. *By applying active contour to all the frames of the video, the representative signal of the cyclic depolarizations of the cell membrane is extracted.*

By having an automatic algorithm to segment the cell, it is possible to calculate the area of the cell instant by instant (Figure 4.8). There is therefore a correlation between the peaks of the fluorescence intensity signal, with peaks in the area vs time curve, demonstrating that the depoolarization of the membrane leads to cell contraction.

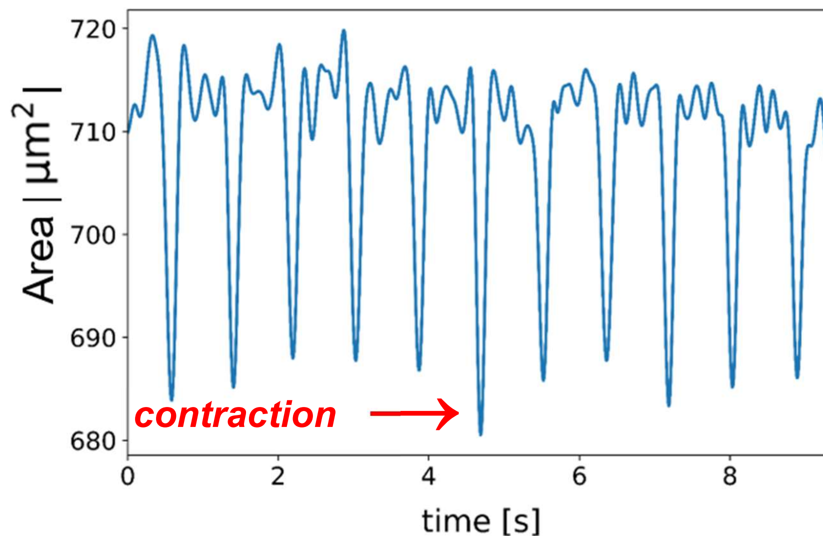

Figure 4.8. *By having an automatic algorithm for the automatic segmentation of the cell, it is possible to extract the trend of the area over time. Cell contractions are visible as a result of membrane depolarization.*

4.4 Analysis of local membrane potential variations

Optical probes for measuring membrane potential, in contrast to electrode-based techniques, offer high spatial resolution. This high spatial resolution depends also on the type of camera used for video/images acquisition.
It is possible to combine the advantage of having high spatial resolution in the measure of membrane potential with the advantage of having an automatic algorithm for extracting the cell boundary in order. This combination paves the way to conduct local analysis of membrane potential variations, and extract new parameters useful for the evaluation of the physiological and pathological state of the cell. As an example which is illustrated below, it is possible to extract the propagation speed of action potentials that are fired from beating cells

4.4.1 Visualization of action potentials

Some cardiomyocytes exhibit beating behaviors which are characterized by action potentials firing. These action potentials arise at a precise point in the cell, and propagate along the longitudinal axis of the cell. It's possible, through image processing, to isolate this rapid variation of membrane potential (action potential) from the resting membrane potential (baseline) by subtracting in each FluoVolt acquired frame the mean value of the fluorescence intensity belonging to the region of the image occupied by the cell. With this procedure it is possible, therefore, to highlight the variations in time of the fluorescence intensity, that is the propagation of an action potential along the longitudinal axis of the cell, causing its contraction (Figure 4.9).

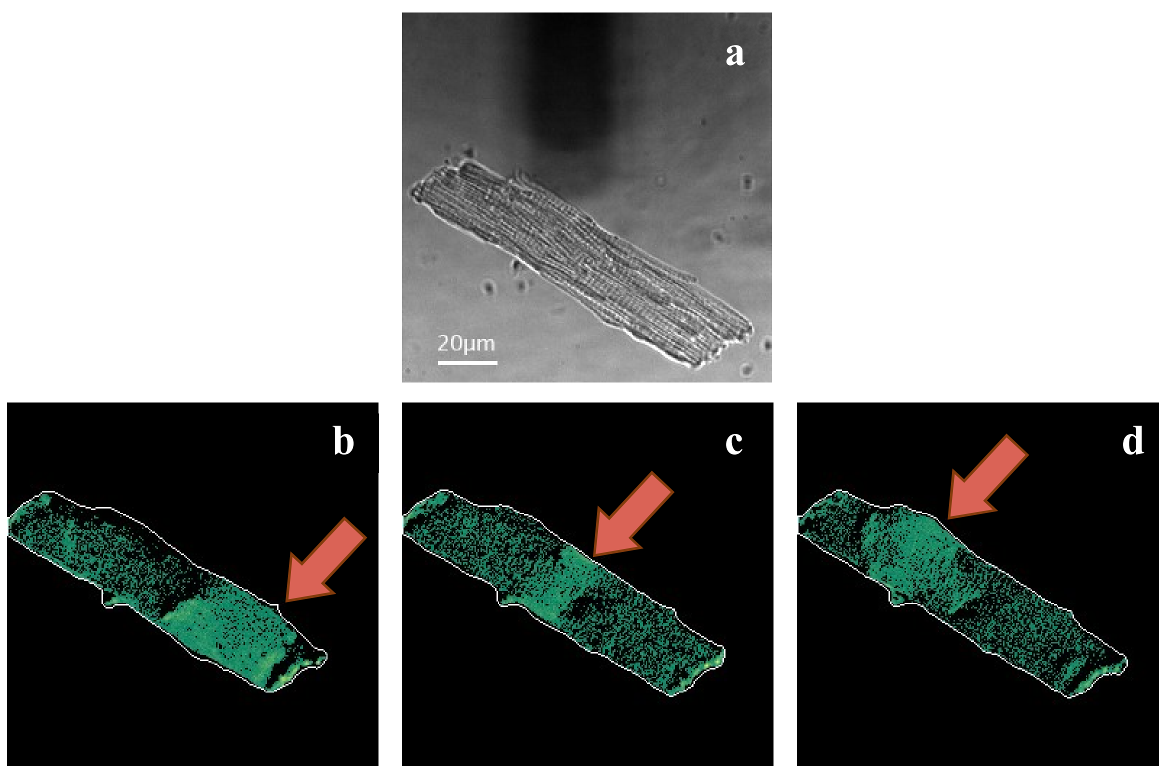

Figure 4.9. *The depolarizing wave fired by the cell travels along the longitudinal axis of the cardiomyocyte. (a) Brightfield image of the cardiomyocyte. (b),(c),(d) Time sequence of images captured in FluoVolt settings: the depolarizing wave is characterized by regions of dense green pixels. The green color is used just to facilitate visualization and it corresponds to a high fluorescence intensity value.*

Changing colormap from grayscale to a green color for purely visual reasons, it can be noticed a homogeneous color zone, where many pixels take a high fluorescence intensity value. This zone represent a local depolarization of the cell membrane, which moves along

the longitudinal axis of the cell with the passage of time. This longitudinal propagation is represented by the images placed in time sequence from left to right (Figure 4.9b,c,d). Noise and part of the artifacts introduced in the image processing have to be associated to spare green pixels distributed on the cell surface.
From the visualization of the action potentials it is possible to extract parameters for the physiological evaluation of the cell, such as the action potential propagation speed and the beating rate, as illustrated in the next paragraph.

4.4.2 Action potentials propagation speed

By having the cell contour on the image of the Fluovolt signal (grayscale colormap again), an algorithm for video stabilization was created, eliminating as much as possible the movements that the cell makes and suffers over time due to contractions. This video stabilization is necessary to allow the extraction of important signals as shown below.

To stabilize the video, firstly it is necessary to fit a geometrical shape such as an ellipsoid on top of the cell in the FluoVolt image (Figure 4.10). From this fitting the algorithm extract the angle between the major axis of the ellipse and the vertical of the image. Pulling out this angle is important because it allows to rotate each frame of the video around this ellipse major axis by a frame-specific angle (Figure 4.10a). In this way the cell is oriented vertically within the video and kept fixed in space and time, despite suffering contractions and movements in the original video.

Keeping the cell fixed in the video thanks to the stabilization algorithm, allows to extract the fluorescence intensity (representative of the membrane potential) in multiple and distinct areas of the cell, by simply summing the pixel intensities belonging to these “independent” zones. Therefore, fluorescence intensity signals coming from areas selected at the extremes of the cell can be extracted (Figure 4.11) The "dist" distance between zones is known because the conversion factor from pixel to micrometer is known.

The two extracted signals are both of oscillatory nature, due to the presence of action potentials that make the membrane potential vary rapidly. These two oscillating signals are out of phase over time: this phase shift interval is due to the time that a single action potential takes to travel from one end of the cell to another.
The peak-to-peak time interval between respective peaks belonging to the two respective curves is therefore calculated to find the time necessary to a single action potential to travel from one end of the cell to another (Figure 4.12).

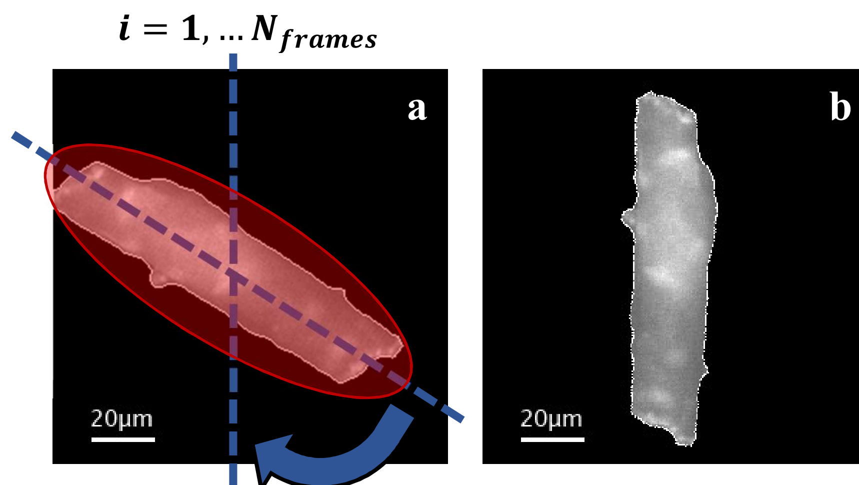

Figure 4.10. *The video stabilization algorithm allows each video frame to be independently rotated at an angle equal to the cell's rotation relative to the image axis. (a) An ellipsoid is fitted above the cell. The angle that the major axis of the ellipsoid forms with the vertical of the image is used to rotate the cell. (b) The cell is stabilized vertically.*

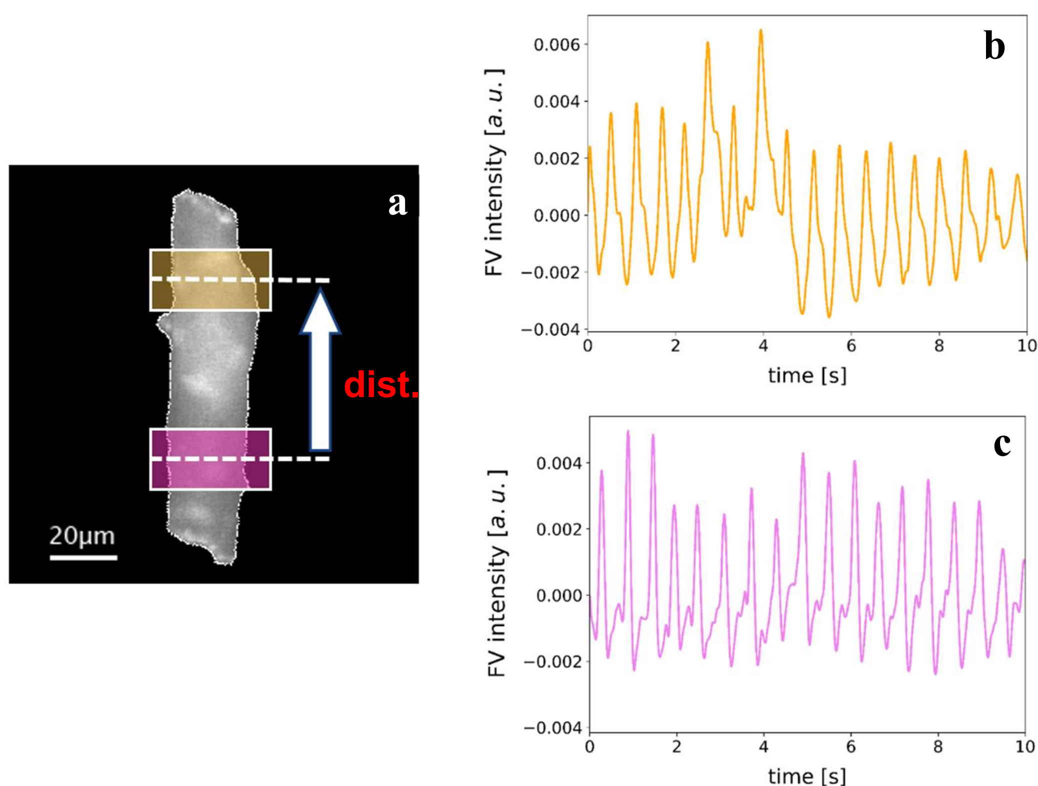

Figure 4.11. *By having stabilized the cardiomyocyte video, the fluorescence intensity signal can be accurately extracted in two different areas of the cell, such as the extremities. (a) Regions, from which signals are extracted, and "dist" the distance between the zones. (b), (c) The extracted signals: the colour of the curve is representative of the area from which they are extracted.*

