UNIVERSITÀ DEGLI STUDI DI PADOVA

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Corso di Laurea in Fisica

Tesi di Laurea

Electrophysiologicalanalysisofhumancells derived from neuronal differentiation ofskin mesenchymal stem cells

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Abstract

Human skin hair follicle is a niche for different kind of adult mesenchymal stem cells (MSCs) such as neural crest stem cells and skin-derived precursor cells. MSCs ability to differentiate in neural and glial-like cells is of great interest for regenerative medicine as well as for the comprehension of molecular mechanisms involved in the development and the cure of neuropathies. Even if with reduced differentiative potential, MSCs have undoubtable economic advantages with respect to reprogrammed stem cells (iPSCs); at the same time, they already have well established therapeutic potential in animal models of stroke, spinal cord injury and multiple sclerosis. Starting from Prof. Bortolozzi's lab protocol for the extraction, proliferation, and differentiation of adult MSCs into neurons, we performed electrophysiology experiments to characterize three different monoclonal populations selected from seven by mRNA analysis before the differentiation step. The patch-clamp technique has been used to depolarize single cells at different neuronal maturation stages to monitor resting potential and voltage-dependent electrical activity. To validate our patch clamp protocol, we used primary cultures of mouse motorneurons to record action potentials, which are the characteristic feature of mature neuronal cell. In MSCs-derived neurons, action potentials were not recorded during one-month differentiation period, despite cells exhibited a typical neuronal morphology. Overall, these results suggest that the mRNA signature expressed by the three MSC clones we analyzed corresponds to a limited neuronal potential, so different signatures should be taken into account in a future work.

I follicoli piliferi umani sono una nicchia per diversi tipi di cellule staminali mesenchimali adulte (MSCs), come ad esempio le cellule staminali della cresta neurale e le cellule precursori derivate dalla pelle. La capacità delle MSCs di differenziarsi in cellule simil neuronali e gliali è di grande interesse per la medicina rigenerativa, così come per la comprensione dei meccanismi molecolari che intervengono nello sviluppo e nella cura delle neuropatie. Anche se con capacità di differenziamento ridotta, le MSCs hanno indubbiamente dei vantaggi economici rispetto alle cellule staminali riprogrammate (iPSCs); nello stesso tempo, sono già stati osservati i benefici terapeutici in modelli animali di ictus, lesioni del midollo spinale e sclerosi multipla. A partire dal protocollo del laboratorio del Prof. Bortolozzi per l'estrazione, proliferazione e differenziamento di MSCs adulte in neuroni, abbiamo eseguito esperimenti di elettrofisiologia per caratterizzare tre diverse popolazioni monoclonali selezionate da sette cloni attraverso l'analisi del mRNA prima di procedere con il protocollo di differenziamento. La tecnica del patch-clamp è stata usata per depolarizzare singole cellule a differenti stadi di maturazione neuronale per monitorare il potenziale di riposo e la dipendenza dell'attività elettrica dal voltaggio. Per convalidare il protocollo di patch-clamp, abbiamo usato colture primarie di motoneuroni derivati da topi per registrare il potenziale d'azione, che è una caratteristica specifica delle cellule neuronali mature. Nei neuroni derivati da MSCs, il potenziale d'azione non è mai stato registrato durante il mese di differenziamento, nonostante le cellule esibissero una morfologia tipicamente neuronale. Complessivamente, questi risultati suggeriscono che la caratterizzazione data dal mRNA espressa

dai tre cloni che abbiamo analizzato corrisponde ad un potenziale neurologico limitato, quindi differenti firme transcrittomiche dovrebbero essere prese in considerazione in un lavoro futuro.

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1. Introduction

Electrophysiology was very important for the development of biology and medicine in the last century as it unveiled how neurons communicate each other. In electrophysiology, the cell is equivalent to an electrical circuit, so a brief introduction of the electrical properties of cell membrane and channels is provided, together with the description of neurons and their specific properties such as action potentials. A short introduction of mesenchymal stem cells and their capacity to differentiate in different type of cells is also provided. Then all the setup used for the experiments is explained in detail.

1.1. Cell membranes and Ion channels

The cell membrane is a barrier that separates the cytoplasm of the cell from the extracellular environment; it is made of two layer of phospholipids that arrange them in order to have the hydrophobic tail face each other (1). The membrane not only separates the intra and extra cellular environment but also regulates the exchange of ion and nutrient and can create tissue structure with other cells. The exchange of substances through the membrane is accomplished by the channels that are in the membrane and are made of proteins.

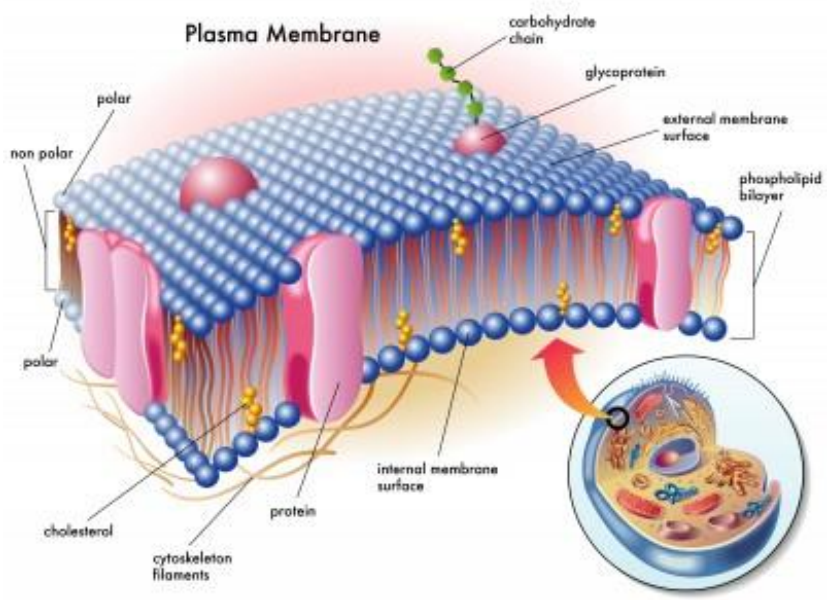


Figura 1: Structure of the plasma membrane

The channels are very important for the exchange of ions through the cell, in particular we can divide channels in two different types: the passive channels that allow the exchange of ions down the electro-diffusive gradient concentration and the active channel that exchange ions against the electro-diffusive gradient and need energy from ATP to work, an example is the Na^+/K^+-ATPase that we will find later.

The phospholipidic membrane has a very high resistivity (10^{15} Ω cm) so the ion does not pass through these two layer and the membrane can be seen as a condenser because the charge on its sides cannot pass, but the membrane has also ionic channel that permits the passage of ion and this can be seen as a resistor, so the cell has two parameters: the capacitance of the membrane C_m and the resistance R_m (2). Each cell has a different resistance due to the different expression of the channel for different ions and their capability of

remaining open or closed. The membrane can be seen as a series of resistor in parallel with condenser and we can create the equivalent circuit of the cell with the C_{eq} and R_{eq} (2) .

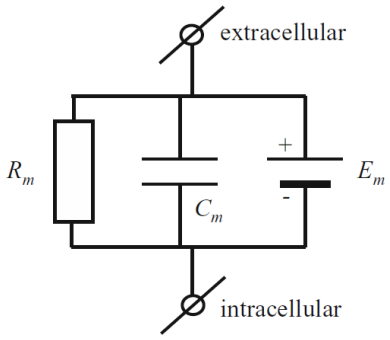


Figura 2: Equivalent circuit for a cell

The battery in the circuit is due to the Nernst equation, in fact we can see that the current goes at 0 not at a voltage 0 but at a value of voltage E_m, that's because of the gradient of the concentration between the two interfaces of the cell and this can be seen as a battery.

The capacity of the cell can be calculated by the formula

$$C_m = \frac{\varepsilon \varepsilon_0 A}{d}$$

where ε is the dielectric constant of the membrane and is around 3 and d is the distance of the two layer and is around 5 nm, so the capacitance of the cell is very high due to the very low distance of the phospholipidic bilayer (2).

1.1.1. Resting potential

The membrane of a cell act as a semipermeable barrier for some ions presents in the intra and extra cellular environment, this behavior leads to have a concentration gradient of ions and so a potential different from zero when the cell is at resting. This can be explained by considering a bath with two solutions of a salt with different concentration separated by a semipermeable barrier that allows the passage of only a type of ion. At time zero the potential in the two sides of the bath is the same because the total charge is zero for both, but at different time the difference in the concentration causes a flow of ions to the more concentrated side to the others and causes a difference of charge and so a difference of potential. The situation reaches its equilibrium where the sum of the force due to the difference of concentration and electric force are zero and it can be described by the Nernst Equation.

$$E_X = RT / zF \ln\left[X\right]_e / \left[X\right]_I$$

where $[X]_i$ and $[X]_e$ indicates the concentration of the ion in intra and extra cellular environment, F is the Faraday constant that is 96500 C/mol, z is the valence of the ion, in case of a monovalent ion is ±1, T is the temperature and R is the constant of gas. Notice that at equilibrium there is always a flow of ion but the sum of the flow in the two direction are the same. This equation cannot be used for the cells because there are more than a ion that contribute to the flow, in this case the potential at equilibrium is given by the

Goldman-Hodgkin-Katz equation that consider also the permeability of different ions for the membrane, in the case of a cell where the principal ions are K^+, Na^+ and Cl^- the equation becomes

$$E = RT / zF \cdot \ln \frac{P_K[K^+]_e + P_{Na}[Na^+]_e + P_{Cl}[Cl^-]_e}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_i}$$

The difference in the permeability for the K^+ and Na^+, at rest the resting potentials are -90 mV for K^+ and +63 mV for Na^+ (3), leads to the fact that for different cells the resting potential can be very different and also that the cells can transfer electric signals thanks to the opening and closure of channels with different permeability to the different ions. With the opening and closure the electric signals that is created can be calculated by the previous equation and can change the resistivity of the cell.

1.1.2. Equivalent circuit for the cell

The Nernst potential can be seen as a battery for the circuit and we can consider the equivalent resistance as the sum of three different channel, two channel for the main ions for the physiology of the cell: $Na^+$and K^+ and a resistance due to all the other ions that we call leakage resistance.

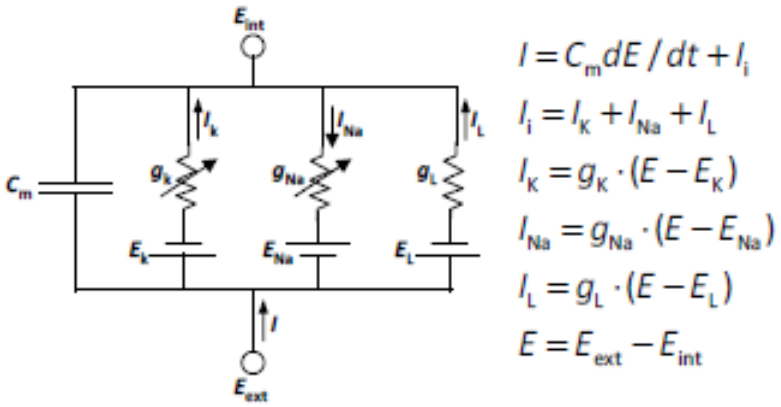


Figura 3: Equivalent circuit for an axon

A battery is in series with every channel to explicit the force due to the Nernst equation.

The passage of all the ions can change due to the full opening or full closing of the channel, so we can draw them in the circuit as a variable resistance. This behavior is the basis for all the cell of the resting potential and is the way for the neurons to create and transmit an electric signal.

1.2. Neurons

Neurons are the basis of the nervous system and they are excitable cells that means they are capable of creating and transmitting chemical and electric signals by changing the ion concentration on the plasmatic membrane as response to external stimulus. A neuron has a very specific shape, we can see a central structure, with the nucleus and all the other structure of the cells, and some elongation of the cell that represent the dendrites and the axon.

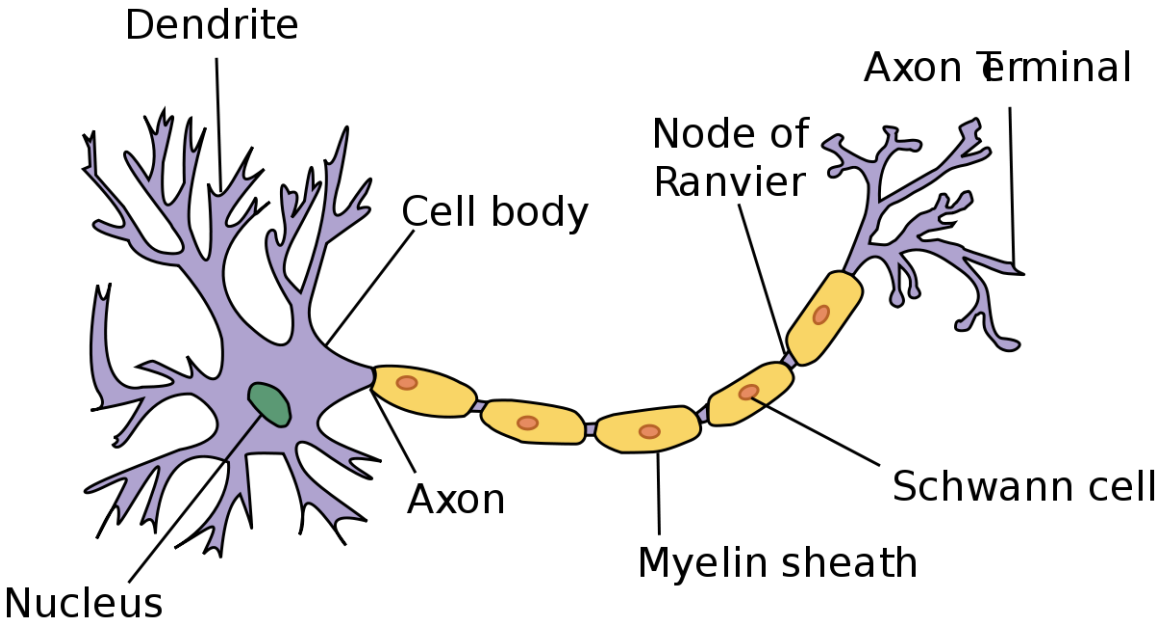


Figura 4: Structure of a neuron

The central body called also soma contains all the structure of a typical cell and has a wide cytoskeleton connected to the dendrites and the axon. The soma has a low concentration of K^+ and Na^+ channel so it cannot create action potential but is important for the neurons for its reparation function when the neurons is damaged in its dendrites or axon and it is also the centre where all the signals are collected.

The dendrites are small and branched structure of the neurons that collect the signals through a synapses that is the link to another neuron or to the external environment as for the olfactory neurons and the retinal photoreceptors. The density of the K^+ and Na^+ channel depends on the neurons but they are all capable of transporting the signal to the soma but most of them cannot create an action potential.

The axon is the part of the neuron that can creates and transmit the signal from the neuron to the target cell where it is connected by the axon terminal. The signal can travel only from the cell body to the termination. The axon has a cylindrical shape with the same diameter for all its length, which is different for all the neurons and it goes from a hundred of µm to more than ten centimeters. The first part of the axon has an high density of channel Na^+ and K^+ and is the region where the action potential is created, the second part of the axon is covered form glial cells called also Schwann cells that preserve and speeds the transmission of the signal, the last part of the axon is made of a separation of the axon in many branches, these branches are also covered from glial cells and only a little termination is in a direct link to another cell and this part is the synapses. In these last branches the electric signal is converted to a chemical signal thanks to a neurotransmitter.

Neurons can be distinguished by the number of dendrites and their position, we can find

- Unipolar neurons: has no dendrites and an only axon
- Bipolar neurons: has a dendrites and an axon
- multipolar neurons: has a different number of dendrites and an axon

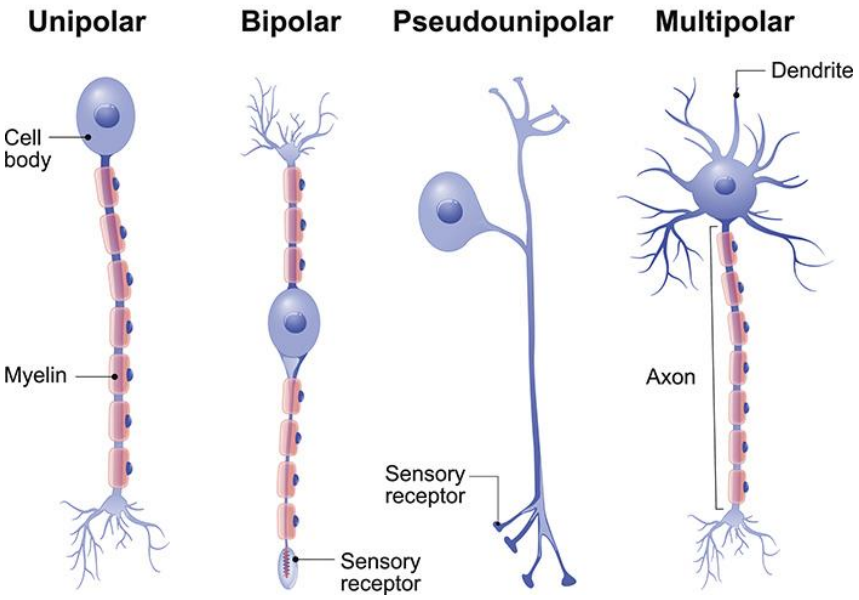


Figura 5: Type of neurons

1.2.1. Action potential

The action potential is a characteristics of neurons, muscle and neuroendocrine cells that are the excitable cells in the human body and is an active response to an external stimulus. These cells possess a voltage dependant channel that is the Na^+ channel that is capable of changing the permeability of the ion to the cells. To describe the mechanism of the action potential let's first consider how the Goldman-Hogdkin-Katz equation changes when the cell is more permeable to a single type of ion while the other is less permeable. In this case we can consider the resting potential for the two types of ion that are involved in the action potential, if we write the equation as dependant from the ratio of the tow permeability of the ion we obtain

$$E = RT / zF \cdot \ln \frac{\left[K^+\right]_e + (P_{Na} / P_K)\left[Na^+\right]_e}{\left[K^+\right]_i + (P_{Na} / P_K)\left[Na^+\right]_i}$$

In this equation we can see that if the permeability to the Na^+ is much greater than the K^+, the contribution to the potential is mainly from the Nernst potential of Na^+, so it will be near 63 mV, in the other case when the permeability to the Na^+ is nearly zero the potential is equal to the Nernst potential of K^+, so around -90 mV (3). The activation and inactivation of the voltage dependent channel modifies the permeability of the ion in the two opposite cases, or channel completely open or completely close, in this way the potential can reach values from -90 to 63 mV. Another important properties of the channel is that he can inactivate while still remaining open and this is fundamental for the refractory period that is when the cell cannot fire an action potential for a short period of time. We will now see how this potential works and its properties and also the changes in the conductivity of the cell to the specific ion.

For a neuron at rest the resting potential is around -70 mV and it comes from a fraction of K^+ channel activated, while the Na^+ channel are all inactivated, when the cell depolarize the potential can reach a threshold where the Na^+ channel start opening, this potential is around -55 mV. When the cell start

reaching the threshold potential there are few Na^+ channel activated, but the electromotive force due to the different potential for the Na^+ is quite high, (V_m-E_{Na}), so the Na^+ start flowing inside the cell and the flow is greater than the exit of K^+ so the cell becomes more positive and the potential increase. This increase leads to a more probability of the activation of the channel so the Na^+ continue flowing from the outside of the cell and the potential constantly increase, at 0 mV the conductance of Na^+ reach its maximum. After around a ms the potential of the cell is near the Nernst potential of the Na^+(+63 mV) so the ion is not flowing anymore also if all the channel are open, the Na^+ channel start inactivating while the retarded K^+ channel start opening, so there is a fast repolarizing of the cell due to the exit of K^+ from the cell thanks to the high f.e.m of K^+, (V_m-E_k), in this phase the conductance of K^+ reach its maximum and the potential reach the Nernst potential of K^+ (-90 mV). In this state we have an iperpolarization of the cell, the channel K^+ start closing and the ion is not flowing anymore, while the Na^+ channel start closing in order to be able to fire a new action potential in less than 15 ms. After the iperpolarization the cell can return to its resting state, few channels of K^+ remain open to reestablish the resting potential and the concentration of the two ions is balanced by the Na^+/K^+-ATP pump that uses energy to exchange ion from the intra and extra cellular environment, this pump is slow and continues working also in the resting state (4).

