POLITECNICO DI MILANO

Master of Science in Biomedical Engineering

Department of Electronics, Information and Bioengineering

Validation and testing of a culture chamber for long-term neurophysiological recordings with multielectrode arrays

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I dedicate this work to my mom, Ana Lúcia, that even far away always stood by my side and gave me the strength I needed for this journey. Muito obrigada mãe. I also dedicate this work to my father, Daniel, I know he would be proud of me for arriving here.

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Abstract

Introduction

One of the main purposes of neuroscience is to understand the link between the functional connectivity-map of neuronal circuits and their physiological or pathological functions.

This relationship is mostly investigated through the study of *in vitro* neuron networks. A method for *in vitro* studies is the use of dissociated neuron cultures from embryonic mouse. In such manner, the cells grow and develop neuron networks *in vitro* similar to the ones *in vivo* if cultivated in a controlled environment, similar to the physiological one, in order to sustain their viability.

The study of in vitro neuronal networks needs suitable technologies to interface with the model itself [10]. For this reason, the method of investigation needs to be carefully chosen in order not to cause disturbances on the cell culture and not to interfere with its growth. Currently, the gold standard for measuring in vitro extracellular electrical activity from neuronal networks are Multi-electrode arrays (MEA). They consist of a device where multiple microelectrodes (few tens of micrometers) are arranged onto a transparent glass or on metal thin film substrate through which it is possible to record neural signals.

Therefore, cell lines or primary cell preparations can be cultivated directly on the MEA constituting a powerful experimental tool. Neurons are then directly connected to the microelectrodes by a neuro-electronic junction, and their extracellular signals can be recorded simultaneously and in a noninvasive way.

Extracellular potentials reveal changes in current or potentials of neuron group cells enclosing a microelectrode. Amplitudes of extracellular action potentials can vary between 30 to 400 µV peak-to-peak [13] and the frequency content ranges from 300 Hz to 5 kHz.

MEA technology has been proven valuable to characterize the neuron networks dynam-

ics. Long-term neuron electrophysiology studies using multi-electrode arrays are only feasible if the cells plated on MEA chip (culture dish) are maintained inside an incubator which provides the correct environmental conditions for they growth such as sterility, appropriate temperature, pH (5 % relative carbon dioxide concentration), osmotic pressure, and high relative humidity to prevent evaporation of cell culture medium.

Mostly, every time cell recordings are necessary the culture is taken out of the incubator to be placed on a standard experimental setup to have their signals recorded by the data acquisition system, undergoing abrupt environmental changes that can alter neurons survival and electrophysiological activity.

Many applications would benefit from uninterrupted long-term monitoring of neuronal activity such as to investigate neuronal plasticity; network development, especially for the following of human pluripotent stem cells neuron differentiation and maturation; drug screening, making possible to study the onset of a pathological mechanism.

The main objective of this thesis was to validate long-term and parallel electrophysiological recordings on an environmental controlled experimental chamber based on Multielectrode arrays (MEA) [14]. In order to achieve this goal, this work was structured in mainly three parts: (i) improvement of anti-water condensation system of the chamber applying an Indium Tin Oxide heater (ITO); (ii) validation of the updated environmental control system; (iii) validation of the device performing long-term acquisitions using hippocampal neuron cells. This work was developed at the Experimental Imaging Center (CIS) Laboratory of San Raffaele Hospital.

Material and Methods

The culture chamber used in this work was previously developed by Giulia Regalia [13] and later improved in previous works [17] [20]. This chamber is integrated with a control unit, a commercial heating water bath, a humidifier, a gas cylinder, anti-condensation solution, environmental sensors, and a data acquisition system.

Temperature is regulated through the heating of the water that circulates in a cavity between chamber inner and outer boxes using a commercial pump; warmed air from a temperature-controlled humidifier flowing into it; heating of the chamber surface in order to avoid water condensation using an ITO heater.