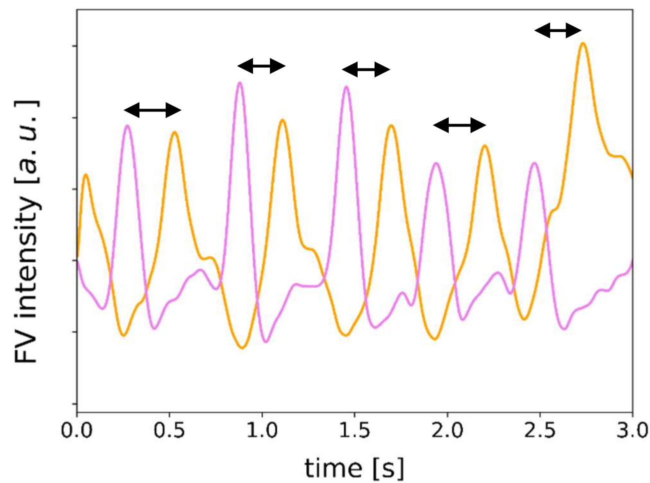

Figure 4.12. *The extracted signals are out of phase over time: the phase shift time is quantifiable between respective peaks and indicates the time needed to the action potential to travel from one part of the cell to another*

This calculation is made for all pairs of detectable peaks. Dividing each time interval found by the distance between the regions used to extract the signals, the output is the propagation speed of the depolarizing wave. By having obtained multiple values, it is visible that these values are distributed in a Gaussian way, and the average value of the distribution can be used as a representative value of the propagation speed of the action potential (Figure 4.13)

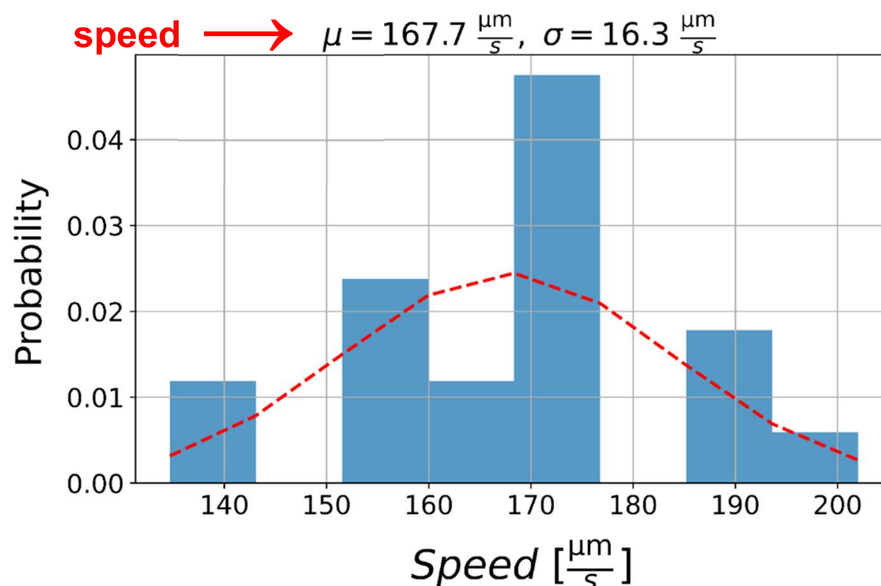

Figure 4.13. *The velocity of propagation of the potential is calculated as the time necessary for the depolarizing wave to pass from one end of the cell to the other over the distance between the two extremes. Calculating this quantity for each action potential gives a Gaussian distribution of this value. The mean value can be considered the propagation speed.*

4.4.3 Cardiomyocytes beating frequency

Due to video stabilization, local extracted signals are very clean and smooth. For this reason, by directly applying the Fast Fourier Transform (FFT) to the signals of figure 4.11, we obtain an easily interpretable signal spectrum: the peak is representative of the frequency with which the cell is firing action potentials (Figure 4.14).

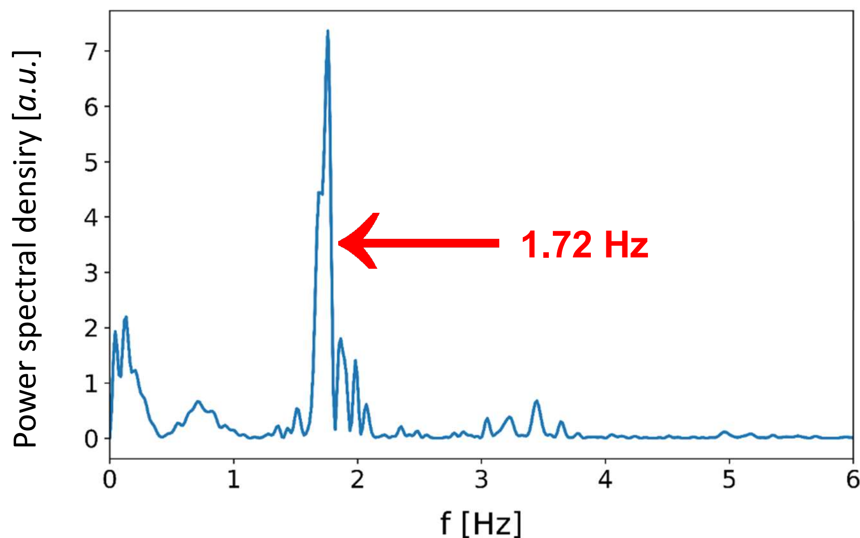

Figure 4.14. *By applying FFT to the fluorescence intensity signals extracted from the video, the beating frequency of cardiomyocytes is obtained.*

4.5 Results and future perspectives

In the previous paragraphs it was shown how to analyze the 2D signal acquired through the camera: a video recorded in the GFP/FITC configuration allows to capture the FluoVolt fluorescence. From the video a one-dimensional function of time is extracted and this signal is representative of the membrane potential. This procedure, in the case of cardiomyocytes, require a dedicated algorithm to automatically extract the contour of the cell in each frame.

Moreover, combining the advantages of an automatic cell segmentation for the realization of a video stabilization algorithm with the advantages of high spatial resolution offered by the use of an optical probe for membrane potential measurements, it is possible to obtain new insights into local membrane potential variations. Therefore, parameters such as beating rate and action potential conduction can be extracted. Key parameters for studying the physiopathology of cardiac cells.

Having acquired knowledge on how to analyze FluoVolt signals representative of membrane potential variations obtained by monitoring cardiomyocytes, the next step is to stimulate the cell with FluidFM.
One possibility is represented by a chemical stimulus, such as injection of an ion-channel blocker drug (which can be membrane impermeable). An alternative is represented by purely mechanical stimuli, through beads indentation anchored to a tipless cantilever by applying an underpressure to the microchanneled cantilever.
In both cases, the simultaneous monitoring of the membrane potential via FluoVolt have to be performed to study the dynamics of stimulated ion channels and to check how extracted parameters varies for different types of stimuli.

This step of cellular stimulation with simultaneous cell membrane potential monitoring is necessary for the continuation of the proof of concept project in which the goal is to demonstrate the feasibility of this simultaneous single cell stimulus and membrane potential monitoring. However, it was not possible to continue in this direction due to multiple problems: firstly, there was not consistent access to cardiomyocytes (delivery problems). They were isolated in another department/building. Secondly, received isolations often led to bad cells. Good cells appear in rectangular shape as in Figure 4.1, with sharp edges, but often received cells had rounded edges, meaning that the cells were close to death. The last reason is due to the technical difficulties encountered using state of the art cantilever specifically designed for cardiomyocytes injection (Figure 4.15).

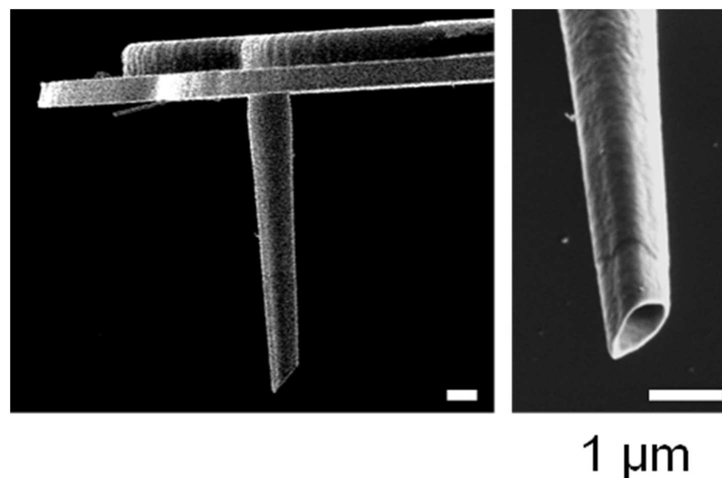

Figure 4.15. *FluidFM tubular tips specifically designed for cardiomyocytes injection. Credits: Elaheh Eelanjegh Zare*

These types of nanosyringes have a tubular tip, with an opening/cut engineered to facilitate the crossing of the tip through the very thick cellular membrane of cardiomyocytes. Often, while using these cantilevers, it was not possible to obtain a sufficiently high intensity AFM

laser reflected on the photodiode in order to conduct the experiment, limiting the progress made at the beginning of the proof of concept project.

4.6 Materials and methods

Cell line and culture

Freshly isolated cardiomyocytes (CM) cells were received in a specific buffer containing 135mM NaCl, 4mM kCl, 1.2mM NaH2PO4, 1.2mM MgCl2, 10mM HEPES, 11mM Glucose, 100uM CaCl2. Cells were progressively resuspended in the same buffer but with a different CaCl2 concentrations: 300uM, 500uM, 800uM. The last step was used to both load FluoVolt and seed the cells on laminin pre-coated dishes (WillCo HBST-5040). FluoVolt (Thermo Fisher Scientific) was loaded (30 minutes incubation) according to Thermo Fisher Scientific protocol using a specific buffer containing 135mM NaCl, 4mM kCl, 1.2mM NaH2PO4, 1.2mM MgCl2, 10mM HEPES, 11mM Glucose, 800uM CaCl2. This buffer was used also during washing steps (before and after loading FluoVolt, with 1ml of buffer) and during the experiments. All the solutions (for preparing and conducting the experiments) are at room temperature (RT).

Microscope setup

The microscope is equipped with a temperature-controlled incubation chamber (kept at RT for the experiment), an EMCCD camera system (Hamamatsu Photonics C9100-13 camera), a Smaract xy-stage which contains 100um piezo on sample holder for long range AFM measurements and a Colibri laser accessory for fast switching fluorescence illumination source.

Live imaging

For all images and videos, a 63x Water Immersion NA 1.3 *DIC* used with Immersol W2010 with 1.4 numerical aperture was used; images are in 16 bit format. Image acquisition was controlled using MicroManager open source software; Image and video were processed with MATLAB R2020a. FluoVolt was imaged using a 470 nm laser and a Zeiss 38HE emission filter. Images and videos dimensions are 512 x 512 pixel with 2px/um (2x2 binning to increase sampling frequency). FluoVolt videos are 8-16sec in length, acquired with 5% laser power (minimal phototoxicity), 16.5msec sampling period and 15msec exposure.

Chapter 5

HEK293-based project

The procedure for extracting a signal from FluoVolt data that is representative of membrane potential variations in cardiomyocytes has been described in chapter 4. However, the goal of the project is to combine this membrane potential measurements with a single-cell stimulation via FluidFM to trigger membrane potential variations. This was not possible for cardiomyocytes, as discussed at the end of chapter 4. In parallel with the project based on cardiomyocytes, the same project was conducted with another type of cells: HEK293 cells. The challenges that did not allow the continuation of the cardiomyocyte-based project did not arise with HEK293 cells and the reasons will be explained in the following paragraphs. In this chapter some basic notions about HEK293 cells will be presented. Successively it will be presented which type of stimulus to was chosen to stimulate the cells, the complete procedure for data acquisition during the experiment with the FluidFM and the troubleshooting that was adressed to reach FluoVolt and FluidFM compatibility. Finally, the results obtained from the experiments and future ideas for the continuation of the project will be presented.