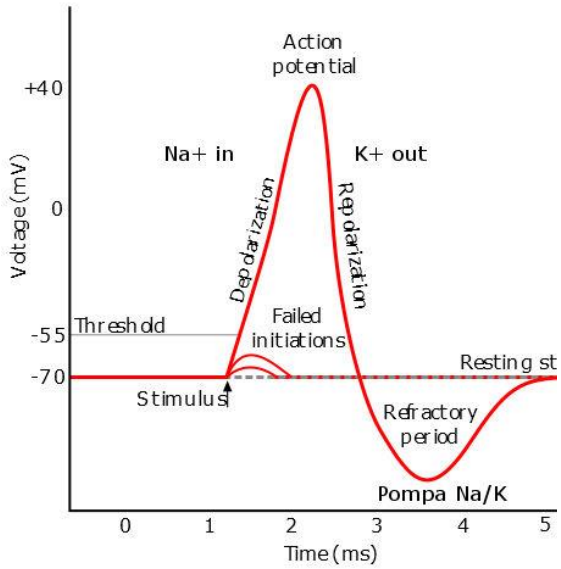


Figura 7: Action potential

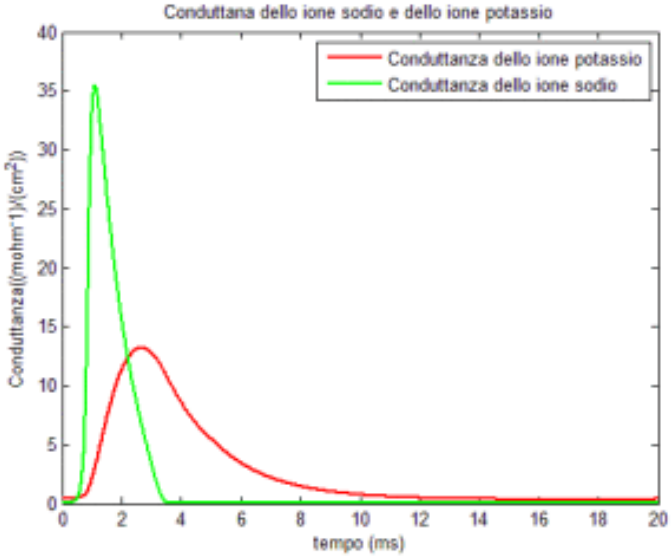


Figura 6: Conductance of the two channels for the specific ions

In the two figures we can see the shape of an action potential during few ms of time and the change in the conductance of the two ions.

After the fire of an action potential the cell must wait for around 10 ms before launching another one, this is due to the channel Na^+ that can exist in an inactivated state where the channel is not permeable to the ion but it is still open, then the closing of the channel is necessary before firing another action potential and this process is much slower than the rise of potential due to the opening.

Most of Na^+ channel are TTX sensitive, that means they are inactivated by a small concentration of TTX, in this case the response of the cell to a stimulus is only passive and so the cell acts as an ohmic circuit.

1.3. Stem cells and Mesenchymal stem cells

Stem cells are cells in the body that has two different characteristics from the other cells: self renewability and differentiation. The first is the ability to generate daughter cells while maintaining the undifferentiated state and the other is the ability to differentiate in different type of cells. The second property, called potency, introduces also a division on the different stem cells based on their ability to differentiate in a vary number of cell. They are classified as totipotent, pluripotent, multipotent and unipotent

- Totipotent: are cells of the first stages of the embryos and can divide in any and all type of human cells, after four days of embryonic cell division they start differentiate in pluripotent
- Pluripotent: are cells of the second stages of the embryos, after 4 days, and can divide in any type of cell but cannot differentiate in a whole human body
- Multipotent: they can differentiate in a limited number of cells and all with the same precursor
- Unipotent: they can differentiate in a single type of cell

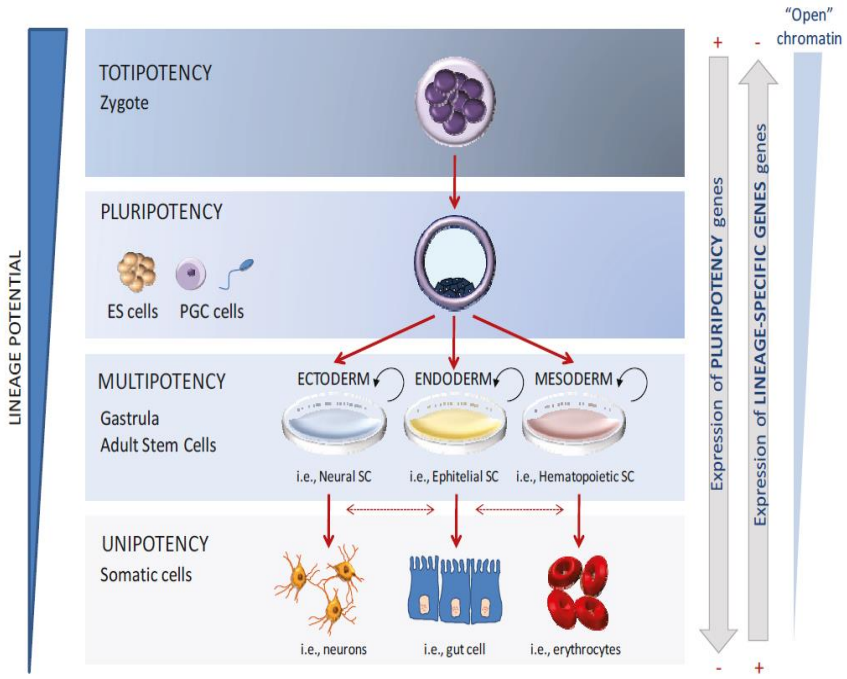


Figura 8: Stem cells and their potency (5)

Mesenchymal stem cells are multipotent stem cells that belong to the mesodermal lineage and are isolated from mesenchymal tissue such as dermis, adipose tissue and peripheral blood. These cells can be isolated and differentiated in adipocytes, osteoblasts, neurons, chondrocytes and muscle cells.

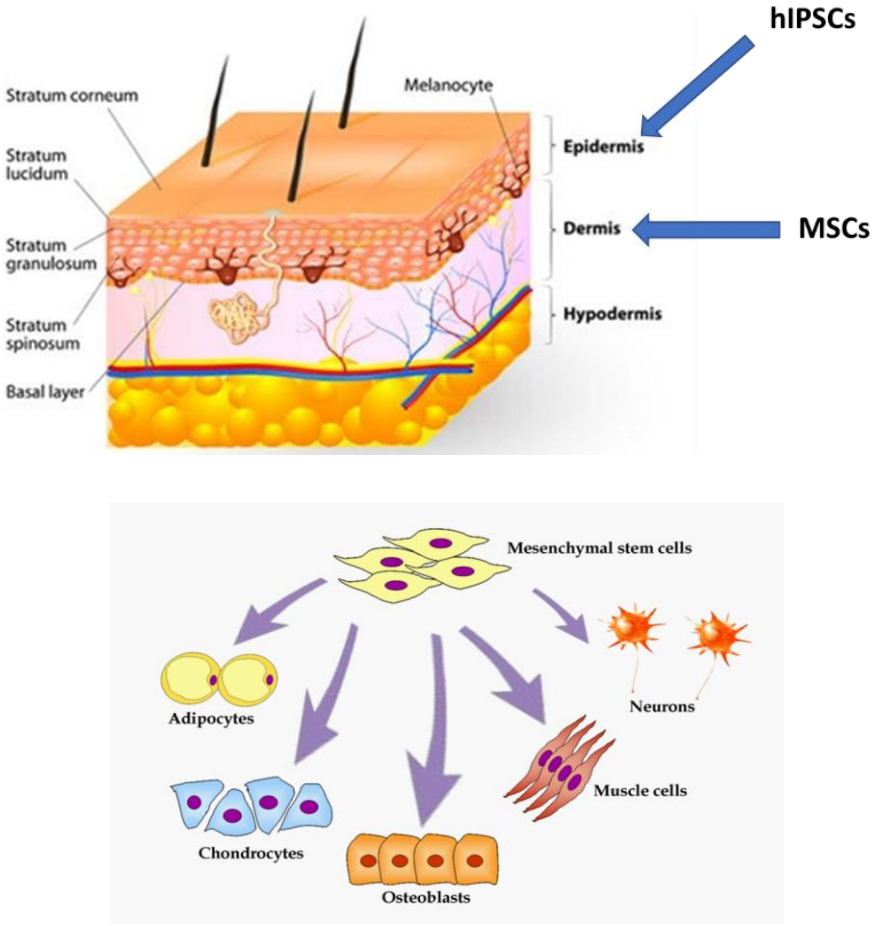


Figure 9: Origin of MSC and their potency

In the first figure we can see the origin of MSCs and a possible origin for the hIPSCs and on the last we can see the possibility of differentiation of our cells.

1.4. Patch Clamp

The patch clamp technique was developed by the physicist Erwin Neher and the physiologist Bert Sakmann in 1976 and it represented a big advance in electrophysiology and had also advancement in biology and chemistry. A patch clamp consist in a seal obtained by pressing a firepolished pipette against a tiny area of the membrane cell and it permits to measure a single channel currents. In 1981 they demonstrated that a clean glass pipette can fuse with a cell membrane to form a seal with an high resistance, of the order of Giga Ohm, and mechanical stability that permits to record the small current (0.1-1.0 pA) of the cell. For their discoveries Neher and Sakmann shared the Nobel prize for Medicine in 1991.

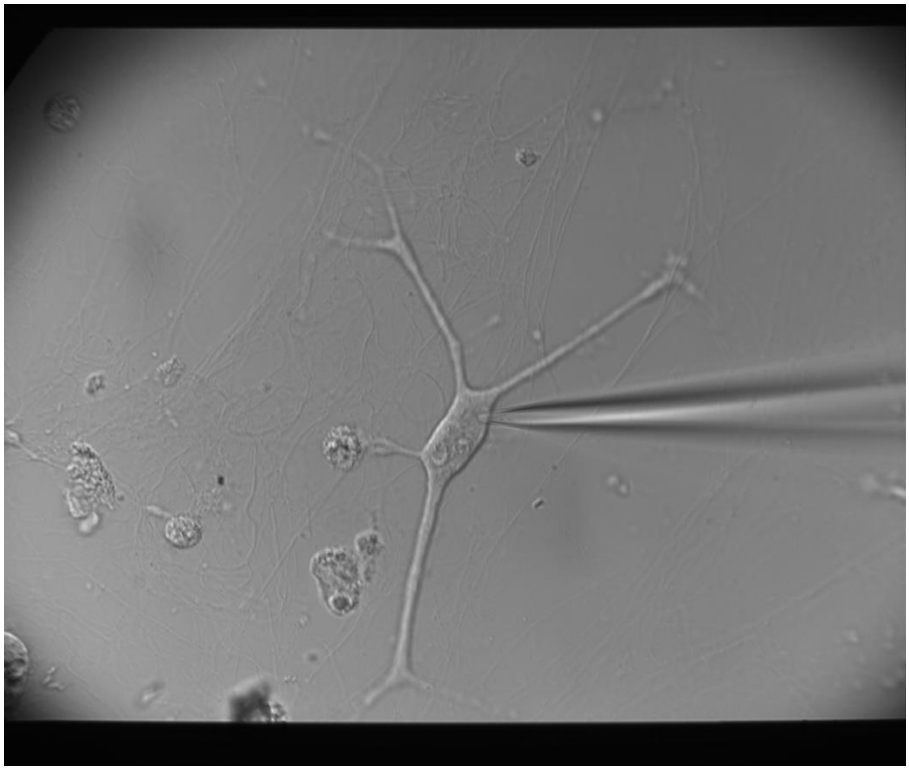


Figura 9: A neuron-like cell in the cell-attachedconfiguration (from our experiments)

The current flow is recorded by an AgCl coated silver electrode through the reaction

$$Ag + Cl^{+} \square \quad AgCl + e^{-}$$

that permits to measure the ionic flows of the Cl^{-} present in the solution.

The interface between cytoplasm and pipette leads to an access resistance that depends from: the different ionic concentration of the intra and extra-cellular solution, pipette resistance (depends on the material) and pipette aperture (around 2 µm). The equivalent circuit of the gigaseal consider the access resistance, the membrane resistance and capacitance, the resting potential of

the cell and an eventual leakage resistance due to a problem in the gigaseal like a not perfect attach to the membrane, a pipette not microforged well, some disfunction in the membrane or the presence of dirt in the pipette.

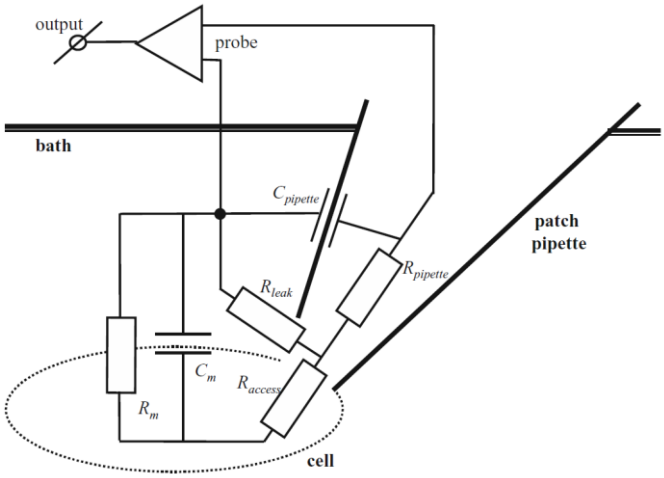


Figura 10: Equivalent circuit of the patch clamp

The patch clamp setup permits to obtain different configuration to study the membrane characteristics and single channel currents.

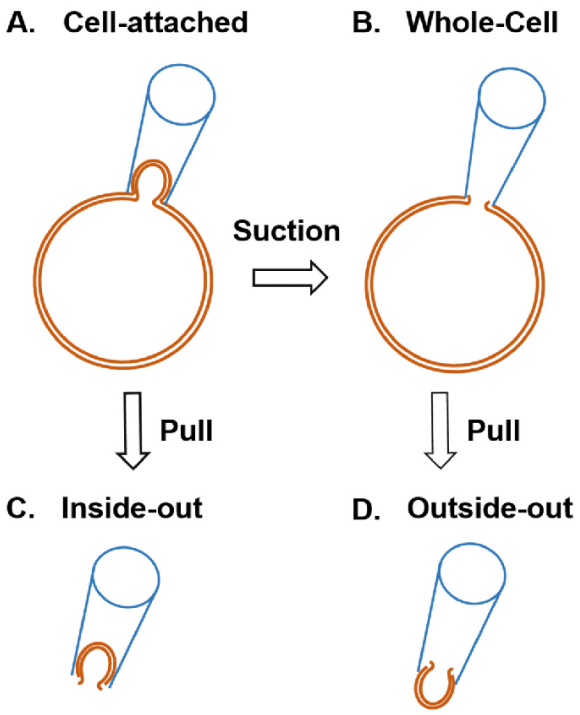


Figure 11: Different type of patch clamp configuration

The cell attached configuration permits to study a single channel currents by changing the voltage difference in the pipette, in this case if there is one or more voltage channels activated in the patch area the setup can register it. In the whole cell configuration we can register the behavior of the entire cell and permits to control the intracellular potential and solution due to the exchange of cytoplasm that flow in the pipette that has a volume far greater than the cell. With this configuration we can register current flowing in all the membrane and deliver specific ions in the cells thanks to the exchange of fluids between the pipette and the cell. The others two configurations: inside out and outside out permits to control the transmembrane potential and can be used to study the membrane with different chemicals stimuli by changing the bath solution.

1.5. Previous studies and literature

Other studies were made on the differentiation in vitro of the MSC and also of IPSC for their importance on future advancement in drug screening and cell therapy and regeneration. Until now there is not any experiment in which a culture of neurons derived from MSC is obtained, but there are studies of differentiated MSC in co-culture with neurons (7) and also a previous study in this lab in which we tried to differentiate MSC and IPSC (6) . In the study made by Martina Nicoletti the aim was to differentiate the IPSC cell in neurons and Schwann cells in order to create a myelinization in vitro of the neurons to study the Charcot-Marie-Tooth disease, in this study Martina reached a differentiation for the IPSC, while for the MSC she didn't obtain a differentiation, in her differentiation protocol she used the same steps for the MSC and IPSC. The study made by the university of Liege (7) succeeded in differentiate MSC into neurons in a co-culture with neurons, they saw the cells fire a single action potential and response to many different neurotransmitter. In our study we try to differentiate the MSC into neurons in vitro and without any type of co-culture.

2. Materials and Methods

2.1. Experimental setup

The patch clamp setup is different for any type of experiments, but some features are common for every setup: a microscope placed on a vibration isolating table and surrounded by a Faraday cage to isolate the electrode from the 50 Hz noise, a patch clamp amplifier and a pulse generator for pulse generating, a micromanipulator holding pipette that can move the pipette with very little step, a perfusion system to maintain the extracellular solution clean in order to create an healthy environment for the cells.

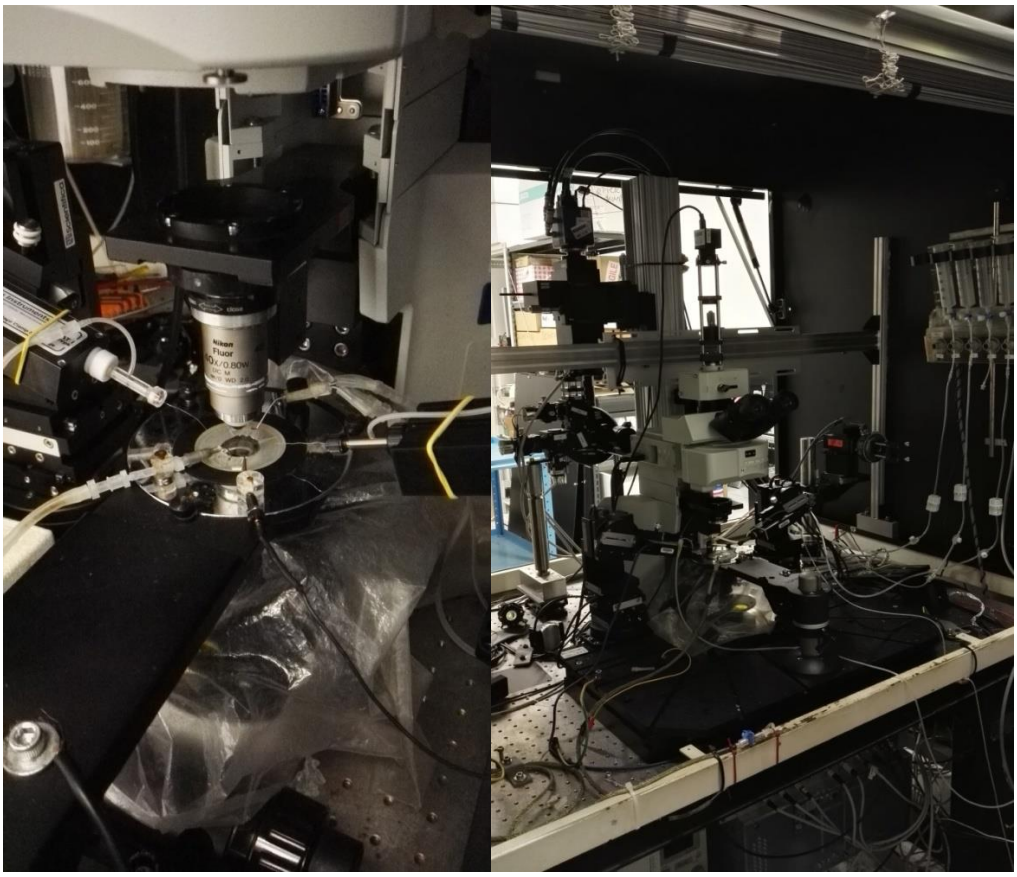


Figura 12: Microscopy setup and pipette holder

The microscopy system uses a differential interference contrast to see the cells, with this technique we are capable of see the cell with a major contrast and to see objects that usually we cannot see with a bright-field microscopy. The DIC uses a polariser to create a beam, then this beam pass to a objective-specific prism that split the beam in two different beams that vibrate perpendicular with each other. These two rays pass through a condenser but as they are perpendicular they do not cause interference. The beam now pass through the specimen, which has varying thickness and index of reflection and so creates two beam with different wave paths. The beams pass an objective and a nosepiece prism which combines them, the beam reaches another polariser which brings the beams vibration on the same axis so they can have destructive and constructive interference and what we can see is the final beam collected by a camera.