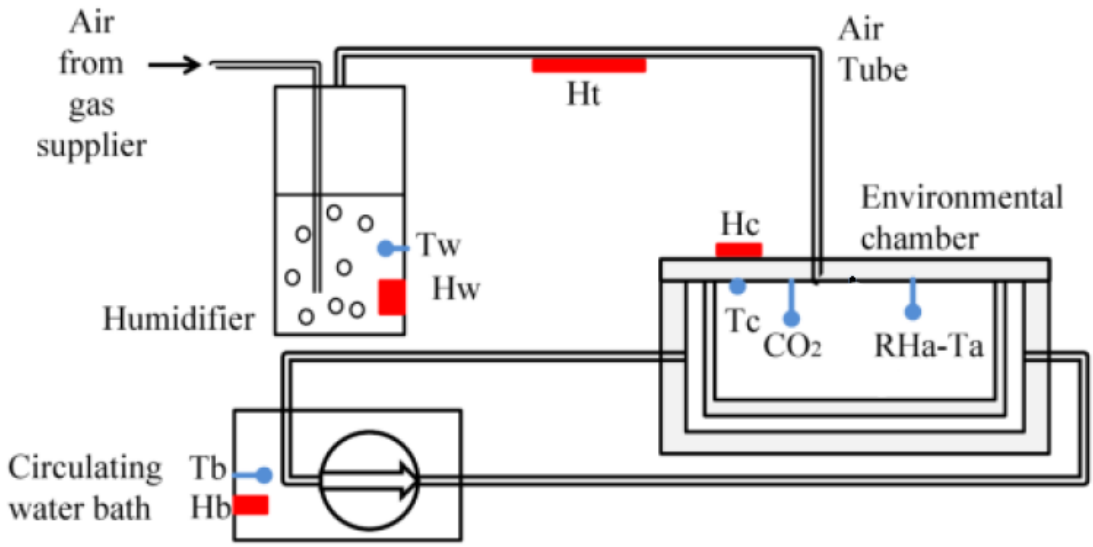

Figure 1: Overview of environmental control

Relative humidity (RH) is controlled by a humidifying bottle and a heating module. A mixture of gas (5% CO_2, 20% O_2, 75% N_2) flows into a humidifying bottle and also provides the ideal carbon dioxide concentration to the chamber. The environmental parameters can be monitored in real time. In the first part of this work, exploratory tests were performed to characterize the Indium Tin Oxide heater (ITO) and evaluate the feasibility of using it in our application.

In the second part, a validation of the environmental control was performed by means of a 2-hours parallel recording inside the chamber was performed using two MEA chips filled with phosphate buffered saline (PBS) solution. The aim of this acquisition was to assure the stability of environmental parameters (temperature, relative humidity, and CO_2) and to verify the absence of water condensation.

Lastly, a validation of the environmental control chamber with hippocampal neuronal cultures was performed. MEA chips were placed inside the environmental chamber and maintained in there during 4 days for recordings. Additionally, on the last day of recordings a pharmacological experiment was conducted administering the neurotoxin TTX (Tetrodotoxin) to one of the MEA-chips.

Results

ITO heater properties observed were non-uniform temperature along the surface, resistance values depending on bus bars and lead wires configuration and also on the presence of openings or cracks [7].

Experiments conducted with the ITO panel applied to the surface of the chamber, demonstrated that he heater was able to reach temperatures in the desired range (around 37 °C) with a short response time and it was successful in retaining heat along the duration of the experiments with no water condensation.

In respect to the acquisition using PBS, some limitations of our system were identified: instability, presence of noise (fake spikes and electric hum 50 Hz/100 Hz) and difficulty to raise relative humidity values. Even with these drawbacks, the environmental control obtained showed to be acceptable.

Regarding the validation of chamber using hippocampal neurons, environmental parameters were maintained within physiological ranges. Although relative humidity in our system was not the appropriate one, a FEP membrane [1] used to cover the cell cultures helped to avoid evaporation of cell culture medium Figure 2.

Data obtained with this long-term acquisition was useful to follow spike activity over time, extract network parameters, and assess their frequency content. The Tetrodotoxin (TTX) application to one of our MEA-chips confirmed existence of spikes, since it blocks neuron spiking activity Figure 2.

Conclusion

The results obtained demonstrated the capability of the system to record long-term activity from *in vitro* neuronal cultures, thanks to the control of environmental conditions that were kept within physiological values throughout the experiments. This device has potential for being a powerful instrument for the study of hippocampal neurons, neuron differentiated stem cells and ultimately to help understand and model disease mechanisms.