5.1 HEK293 $Na_V1.5$

Human embryonic kidney 293 (HEK293, HEK-293, or HEK) cells are one of the most common cell lines used for research purposes, second only to HeLa cells. The HEK293 cell line was initially produced in 1973 from normal fetal human embryonic kidney cells. These cells were created following transfection of adenoviral genome parts into human chromosomes of the fetal cell's genome. The number 293 came from the scientist discoverer habit of numbering his experiments.

HEK293 cells are frequently utilized in research for several reasons. Firstly, they are very easy to grow and to maintain, with high reproducibility, which makes them preferable over

other less-robust and slow-growing cell lines. This is also the first reason why was possible to continue with the proof-of-concept project part of this thesis (not the case with cardiomyocytes, which are extremely more delicate cells).

Furthermore, HEK293 cells are very efficient at protein production and accessible for transfection. Through the insertion of plasmid vectors, the cell's synthetic protein machinery can be influenced to make the transfected protein, utilizing the inserted genetic information. In fact, the HEK293 cells used in this project are modified to overexpress the voltage-dependent sodium channel $Na_V1.5$, and these cells are called HEK293 $Na_V1.5$. This $Na_V1.5$ channel, encoded by SCN5A gene, is typically considered a cardiac-specific isoform and represents the majority of channels in adult cardiomyocytes. $Na_V1.5$ is intimately involved in cardiac action potential conduction because it mediates the fast influx of Na^+ ions across the cell membrane, resulting in the fast depolarization phase of the cardiac action potential. Accordingly, mutations to this protein are associated with many different types of cardiac arrhythmias. Therefore, there is this parallelism between cardiomyocytes and HEK293 $Na_V1.5$ cells and the initial idea was to append the study of cardiomyocytes with the study of HEK293 $Na_V1.5$.
HEK293 have huge nuclei: approximately two-thirds of HEK293 cell is nucleus and they have a diameter of around 15-20um. HEK293 cells are typically grown as a semi-adherent monolayer (Figure 5.1, green box). Due to their characteristic semi-adhesion, HEK293 requires coated dishes (e.g., fibronectin) otherwise, during the cells washing steps required to load FluoVolt, some cells detach from the substrate and end up on top other cells (Figure 5.1, red box) leading to more complicated experiments and data analysis.

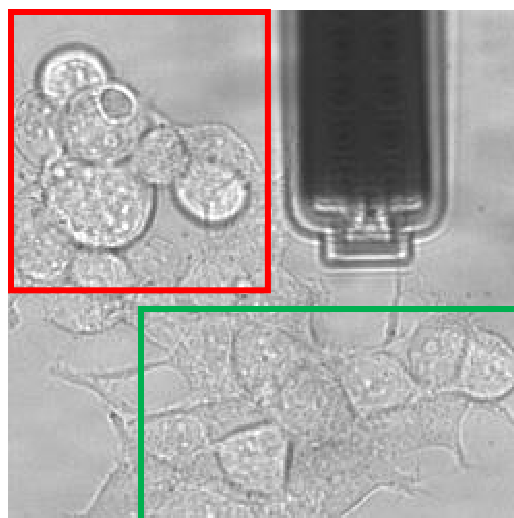

Figure 5.1. *HEK293 tends to grow in monolayer (green box), but without dish coating procedures, some cells detach and fall on top of other cells.*

Their volume ranges from 3000 to 5000 femtoliters. Since HEK293 grow fast, when they are ready for the experiments, HEK293 already formed colonies (Figure 5.2).
In FluoVolt images as figure 5.2, it is evident that the edges of the cells are not clearly visible as for single cells (e.g., cardiomyocytes of Chapter 4). For this reason, active contour based analysis has not been applied to extract the fluorescence signal, but another technique has been used and it will be explained in the following paragraphs.

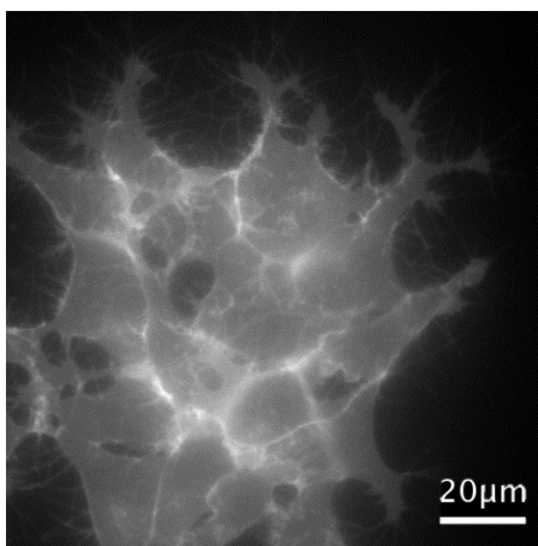

Figure 5.2. *FluoVolt image of HEK293 forming colonies. This complicate the data analysis.*

5.2 Chemical stimulation via FluidFM injection

The goal is to stimulate the cell during monitoring of membrane potential. Among all the possible stimulations, it was chosen to proceed with a chemical stimulation, that is the injection of molecules inside the cell with the aim of triggering membrane potential variations that are quantifiable, in order to reach the goal of the proof of concept project.
Multiple candidates are eligible for an injection that triggers changes in membrane potential. It's possible to inject drugs called ion channel blockers, and depending on the type of ion channel that they affect, they can induce changes in resting membranes potential, or in the dynamics of excitable cell. In this class of drugs there are molecules impermeable to the cell membrane, for which injection is the only way to access the intracellular fluid and the FluidFM represents a solution to this limitation. Another possibility for triggering potential changes through injection is represented by injection (active delivery) of small charged molecules, such as ions. Ionic charges are the main

molecules responsible, at the physiological level, for the creation of the resting membrane potential as well as its rapid variations, the action potentials. Ion injection is therefore a reasonable choice to trigger changes in the membrane potential by directly disrupting the homeostasis of intracellular ion concentrations. The ions mainly involved in this homeostasis are calcium(Ca^{2+}), potassium (K^{+}) and sodium (Na^{+}). It is known that Ca^{2+} is very important not only in determining the static and dynamic characteristics of the membrane potential, but also plays a fundamental role in many cellular processes involving signaling mechanisms and in determining the mechanical properties of the cell. Instead, in the current state of knowledge, Na^{+} and K^{+} have a privileged role in influencing the electrical activity of the cell. For example, the resting membrane potential (RMP) of the cell is very close to the Nerst potential for K^{+} ions, indicating that the latter make the greatest contribution to RMP formation. However, in this case, HEK293 available cells were overexpressing a specific $Na_V1.5$ ion channel for sodium. Aware of this feature, active delivery (i.e., forced injection into the cytoplasm) of Na^{+} ions in a cell line that overexpresses sodium channels is expected to cause a measurable response already from the first seconds after stimulation. For these reasons, it was chosen to inject ions into the intracellular fluid of HEK293 cells, and in particular Na^{+} ions. From a practical point of view, this is equivalent to filling the microchannel of the cantilever with a physiological solution whose concentration of Na+ is known, and this solution is what is injected into the cell during the experiment.

The following experiments were therefore designed:

- control experiment: inject only $[Na^{+}]=0$ physiological solution into some cells. The control group allows to isolate as much as possible the effects of the treatment (Na^{+} injection) from all the others. This makes it easier to exclude alternative explanations of the results obtained from the experiment.
- inject the same physiological solution into some cells, with $[Na^{+}]=150mm$
- inject the same physiological solution into some cells, with $[Na^{+}]=600mm$

in order to obtain 3 different classes of injected cells, to be compared between them.

In parallel with the attempt to stimulate the cell during simultaneous measurement of membrane potential, it is relevant to understand if any visible variation in membrane potential depend significantly on the type of stimulus, in this case the sodium concentration. his is the reason why two sodium concetrations were tested.

During the experiment, HEK293 cells loaded with FluoVolt are kept in a buffer whose Na^+ concentration is 150mm. For this reason, this concentration was chosen as the target of one of the two injections, with the aim of increasing the concentration of intracellular Na^+ causing depolarization of the cell membrane. The second target, equal to 600mM, represents an increase in the concentration of intracellular Na^+, above the physiological limit, with the purpose of testing the cells in a condition of stress.

Injecting into HEK293 does not require tubular nanosyringes (Figure 4.15) needed to overcome the thick cell membrane of cardiomyocytes. Pyramidal tip nanosyringes are commercially available from CytoSurge (Figure 5.3) with a lateral opening and nominal spring constant equal to 2.2 N/m. Gently approaching this pyramidal tip above the cell and pressing it against the cell membrane with a force that for HEK293 is around tens of nN, it is possible to insert the opening of the tip inside the cell and inject the solution contained in the cantilever. The use of commercially available probes has avoided the challenges encountered with tubular nanosyringes (difficulties in obtaining the reflected AFM laser on the photodiode). This is the second reason why the project based on HEK293 cells and not the cardiomyocyte project has been successful.

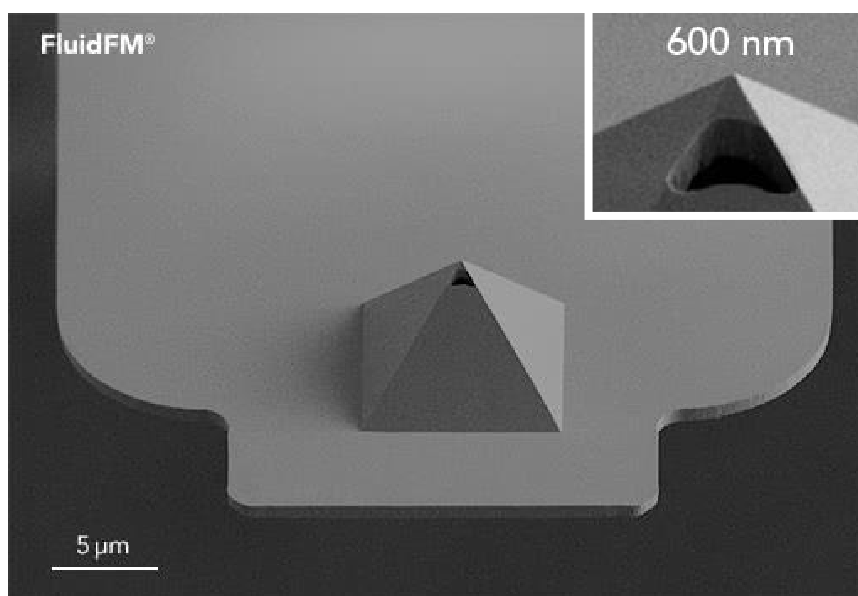

Figure 5.3. *FluidFM nanosyringes used for injecting Na^+ into HEK293 cells. These nanosyringes have a pyramid-shaped tip, with a small aperture on the side of the pyramid through which the solution contained into the cantilever exit. Tip dimensions: 10um x 10um (pyramid base) x 7um (pyramid height).*

Injection into the intracellular fluid then involves bringing the tip of the cantilever in contact with the cell membrane. During this contact, parts of the membrane and other organic material present on them (e.g., proteins) can remain adsorbed to the surface of the cantilever and in particular to the pyramidal-shaped tip, causing the openings to become clogged. To avoid this phenomenon as much as possible, the hydrophobicity of the surface of the cantilever is increased by coating overnight with SigmaCote (Sigma-Aldrich) in vapor.
Finally, it should be made clear that whenever an injection attempt is made into the cells, there must be evidence that the injection actually took place. Sometimes, in fact, because of the partial clogging of the tip openings or because of the not perfect coordination of all the steps to be done manually and quickly in sequence to make the injection, it happens that injection fail. For this reason, in addition to filling the cantilever with Na^+ to be injected, there is another important substance to consider, necessary for the success of the experiment. This substance should be a fluorescent impermeable molecule to the cell membrane, visible with the correct settings for lasers and filters on the microscope, and should be injected simultaneously with the solution containing Na^+. Only injected cells will appear fluorescent in the correct settings to capture the signal of this substance.