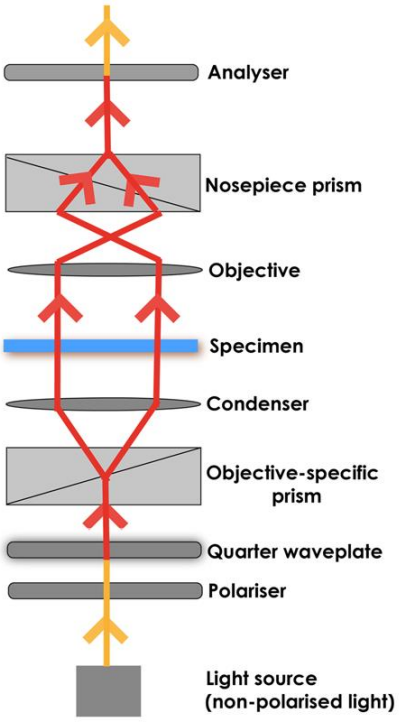
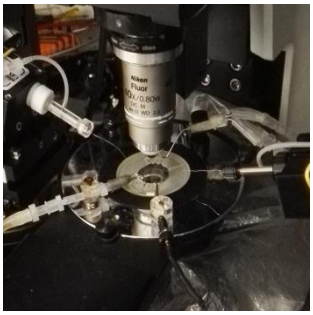


Figura 13: Path of a beam in a DIC setup

The perfusion system in the setup allows to maintain an external environment healthy for the cells and to select the solution to be used during the experiments and in case to change the solution during the recording, for example to insert a blocking substance to stop a specific channel. The system consists of four syringe of 50 ml connected to the chamber plate by plastic tubes, that can select also the flux of liquid; a controller allows to open the different syringe. The other site of the chamber plate is connected also to a nozzle which can collect the solution in excess in order to maintain the volume of liquid in the chamber quite constant .



Figures 14: In the first picture we can see the syringe, while on the other two we can observe the chamber with the pipette and the nozzle and the controller where we see also the controller for the holders

The input and aspiration can be left opened during all the recording in order to avoid any problem to the health of the cell due to a starve of substance through consumption.

The pipette are made by a Patch glass-thin wall, OD =1.5mm, ID=16 mm, length= 10 cm (WARNER INSTRUMENTS) that can be pulled by heating the pipette, the two pipette obtained have the tip that can

present some irregularities, so the tip is microforged by a resistance covered in glass to eliminate them. After the preparation of the pipette the intracellular solution is poured inside thanks to a syringe, the solution should not fill the entire pipette, but only the tip should be covered enough to let the electrode be connected to the solution, in case of solution in exceed there is a problem of compensating the capacity of the pipette.

Figura 15: Heating puller

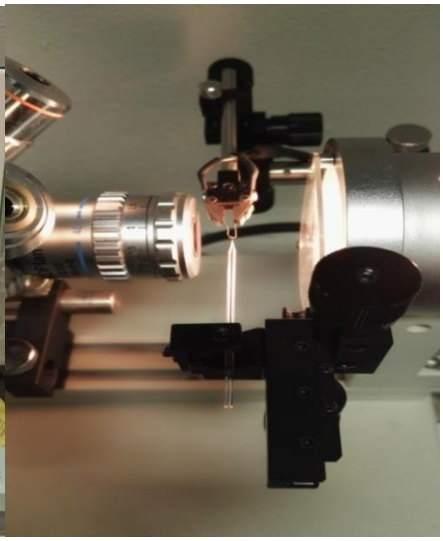


Figura 16: Microforge

2.2. Differentiation protocol

For the growth of the MSC two protocol have been followed: one for the isolation of the stem cells from the human skin and one for the growth and differentiation of the stem cells. All the single cells were cultivated in two kind of cover slips: one in german glass and one in plastic, in the each case a layer of matrigel was added to the cover slips in order to make the cell adhere. Each clone had two different protocol for the differentiation and a control which was a cover slip without any factors that can induce a specific differentiation, so that the cells can grow freely.

2.2.1. Differentiation protocol for MSC

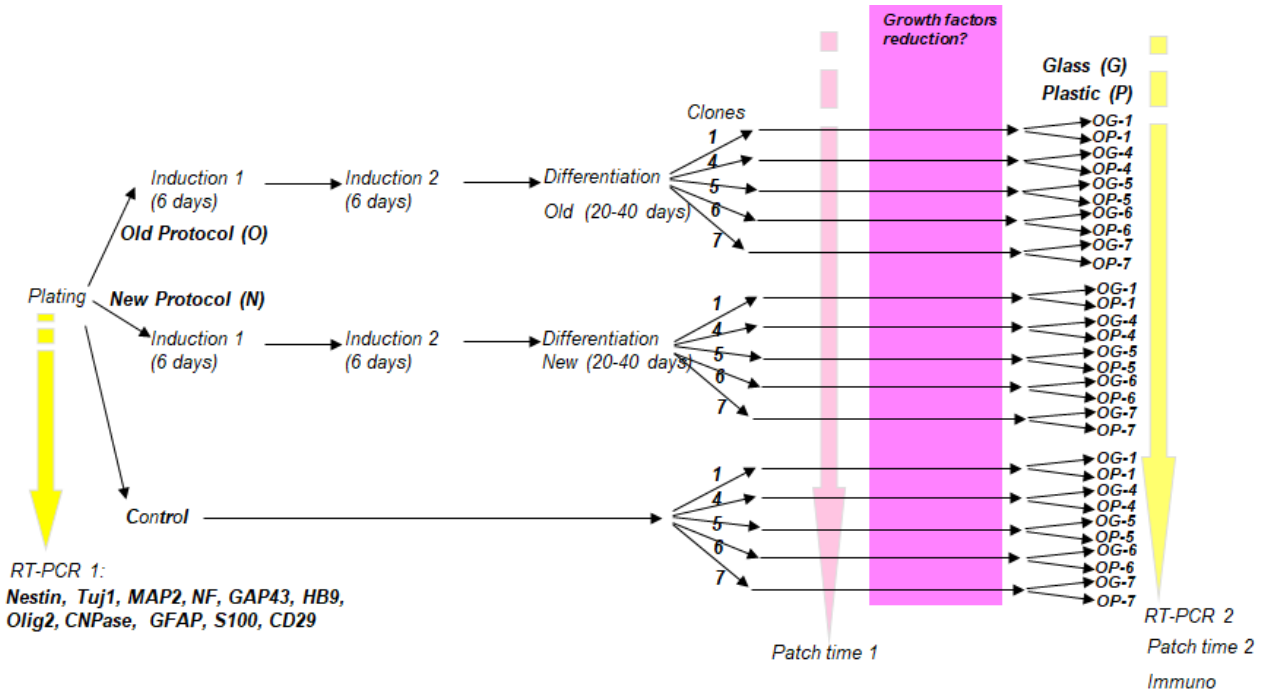


Figura 17: Differentiation protocol for MSC

The cells have been cultivated in german glass and plastic and the results of the differentiation was different on the two types of substrate, we studied the different types of substrate through an AFM analysis to see why the cells preferred to differentiate and grow in glass. We obtained four types of figures for the analysis: two figures of the different materials without matrigel and two figures with matrigel.

The differentiation started on different clones: 1, 4, 5. 6 and 7; but only two clones were selected for the patch clamp experiments at the end of the induction: clone 4 and 7 because they were the most morphologically differentiated and clone 5 was selected for practice before experiment on the other clone. The specific protocol followed for the differentiation can be seen in appendix.

2.3. Patch Clamp protocol

For the patch clamp we first try different intracellular and extracellular solution in order to compensate the shrinking or growing of the cells due to the different osmolarity between the intracellular environment before and after the whole cell configuration. We stated that the best combination of solutions were

Intracellular solution

Nome	Peso (g/mol)	Molarità (mM)	Dose (mg/100 ml)
K-Glu	234.25	125	2928.1
KCl	74.56	5	37.28
CaCl2	147.01 40mM	2	29.4 5 ml
$MgCl_2$	95.21	2	9.52
ATP-2Na	551.14	4	220.45
HEPES	238.1	10	238.1
EGTA	380.35	10	380.35
GTP – Na	523.18	0.3	15.70

Extracellular solution

Nome	Peso (g/mol)	Molarità (mM)	Dose (g/l)
NaCl	58.44	140	8.18
KCl	74.56	5	0.373

$CaCl_2$	147.01	2	0.294
$MgCl\text{-}2H_2O$	95.21	1	0.0952
HEPES	238.81	10	2.383
Glucosio	180.2	10	1.802

With these combination the volume of the cells remains quite the same during the experiments.

The cover slip was surrounded by the extracellular solution that during all the experiments was also washed away with the perfusion system in order to avoid any type of contamination in the extracellular environment due to the leakage of liquid from the pipette with the intracellular solution. Each covers slip remained under the microscopy for two hours so that for each cover slip from 10 to 12 cells were patched. The protocol for the experiment can be reassumed in a number of passage

1. Prepare the pipette and the bath for the experiments
2. Select the cell to patch
3. Add the intracellular solution in the tip of the pipette through a syringe with a small tube made with a plastic pipette and avoid inserting too much liquid or making bubble that can alter the measure
4. Through the pipette holders insert a positive pressure and hold it to avoid any type of contamination due to the insertion of the pipette in the bath
5. Insert the pipette in the bath and focus on the tip to see if there is any dirt or bubble. If the pipette hasn't got any type of deformation or dirt the resistance should be around 10MΩ (the resistance changes also with the aperture of the pipette).
6. Go down with the pipette until you can nearly see the cell, focus on the cell and maintain positive pressure going down until you can reach the cell membrane.
7. After touching the membrane remove the positive pressure and go in a negative pressure by passing for a moment in zero pressure and hold the negative pressure in order to aspirate the cell membrane
8. Wait until a gigaseal is formed, that is when the access resistance is around a few GΩ, 10 GΩ would be a perfect gigaseal.
9. Compensate the capacitance of the pipette through the compensation of the Clampex
10. Open the pressure, after this we are in cell attached configuration, to obtain a whole cell we need to apply a little negative pressure through a "kiss", to see when a whole cell is formed the response of the cells must be seen, in whole cell the current grow very faster before reaching the stationary state.

11. After reaching the whole cell configuration we can see the resting potential of the cell by setting the holding so that the initial current without any step is 0 pA.
12. The different protocol now can be launched to register the behavior of the cell through a current clamp or a voltage clamp.
13. If any behavior is out of the ohmic one a whole cell compensation may be necessary to see if a Na current is visible in a voltage clamp. The whole cell compensation compensate the capacitance of the membrane so that a small and fast currents like the Na can be seen and is not cancelled by the signal of the capacitance.

The protocols used may vary due to the different response of the cell, for example the current clamp protocol should be set for reaching a positive voltage in few step of current and to remain in a voltage field under the 100 mV that is already impossible for the cells to reach in natural way. Usually the Voltage clamp protocol ranges from -40 mV to +80 mV.

In case that a Na current is seen we can be sure that is a Na current by bathing the extracellular solution with TTX. To do so another pipette must be inserted in the bath right upper the cell, then a positive pressure is made and the TTX is inserted in the bath. Recording the response of the cell at the current step that show the Na current should show the erasing of the current after inserting the TTX.

2.4. Experiments

2.4.1. MSC

The MSC after the differentiation were incubated and used for the experiments, the experiments on the different clone were made in two or three days and the next after ten days, in order to create a time lapse to see how the differentiation protocol was going. The experiments were made on glass and plastic on clone 4 and 7 but only on glass on clone 5 because the cells on plastic were not differentiated.

2.4.2. AFM analysis

In order to understand why the cells preferred to differentiate on glass and were not differentiated or only partially on plastic we used an AFM analysis of the different cover slips to see the roughness of the surface with and without the matrigel layer.

2.4.3. Primary motorneurons

Motorneurons were extracted from mouse embryos by our collaborator Prof. Pennuto and cultured for three weeks at VIMM in incubator at 37 °C. The sequence of protocols for the neurons was to start the acquisition of the data with a signal maintaining the current at 0 pA in order to see if any spontaneous action potential can be seen, then a current clamp was run to see the action potential and a voltage clamp to see the K^+ and Na^+ currents, in order to see the second type of current a whole cell compensation was made to eliminate any type of suppression of the signal due to a capacitance not compensated. In some cases a solution with TTX were sprayed over the cell in order to see if the action potential was blocked by this neurotoxin. The solution with TTX was prepared by adding 0.5µM of TTX to the intracellular solution.

3. Results

3.1. AFM

For each cover slips we did an AFM analysis of a square of dimension 5×5 µm with an high resolution and a 50×50µm with less resolution to see the roughness on an higher surface. The cells have a diameter about 10µm so the first area of analysis is much smaller of the area covered by a cell and so we can analyze well which type of roughness preferred the cell. For each cover slip we did also an analysis of the square root of the height along the z axis to understand how the roughness were distributed on all the surface, the results are shown in the table, where we marked the presence of matrigel with coat

	5×5µm	5×5µm	50×50µm
Glass no coat	0.43946 nm	0.37103 nm	5.0023 nm
Glass coat	2.8388 nm	6.4738 nm	7.2768 nm
Plastic no coat	8.1871 nm	3.4484 nm	4.0485 nm
Plastic coat	3.1022 nm	4.3084 nm	8.782 nm

We took two measures of the smallest square on different position on the cover slips to see how the roughness can change, by this first measures we can see that for the plastic and glass with matrigel the results were similar and also the plastic without the matrigel had similar values while the glass without the coat had a square root of the roughness much smaller. We see now the histogram of the height and the 3D graphs for the different cover slips.

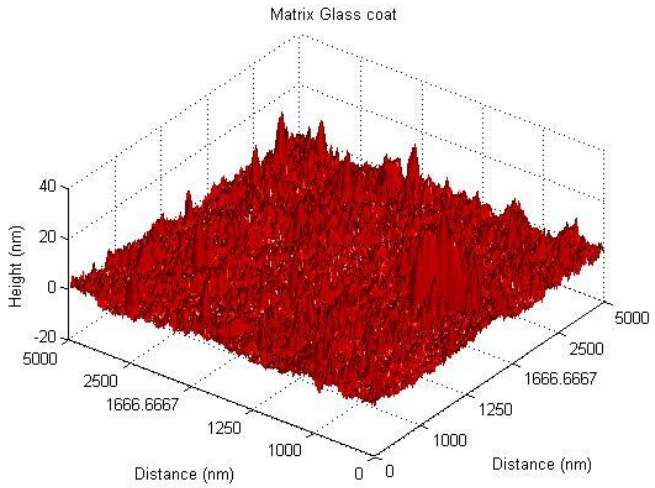


Figure 18: Histogram 5×5 µm glass coat

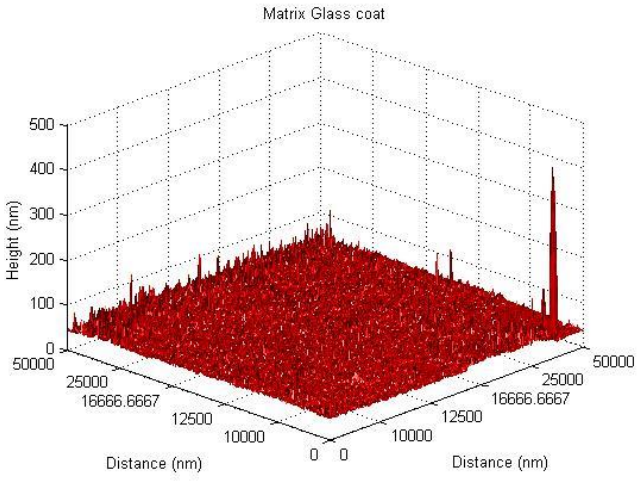


Figure 19: Histogram 50×50µm glass coat

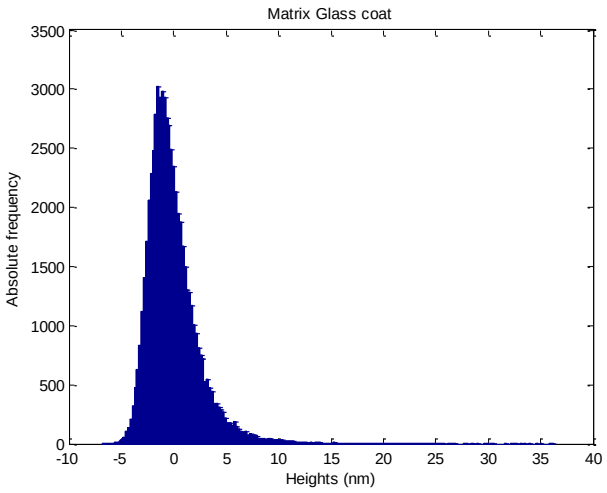


Figure 24: Histogram 5×5 µm glass coat

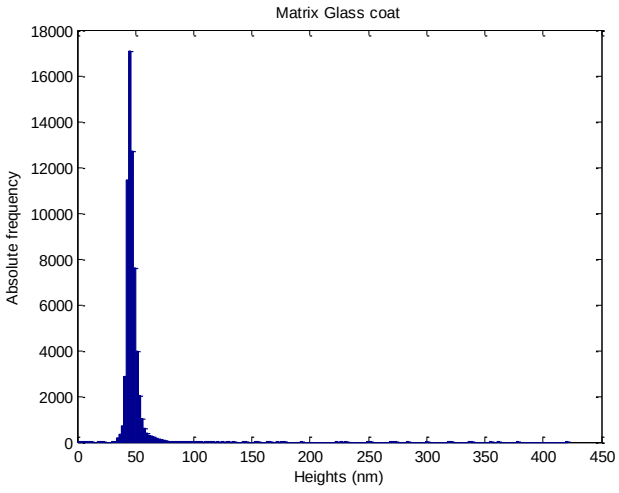


Figure 25: Histogram 50×50µm glass coat

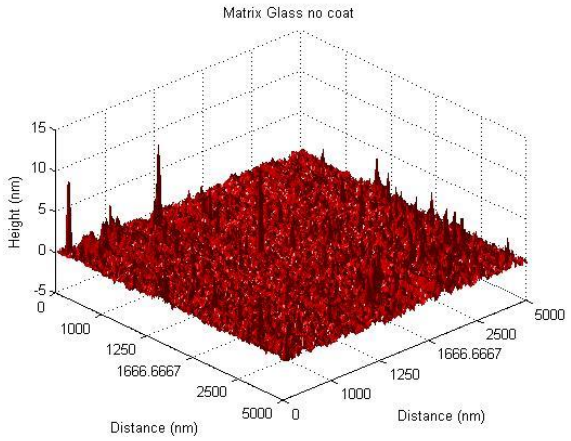


Figure 26: Glass no coat5×5µm

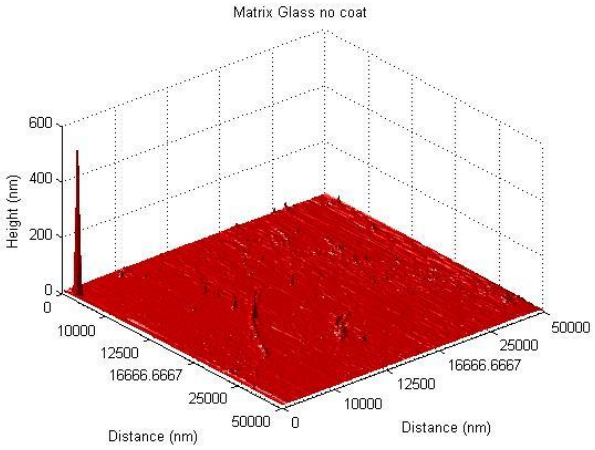


Figure 27: Glass no coat 50×50µm

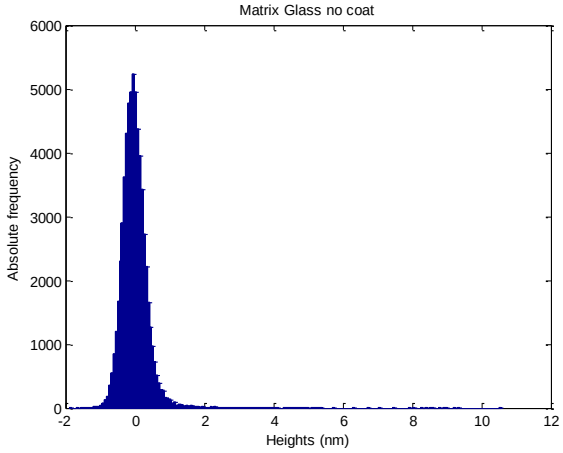
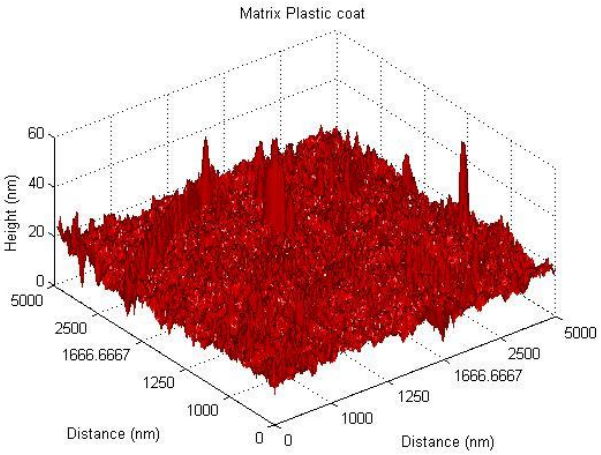
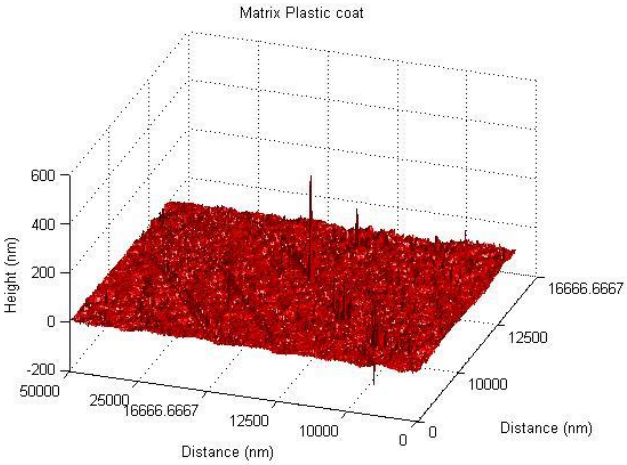