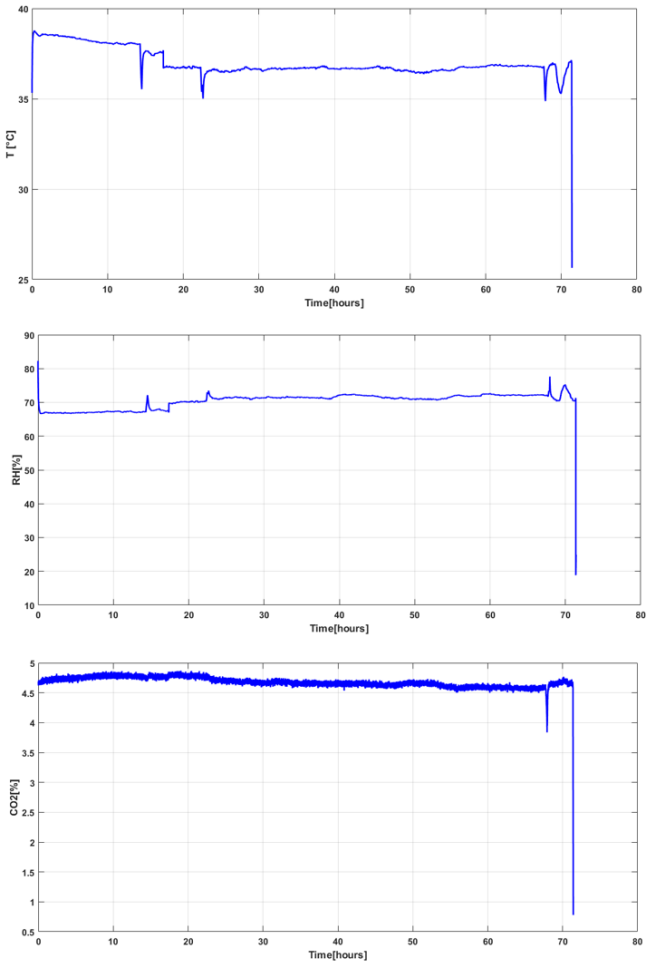

Figure 2: Performance of environmental chamber: temperature, relative humidity and CO2 concentration values

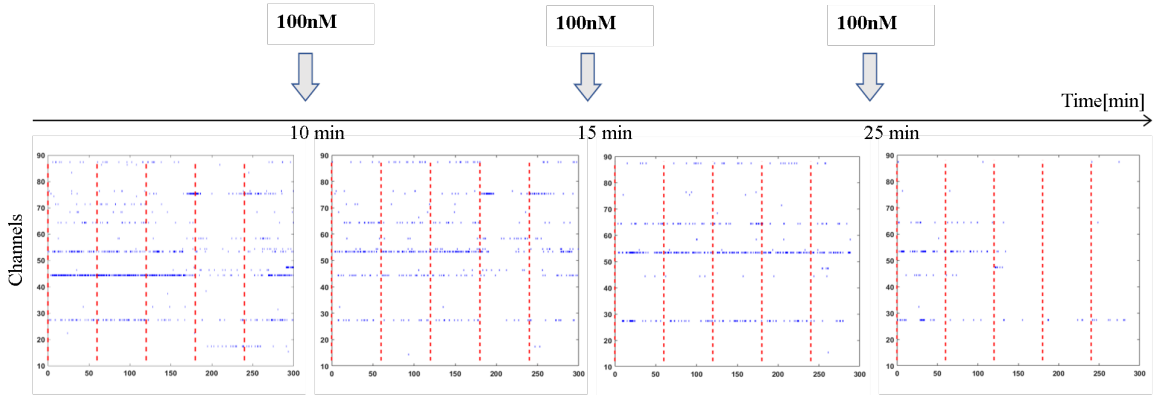

Figure 3: Raster plots obtained before and after events of 100 nM Tetrodotoxin (TTX) administration, indicated in the figure

Sommario

Introduzione

Uno degli scopi principali della neuroscienza è capire il legame tra la connettività funzionale dei circuiti neuronali e le loro funzioni fisiologiche o patologiche [10].

Questa relazione viene principalmente studiata attraverso la coltura *in vitro* di reti neuronali. Uno dei metodi di studio *in vitro* è l'uso di colture di neuroni dissociati da embrioni di topo. Se coltivate in un ambiente controllato, simile a quello fisiologico, le cellule crescono e sviluppano in vitro reti neuronali simili a quelle *in vivo*.

Lo studio *in vitro* delle reti neuronali richiede tecnologie adeguate per interfacciarsi con il modello stesso [10]. Per questo motivo il metodo di indagine deve essere scelto con cura per non causare disturbi alla coltura cellulare e non interferire con la sua crescita. Attualmente lo standard di riferimento per la misurazione dell'attività elettrica extracellulare di reti neuronali in vitro è rappresentato dai sistemi Multi-electrode arrays (MEA). Questa tecnologia è costituita da un dispositivo in cui più microelettrodi (lunghi poche decine di micrometri) sono disposti su un vetro trasparente o su un sottile film metallico attraverso il quale è possibile registrare segnali neuronali.