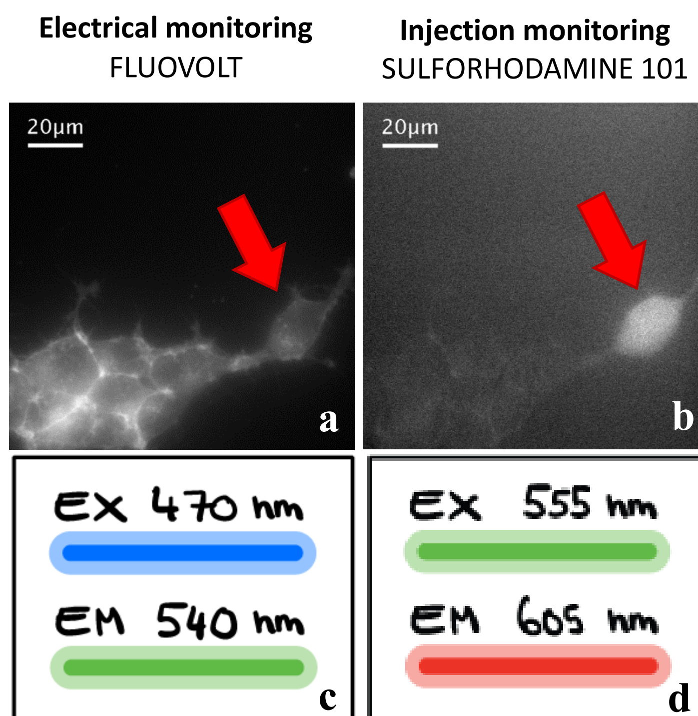

Figure 5.4. *(a) Fluovolt signal: red arrow indicates the cell that will be injected. (b) The same cell after injection is visible with Sulforhodamine, not the other ones in the colony. (c), (d) Excitation and Emission respectively from Fluovolt and Sulforhodamide. Their emissions cover different parts of the electromagnetic spectra, allowing for multiplexing*

Obviously this substance used to monitor the injection must emit the fluorescent signal in an area of the electromagnetic spectrum that does not interfere with the signal emitted by FluoVolt.
The substance used in this work is called Sulforhodamine101, it is impermeable to the cell membrane, and emits the fluorescent signal in the red light part of the electromagnetic spectrum, unlike FluoVolt. In this configuration it is therefore possible to chemically stimulate the cell by injection and simultaneously monitoring the membrane potential through FluoVolt, and monitoring the injection by Sulforhodamine101 (Figure 5.4).

5.2.1 Experiment procedure

The procedure followed during the experiments is illustrated below, starting from the setting up of the FluidFM to the realization of an injection inside the cell.

FluidFM tool setup
The first step is to fill the resevoir with 10uL of the solution to be injected into the cell. The probe is then mounted on a support that is magnetically attached to the AFM head. Then the laser spot of the AFM is positioned manually near the tip of the cantilever and the offset measured by the photodiode is reset. Consequently, it is necessary to calculate the true cantilever spring constant because, due to manufacturing reasons, the nominal value provided by the manufacturer does not correspond exactly to the actual value. This calculation is done automatically through the software supplied with the AFM head. Next, by applying an overpressure on the fluidic channel, typically 1 bar, and waiting around a minute,causes the microchannel of the cantilever to be filled with the liquid previously loaded into the reservoir. This filling process is performed when the cantilever has not yet been immersed in liquid. It's a process that must be done in the air, otherwise immerging the cantilever with the empty microchannel in liquid, increase the risk that bubbles form inside the microchannel, resulting in clogging of the microchannel.
Then it must be verified that the microchannel of the cantilever is actually filled. This can be done by looking at the cantilever in the laser configuration and microscope filters to capture the fluorescence of sulforhodamine101. If in this configuration the cantilever appears fluorescent the filling of the microchannel is completed. When the microchannel is filled, the cantilever can be immersed in the liquid. Finally, the last calibration step of the instrument is necessary: with a process that happens automatically, the cantilever approaches a rigid substrate five times (the bottom of the plate) in force spectroscopy mode and through this process the software extracts a parameter called sensitivity. It is through these two factors, spring constant (N/m) and sensitivity (m/mV) that the software converts

the measured signal on the photodiode (mV) into a force-feedback signal (nN) used by the PID controller. The instrument is ready for the experiment and the goal is to inject the solution in the cantilever into the cells.

Preliminary steps: choosing the cell and cantilever relocating

The following is a description of the procedure used to perform the injection via FluidFM:

1) Choosing the cell to inject (Figure 5.5a)
2) Bring the cantilever in contact with the dish and take an image (Figure5.5b): this image is necessary because during data analysis, as will be shown below, the selection of the injection point is required. An image with the cantilever in contact with the dish shows the point inside the image where the cantilever will touch during the single cell force spectroscopy
3) Distance the cantilever (about 20um) from the dish and relocate the cells under the cantilever openings, moving the XY plane/stage on which the cells lie. In order to be able to relocate the cells under the cantilever, the cantilever must be sufficiently far away not to touch the cells during the relocation. (Figure 5.5c)
4) Further distance the cantilever from the surface so that the total distance between the cantilever and the dish is (at least) 70um. The cantilever gradually exits the focus plane, resulting more blurry.(Figure 5.5d)

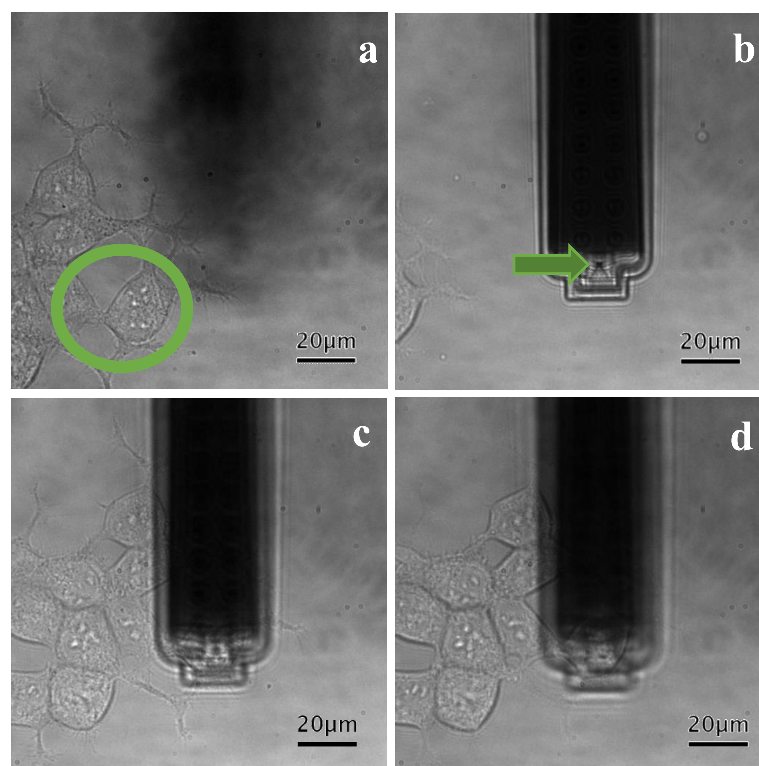

Figure 5.5. *(a,b,c,d) Preliminaries step before injection*

Performing injection

1) Start recording signals from the FluidFM: the force with which the cantilever is deflected and the pressure applied to the microchannel. Start video recording in Fluovolt settings to monitor membrane potential. Start single cell force spectroscopy. During this first phase, the dish with the cells moves towards the cantilever, coming out of the focus plane (Figure 5.6).

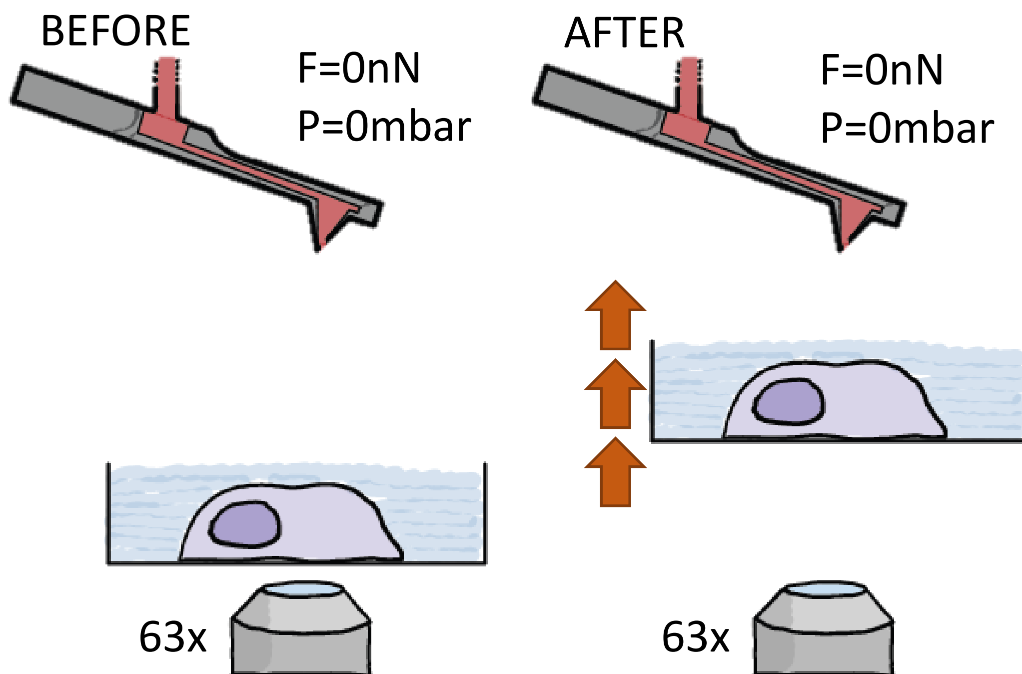

Figure 5.6. *During this first phase, the dish with the cells moves towards the cantilever, coming out of the focus plane*

2) During the phase in which the cantilever moves towards the cell, called approaching phase, the deformation of the cantilever is (practically) zero. In the moment when the cantilever touches the cell it begins a phase of rapid growth in the time-deformation curve, indicating that the contact happened with the substrate and the subsequent crushing of the membrane with the aim of causing a very small rupture such that by applying an overpressure, a substance can be injected. is happening. FluoVolt video is recorded (Figure 5.7).
3) The force necessary for the injection is monitored in live to see when the force setpoint (which is a parameter to be fixed by the operator) is reached. For the HEK293 it is about 60nN. Once this setpoint is reached, it is maintained for a fixed period of time decided by the operator (3s), and then an overpressure of 20mbar is applied for 1s (parameters set by the operator) on the microchannel to perform the injection. (Figure 5.8)

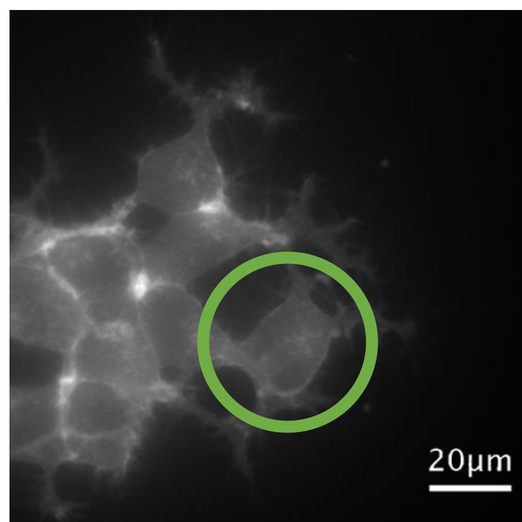

Figure 5.7. *FluoVolt video is acquired during the force spectroscopy.*

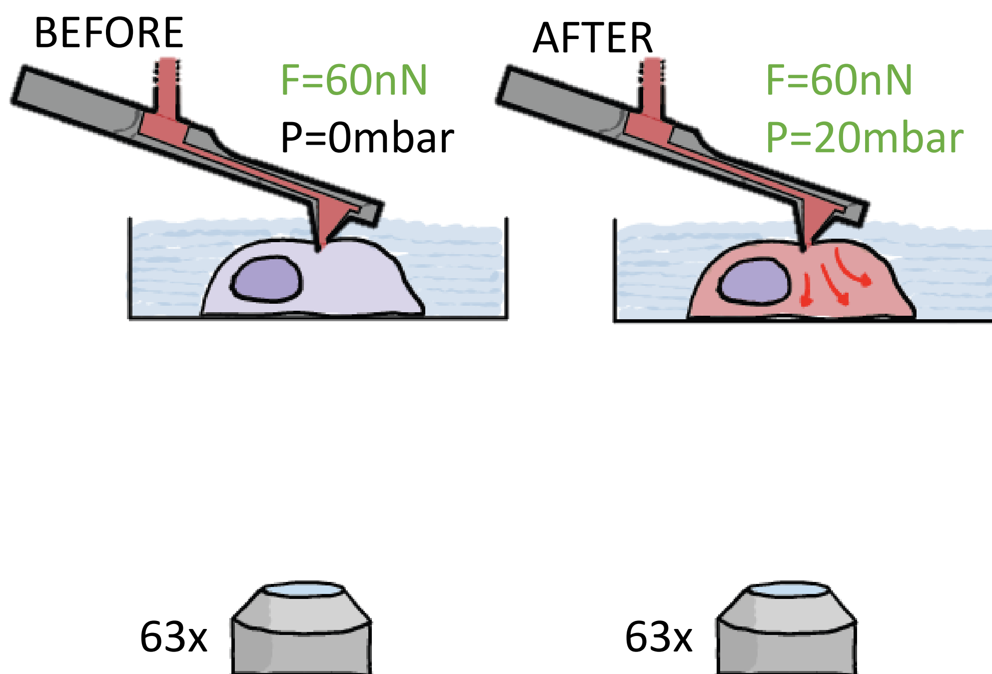

Figure 5.8. *Injection is performed with a 20mbar pressure impulse during the 3 seconds in which the tip stays in contact with the cell, keeping 60nN constant.*

4) The cells return in the focus plane (Figure 5.9). This ends the force spectroscopy and the video is kept recording for monitoring membrane potential after cell stimulation. After a certain amount of time, set by the operator, the video recording is stopped. It's necessary to look at the fluorescence of Sulforhodamine101 to determine if the injection happened (Figure 5.10).