Figure 28: Histogram 5×5µm glass coat





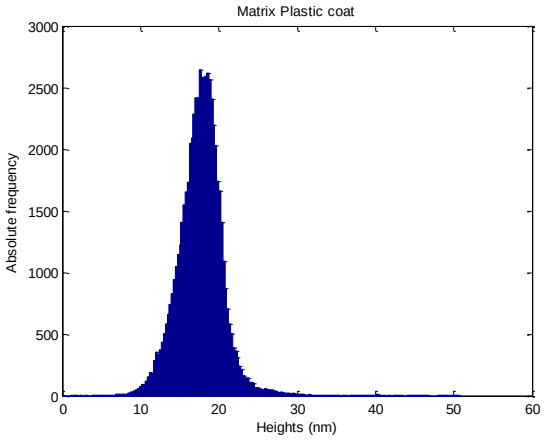
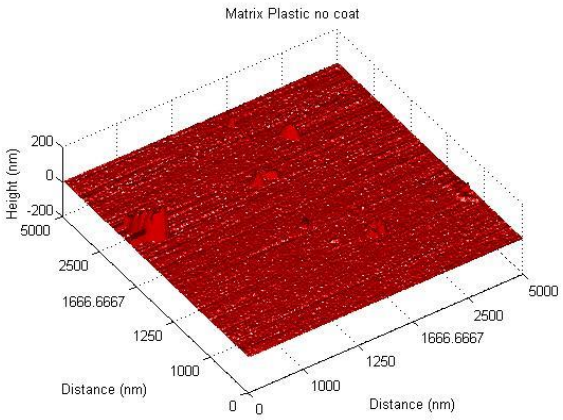


Figure 29: Plastic coat 5×5µm

Figure 30:Plastic coat 50×50µm

Figure 31:Histogram 5×5µm plastic coat



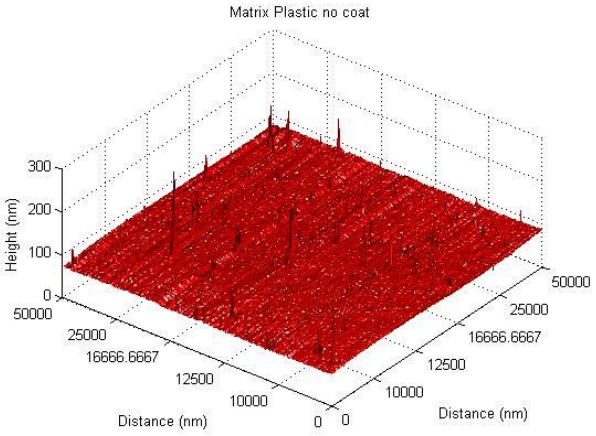


Figure 32: Plastic no coat 5×5µm

Figure 33: Plastic no coat 50×50µm

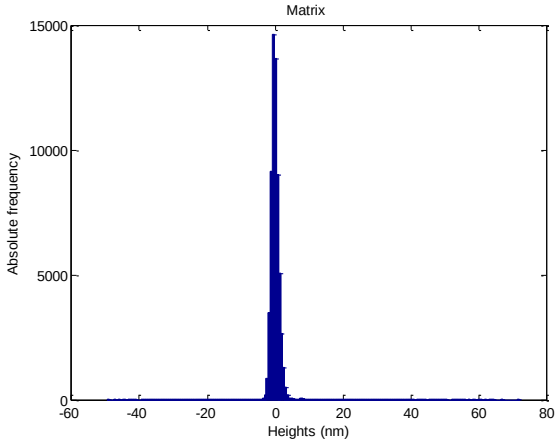


Figure 33: Histogram 5×5µm plastic no coat

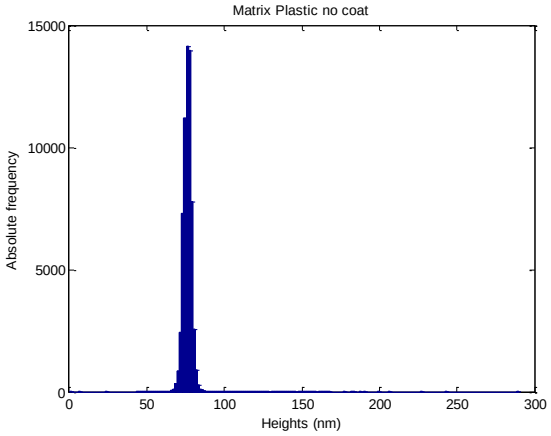


Figure 34: Histogram 50×50µm

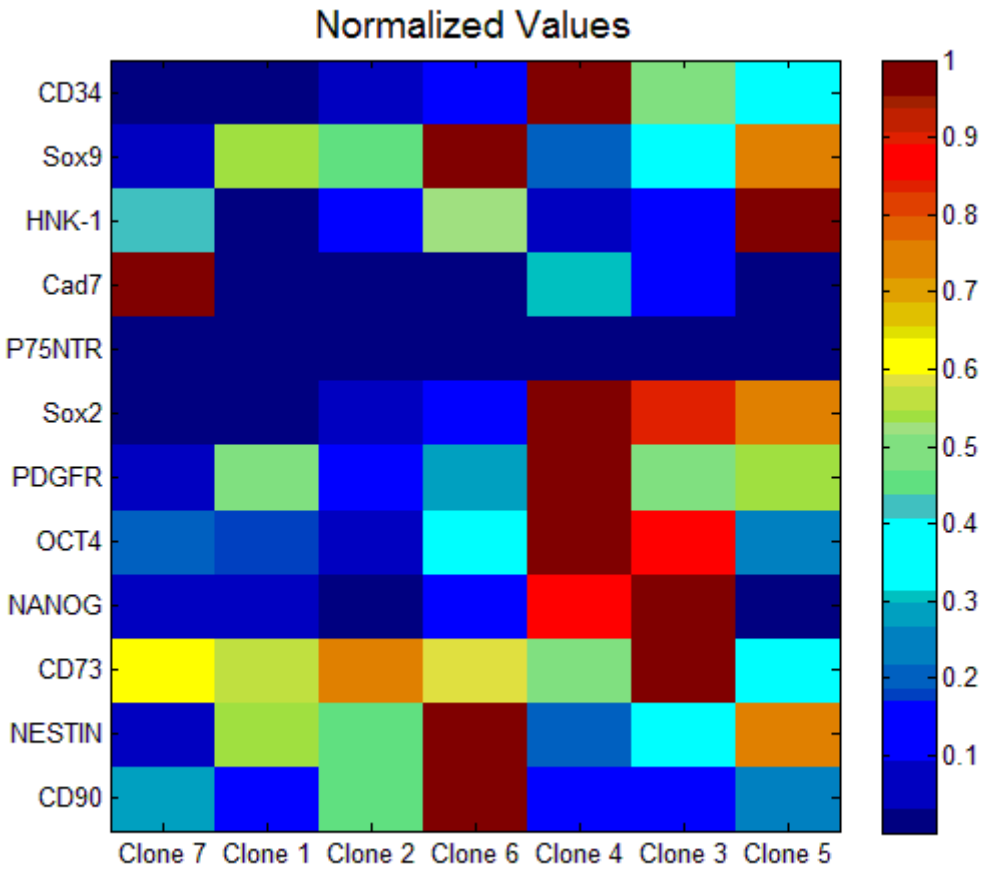
As we can see from the 3D graphs of the surface we can point out the difference between the glass without the matrigel, which is the one with less impurity on the surface, and the other three cover slips which had some impurity on the surface. We can see that the impurity on the glass with matrigel is due only to the new layer, while the impurity on plastic comes from the natural roughness of the cover slips. We cannot see a large difference between the two cover slips with matrigel but we had seen a huge difference on the differentiation of the cells, this difference can derive from the initial difference of the surface between the two materials. In the case of glass the initial surface has very little roughness so the matrigel can cover homogeneously the glass and the final roughness is due to the matrigel, while on plastic the initial roughness of the cover slip may cause a difference in the cover of the matrigel. A difference can also be that as we can see on plastic we can have a small region with an height quite high, higher than glass, and this structure can influence the attach of the cell to the matrigel and its growth.

3.1. MSC

3.1.1. RT-PCR

In our study we developed a system of selection on monoclonal colonies of stem cells based on the signature of specific genes analyzed by a RT-PCR analysis.

RT-PCR



In the figure we can find the normalized levels of mRNA transcribed from different genes (that can be detected thanks to specific primers) for different clones. We can see there was a big difference in the levels of neuronal markers. A big difference was found also at the morphological level after the induction periods, so we decided to select clones 4 and 7 for further analysis.

3.1.2. Patch clamp

Patch-clamp permitted to measure the resting potential at different time points. The results on the resting potential were different for each clone, clone 7 was the one with the lowest mean resting potential and the nearest to a neuron resting potential while the other were too low, but a current different from the ohmic behaviour was seen for some cell on all the clone, but none of the clone fired an action potential.

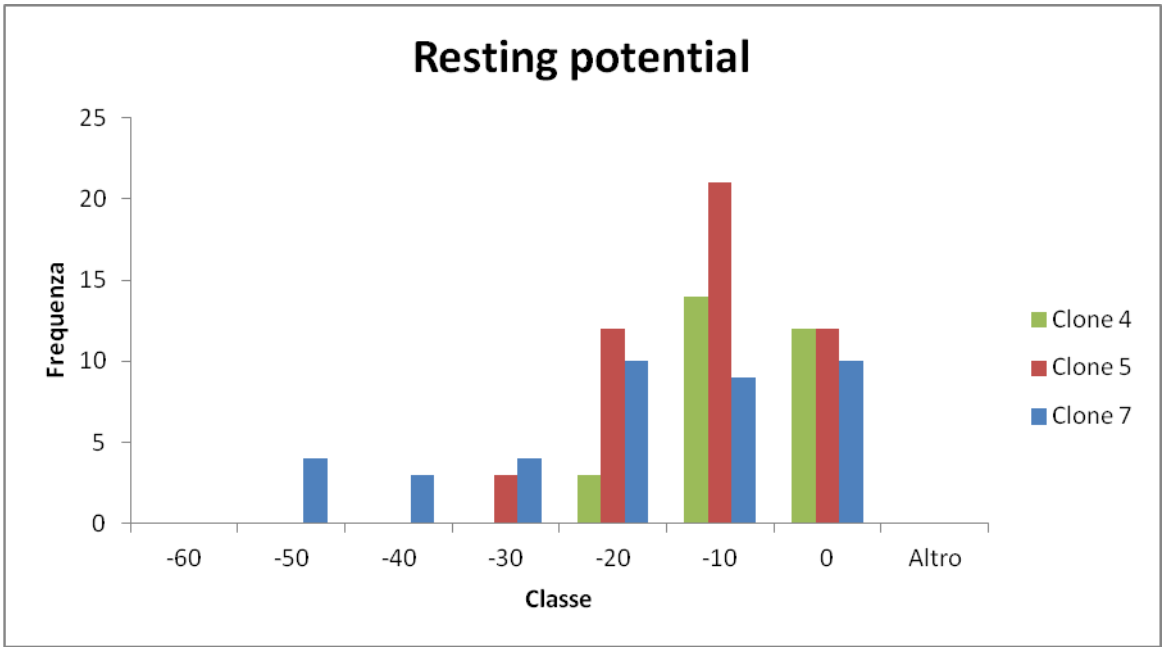


Figure 20: Histogram of the measured resting potentials

The mean resting potential for clone 4 was -9,5 mV, for clone 5 was -16,7 mV and for clone 7 was -22,2 mV. All the values found were quite low for a neuron and only clone 7 had a resting potential near the resting potential found later for the neurons with a firing action potential.

On the next figures we can see the resting potentials for the different clones at different stages of maturation.

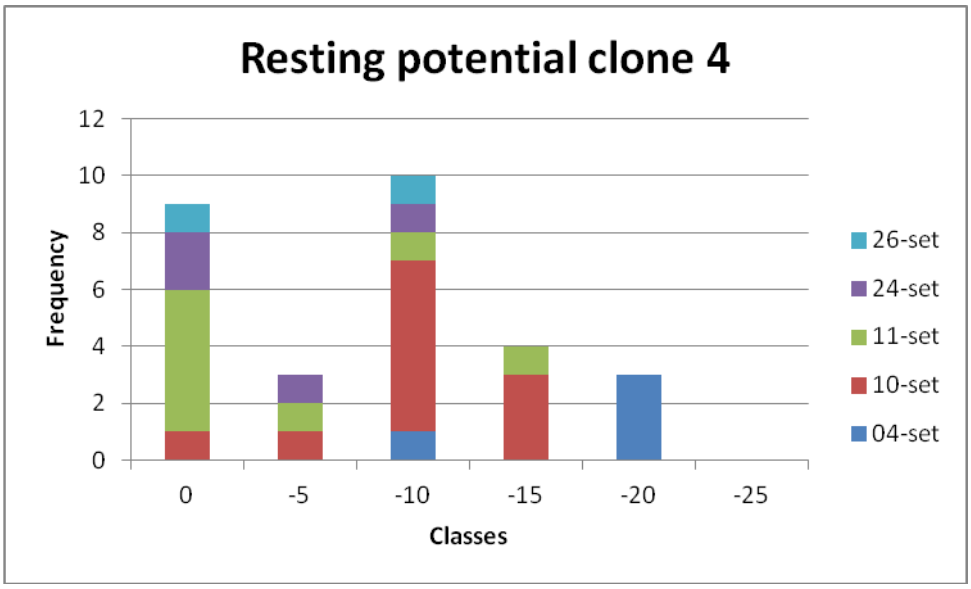


Figure 21: Histogram of the measured resting potentials clone 4

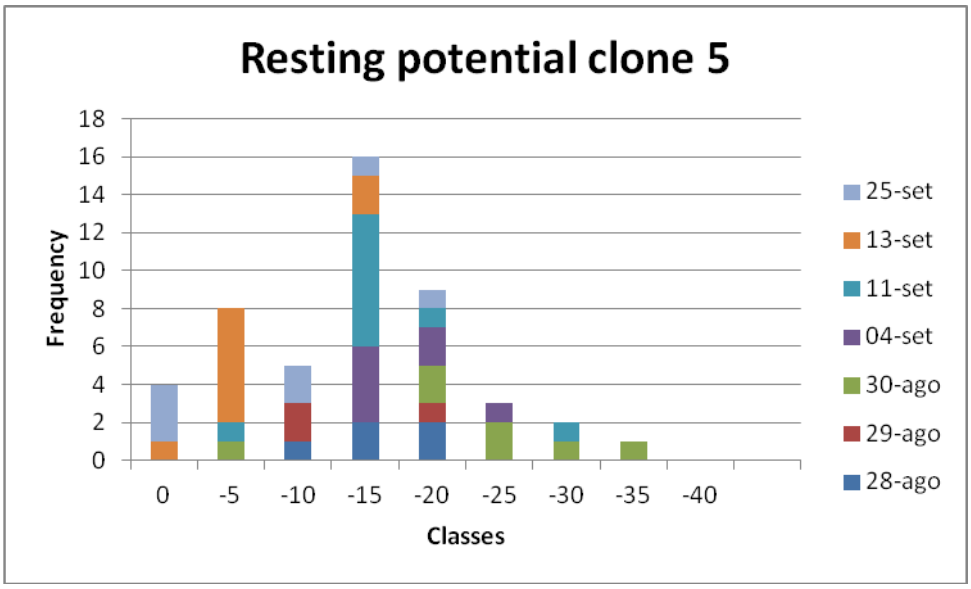


Figure 22: Histogram of the measured resting potentials clone 5

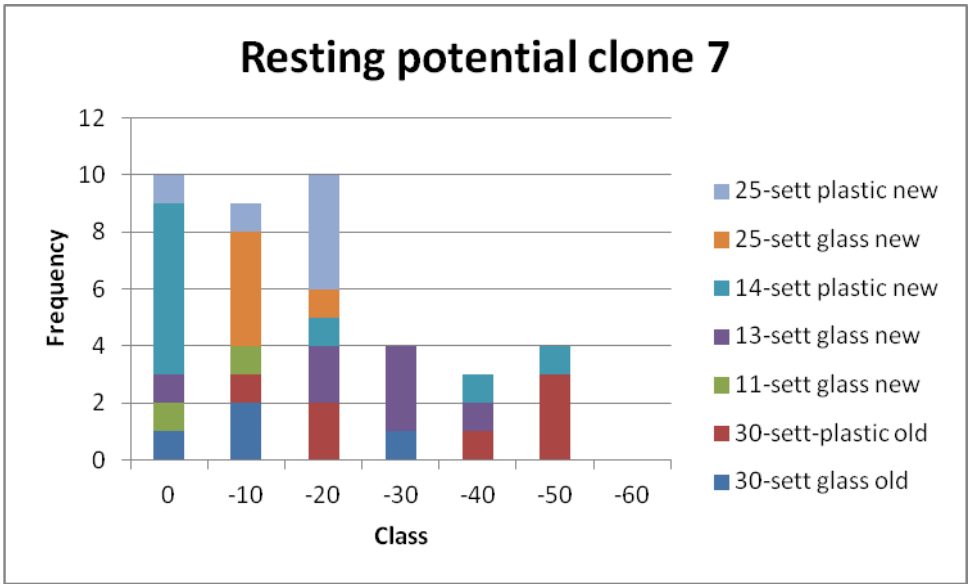


Figure 23: Histogram of the measured resting potentials clone 7

In the last histogram we can see the results found for the clone 7 on plastic and on glass, we can see that the best result were on plastic with the old protocol of differentiation, but for this type of covers slip we have only a set of experiments performed on the same day. In general the lowest mean potential were found on plastic but the mean resting potential is lower on glass.

3.1.3. Clone 4

First we analyze the behavior of the cell in response to a voltage step on different days in order to show changes of the cells after some days where the differentiation protocol continues to be administered (the day of differentiation is indicated below the graphs) to the cell, then we show the difference in the two protocol to understand which was the best for the differentiation. Then the same study is made for the current clamp.