Pertanto, le linee cellulari o le colture di cellule primarie possono essere coltivate direttamente sul MEA che diventa così un potente strumento sperimentale. I neuroni sono quindi direttamente collegati ai microelettrodi per mezzo di giunzioni neuro-elettroniche e i loro segnali extracellulari possono essere registrati simultaneamente e in modo non invasivo.

I potenziali extracellulari registrati indicano cambiamenti nella corrente o nei potenziali di membrana di gruppi di cellule neuronali che sono in contatto con un microelettrodo. Le ampiezze dei potenziali di azione extracellulari possono variare da 30 a 400 μV da picco a picco [13] e il contenuto in frequenza varia da 300 Hz a 5 kHz.

La tecnologia MEA si è dimostrata preziosa per caratterizzare le dinamiche delle reti neuronali. Gli studi di elettrofisiologia sui neuroni a durata prolungata che utilizzano sistemi di registrazione a più elettrodi, sono possibili solo se le cellule piastrate sui chip MEA sono mantenute all'interno di un incubatore che fornisce le condizioni ambientali corrette per la loro crescita come sterilità, temperatura appropriata (37 °C), pH (concentrazione relativa di anidride carbonica del 5 %), pressione osmotica e elevata umidità relativa per impedire l'evaporazione del terreno di coltura.

Inoltre, ogni volta che le registrazioni cellulari sono necessarie, la coltura viene estratta dall'incubatore per essere collocata su un setup sperimentale standard subendo improvvisi cambiamenti ambientali che possono alterare la sopravvivenza dei neuroni e l'attività elettrofisiologica.

Molte applicazioni trarrebbero beneficio da un monitoraggio ininterrotto e a lungo termine dell'attività neuronale, come ad esempio gli studi sulla plasticità neuronale, sullo sviluppo delle reti neuronali, in particolare per quanto riguarda il differenziamento e la maturazione neuronale delle cellule staminali pluripotenti umane, e gli screening farmacologici, rendendo anche possibile studiare l'insorgenza di un meccanismo patologico.

L'obiettivo principale di questa tesi è di convalidare le registrazioni elettrofisiologiche a lungo termine e in contemporanea su più chip MEA all'interno di una camera in cui le condizioni ambientali sono controllate [14]. Per raggiungere questo obiettivo, questo lavoro è stato strutturato principalmente in tre parti: (i) miglioramento del sistema che previene la condensazione dell'acqua nella camera applicando un pannello riscaldante di Ossido di Indio-Stagno (ITO); (ii) convalida delle implementazioni apportate al sistema di controllo ambientale; (iii) validazione del dispositivo che esegue acquisizioni a lungo termine usando cellule di neuroni ippocampali. Questo lavoro è stato sviluppato presso il laboratorio del Centro di Imaging Sperimentale (CIS) dell'Ospedale San Raffaele.

Materiali e Metodi

La camera di coltura utilizzata in questo lavoro è stata precedentemente sviluppata da Giulia Regalia [13] e in seguito migliorata nei lavori successivi [17] [20]. Questa camera è stata integrata con un'unità di controllo, un bagnetto commerciale riscaldante ad acqua, un umidificatore, una bombola a gas, una soluzione anticondensa, sensori ambientali e un sistema di

acquisizione dati.