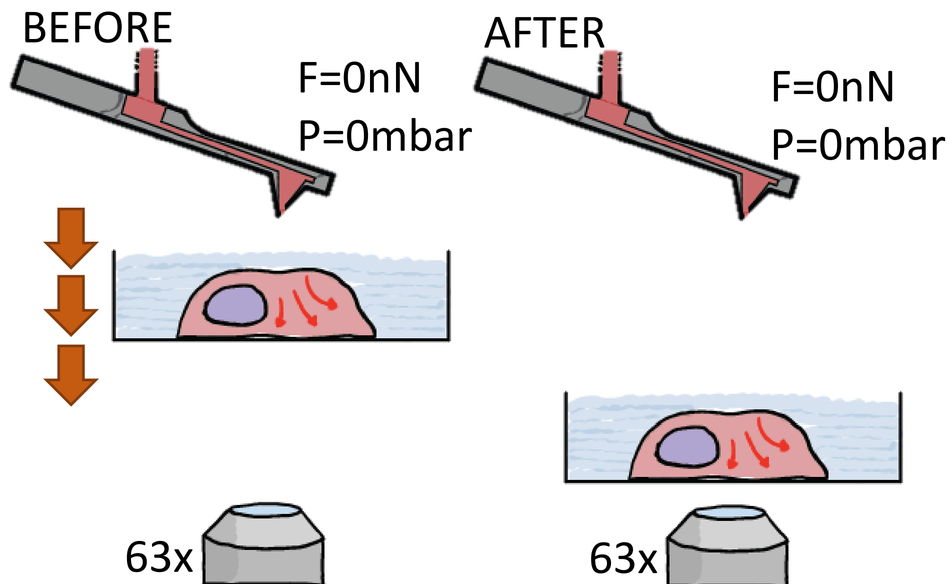

Figure 5.9. *Cells return in the focus plane after injection, allowing membrane potential monitoring right after the stimulus*

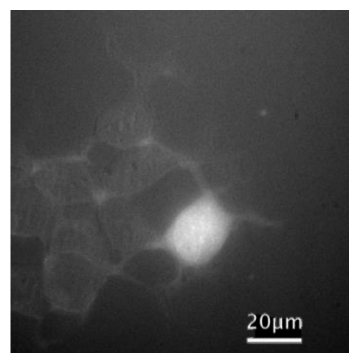

Figure 5.10. *Injection validation through Sulforhodamine101*

5) All the data needed for the analysis have been acquired: the monitoring of the force applied by the cantilever on the cell vs time (F vs t), the monitoring of the value of the pressure applied in the microchannel (P vs t) and the monitoring of membrane potential by capturing FluoVolt fluorescence video. This process have to be repeated again for a new cell.

5.2.2 Electrical and mechanical signals from injected cells

To get the representative signal of membrane potential variations from a FluoVolt video, you must select in each frame of the video, the portion of the image in which the cell is present. For cardiomyocytes, a special algorithm was developed that could select the cell correctly even in the presence of its contractions. In the case of HEK293 this algorithm is not applicable: by not being single cells, but being in colonies, the algorithm could not reliably find the cell boundary due to the lack of clearly visible cell edges (Figure 5.1). This in principle is not a problem, since the HEK293 do not have the ability to contract and therefore the portion of the image they occupy should remain constant throughout the video. A manual selection of the contour of the cell, extracted looking at the first frame of the video is the method used for extracting the signal. The binary mask that is obtained (Figure 5.11) is used to extract the cell's fluorescence 1D signal from the FluoVolt video.

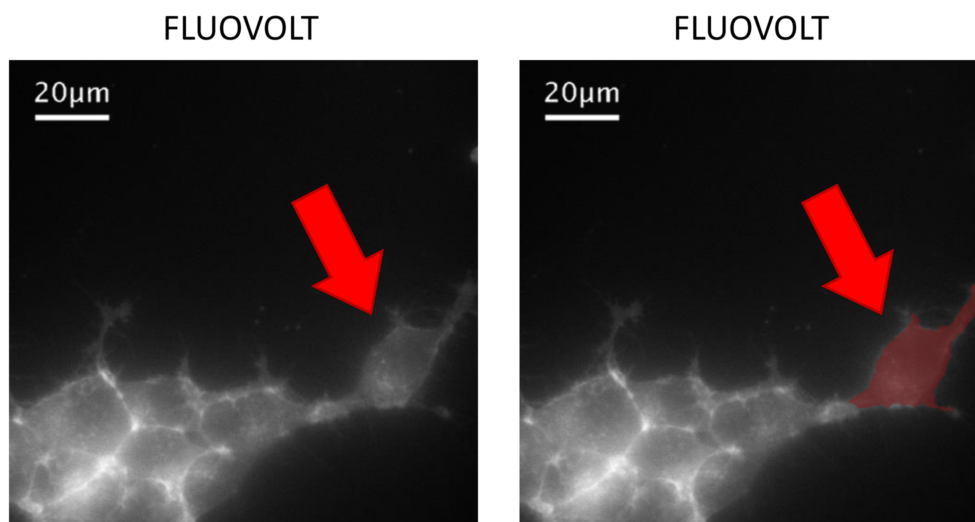

Figure 5.11. *Right: cell to be injected. Left: manually selected cell boundary to extract the fluorescence intensity signal from the video.*

Finally, it is useful to visualize the signals extracted during the experiment, to understand the dynamics of the quantities involved. In particular, it is convenient to visualize the trend over time of the following quantities: the intensity of FluoVolt fluorescence, the force applied by the cantilever on the cell, and the pressure applied to the microchannel (Figure 5.12) for the duration of the entire FluoVolt video.

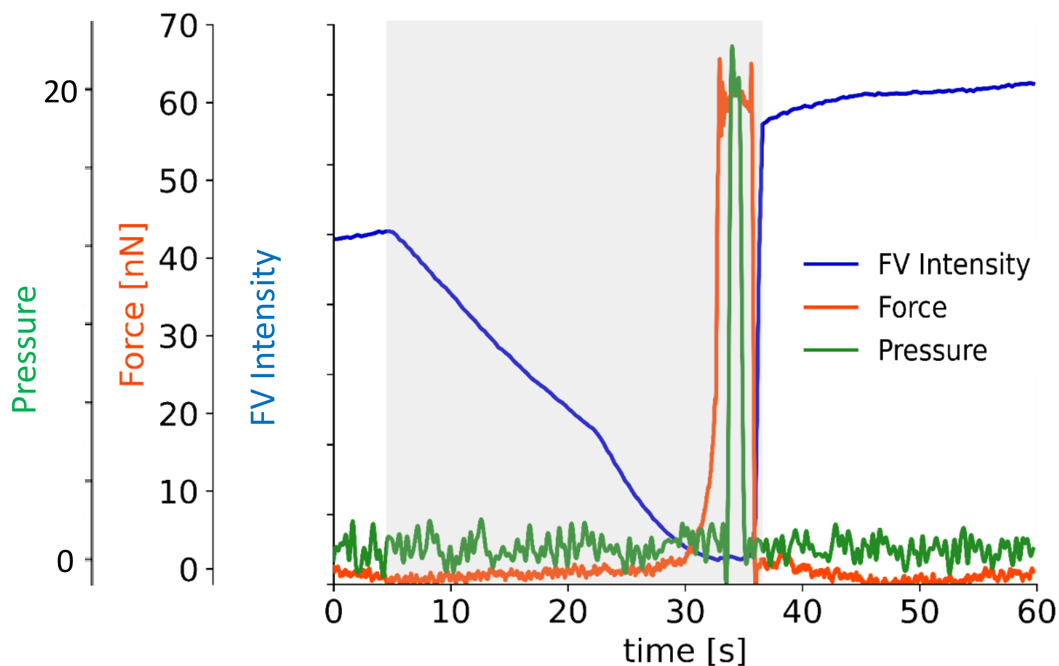

Figure 5.12. *Signals extracted during the experiment*

First of all, it is noticeable an area of the graph highlighted in grey: this is the time window in the video during which the cells are not in focus, and therefore the fluorescence signal is not representative of the membrane potential. In this time window, therefore, it is not significant to evaluate the fluorescence signal. However, it is useful to note that it is in this "blind" area (from the point of view of fluorescence), that the injection takes place in the cell: it can be noted the rapid increase in force up to 60nN fixed, and during the pause time of 3 seconds, a pressure pulse of 20mbar occurs such that the injection takes place.

The intensity of the fluorescence remains the focal magnitude of the analysis. It is meaningful to know whether the chemical stimulus that is being applied to the cell causes a response in the membrane potential, and whether this response has a dependence on the different sodium concentrations tested. With HEK293 there is a static fluorescence response (Figure 5.13) that does not show rapid variations as cardiomyocytes were showing during contraction. Here, the fluorescence curve is characterized by an initial value, almost in steady state, which undergoes a visible change during post-stimulus. In fact, after the stimulus, it is still noticeable an almost constant value, but shifted towards higher values. This is representative of a depolarization of the membrane.

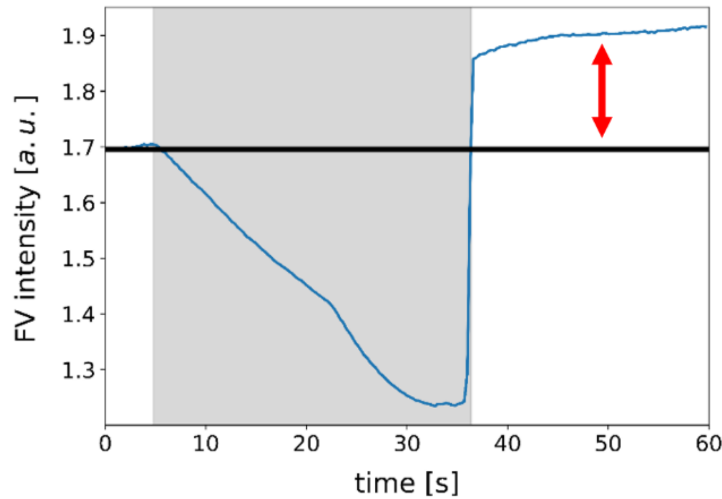

Figure 5.13. *HEK293 extracted fluorescence intensity shows a static curve, and shifted values after the stimulus*

To understand if this depolarization is due to sodium injection, it is necessary to quantify the variation that is obtained between (approximately) the initial value, or the value of fluorescence before the stimulus, and the final value, (the value of fluorescence after stimulation). However, the raw data are not directly usable as they are not representative of the absolute value of the membrane potential. By having no calibration of FluoVolt it is possible to study only the variations that the signal undergoes, knowing that about 25% of relative variation ΔF/F corresponds to a change of 100mV of the membrane potential, according to ThermoFisher. For this reason the data is normalized (Figure 5.14) as follows:

$$\Delta I_{\%} = \frac{I - I_0}{I_0} * 100$$

dove I è l'intensità della fluorescenza misurata istante per istante, e I_0 l'intensità iniziale.