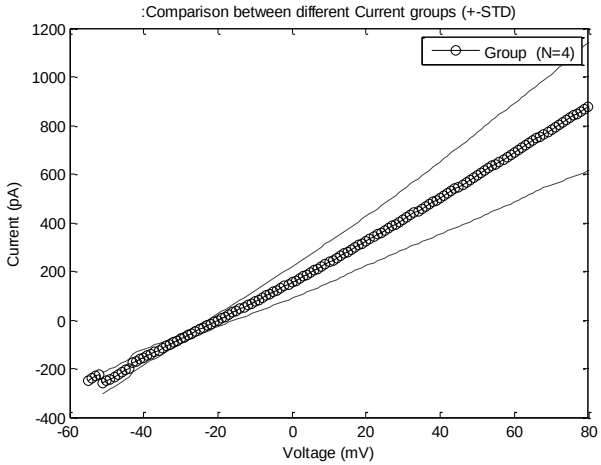


Figure 24: Voltage clamp old protocol 4 September-Day 17

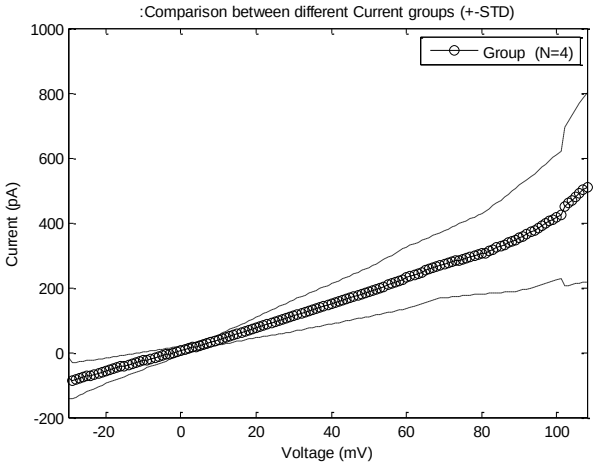


Figure 25: Voltage clamp old protocol 24 September-Day 37

In this two figures we can compare the response of the cell to a Voltage step in the first week of September and on the last, we can see how the resistivity on the last week was lower.

A time lapse of Voltage clamp for the new protocol was not performed due to problem of attaching to the cell for the reason explained before. Also the for the old protocol the measure were difficult to do.

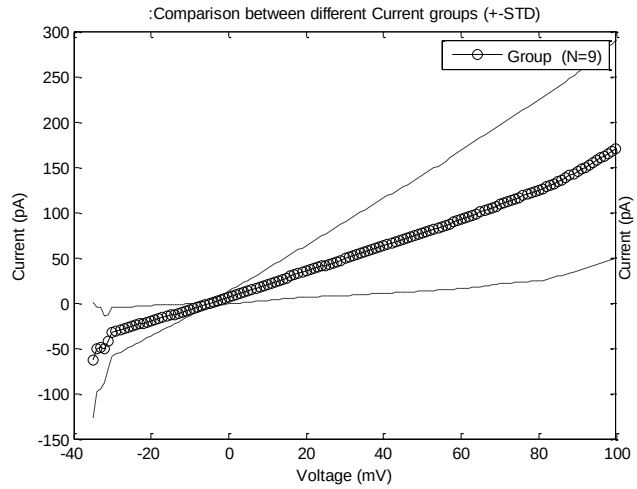


Figure 26: Voltage clamp old protocol 10 September-Day 23

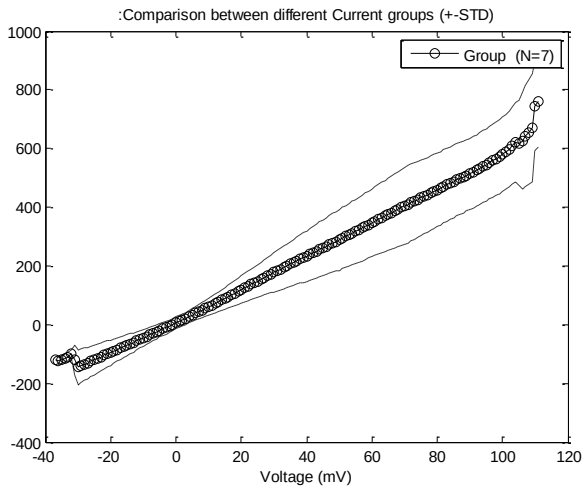


Figure 27: Voltage clamp new protocol 12 September-Day 25

In these two figures we can see the difference between the two protocol on the same step of differentiation, we can observe how the resistivity for the new protocol is lower than the old protocol

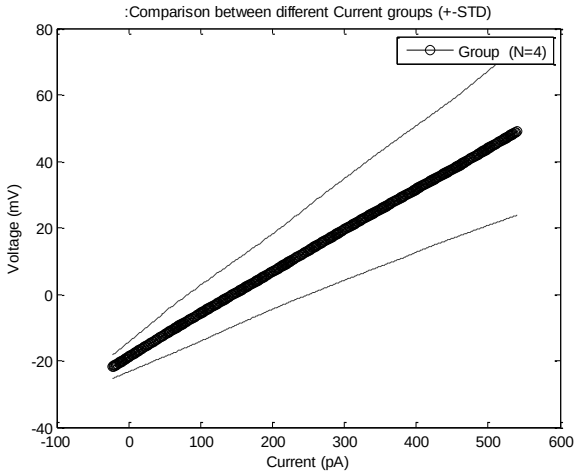


Figura 31: Current Clamp old protocol 10 September-Day 23

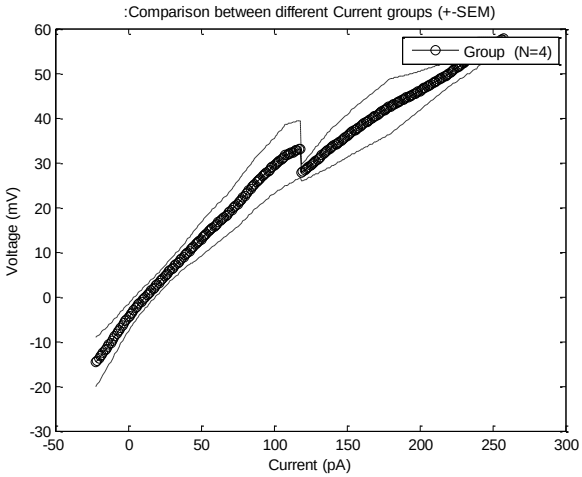


Figura 30: Current clamp new protocol 12 September-Day 25

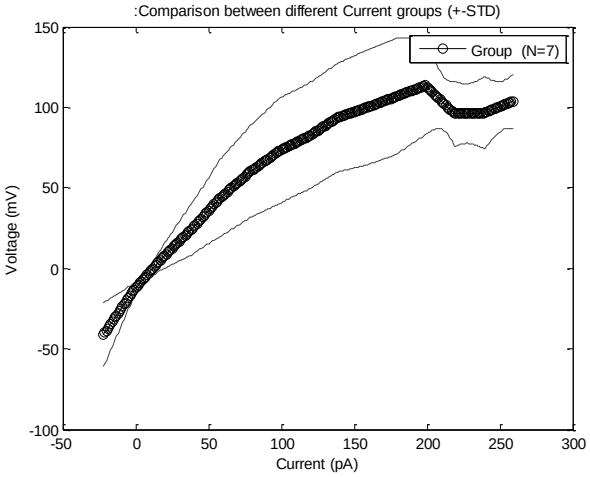


Figure 29: Current clamp old protocol 4 September-Day 17

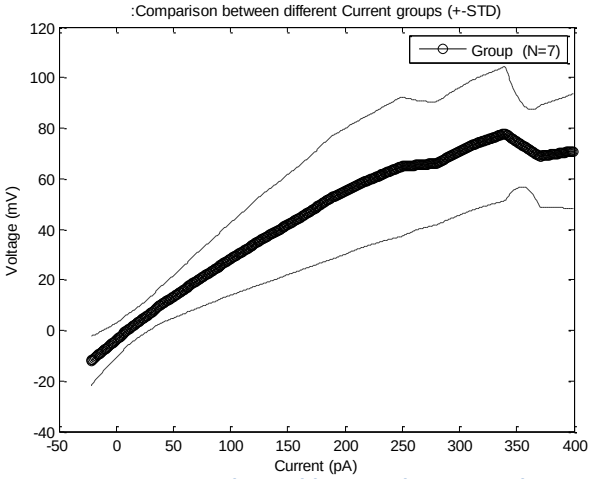


Figure 28: Current clamp old protocol 24 September-Day 37

In these figures we can see first a time lapse for the old protocol and also the difference the difference for the old and new protocol at the same stage of differentiation. In the first figures we can see that on the last stage of differentiation we can reach a voltage of around 50 mV at 250 pA while on the first stage we need more current to reach the same voltage. On the first stage of differentiation the two protocol presented a similar behavior but we can figure out a resistance quite lower for the new protocol.

After seeing the mean behavior of the cells we now highlight some interesting response to a current clamp protocol of some cells that seemed to have some type of channel.

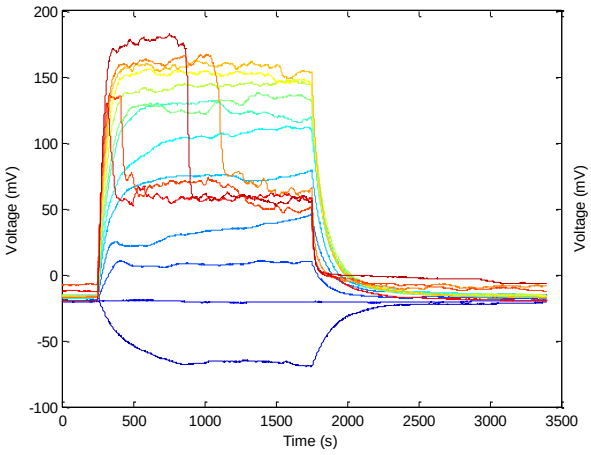


Figure 32: Current clamp 12 September-Day 25

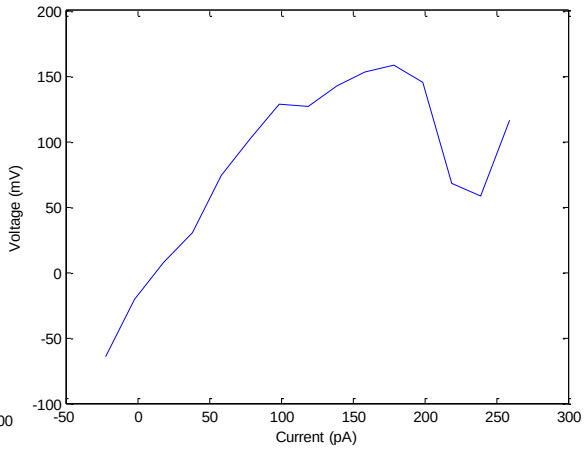


Figure 33: I-V chart-Day 25

As we can see at an higher value of current the voltage decrease in a non ohmic way, this is probably due to some type of channel that are closing and so the voltage decrease.

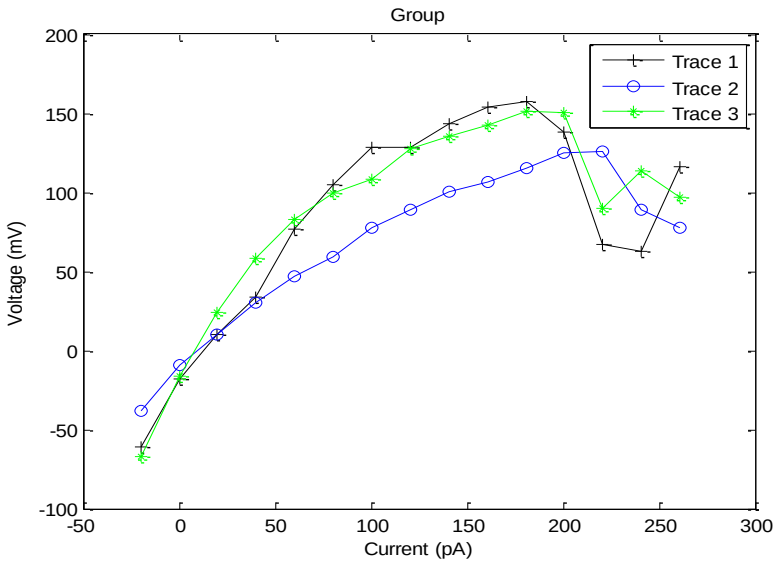


Figure 34: Current clamp 12 September-Day 25

This behavior was seen on three cells on the same day and the figure above shows the three traces found for the I-V graphs, the three traces are very similar.

3.1.4. Clone 5

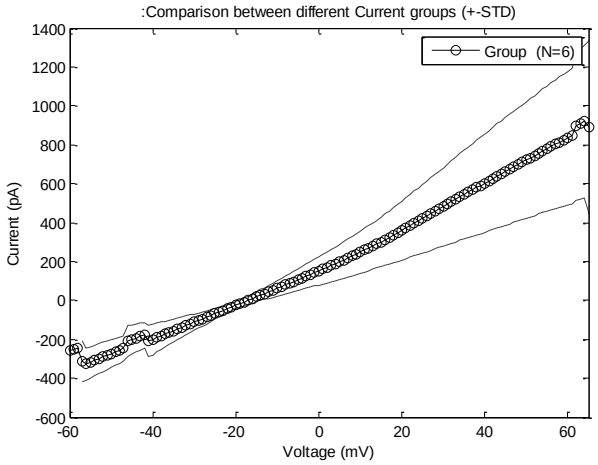


Figure 36: Voltage clamp 28 August-Day 10

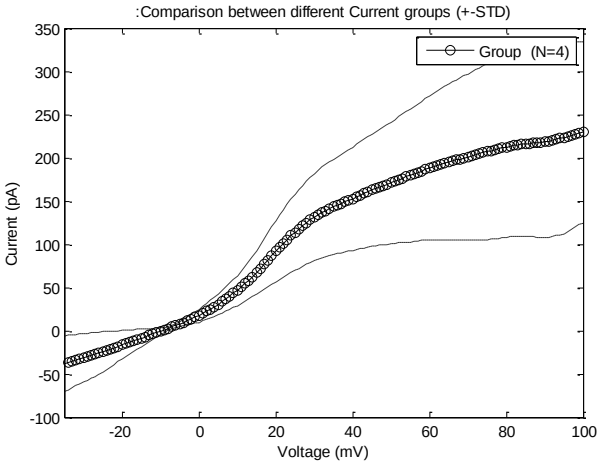


Figure 35: Voltage clamp 25 September-Day 38

These figures shows the difference between the behavior of the cells in two different time lapses, as we can see from the X axis the protocol used for the measurement has changed, the first goes from -60 mV to 80 mV while the other goes from -40 to 100 mV, each of them with a step of 10 mV. As one can notice the difference in the current is very high since the maximum currents in the two figures are 1200 pA in the first and around 200 pA in the second (with a voltage step of 80 mV). The little curve on the second figure is something that can be seen on many experiments and is a characteristic of the MSC

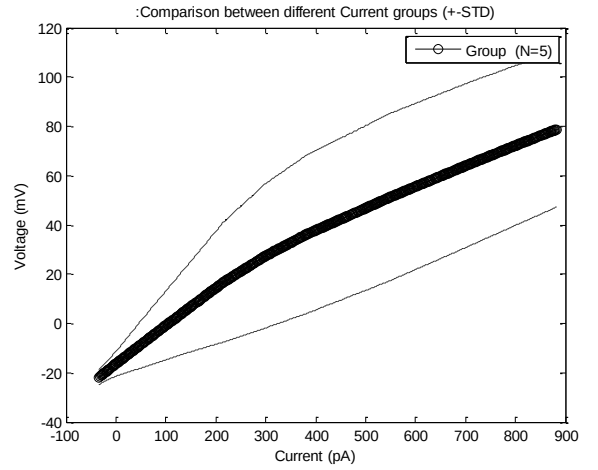


Figure 38:Current clamp 28 August-Day 10

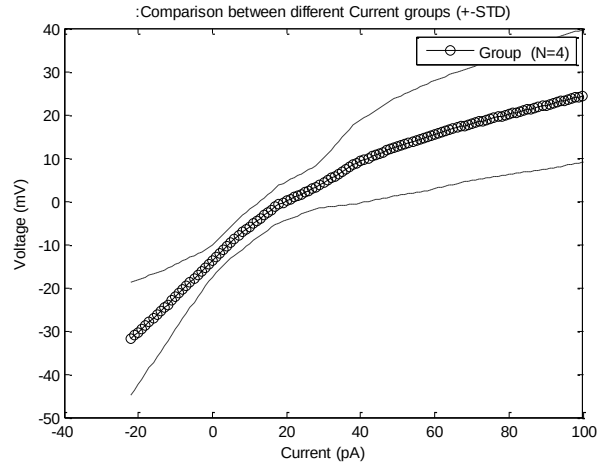


Figure 37: Current Clamp 25 September-Day 38

The Current clamp outlines a very different behavior of the cells in the two time lapse, the protocol is very different and we can see how a larger current is needed to have the same voltage, for example to obtain a potential of 30 mV the current at the 28 August was around 250 pA while at 25 September was 100 pA.

Clone 5 on the last days of experiments had a behavior similar to an action potential but the resting potential were too low to be a neuron.

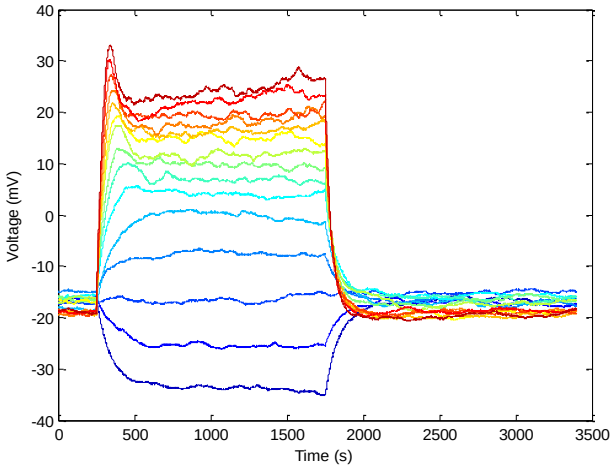


Figure 39: Current clamp 25 September-Day 38

The spikes at the start of the figure are similar to a non mature neuron, but the resting potential of the cell is too low for a neuron.

3.1.5. Clone 7

With clone 7 we can compare the old and new protocol on different weeks of analysis but a time lapse is possible only on new protocol due to the lack of cover slip of the old one. with new protocol is possible also to do a time lapse of the two different materials used for the cover slip. First let's consider the difference between the new and the old protocol.

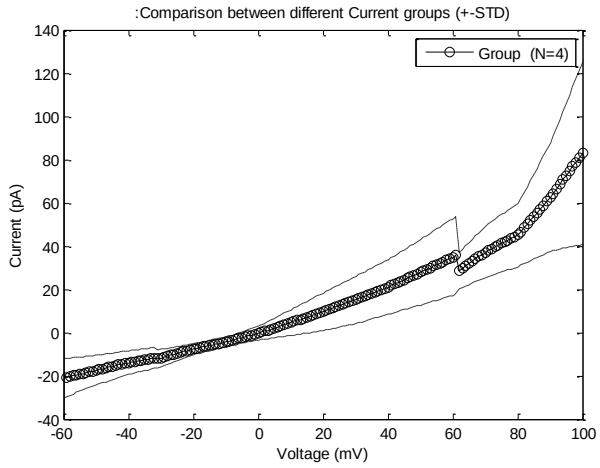


Figure 40: Voltage clamp old protocol 30 August-glass-Day 12

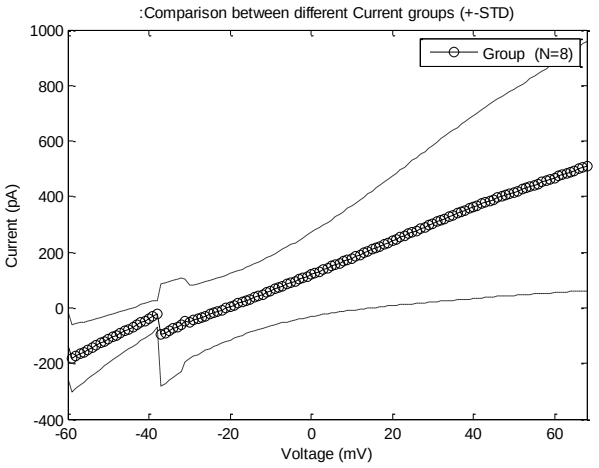


Figure 41: Voltage clamp old protocol 13 September-glass-Day 26

We can see that the protocol used for the two experiments were different, but also we can highlight a huge difference on the current at the same voltage, the old protocol has an higher resistance. Let's now consider the same voltage clamp but on plastic.