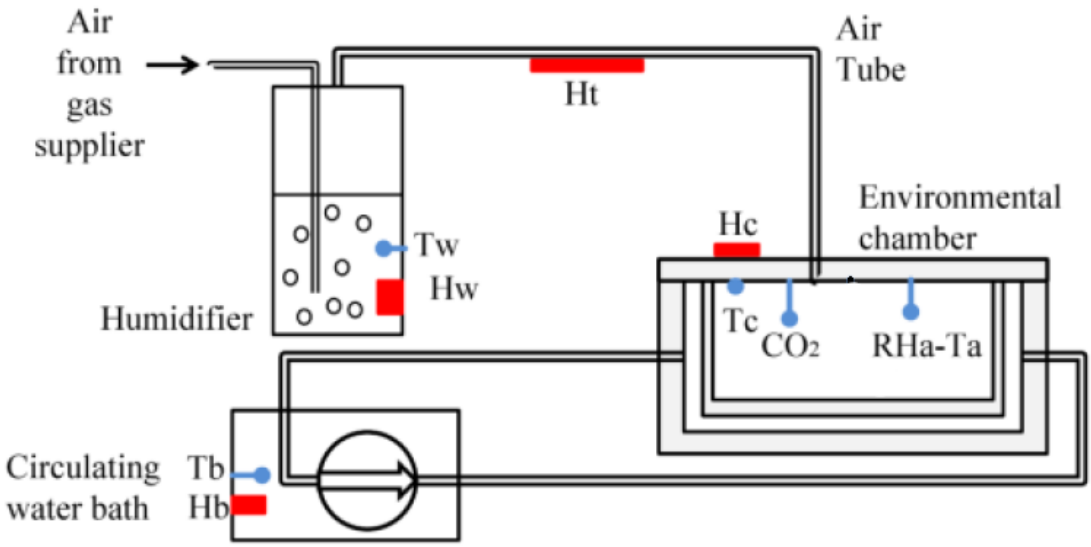

Figure 1: Sistema di controllo dei parametri ambientali

La temperatura viene regolata attraverso tre sistemi diversi: (i) il riscaldamento dell'acqua che circola in una cavità tra la parte interna ed esterna della camera grazie ad una pompa commerciale; (ii) l'aria riscaldata che da un umidificatore a temperatura controllata fluisce nella camera; (iii) il riscaldamento della superficie della camera mediante un pannello riscaldante ITO al fine di evitare la condensazione dell'acqua.

L'umidità relativa (RH) viene controllata da una bottiglia umidificante, cioè da una bottiglia contenente acqua in cui viene fatta gorgogliare una miscela di gas, e da un modulo di riscaldamento. La miscela di gas (5% di CO_2, 20% di O_2, 75% di N_2) che gorgoglia nell'acqua della bottiglia umidificante fornisce anche la concentrazione di CO_2 ideale per la camera. I parametri ambientali possono essere monitorati in tempo reale.

Nella prima parte di questo lavoro sono stati eseguiti test sperimentali per caratterizzare il pannello riscaldante ITO e valutarne l'eventuale utilizzo nella nostra applicazione. Nella seconda parte è stata eseguita una convalida del controllo ambientale mediante una registrazione di due ore all'interno della camera utilizzando in contemporanea due chip MEA riempiti con soluzione di tampone fosfato salino (PBS). Lo scopo di questa acquisizione è assicurare la stabilità dei parametri ambientali (temperatura, umidità relativa e CO_2) e verificare l'assenza di condensa.

Infine, è stata eseguita una convalida della camera di controllo ambientale con colture di neuroni ippocampali. I chip MEA sono stati collocati e mantenuti all'interno della camera ambientale per 4 giorni allo scopo di effettuare registrazioni a lungo termine. Inoltre, durante l'ultimo giorno di registrazione è stato condotto un esperimento farmacologico somministrando la neurotossina TTX (Tetrodotossina) alla coltura neuronale di un solo chip MEA.

Risultati

Il pannello riscaldante ITO ha mostrato temperature non uniformi lungo la sua superficie e valori di resistenza dipendenti dalla configurazione delle barre di distribuzione e dei fili di piombo e anche dalla presenza di aperture o crepe [7].

Gli esperimenti condotti con il pannello ITO applicato alla superficie della camera hanno dimostrato che il suo riscaldamento è in grado di raggiungere temperature nell'intervallo desiderato (intorno ai 37°C) con un breve tempo di risposta e a mantenere il calore costante lungo la durata degli esperimenti senza condensa d'acqua.

Per quanto riguarda le registrazioni dei chip MEA riempiti con PBS, il nostro sistema ha mostrato alcune limitazioni: instabilità, presenza di rumore (falsi spikes e rumore elettrico di rete a 50Hz/100Hz) e difficoltà nell'aumentare i valori di umidità relativa. Nonostante questi inconvenienti, il controllo ambientale ottenuto si è comunque rivelato accettabile.

Per quanto riguarda la convalida della camera utilizzando i neuroni ippocampali, i parametri ambientali sono stati mantenuti entro gli intervalli fisiologici. Sebbene l'umidità relativa nel nostro sistema non fosse quella appropriata, una membrana FEP [1] utilizzata per coprire le colture cellulari ha contribuito ad evitare l'evaporazione del mezzo di coltura cellulare. I dati ottenuti con queste registrazioni a lungo termine sono stati utili per seguire l'attività degli spikes nel tempo, estrarre i parametri di rete e valutare il loro contenuto in frequenza. La somministrazione di TTX a uno dei nostri chip MEA ha confermato la presenza specifica di spikes in quanto l'attività neuronale viene bloccata in presenza della tossina.