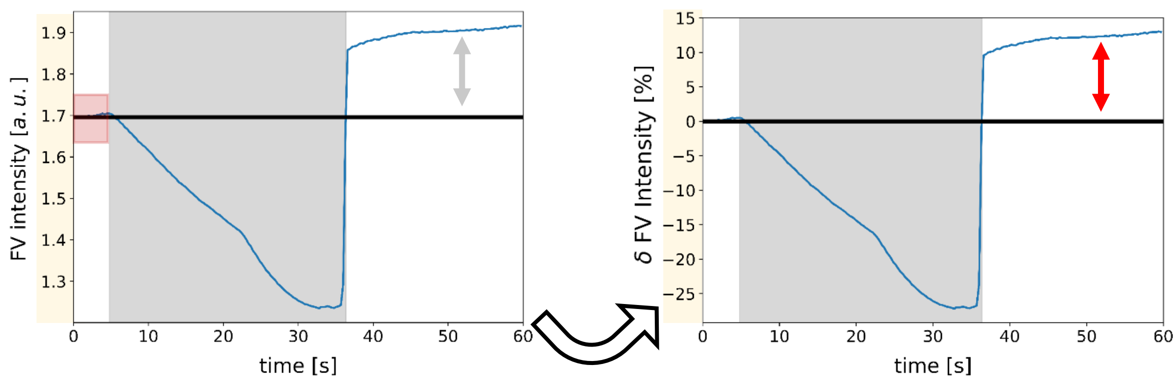

Figure 5.14. *The values in the y-axis are normalized to percentage variations with respect to pre-stimulus value.*

Therefore it is meaningful to quantify if the variation of intensity between pre and post stimulus value, and therefore the variation of the membrane potential, indicated in figure 5.14 by the double pointed red arrow, depends on the sodium injection.

5.3 Results

The question is: does this highlighted ΔI in figure 5.13 depend on what we injected? This quantification of the cell response is calculated upon intensity normalization $\Delta I/I$, as depicted in figure 5.14

5.3.1 Increased intracellular sodium depolarizes the cell membrane

To assess the response of cells to different sodium concentrations, it was considered the response at 12 seconds post-stimulus. The reason why it was chosen 12 seconds after the stimulus is due to the fact that not all cells had monitoring data greater than 12 seconds post stimulus. In order to evaluate the greatest number of datapoints, a post-stimulus time was chosen such that it was present in all datapoints. The results can be represented through a boxplot (Figure 5.15). It is therefore visible that as the concentration of sodium in the injected solution increases, the cell membrane of the stimulated cell undergoes a greater depolarization. The percentage of intensity change can be converted to membrane potential change using the conversion factor provided by ThermoFisher. However, being it an inaccurate value (from scientific literature), it was considered appropriate to maintain the variations expressed in percentage, and not in mV.

It is not necessary to limit the evaluation of the cellular response to 12 seconds post-stimulus. In fact, it is possible to mediate the entire dynamics of the cellular response of cells belonging to the same class of stimulus (same concentration of sodium injected) in order to obtain the average cell response for each class (Figure 5.16). It is therefore noted that in post-stimulus dynamics, the variation of the membrane potential with respect to the initial potential, moves in the direction of a depolarization of the cell membrane.

This depolarization is higher in magnitude with higher concentration of injected sodium already during the first moments after post-stimulus. And this behavior remains throughout video monitoring.

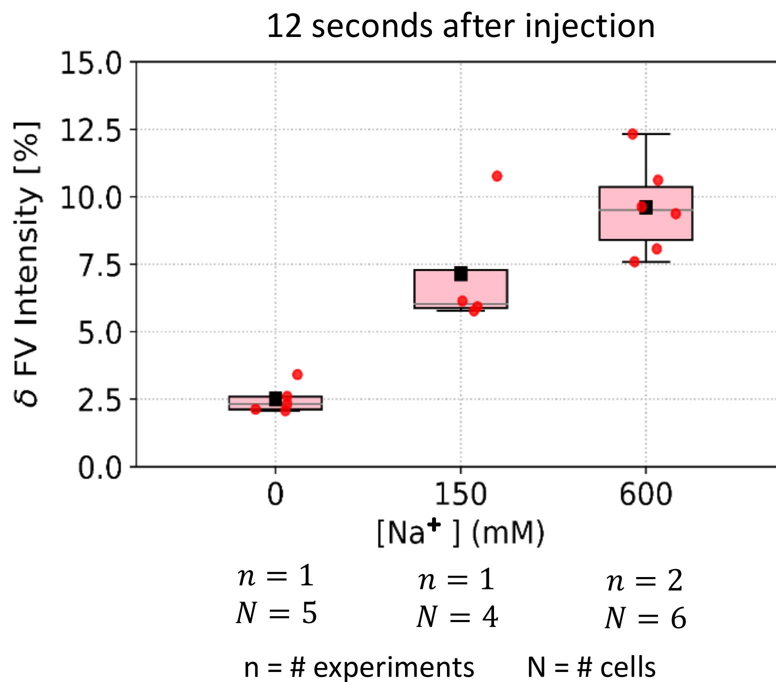

Figure 5.15. *Higher concentration of injected sodium results in a higher (in terms of magnitude) depolarization of the cell membrane. Each red dot is one cell. Black dot: average of the red dots. Gray line: medium of the distribution*

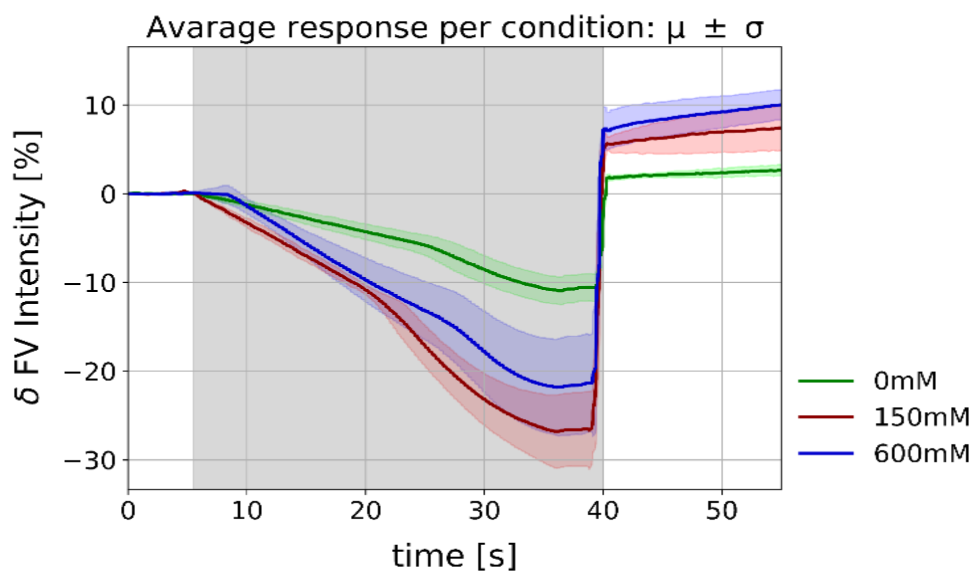

Figure 5.16. *Avarage dynamic for each tested condition. Each curve is the mean ± std. After stimulus, the depolarization remains higher for cells injected with higher sodium concentration*

With this proof of concept project, which triggers changes in the membrane potential against an active delivery of sodium, the possibility of stimulating individual cells with simultaneous monitoring of changes in the membrane potential (respectively through the use of FluidFM and FluoVolt) is demonstrated. It is therefore possible to combine in a single methodology the versatility that both these instruments offer, expanding the possibilities of study cellular processes that, triggered by mechanical and chemical stimuli, involve changes in membrane potential.

5.3.2 Including injection site information into the analysis

To extract the fluorescence intensity signal from the FluoVolt video, a manually selected mask was used on the first frame of the video. This is because, unlike cardiomyocytes, HEK293 cells do not undergo contractions, and therefore the space they occupy in the image does not vary over time, at least within the minute during which they are monitored. However, it has been noted that, during injection, the pressure of the pyramidal tip against the cell with a force equal to 60nN cause crushing of the cell and a consequent deformation of the cell (Figure 5.17). Such deformation has consequences in the analysis: the area that the cell occupies in the image varies from pre-stimulation to post-stimulation, not in negligible values. It is about 20%, as shown in the example.

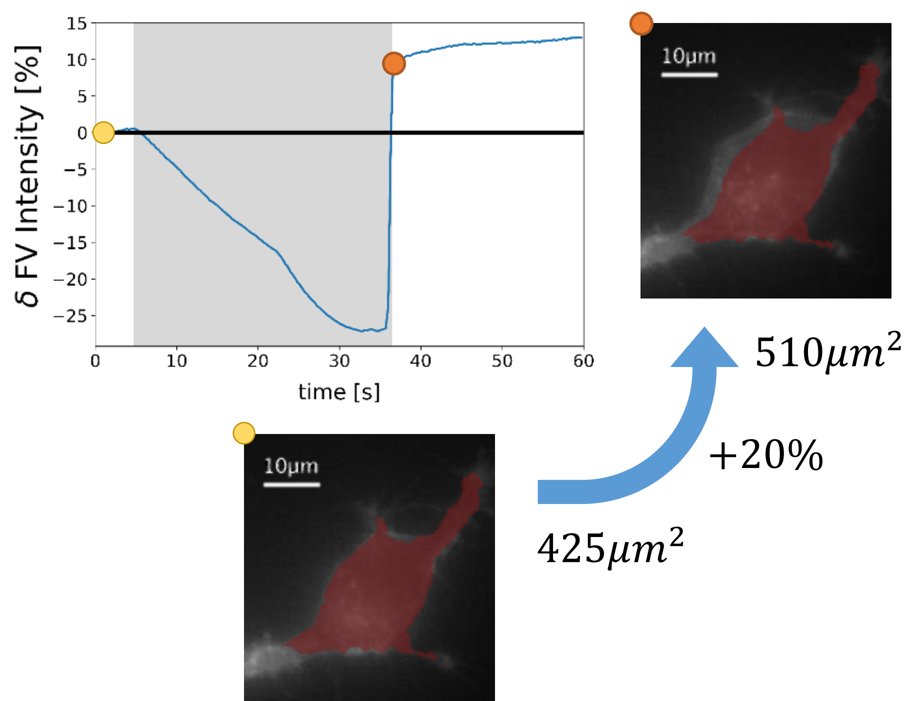

Figure 5.17. *Cell undergoes a deformation caused by the cantilever crushing on it. For this reason, the mask selected in the first frame of the video is not fitting the same cell after the stimulus*

It is therefore clear that using the same mask (visible in red) to extract the intensity signal throughout the duration of the video, can introduce bias in the signal. Sometimes, in fact, the cell comes out of the mask and in this process some regions of the background are wrongly considered in the calculation of the membrane potential, artificially lowering its value. Other times the opposite happens: there are regions of the cell that appear extremely whiter (high intensity) and due to the crushing of the cell, it changes the proportion of space they occupy inside the mask, causing a bias towards higher values.
The crushing effect can therefore cause bias in the measure of variations in the fluorescence intensity, and this is due to the asymmetric way in which the cell exits the edges of the manually pre-selected mask. It is therefore necessary to have an analysis technique that is invariant with respect to the asymmetric deformations of the cell caused by its squeezing. The idea is to exploit the available information on where injection point is, using the image of the cantilever in contact with the dish (Figure 5.18) that is acquired during the experiment, as already illustrated above. Around the injection point the deformation that the cell undergoes is geometrically symmetrical due to the geometry of the pyramidal tip.

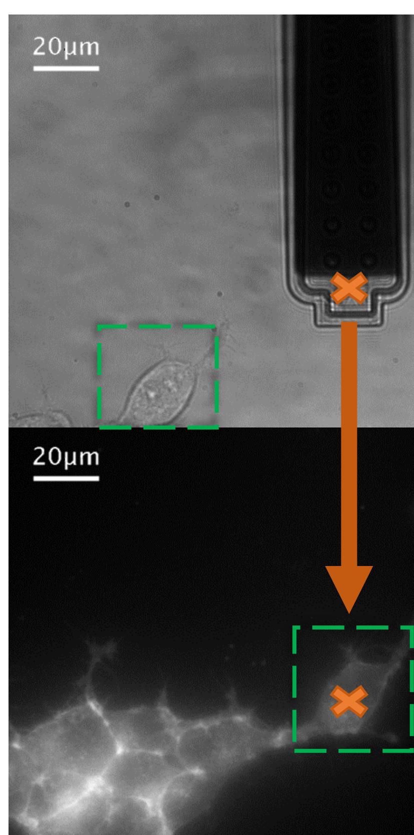

Figure 5.18. *It is possible to get the injection point information by selecting injection point in the image above (Brightfield), and returning the point in the image below (FluoVolt). What moves from one image to another are the cells, which are relocated under the cantilever. In the green box the cell to be injected*

For this reason it has been chosen to use a mask that was symmetrical: a ring centered in the injection point. Rings with rays of various sizes were tested (Figure 5.19) to extract their respective fluorescence intensity signal (Figure 5.20).