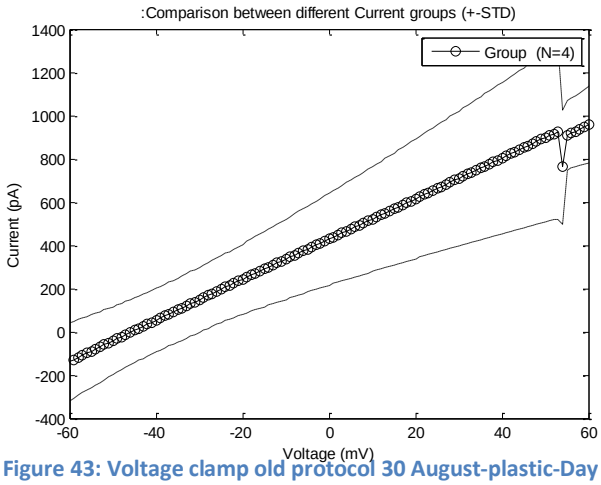


Figure 43: Voltage clamp old protocol 30 August-plastic-Day 12

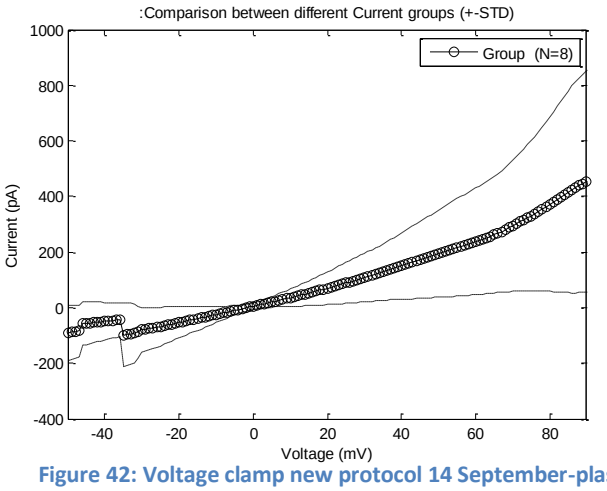


Figure 42: Voltage clamp new protocol 14 September-plastic-Day 27

In this case were we have more data to compute the mean we can see that the new protocol has an higher resistivity than the old one. More in general we can see that experiments made on the same day on the old protocol had a very different results because on glass the resistivity is greater and this can be seen also from the two resting potential, on glass we have a mean resting potential of -16 mV while on plastic the mean resting potential is -41 mV. For the new protocol there is less difference for the different materials and also the resting potential is similar: -28 mV for glass and -31 mV for plastic.

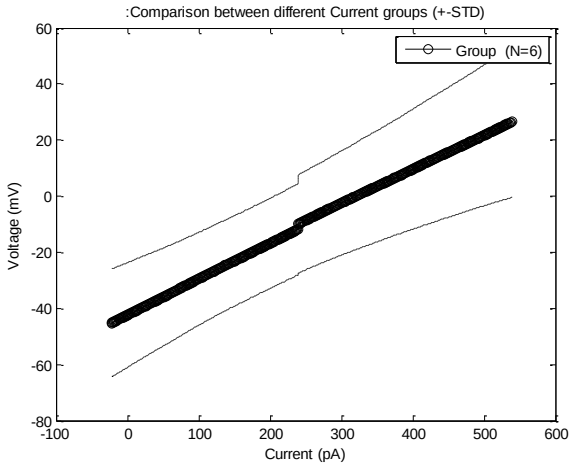


Figure 44: Current clamp old protocol 30 August-plastic-Day 12

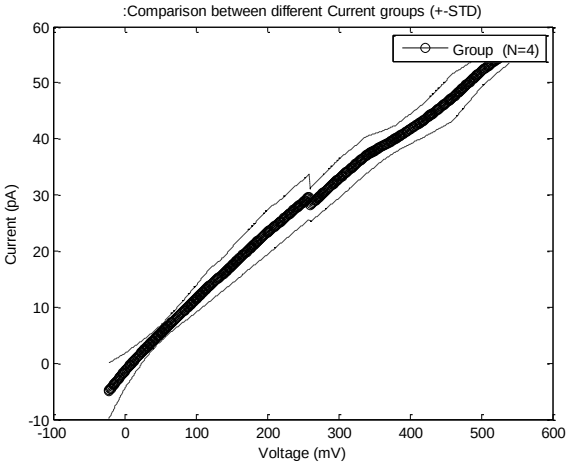


Figure 45: Current clamp new protocol 14 September-plastic-Day 27

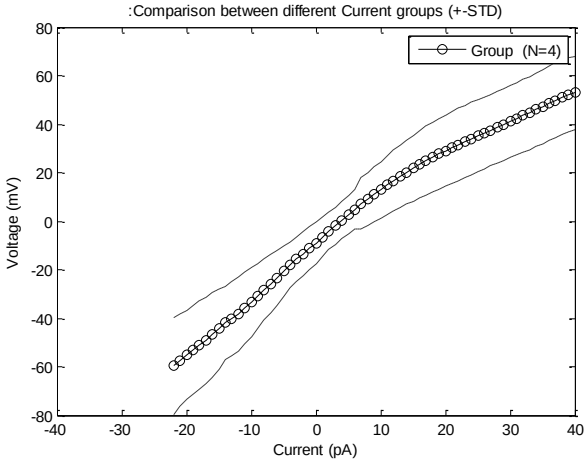


Figure 46: Current clamp old protocol 30 August-glass-Day 12

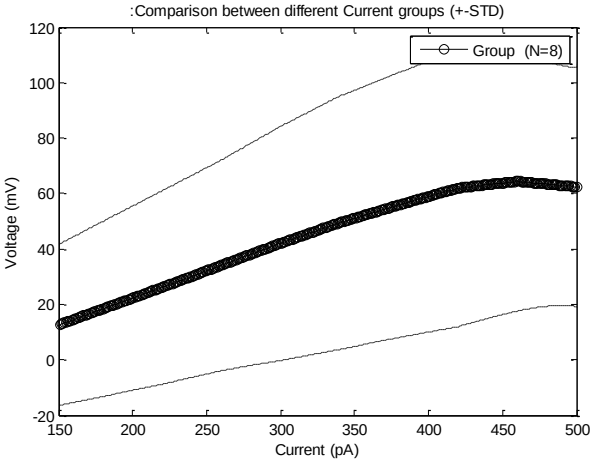


Figure 47:Current clamp new protocol 13 September-glass-Day 26

For the current clamp protocol we can notice that while there isn't a great difference between the plastic and glass of the new protocol, for the old protocol the glass has a very different behavior from the other and also the plastic never reaches a voltage around 50 mV also at higher currents.

Now we see a time lapse for the new protocol to see how the behavior of the cells changes during time and we outside also the difference between plastic and glass.

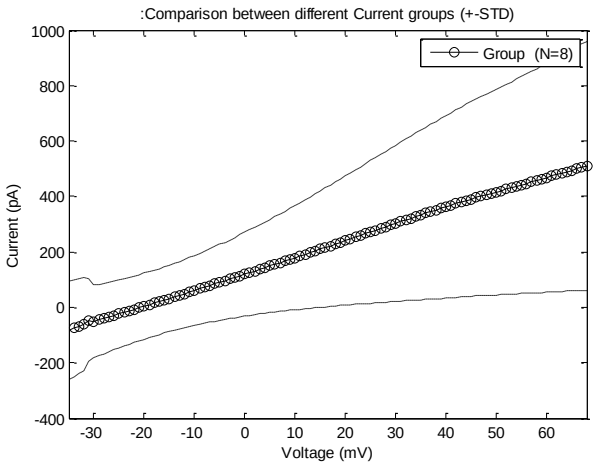


Figure 48: Voltage clamp 13 September-glass-Day 26

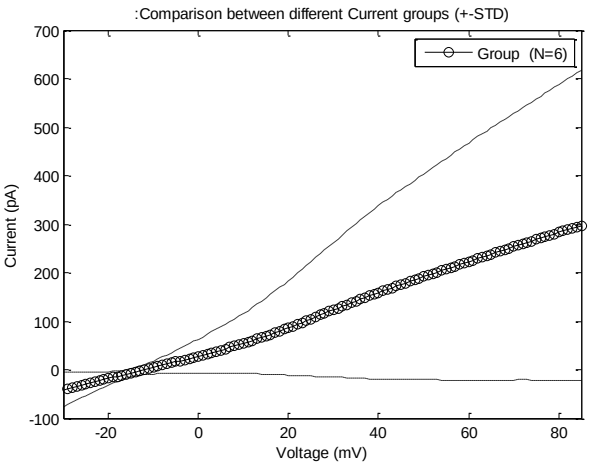


Figure 49: Voltage 25 clamp September-glass-Day 38

For the Voltage clamp we can see that there is an increase of the resistivity on both materials.

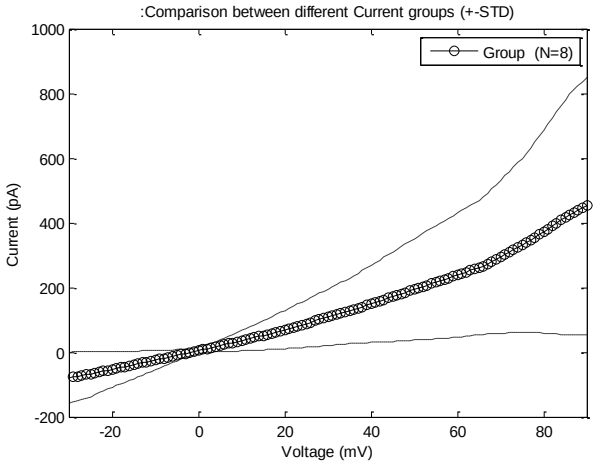


Figure 50: Voltage clamp 14 September-plastic-Day 27

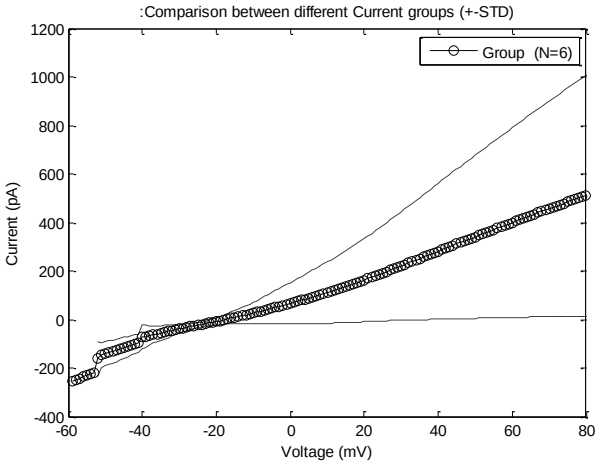


Figure 51: Voltage clamp 25 September-plastic-Day 38

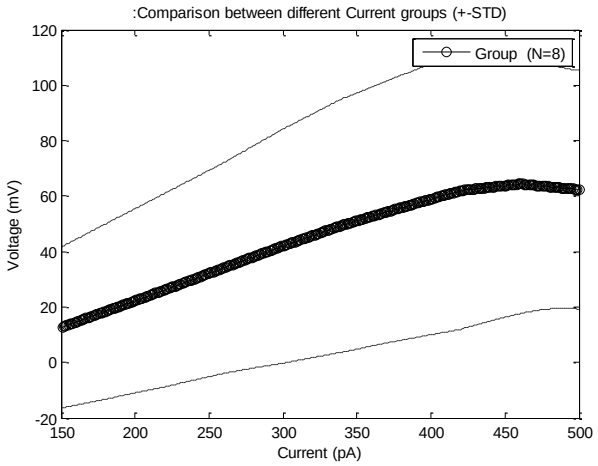


Figure 52:Current clamp 13 September glass-Day 27

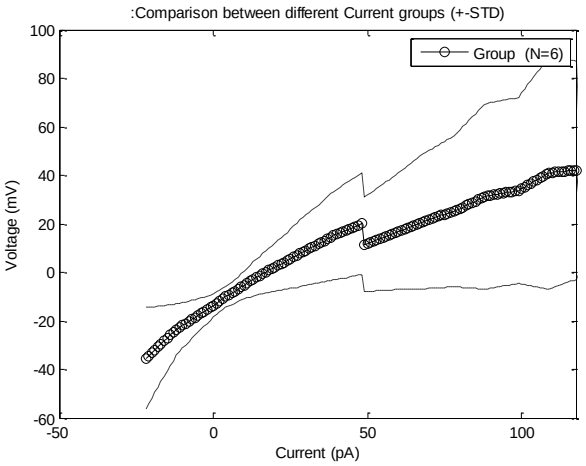


Figure 53:Current clamp 25 September-glass-Day 38

We can see that the range of the current step is very different in the two figures and we can highlight that the final part of the graph is only due to the specific behavior of some cell.

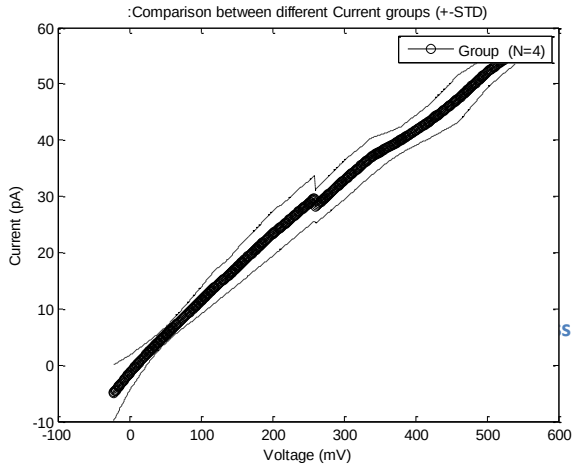


Figure 55: Current clamp 14 September-plastic-Day 27

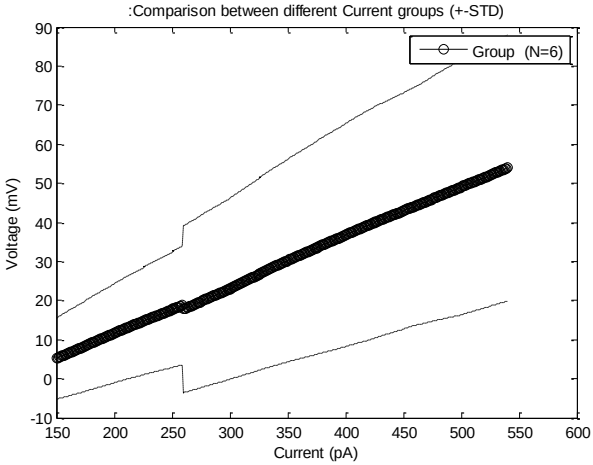


Figure 56: Current clamp 25 September-plastic-Day 38

For both voltage and current clamp we can see that on plastic the behavior of the cells are similar while the difference is in the glass where the resistivity increase with the time.

Also on clone 7 we can find something similar to the channel observed on clone 4.

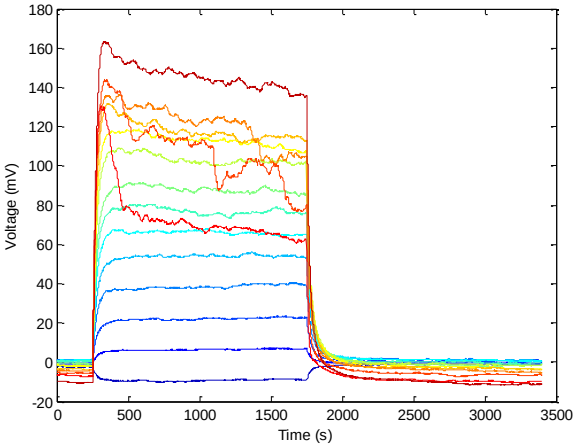


Figure 57: Current clamp 13 September-Day 27

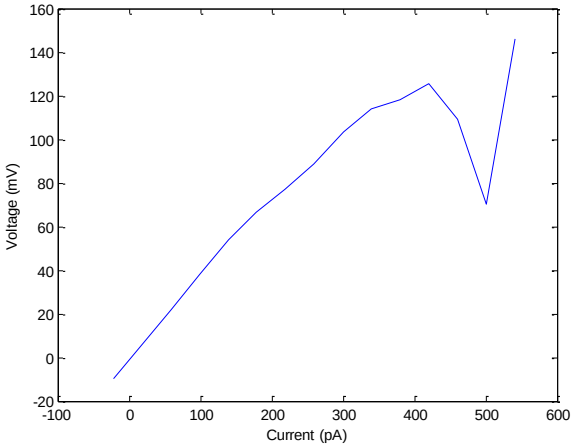


Figure 58: I-V chart-Day 27

Also in this case we can see that at higher current the voltage decrease at the increasing of the current, but naturally the cells cannot reach a potential so high so all the behavior found over 100 mV is not characteristic for the cell.

3.2. Motorneurons

First we present the results of a single cell to see the behavior of a neurons and then we present the result obtained from all the different measurement.

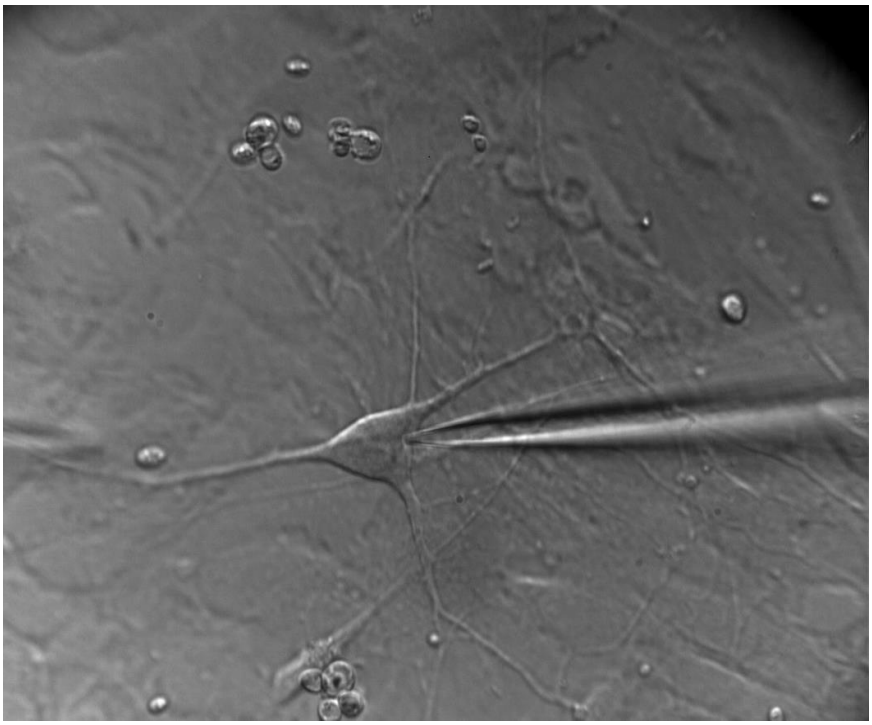


Figure 59: A neurons with two pipettes, the blurred one on the right has the TTX

The measure at resting potential of the neurons allows to visualize the spontaneous action potential of the cell, in this case we can see that all the spike has the same height and the same time, but there is no pattern in where the action potential start. In this case we can also see that the resting potential of the cell is around -53 mV which is consistent for a neuron considering the liquid junction potential that is the potential due to the different velocity of drift for the different ions present in the pipette.

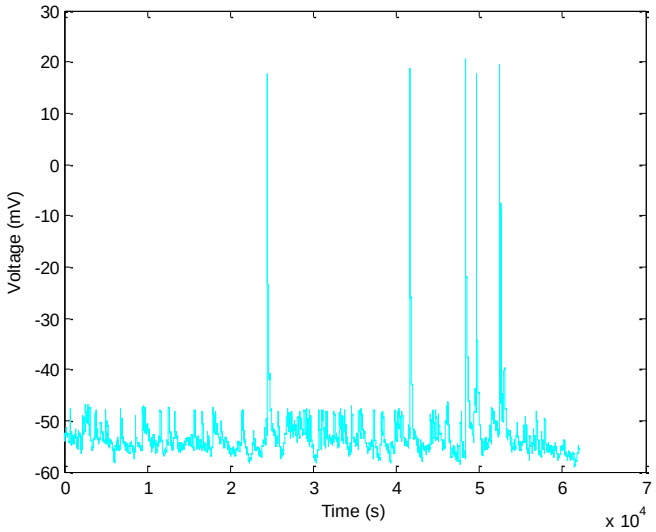


Figure 60: Spontaneous action potential

After seeing a spontaneous action potential we ran a current clamp to see a cascade of action potential, in this case the theorical behavior of the cell was to launch a series of action potential when stimulated by a current step and to increase the rate of firing and decrease the height of the spike when increasing the current. This can be seen in the next figure and is predicted by the Goldman-Hodgkin-Katz equation.