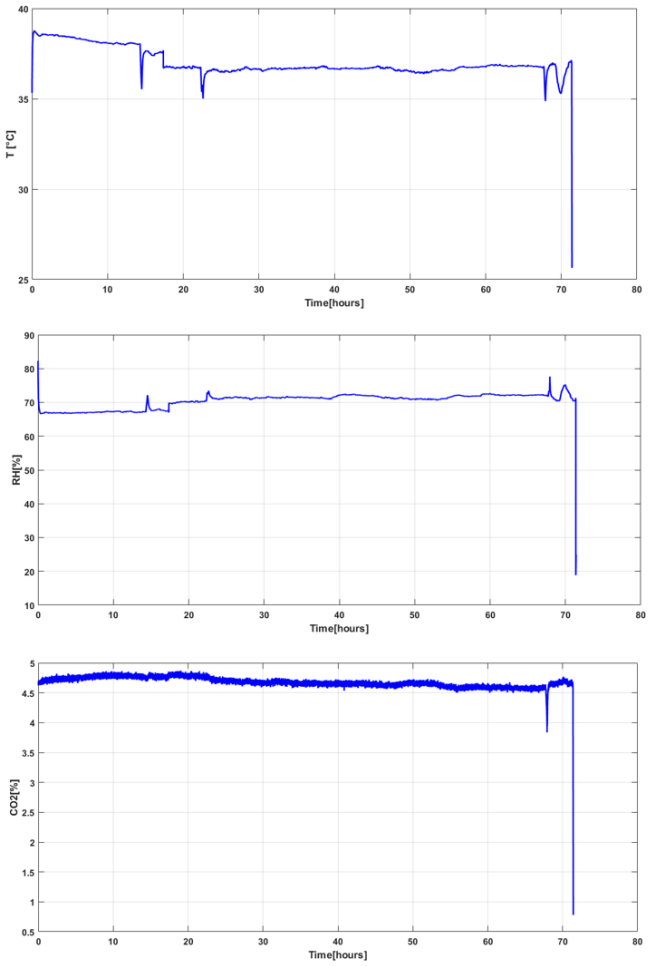

Figure 2: Performance della camera ambientale: temperatura, umidità relativa e concentrazione di CO_2 concentration values

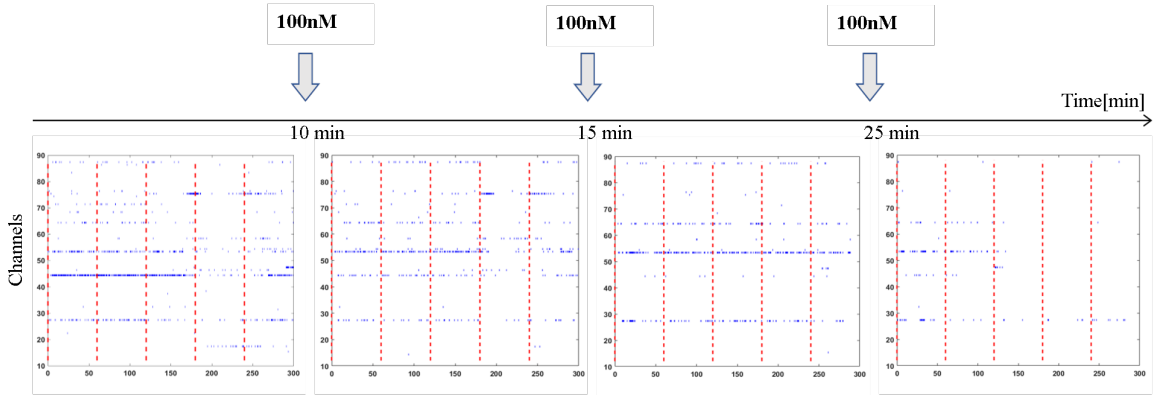

Figure 3: Raster plots ottenuti prima e dopo gli eventi di somministrazione di 100nM Tetrodotoxin (TTX)

Conclusioni

I risultati ottenuti hanno dimostrato la capacità del nostro sistema di registrare a lungo termine l'attività in vitro delle colture neuronali, grazie al controllo delle condizioni ambientali che sono state mantenute entro i valori fisiologici durante gli esperimenti.

Questo dispositivo ha il potenziale per essere un ottimo strumento per lo studio dei neuroni ippocampali, del differenziamento e della maturazione neuronale delle cellule staminali pluripotenti umane e, infine, per aiutare a comprendere i meccanismi di malattie neuronali utilizzando modelli cellulari.