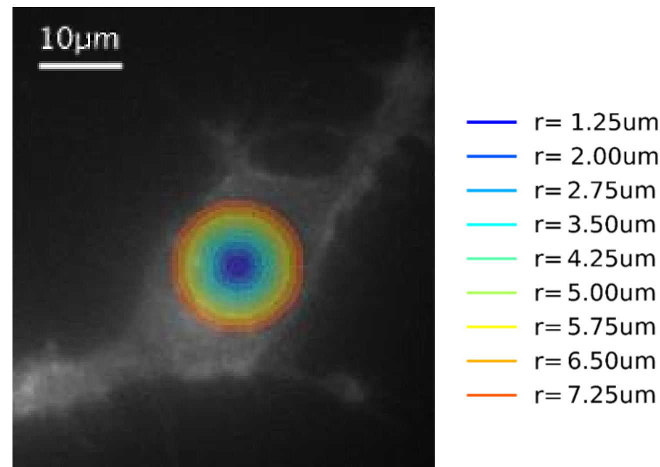

Figure 5.19. *0.75um thick rings were used as symmetrical masks centered on the injection point to extract FluoVolt signals*

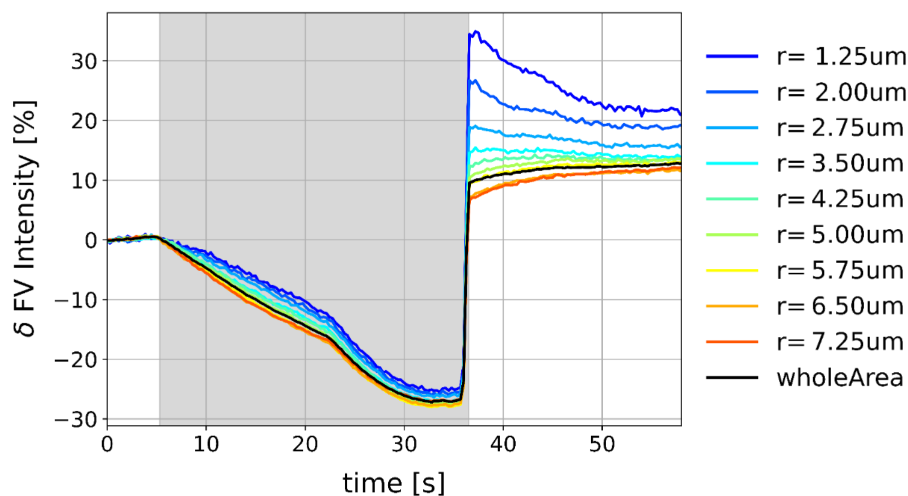

Figure 5.20. *Extracted signals for different ring masks*

To choose which mask to use to analyze the overall membrane potential, a ring that was representative of both the pre-stimulation and post-stimulus membrane potential was sought. For some injected cells, pre-stimulation and post-stimulation video were analysed independently, using two manually selected masks on the pre-stimulation video portion and the post-stimulation video portion. The obtained fluorescence intensity curve was qualitatively compared with the result obtained from ring-based analysis centered at the injection point. As a result, analyzing the membrane potential through 0.75um rings whose

inner radius is greater than (at least) 5um, provides an output fluorescence intensity signal more representative of the membrane potential, compared to what is achieved with an analysis based on the selection of the cell boundary in the first frame of the video. This analysis based on the rings allows, therefore, in a simple way (but facing a few more steps during the experiment and during the acquisition of the image of the cantilever in contact with the dish) to obtain a more precise measurement of the variations of the membrane potential, even in the presence of cell squeezing caused by the pyramid tip, without having to separate the FluoVolt video in pre-stimulation and post-stimulation. The advantage is also due to the fact that in post-stimulus, due to the elasto-plasticity properties of the cell, the cell goes into a relaxation deformation phenomena (changing shape) but with ring-based analysis the mask continues to be geometrically invariant to deformation, while even a manually segmented frame right after the injection, is still not good enough because it can not follow these mechanical relaxation phenomena.

It is sufficient to choose only one of all the rings previously shown for the extraction of the FluoVolt signal, with the constraint that its internal radius is greater than 5um. It was chosen to use the 5um radius to redo the analysis shown in paragraph 5.3.1, as using larger radiuses does not guarantee that the signal can be extracted from all cells. Because of cell variability, some cells are smaller and others larger, so choosing the smallest radius that meets the constraint allows to be sure that it will works in both large and small cells. Ring-based analysis, 12 seconds after injection, shows the same trend as before (Figure 5.21), that is, higher membrane depolarization depending on the injected sodium concentration.

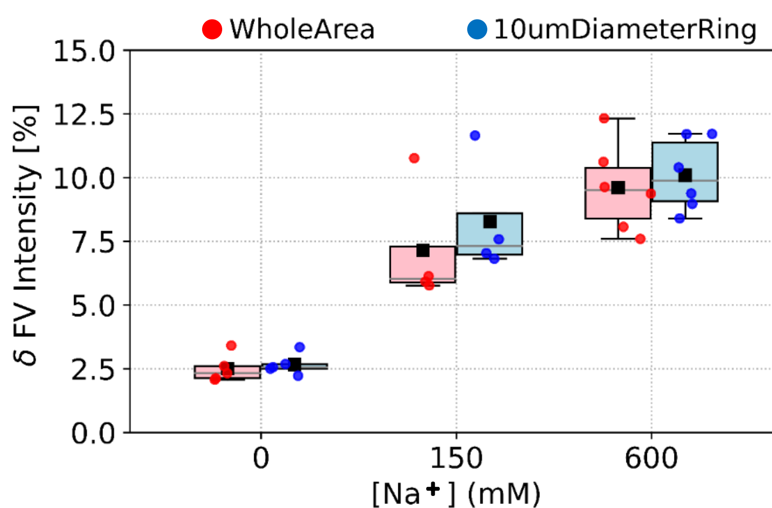

Figure 5.21. *Red: results from analysis based on a mask segmented on the first frame of FluoVolt video. Blue: results obtained using 5um radius ring centered in the injection point as a mask to extract the fluorescence intensity signal*

Moreover, it is noted, especially for the 600mM condition, a decrease in the variability of the result: the datapoints are less scattered around the average value. Probably this is due to the reduction of the artifacts (random) introduced due to the squeezing of the cell in a non-symmetrical way out of the manually selected mask on the first frame of the video, from which the ring-based analysis is exempt, This represent a useful and smart technique for extracting the Fluovolt signal.

5.4 Future perspectives

During extraction of FluoVolt signal through ring-based analysis, a different dynamics emerged depending on the distance from the injection point where the pixels considered for signal extraction are located. Frequent phenomenon in the analyzed data is the presence of a large peak in the curve obtained using the smallest ring, with radius 1.25um (Figure 5.22). Highlighting three frames extracted from the video, indicated with 3 colored dots on the graph, it can be noticed also visually thanks to a suitable color scale, the presence of a high-intensity spot gradually decreasing its brightness. This phenomenon appears in all tested conditions of different sodium concentrations (0mm, 150mm , 600mm).

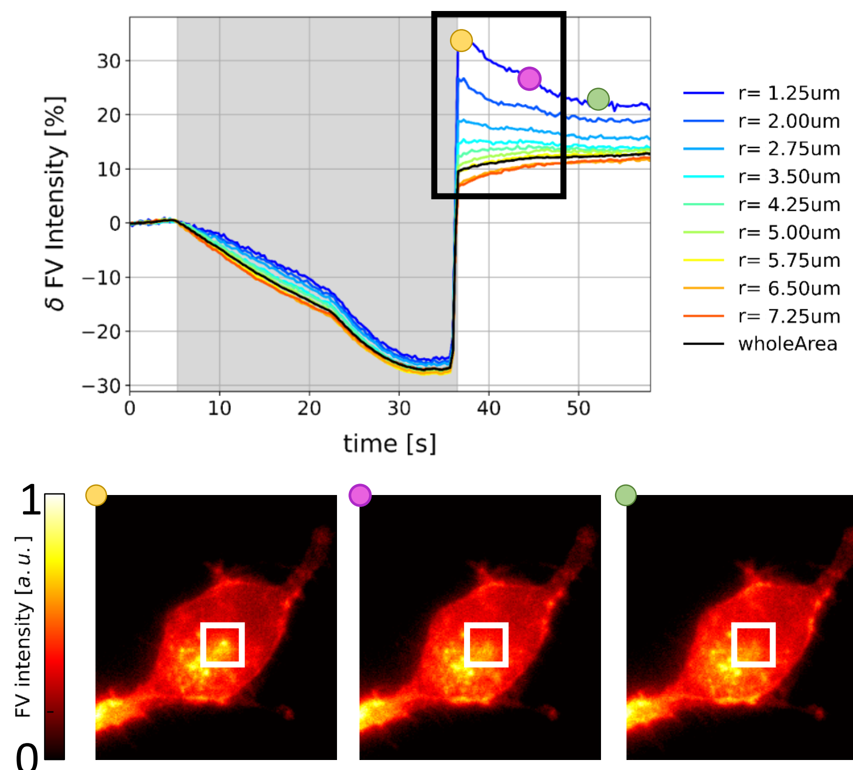

Figure 5.22. *Smallest circle analysis shows peak-shape dynamic, whose reasons are still unknown*

It is not clear at the moment the reasons behind this different dynamics in the first seconds after the injection, which depend also at the spatial level, in particular to the distance from the injection point. It can actually be a true membrane potential response, which change locally. It could be also due to a redistribution of the surface charges of the cell to which FluoVolt is sensitive. However, it cannot be excluded that this particular dynamic is actually due to an artifact introduced by the method by which the signal is acquired. In the latter case, a possible explanation is based on the following hypothesis (Figure 5.23). FluoVolt is a membrane staining dye, and the camera lens used for imaging, having a very large aperture, allows to focus the image on just a narrow plane containing a section of the cell. At the time of injection, the pyramidal tip causes a deformation of the membrane, which is very likely to closer to the focus plane. Since the membrane is the colored (and therefore bright) part, this can cause that large peak of intensity visible near the injection point. To verify if this hypothesis is true, it would be sufficient in principle to try the same experiment but with a membrane staining dye not sensitive to the membrane potential. If the same dynamic appears with a peak in the extracted fluorescence intensity signal near the injection point, then it can be concluded that this dynamic is not representative of the membrane potential but only representative of the membrane deformation entering the focal plane. The exponential decrease, at that point, would indicate a stress-relaxation phenomenon, due to the relaxation of an elasto-plastic substrate to which the cell can be compared.

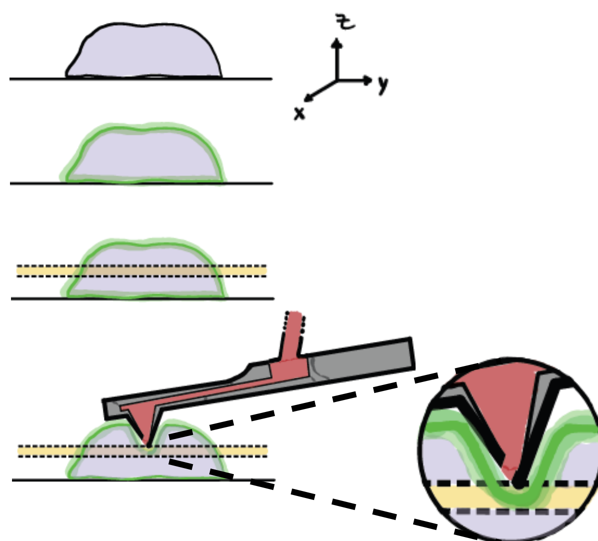

Figure 5.23. *Mechanical-based hypotesis to explain peak-shape fluorescence intensity curves appearing when analyzing FluoVolt data close to the injection point. Green: FluoVolt staining the membrane. Yellow stripe: focal plane*

5.5 Experiment parameters optimization

Combining FluoVolt and cell stimulation via FluidFM required tuning of many parameters in a troubleshooting process (Figure 5.24). Each chosen value therefore represents the solution to a given problem. Some of the challenges addressed are discussed below.

Parameters optimization

Initial position	70 µm
Final position	100 µm
Approach speed	2 µm/s
Retraction speed	100 µm/s
Force setpoint	60 nN
Pause time	3 s
Pressure protocol	20 mbar for 1 s
PID controller	I = 200
Piezo	100 µm
Laser Intensity	7%
Ts	300 ms
Exposure	50 ms
Dish coating	fibronectin

Figure 5.24. *Optimized parameters for force spectroscopy (red) and imaging (blue).*

The FluidFM used in the laboratory has two piezo available to move the apparatus in order to create a force spectroscopy: one piezo is 12um and the other one is 100um. In principle the first can be used, because it allows to have a maximum distance between dish and cantilever of 12um, and HEK293 are less than 12um thick. With this piezo, the stage on which the dish is mounted with the cells does not move during the force spectroscopy, allowing to stay in the focal plane with the cells for the entire duration of the video.
However, it has been verified that under these conditions, the cantilever being very close to the camera lens, reflects part of the light emitted by FluoVolt fluorescence that is then recaptured by the camera. The position of the cantilever thus affects the FluoVolt signal (Figure 5.25).