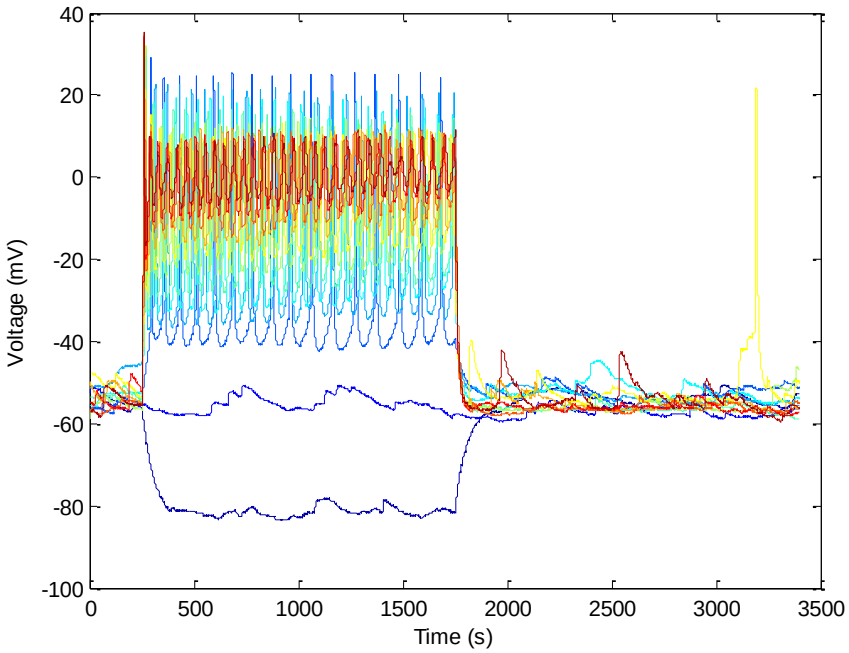


Figure 61: Action potential due to current step

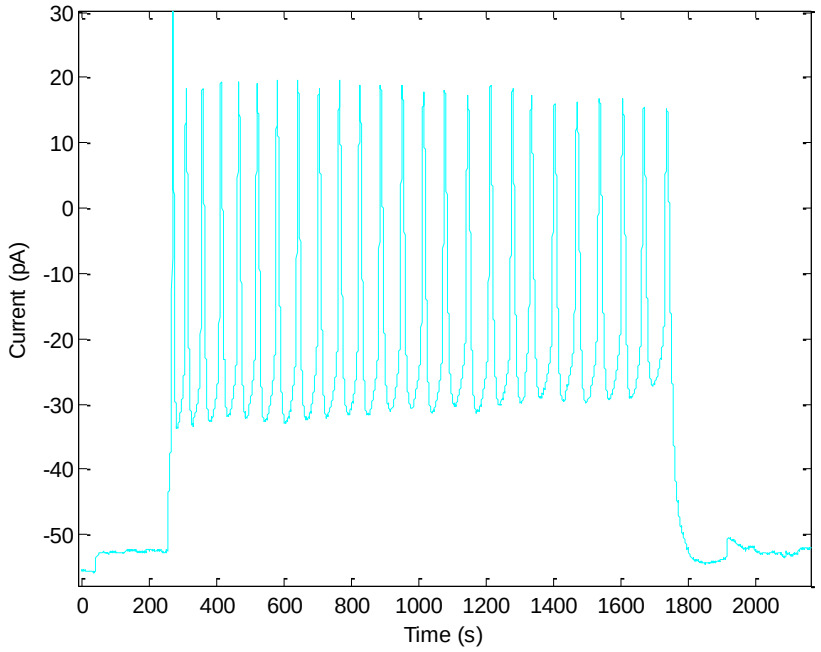


Figure 62: Action potential at 120 pA of current

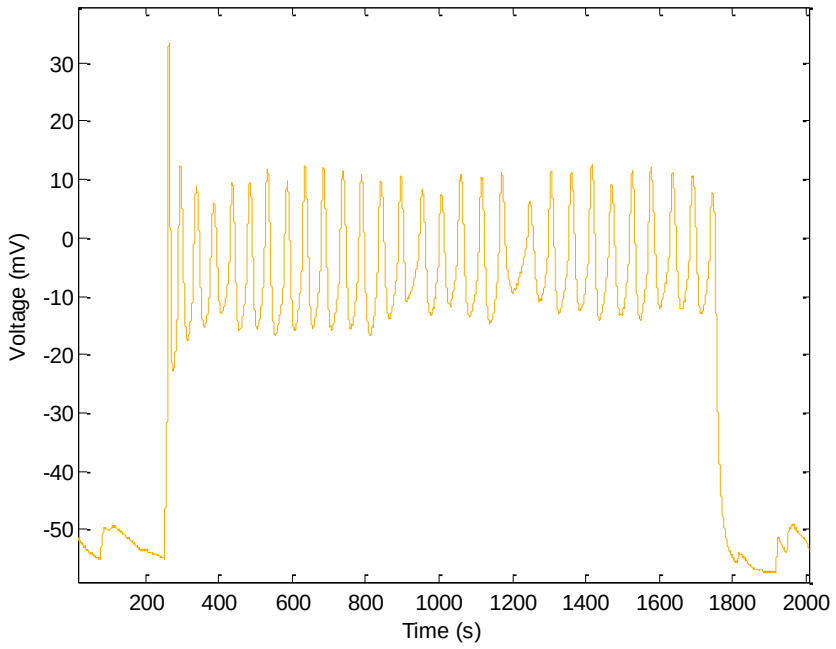


Figure 63: action potential at 280 pA

We can see that at different current the height of the spike decrease, from the 20 mV at 120 pA to the 10 mV at 28 pA, and also the frequency of the spike slightly increase.

Now to see if the voltage measured are action potential we insert in the bath the TTX solution by applying a positive pressure to the pipette near the cell, in this way we stop the Na^+ and we should not see anymore the action potential because the response of the cell is only passive.

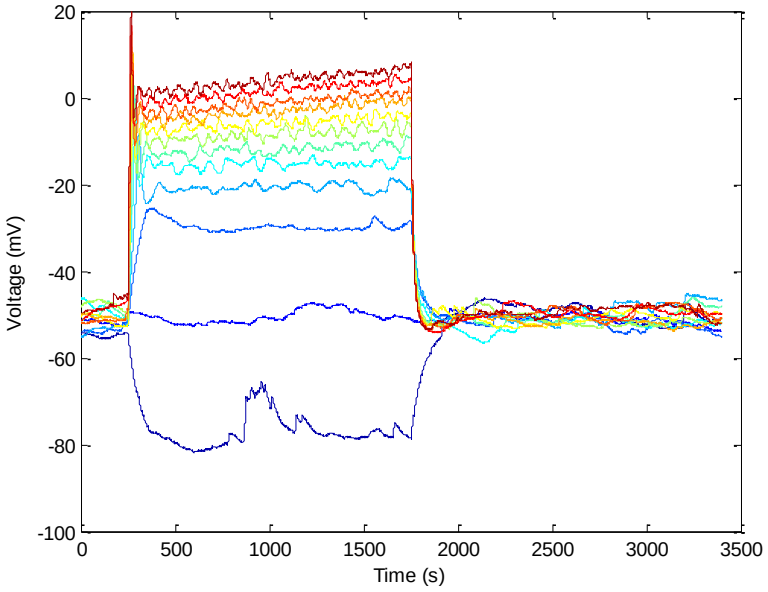


Figure 64: Current clamp with TTX

The spike are now suppressed for the presence of the toxin, if we wait for some minutes the perfusion system is able to wash out the toxin from the bath and so we should be able to see again the spike of action potential.

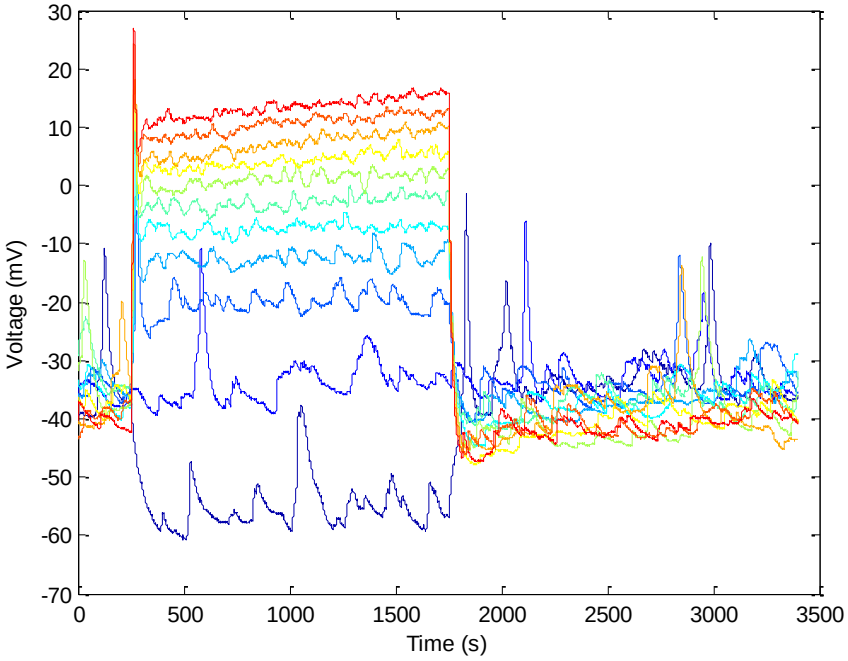


Figure 65: Action potential after washout

We can see the spikes again but many of them are not visible for a not perfect wash out of the toxin or for some not reversible damage to the channel .

We can now see the voltage clamp measure to see the Na^+ and K^+ currents.

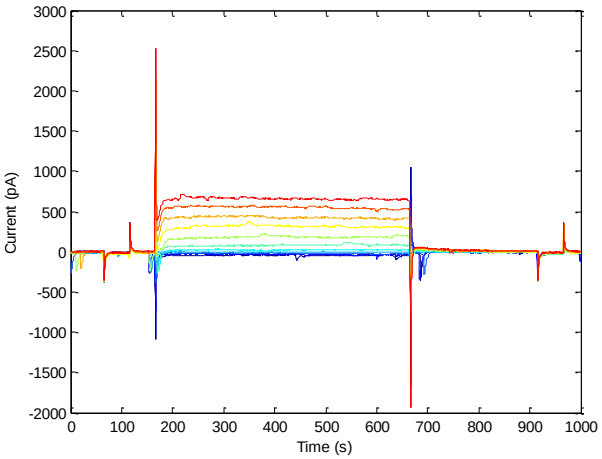


Figure 66: Voltage clamp

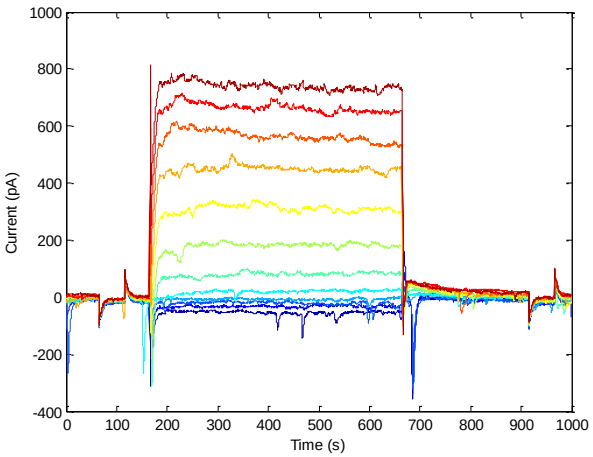


Figure 67 :Voltage clamp with whole cell compensation

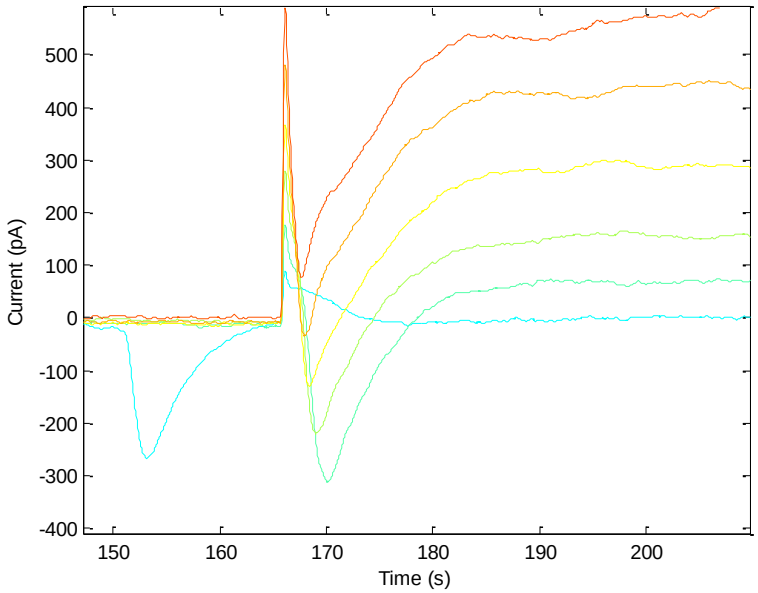


Figure 68: Whole cell configuration with a zoom on the Na^+ current spike

In the last figure we can observe how the Na^+ current change with different voltage, the depolarization of the cell is fast for every step but the height of the spike decrease at the increasing of the voltage.

This is the protocol followed for every cell, now we analyze the mean resting potential.

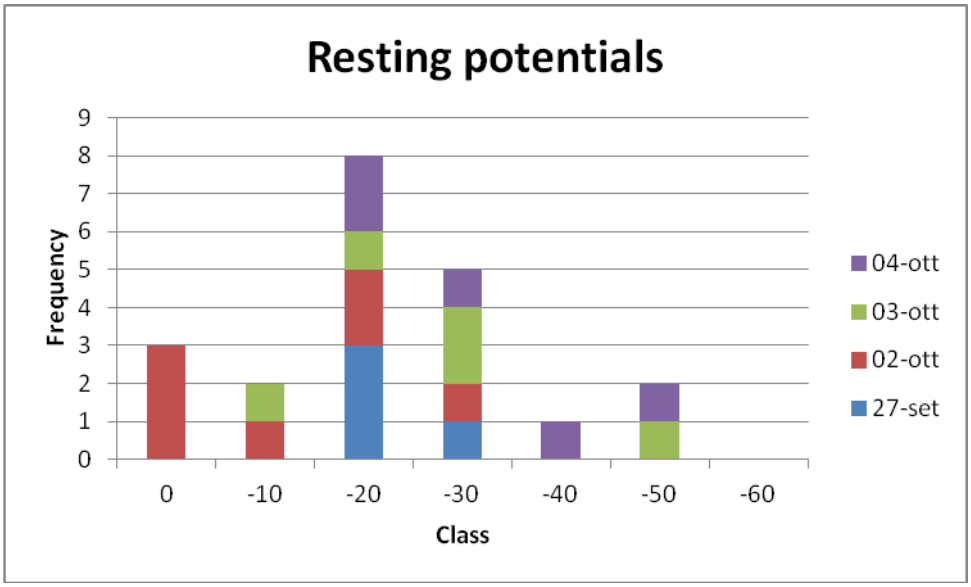


Figure 69: Histogram of the resting potentials

The mean resting potential of the cells was around -26 mV, and the cells that had an action potential had a mean resting potential of -47 mV.

4. Conclusions

On the experiments on the clones we did not manage to see an action potential and a resting potential quite low as the neurons, this can be due to some problem in the selection process and to some technical problem found during the analysis. In this chapter we list the advances in the research on the differentiation of MSCs into neurons and all the problems found and how we can avoid them in a future work.

During our study we notice that the neurons on plastic were not differentiated at all (clone 5) or only partially (clone 4 and 7) so we try to compare the behavior on the different material.

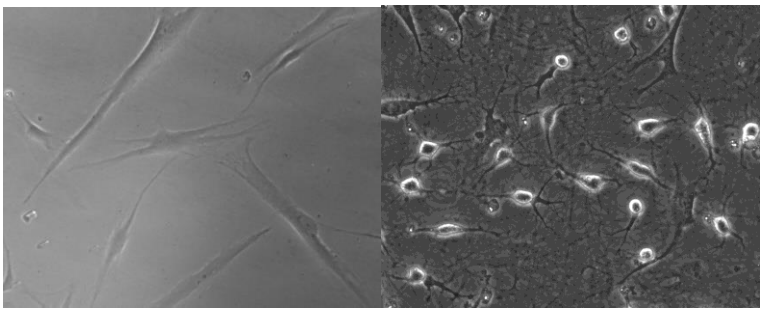


Figure 70: Clone 5 on plastic and glass

In the figures we can see the cells on clone 5 on plastic and on glass and notice that on plastic the cells had not a neuronal appearance.

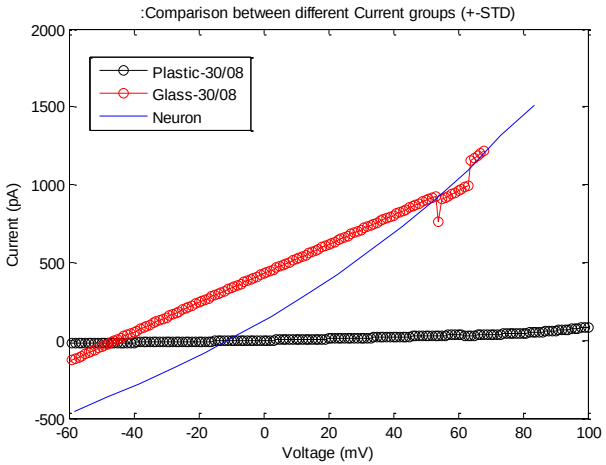


Figure 71: Comparison between glass and plastic-Voltage clamp

On the figure we can notice the behavior of the cells of clone 7 to a voltage clamp protocol and compare with a neuron, we can see that on glass the response to electrical stimuli is similar to the neuron while there is a huge difference on plastic. This comparison is made on the first stage of differentiation (day 12) but we keep this results because the experiments made on plastic at later stage of differentiation had a resting potential near 0mV so we do not consider them.

We find that clone 5 and clone 7 had more neuronal characteristics respect clone 4, from a point of view of morphological appearance and from the electric characteristics.

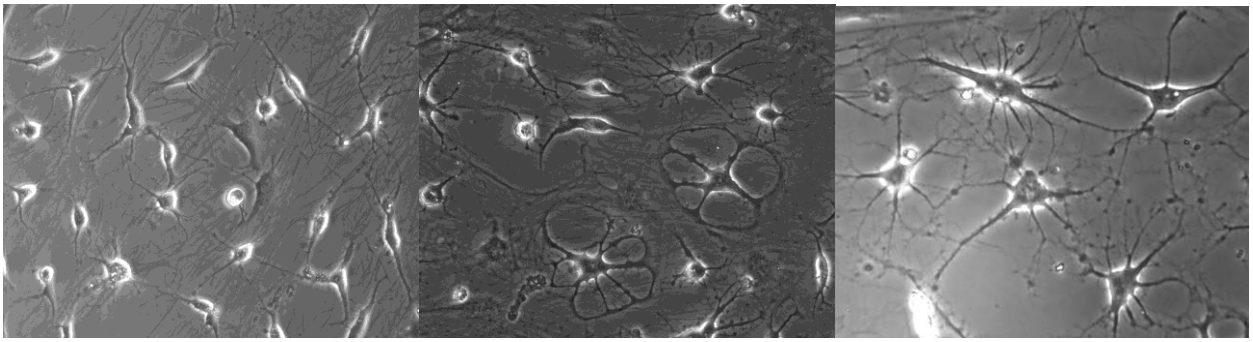


Figure 72: Clone 4 Figure 73: Clone 5 Figure 74:Clone 7

In these figures we can see the neurological appearance of clone 5 and clone 7 with the soma and well defined dendrites while on clone 4 we can see some cells with dendrites but most of them have a not well defined central body or fewer dendrites. We have also analyzed the results obtained for the resting potential on the different clones seeing that clone 4 had a resting potential quite higher than the others.