Chapter 1

Introduction

Neurons are cells that constitute the basic units of the nervous system. These cells are able to generate and transmit electrical signals due to the presence of voltage-gated ionic channels in the cell membrane. Structure of a neuron can be divided into soma – cell body, where the nucleus of a neuron is located, dendrites and axons. Dendrites are considered neuron inputs because they receive electrochemical signals that can be either excitatory or inhibitory. Axons allow the propagation of these signals along the cells including over long distances. The termination of the axon functions as an output, where electrical signals provoke the release of chemical signals, neurotransmitters, into the synaptic cleft – allowing neuronal communication.

Neurotransmitters bind to the receptors located in the membrane of the postsynaptic neuron, which allows ions to flow in or out of the cell promoting electrical changes. The membrane at rest is negatively charged and the voltage difference generated by the movement of ions can depolarize or hyperpolarize its potential. These signals are called excitatory postsynaptic potential (EPSP) or inhibitory postsynaptic potential (IPSP), respectively.

When the summation of all the neuron inputs – synaptic potentials, equals or exceeds a certain threshold (-45 mV) [6] an action potential occurs [Figure 1.2].

An action potential is characterized by three phases [6]: a rapid depolarization of the membrane, a repolarization, towards the resting state and the third phase is a recovering of the resting potential after a hyperpolarization.

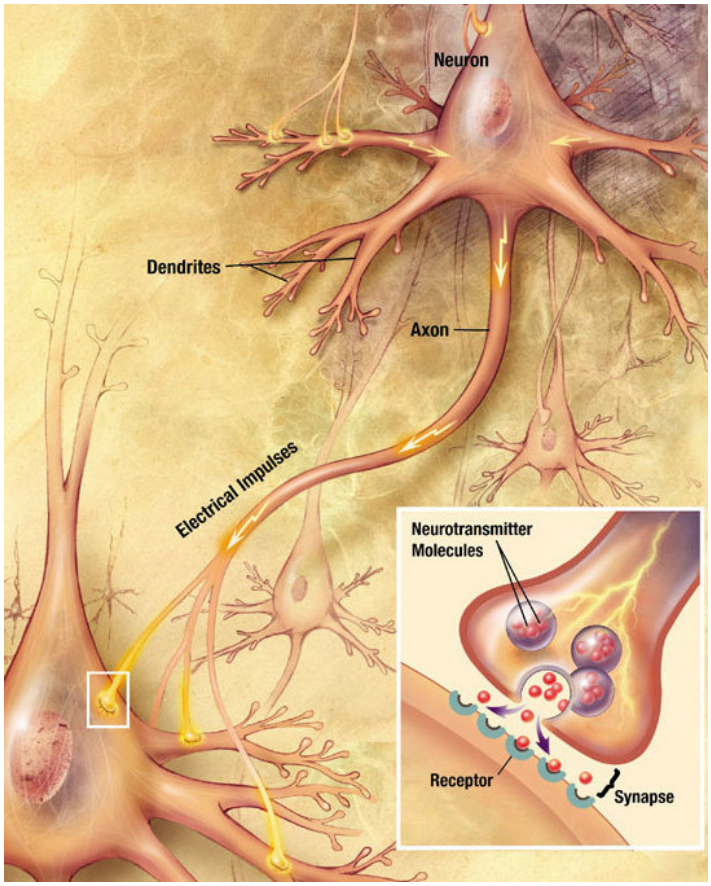

Figure 1.1: Neuronal communication

In the first phase, sodium (Na+) voltage-gated channels triggered by depolarization, activate through a conformational change in the channel that allows sodium to enter the cell, higher the influx of Na+ ions, more sodium channels open which contributes even more to the depolarization. When the depolarization of the membrane potential reaches the threshold there will be an opening of all Na+ channels, inducing a fast depolarization phase of the action potential. Once the threshold is crossed the full action potential is generated otherwise there is no action potential.

If Na+ ions for some reason cannot enter the cell, for example using the neurotoxin – tetrodotoxin (TTX), that blocks the Na+ channels, this culminates in a non-generation of the action potential.

In the second phase, Na+ channels inactivate, switching conformation and the potassium (K+) voltage-sensitive channels open, resulting in an influx of K+ ions out of the cell in order to restore the resting potential. Those channels are slowly activated and open after a higher level of depolarization.