In figure 5.25a it is marked with a yellow circlet the instant of time in which the contact between the cantilever and the cell happens and with a blue circlet the moment in which the intensity of the fluorescence begins to increase. It is noted that the fluorescence rate begins to vary long before the cantilever touches the cell, suggesting that the vari ation in fluorescence intensity is due to some other reason. The response becomes clear by looking at figure 5.25b, where the instant of time marked by the blue circlet on the fluorescence intensity curve indicates the beginning of the fluorescence intensity variation, and this instant of time coincides with the yellow circlet in which the piezo signal begins to vary (that is the moment in which the force spectroscopy begins)The changes in fluorescence intensity measured by holding the cantilever close to the camera lens are not representative of the membrane potential but rather of the cantilever position. Furthermore, in this specific experiment, a manual (and therefore not visible in the piezo signal of figure 5.25b) removal of the cantilever from the dish was carried out. These manual distances are marked in figure 5.25b on the time axis with green circlets, and they show a correlation in the variation of the intensity of fluorescence, once again demonstrating that using the 12um piezo is not possible, with this camera setup

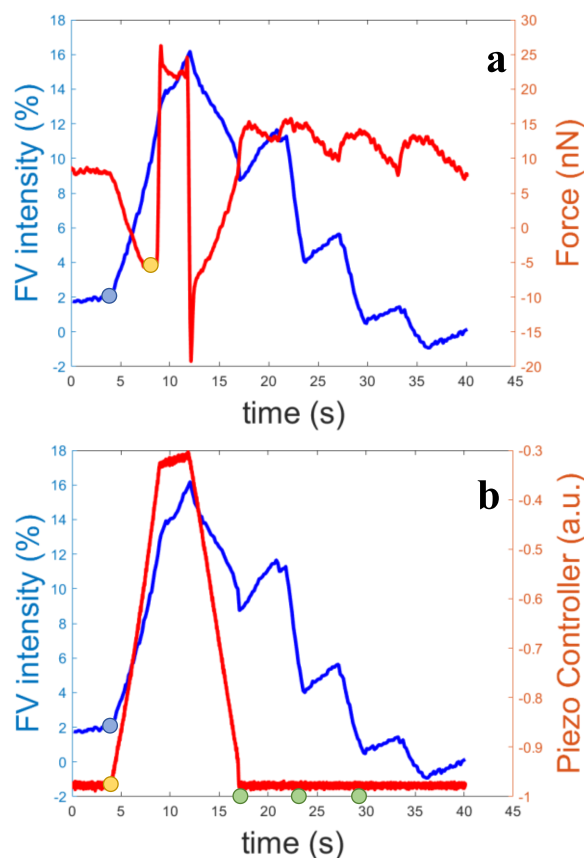

Figure 5.25. *Proofing the necessity of 100um piezo, because the 12um piezo introduces artifacts in the measurement of FluoVolt fluorescence*

For this reason it is necessary to switch to the 100um piezo, which allows to have an initial position and a final position of the single cell force spectroscopy large enough to eliminate the bias in the FluoVolt signal due to the position of the cantilever. However, the central part of the video is lost because in the configuration with piezo 100um it is the stage on which the cells are mounted to move in the direction of the cantilever during the force spectroscopy, leaving the focus plane of the lens.

Both laser imaging and force spectroscopy parameters have an impact on cell viability. In particular, the parameters "force setpoint", "pause time", "pressure protocol" have been optimized to minimize the mechanical stress on the cell, but at the same time maintain a good effectiveness/ efficiency in making the injection. Even "approach speed" has been optimized to not affect cell viability too much: a lower approach speed is less stressful for the cell. However, this value also represents a compromise with the distance to be travelled before the cells reach the cantilever. In fact, it is not desirable a very low a speed, otherwise it takes too long for the cells to reach the cantilever, and the damaging effect of prolonged imaging is obvious. In fact, imaging parameters such as "Sampling period Ts", "laser intensity", and "exposure" have also been optimized to ensure better cell viability and less imaging stress (Figure 5.26)

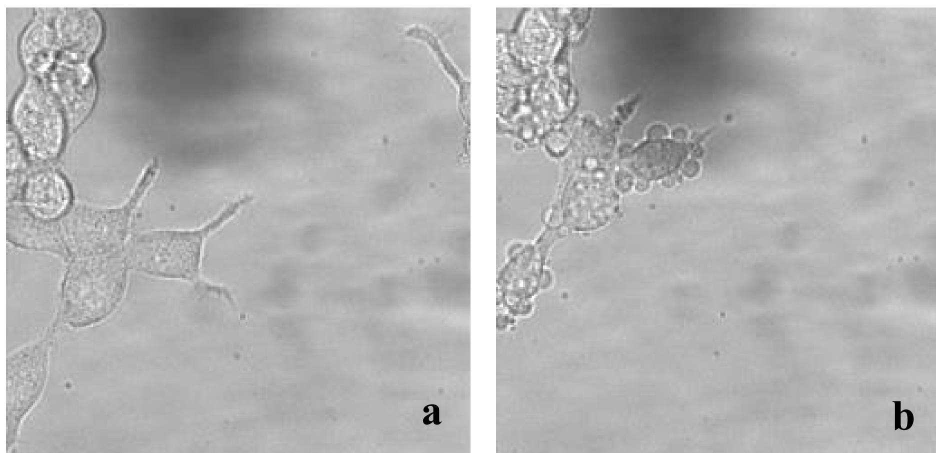

Figure 5.26. *An example of bad imaging: 50 sec imaging with continuos 15% laser. (a) before fluorescence imaging, (b) after fluorescence imaging*

5.6 Materials and methods

Cell line and culture

HEK cells were maintained in high-glucose DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), and 1% penicillin/streptomycin solution (Thermo Fisher Scientific) in a 5% CO2 humidified atmosphere at 37 °C. HEK cells were cultured in plastic T25 flasks, with $2*10^3$cells/cm^2 seeding density and splitted when they reached about 80% confluency. For FluidFM injections cells were seeded 48 hours preceding the experiments onto 40-mm aperture glass bottom dishes (WillCo HBST-5040) with $4*10^3$cells/cm^2 seeding density. The dishes were pre-coated (overnight incubation) with fibronectin (20uL fibronectin in 1mL of mQ water). Before the experiments, FluoVolt (Thermo Fisher Scientific) was loaded (30 minutes incubation) according to Thermo Fisher Scientific protocol using a specific buffer containing 145 mM NaCI, 20 mM glucose, 10 mM HEPES, 3 mM KCI, 2 mM CaCl2, 1 mM MgCl2 (pH 7.35, 310 mOsm) in mQ water. This buffer was used also during washing steps (before and after loading FluoVolt, with 1ml of buffer). For the experiments, the culture media was replaced with LCIS (Thermo Fisher Scientific). All the solutions are pre-warmed at 37°C.

Reagents for injection

Injected solution was based on intracellular mimicking cardiomyocyte solution made of 60 mM CsCl, 50 mM aspartic acid, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 11 mM EGTA, 5 mM Na2ATP, 5 mM Glucose, pH 7.4 (with CsOH) and 15mM Sulforhodamine101 (Thermo Fisher Scientific) to have the proof of the injection. This is the control injection. By dissolving NaCl in this solution, the remaining 150 mM Na+ and 600mM Na+ conditions to test were obtained.

FluidFM setup

The FluidFM system is composed of a FlexAFM scan head (Nanosurf) and a C3000 controller driven by its original software (Nanosurf C3000 v. 3.3835), and a digital pressure controller unit (ranging from −800 to +1000 mbar) operated by a digital controller software (Cytosurge CORA). FluidFM Nanosyringe with 600nm aperture and 2.2N/m cantilever spring constant were obtained from Cytosurge. The probes were coated overnight with the hydrophobic, anti-fouling SL2 Sigmacote (Sigma–Aldrich) on the inside and outside with heat stabilization for ≥ 45 min at 100 °C prior to use. They were then mounted onto a cytoclip holder by Cytosurge. The FlexAFM head is mounted on top of an inverted AxioObserver microscope (Zeiss) controlled by MicroManager open source software. The microscope is equipped with a temperature-controlled incubation chamber, an EMCCD camera system (Hamamatsu Photonics C9100-13 camera), a Smaract xy-stage which

contains 100um piezo on sample holder for long range AFM measurements and a Colibri laser accessory for fast switching fluorescence illumination source.

Live imaging

For all images and videos, a 63x Water Immersion NA 1.3 *DIC* used with Immersol W2010 with 1.4 numerical aperture was used; images are in 16 bit format. Image acquisition was controlled using MicroManager open source software; Image and video were processed with MATLAB R2020a. Sulforhodamine101 were imaged using a 590 nm laser and Zeiss 43 emission filter. FluoVolt was imaged using a 470 nm laser and a Zeiss 38HE emission filter. Images and videos dimensions are 512 x 512 pixel with 4px/um. FluoVolt videos are 60sec in length, acquired with 10% laser power (minimal phototoxicity), 300msec sampling period and 50msec exposure.

Injection experiments

Cantilever spring constant was measured using a software implemented calibration module (Nanosurf), with the resonance frequencies and quality factors obtained from thermal noise spectrum acquired in air, before filling of the probe microchannel. The solution to inject was loaded in the probe reservoir, and the probe was then connected to the pressure controller unit to apply an overpressure Δp of 1000 mbar to flow the solution from the reservoir into the microchannel. The cantilever sensitivity was then calibrated in the sample experimental medium (LCIS). The cell to be injected was visualized by phase contrast microscopy, and the FluidFM probe was approached on the substrate in the vicinity of the targeted cell with a force set point of 100 nN and retracted with the Z-piezo by 70um. The probe was then laterally displaced above the desired point of insertion, under observation by phase contrast microscopy. The tip of the probe was then inserted into the cell through a forward force spectroscopy routine driven by the Z-piezo with a velocity of 2 um/s. The forward force spectroscopy was set to stop when reaching a maximal force (F_{max}) of up to 60 nN. The probe was then maintained in the cell by keeping active the force feedback based controller interior at constant F_{max} for 3sec. During this pause, an overpressure pulse Δp of 20mbar was applied to deliver the solution. The probe was then retracted through a backward force spectroscopy with a velocity of 100um/s. The entire injection process was monitored by fluorescence microscopy, and force spectroscopy. All injection experiments were conducted in LCIS media, with 37°C warmed chamber.

Conclusions

The thesis began by clarifying the context in which the research project conducted at the Laboratory of Biosensors and Bioelectronics of ETH Zurich was developed. In particular, it was highlighted how the transmembrane potential and the activity of ion channels are the fulcrum of a wide range of cellular processes and for this reason they become the subject of numerous studies. Experiments aimed at discovering the mechanisms that regulate their functioning typically take place at the single cell level, using sophisticated instrumentation.

In this context, the proof of concept research project of this thesis is to combine two emerging technologies, FluoVolt and FluidFM, to compensate for the disadvantages suffered by the most common technologies used in single cell studies, paving the way for new approaches to the study of cellular mechanisms.

To demonstrate the feasibility of this idea, a series of experiments have been designed. They consist in monitoring the membrane potential (via FluoVolt) while simultaneously stimulating the single cell (via injection with FluidFM). The aim is to capture and record the cellular response to different types of stimuli (different concentration of sodium injected). This idea has been shown, in principle, to be feasible thanks to the result obtained from experiments with HEK293 cells (Figure 5.15), in which to a precise cellular stimulation, a corresponding and precise response involving membrane potential variations has been obtained.

The final outcome is, however, the result of small steps and intermediate successes: during the project, data analysis allowed to highlight how Fluovolt enable to extract information both in static mode (with HEK293, Figure 5.13) and in dynamic mode (with CMs, Figure 4.7). In addition, it has been highlighted the possibility of analyzing FluoVolt data in different ways: with algorithms based on manual segmentation (for HEK293, Figure 5.11) or automatic segmentation (for CMs, Figure 4.6) for cell boundary identification allowing in all the cases the extraction of FluoVolt derived signals. Finally, it has been shown how

it is possible to obtain, by exploiting the high spatial resolution of FluoVolt, new insights (with HEK293, Figure 5.22) and important electrophysiological parameters (with CMs, Figure 4.16).

Despite the results achieved, this project to combine FluoVolt and FluidFM is only in its infancy. It is still in early stages. The next steps will certainly be in the direction of a development that will make this methodology simple and reliable, allowing researchers to use it for investigating biological issues of fundamental importance.

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