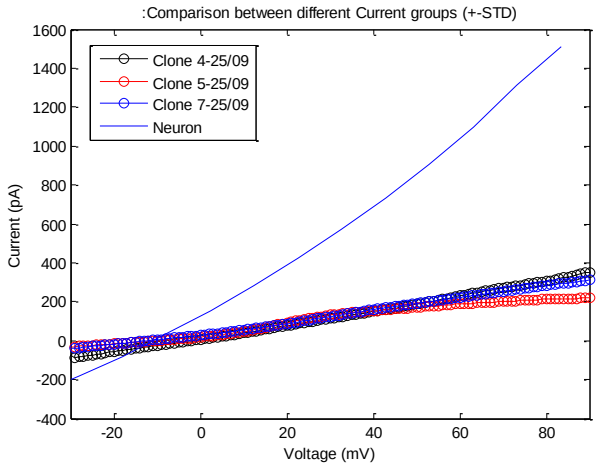


Figure 75: Voltage clamp comparison

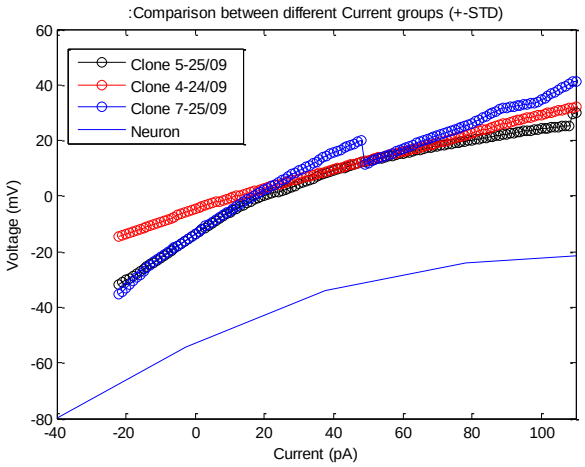


Figure 76: Current clamp comparison

On these figures we can see the behavior of the different clones on the same stage of differentiation, as we can notice there is not a huge difference between the different clones, but all the clones differs highly from the behavior of a neurons with an action potential. The major difference between the clones can be seen on current clamp protocol, where clone 5 and 7 has nearly the same behavior but clone 4 has a slightly different response at negative values of currents. As we can notice also from the figure this difference leads to different resting potential of the cells (the resting potential is the potential at 0 pA current) and so clone 5 and 7 has more neuronal characters respect clone 4, but the difference with the neuron behavior is very high so also these clones has a limited potential on their neuronal differentiation. On Voltage clamp protocol we can see the completely different behavior of the cell and the neuron, this is due to the action potential that is an active response to the change of voltage, while the cells has only a passive response and they act in an ohmic way.

Technical problems came from taking the cover slip and the detachment of the matrigel, this was solved during the weeks of analysis using a special tweezers which maintain the cover slip horizontal during the transport. This problem was seen also before on different colture of MSC, this issue coming from the matrigel properties represents a limit on the time of cultivation in vitro before the detachment, so experiments can be performed until a fixed day of differentiation, after this we cannot maintain the cells on the cover slips.

The last consideration is on the number of clones selected, the selection did during the differentiation was based on the morphological appearance of the cells but as we saw on clone 5 the differentiation for the clones can be achieved on different days. This premature selection may have eliminated some clones with a neuronal potential and clones with different type of MSC, in facts there are three types of different MSC that can be obtained from the dermis but we are not able to establish which type of MSC we are differentiating.

The research in MSC is fundamental for analysis on disease and drug screening but also for the regeneration of tissues and damaged cells on the patient, the future study on these cells will try to use more clones in order to select more MSC to differentiate, extend the signature of the clone selected and will try to improve the differentiation by using more cover slip on german glass.

5. References

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5. *DNA methylation in stem cell renewal and multipotency.* **M. Berdasco and M. Esteller.** 5, s.l. : M. Berdasco and M. Esteller,, 2011, Vol. 2.

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7. *Plasticity of Cultured Mesenchymal Stem Cells: Switch from Nestin-Positive to Excitable Neuron-Like Phenotype.* **Sabine Wislet-Gendebien, Grégory Hans, Pierre Leprince, Jean-Michel Rigo,.** 2005.

6. Appendix

Differentiation of Neuron like Cells from Skin Derived Precursors (SKPs) Cells isolated from Human Skin

MATERIALS

REAGENTS

2-Propanol (Sigma-Aldrich, cat. no. 650447-1L)

Antibodies for characterization (**Table 1**)

Ascorbic acid (Sigma, cat. no. A5960)

B27 supplement without vitamin A 50× (Life technologies, cat. no. 17504044)

BDNF (Life Technologies, cat. no. RP8642)

bFGF (Human) (Peprotech, cat. no. 100-18B-10UG)

CHIR-99021 (Sigma-Aldrich, cat. no. SML1046-5MG)

Chloroform (Sigma-Aldrich, cat. no. 288306)

CNTF (PeproTech, cat. no. 450-13)

DAPI (Sigma-Aldrich, cat. no. D9542)

DMEM HG (Life Technologies, cat. no. 41965039)

D-MEM/F12 (1:1) Dulbecco's modified eagle medium/Nutrient mixture F-12 (Life Technologies, cat. no. 11320074)

DMH1 (Quinoline) (Sigma-Aldrich, D8946-5MG)

DMSO Hybri-max sterile (5× 10 ml, Sigma, cat. no. D265X10ML)

DPBS without Ca and Mg (Euro Clone, cat. no. ECB4004L)

Fetal bovine serum (FBS) South American (Life Technologies, cat. no. 10270106)

GDNF (Life Technologies, cat. no. RP8602)

GlutaMAX I, 100× (Life Technologies, cat. No. 35050061)

HCl (J.T.Baker, cat. no. 6081)

IGF1 (Life Technologies, cat. no. PHG0071)

Matrigel matrix phenolred free (Corning, cat .no. 356237)

MEM non-essential amino acids solution (Life Technologies, cat. no. 11140035)

Mowiol 4-88 (Sigma-Aldrich, cat. no. 81381-250G)

N2 supplement 10× (Life Technologies, cat. no. 17502048)

Neurobasal medium (Life Technologies, cat. no. 21103049)

Paraformaldehyde prills, 95% (Sigma-Aldrich, cat. no. 441244-1KG)

Penicillin Streptomycin sol (P/S) (Life Technologies, cat. no. 15140122)

Poly-D-Lysine (Sigma-Aldrich, cat. no. A-003-E)

Power SYBR Green PCR Master Mix (Applied Biosystems, cat. no. 4367659)

Primers for RT-PCR (**Table 2**)

Purmorphamine (Sigma-Aldrich, SML0868-5MG)

Retinoic Acid (Sigma-Aldrich, cat. no. R2625-1G)

SB-431542 (Sigma-Aldrich, S4317-5MG)

SuperScript IV First Strand Synthesis System (Invitrogen, cat. no. 18091050)

TRI reagent (Sigma-Aldrich, cat. no. T9424-100ML)

Triton X-100 (Sigma-Aldrich, cat. no. T8787-100ML)

Tryple express (Life Technologies, cat. no. 12604021)

EQUIPMENT

15- and 50-ml conical tubes (Falcon, cat. nos. 352096 and 352070)

5-, 10- and 25-ml serological pipettes (Falcon, cat. nos. 4487, 4488 and 4489)

6-, 12- and 24-well plates (Falcon, cat. nos. F3046, F3043 and F3047)

60 mm Petri dishes (Falcon, cat.no. F3004)

7900 HT Fast Real-Time PCR System (Applied Biosystems)

Coverslips (12 mm Ø German Glass; Chemglass, cat. no. CLS-1760-012)

Falcon round-bottom tube (5 ml; cat. no. 352058)

Humidified tissue culture incubator (37°C, 5% CO_2; Heraeus, HERA cell)

Inverted phase-contrast microscope (Olympus, model no. CK30-F200)

Leica TCS SP5 confocal laser scanning microscope (Leica Microsystem)

Microcentrifuge tube (0.5 ml; Sarstedt, cat. no. 50-809-207)

Microcentrifuge tube (1.5 ml; Sarstedt, cat. no. 72.706)

NanoDrop 2000 Spectrophotometer (Thermo Scientific)

Polypropylene conical tubes (15 ml; Falcon, cat. no. F2096)

QuantStudio Real Time PCR (Applied Biosystems)

Sterilized Pasteur pipettes (Fisher Scientific, cat. no. Y10730)

T25 and T75 flasks (Falcon, cat. no. F3108 and F5001)

Water bath incubator

70 µm nylon cell strainer.

REAGENT SETUP

4% PFA (wt/vol) (100 ml) ***For 1 L of 4% Formaldehyde, add 800 mL of sterile de-ionized water to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60 °C. Take care that the solution does not boil. Add 40 g of paraformaldehyde powder to the heated de-ionized water. The powder will not immediately dissolve into solution. Slowly raise the pH by adding 1 N NaOH dropwise from a pipette until the solution clears. Once the paraformaldehyde is dissolved, the solution should be cooled and filtered. Adjust the volume of the solution to 1 L with de-ionized water. Recheck the pH, and adjust it with small amounts of dilute HCl to approximately 6.9. The solution can be aliquoted and frozen or stored at 2-8 °C for up to one month.***

Ascorbic acid (200 µg ml^{-1}) ***Dissolve 2 mg of ascorbic acid in 10 ml of DPBS. Aliquot and store at −20 °C for up to 3 months.***

BDNF, GDNF and IGF1 (10 µg ml^{-1}) ***Dissolve 10 µg of growth factor in 1 ml of sterilized distilled water, aliquot and store at −20 °C for up to 3 months.***

bFGF (10 µg ml^{-1}) ***Dissolve 10 µg of bFGF in 1 ml of sterilized PBS. Store at −20 °C for up to 3 months.***

Blocking buffer ***(1X PBS/5% normal serum/0.3% Triton™ X-100) To prepare 10 ml, add 0.5 ml normal serum from the same species as the secondary antibody (FBS) and mix well. While stirring, add 30 µl Triton X-100.***

DAPI (1mg/ml) ***Dissolve 5 mg of DAPI in 5 ml of DPBS. Aliquot and store at −20 °C for up to 3 months.***

Heparin (1mg ml^{-1}) ***Dissolve 10 mg of heparin in 10 ml of DMEM medium, aliquot and store at −80 °C for up to 3 months.***

Mowiol Add 4.8g Mowiol 4-88 and 12 g glycerol to a 100ml beaker. Mix well using a stir bar, meanwhile add 12ml ddH_2O and continue stirring for several hours at room temperature. Add 24 ml 0.2 M Tris HCl (pH 8.5), heat occasionally to 50°C in a water bath for approximately 10 minutes and continue stirring until Mowiol is dissolved. Once dissolved, centrifuge the solution at 2000 rpm for 15 minutes to clarify the solution. Carefully remove the supernatant; store aliquots at -20°C. NOTE. Once Mowiol is thawed, do not re-freeze again.

Purmorphamine (5 mM) ***Dissolve 13 mg of purmorphamine in 5 ml of DMSO, aliquot and store at −20 °C for up to 8 weeks.***

RA (10 mM) ***Dissolve 15 mg of RA in 5 ml of DMSO. Aliquot 50 µl into brown microtubes. The concentrated stock can be stored at −80 °C for up to 6 months. NOTE! RA is extremely sensitive to UV light, air and oxidizing agents, especially in solution.***

SB431542 (2 mM) ***Add 260 µl of DMSO to 5 mg of SB431542. Prepare aliquots and store them at −20 °C for up to 1 year.***

Y27632 (5 mM) ***In a sterile environment, add 6.24 ml of PBS to 10 mg of Y27632. Prepare 50-µl aliquots in 0.5 ml tubes, and store them at −20 °C for up to 1 year.***

EQUIPMENT SETUP

Matrigel coating **In a sterile hood, add cold (4 °C) DMEM/F12 to a 15-ml conical tube and keep it cold by placing it on ice. Remove Matrigel aliquots from the freezer and place on ice until it thaws. Gently pipette the Matrigel dissolved into cold DMEM-F12. Immediately add appropriate volume to Petri dishes or multi-well plates to cover completely the surface. Allow the Matrigel to set for at least 30 min into the incubator at 37°C. The Matrigel-coated plates can be stored at 4 °C for up to 3 weeks.**

PDL-coated coverslips **In a sterile hood, put one sterilized coverslip in each well of a 24-well plate. Add 75 µl of 0.1 mg ml^{-1} poly-D-lysine onto each coverslip. Incubate the plates at room temperature overnight. The next day, aspirate the PDL, wash approximate 10 times with sterile de-ionized water and let the coverslips dry at room temperature for ~30 min. Leave the plate open in the hood until completely dry. Cover the plates with Parafilm, wrap in foil, label with date and store at −20 °C for up to 4 weeks.**

ISOLATION AND CLONING OF SKPs

Isolation of hSKPs

Unless stated otherwise, perform all steps on ice (i.e., 4 °C).

Collect fresh human skin tissue samples and preserve samples Eurocollin's Solution on ice (4 °C) until further use.

Cut the human skin tissue samples in small pieces of 5–8 cm^2

Bring each sample in a 10 cm tissue culture dish filled with 25 mL of hSKP dissociation solution.

Cut each sample in 3–5 mm^2 pieces and float them epidermis side up

Incubate the samples overnight at 37°C in incubator overnight.

Remove the epidermis from the samples and discard it.

Cut the remaining dermis into smaller pieces (i.e., 1–2 mm^2) and transfer, including hSKP dissociation solution, to a 50 mL tube.

Incubate the samples for 20 min in a thermostated bath at 37 °C.

Incubate on ice (i.e., 4 °C) for 5 min or wait until all large pieces of dermal tissue are sedimented by gravitation

Gently discard the supernatant, but leave 2 mL and transfer to a 15 mL tube.

Start grinding the samples for about 2 min with a sterile 10 mL pipette and, when finished, add 8 mL of hSKP wash medium.

Spin for 10 s at 200 × *g* and room temperature to pellet down the large sample pieces.

Collect supernatant over a 70 µm cell strainer in a 50 mL tube

Spin the collected supernatant for 6 min at 200 × *g* and room temperature.

Remove the remaining medium and add 1 mL of hSKP growth medium.

Resuspend the cell pellet with a 1,000 µL pipette tip by triturating 50–100 times

Plate cells with 2 mL of hSKP growth medium in a 1-2 well of 6-wells plate

Feed the primary hSKP cultures twice a week by replenishing the medium with 2 mL of fresh hSKP feeding medium until sufficient cells are started to proliferate normally.

Primary hSKP will be visible from day 5–7 onwards

Dissociation of Primary hSKP

Unless stated otherwise, perform all steps at room temperature.

Remove the medium from the wells with sufficient confluency approximate about 80%.

Add 0.5 ml of TrypLE™ Express solution in each well.Incubate the plate at 37C incubator and wait for 3-5 minutes. Scrap cells by using P200 tilted tip or by scraper.

Neutralize enzyme activity by adding 1ml of the hSKP medium and pipette into 15ml Falcon tube.

Add 3-4 ml of fresh medium into the tube and centrifuge at 1500 RPM for 5'.

Remove the supernatant and resuspend the cells hSKP medium.

Use trypan blue solution to count the cells and estimate the cell viability.

To create hSKP cultures, plate cells in hSKP growth medium in T75 Flask.

Change medium every 2–3 days with hSKP growth medium until 90 % of confluency is reached.

Cloning of the hSKPs

The confluent adherent cells were harvested and single-cell colonies were generated by serial dilution.

The cells were serially diluted into hSKP medium containing 20% FBS and seeded in 96-well plastic culture plates. Each well contained 200 µl hSKPs medium was assessed microscopically.

Wells containing more than one cell were discarded, and only wells containing a single cell were used.

A cell population that produced cell clones of more than 20 cells was considered to be a colony and was passaged and expanded as usual.

DIFFERENTIATION PROTOCOL

Neuronal Induction 1 of SKPs ● TIMING 6 d (days 0-6)

Culture the SKPs on a Matrigel-coated (1:100 dilution with D-MEM/F:12) glass or plastic coverslips in 24 well plates with SKPs Proliferation medium. Incubate the cells at 37°C, 5% CO_2 for next 24 hrs.

The next day replace the Proliferation medium with Induction 1 medium and change this medium every other day for next 6 days.

Neuronal Induction 2 of SKPs ● TIMING 6 d (days 7-12)

On Day 7, replace the Induction 1 medium medium with Induction 2 medium and change this medium every other day for next 6 days.

Observe the change in morphology of differentiating cells. Cells change their shape which is similar to the neurons wihin this time points

Differentiation and maturation neurons like cells ● TIMING 20-40 d (days 13-52)

In a 24-well plate, place PDL-coated German glass coverslips and coat with Matrigel (1:50). Aspirate the entire medium and change with either Old Differentiation medium or New differentiation medium.

Measurement of electrophysiology and other analytical tests can be performed at different time points.

Downstream assays ● TIMING variable

Immunostaining analysis

Remove the culturing medium and rinse the coverslips-containing cells three times in 1X DPBS for 5 minutes each, directly inside the plate. CRITICAL STEP! During the washing, put DPBS carefully over the cells in order to do not destroy them and use a low-pressure vacuum to aspirate the DPBS.

Remove DPBS, then cover cells to a depth of 2–3 mm with 4% paraformaldehyde and allow cells to fix for 15 minutes at room temperature. NOTE! Formaldehyde is toxic, use only in a fume hood.

Aspirate fixative and rinse other three times in 1X DPBS for 5 min each.

Block specimen in blocking buffer for 60 minutes. NOTE! All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

PAUSE POINT. Cells can be stored at 4°C in blocking buffer and covered with Parafilm for up to 1-2 weeks.

While blocking, prepare primary antibody by diluting as indicated on **Table 1** on blocking buffer.

Aspirate blocking buffer, place the coverslip into a humid chamber, apply 50 µl diluted primary antibody and incubate overnight at 4°C. CRITICAL STEP! After placing one coverslip into the humid chamber, add a drop of blocking solution to prevent the coverslip to dry out and aspire the drop before the addition of the primary diluted antibody.

Rinse three times in 1X PBS for 5 min each.

Dilute the secondary antibodies specific to the primary one at 1:1000 in blocking solution. Add 50 µl of secondary solution to each well and then incubate for 1-2 hours at room temperature in the dark.

Add DAPI for 15-30 minutes before the end of the secondary staining.

Rinse three times in 1X DPBS for 5 min each.

Aspirate the DPBS and let the coverslip to be dried out before using Mowiol to mount it. For best results, allow mountant to stay overnight at room temperature. For long-term storage, store slides flat at 4°C protected from light and sealing the coverslips borders with nail color.

RT-PCR analysis

RNA preparation

Collect the cell lysate at different intervals of the differentiation from motor neurons and Schwann cells, by using 1 ml of TRI reagent.

Pass the lysate through a pipette several times.

Incubate the homogenized samples for 5-10 minutes at room temperature.

Add 0.2 ml of chloroform per ml of TRI, shake tubes vigorously by hand for 15 seconds and incubate them at RT for 5 minutes.

Centrifuge the samples at 13,000 rpm for 15 minutes at 2 to 8°C.

Transfer the colourless upper aqueous phase to a fresh tube.

Precipitate the RNA from the aqueous phase by mixing with 0.5 ml 2-Propanol. Incubate samples at RT for 10 minutes and centrifuge at 13,000g for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

Remove the supernatant. Wash the RNA pellet once with at least 1 ml of 75% ethanol (prepared using RNase-free water). Mix the sample by vortexing and centrifuge at no more than 12,000g for 5 minutes at 2 to 8°C.

Briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). NOTE. Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility.

Dissolve RNA in RNase-free water by passing the solution a few times through a pipette tip.

Measure the concentration of RNA (ng/µl) by using NanoDrop.

RT reaction

Perform the RT reaction using SuperScript IV First-Strand Synthesis System protocol. The procedure should be applied by following the manufacturer's instruction.

Real Time PCR reaction

Set up the following components according to manufacturer's instruction in which a single reaction in 384-well plate should contain 1 µl of cDNA, 2 µl of RNase-free water, 1 µl of primer mixture and 4 µl of Power SYBR Green PCR Master Mix.

Perform PCR with 40 cycles of denaturation: 15 seconds at 95°C; annealing: 1 minute at 62°C; and extension: 15 seconds at 95°C.