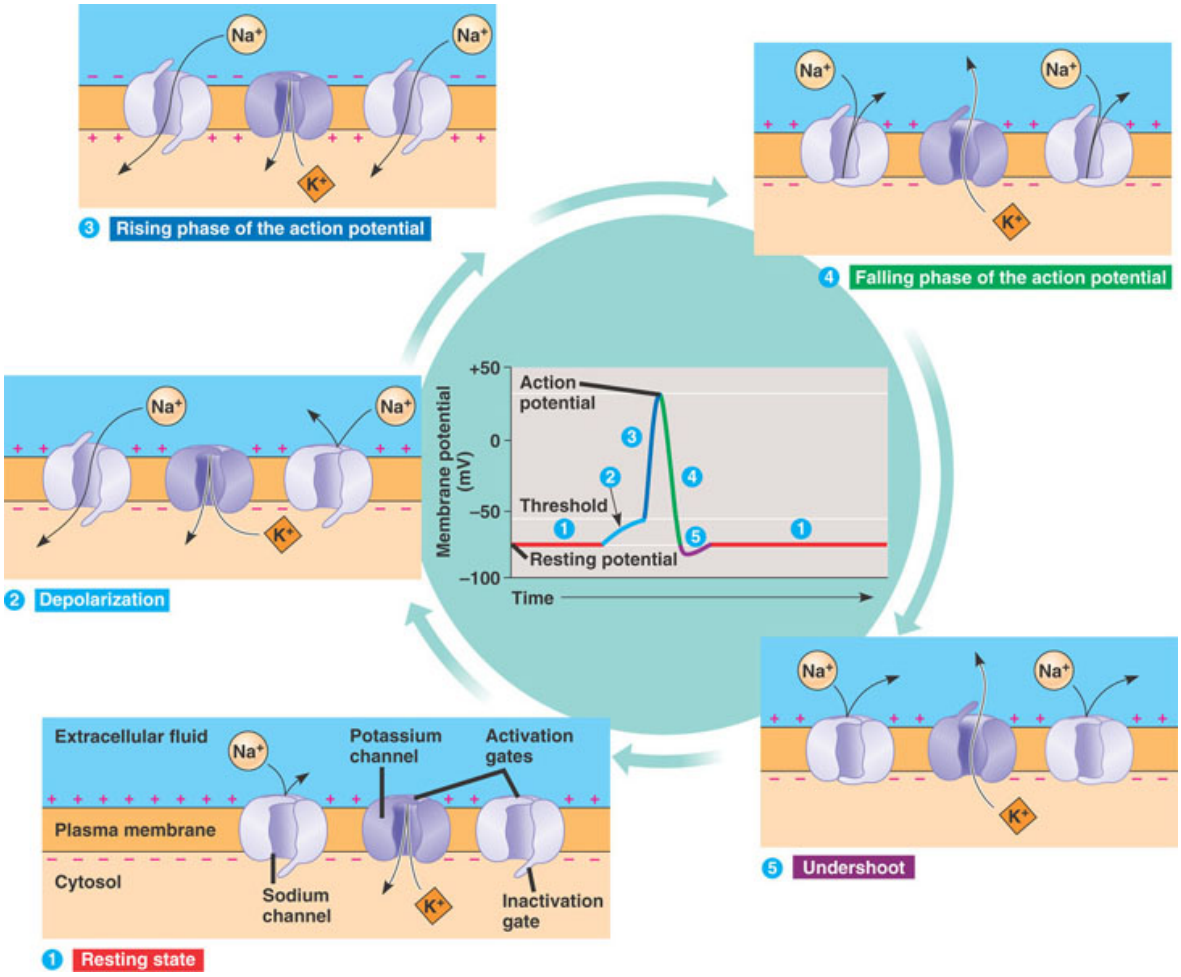

Figure 1.2: Action potential and its mechanism

In the third phase, K+ channels are still open, promoting a hyperpolarization of the membrane; as it hyperpolarizes the K+ channels deactivate, driving the potential back to rest.

The resting state is maintained by the Na+/K+ pump with ATP consumption, that exchanges 3 Na+ molecules from inside of the cell with 2 K+ from outside of the cell.

Action potentials, also referred to as spikes, are essential for the neural communication, they are able to propagate along the axon transmitting signals for other neurons and their frequency encodes information.

1.1 Neuronal networks

Information can flow among neurons in our brain through countless ways. Furthermore, it is remarkable how the brain manages to perform quite complex tasks such as perception, movement, and memory, starting from a vast number of simple processing units.

This is only possible thanks to the existence of neuronal networks. A single neuron on its own cannot fulfill its function if it is not connected to other neurons. Neuronal circuits can be quite simple, with only a few neurons, or they can comprise more complex neuronal networks. The nervous system function depends on that connectivity to process information that travels, within, to or from it.

Although knowledge about single neurons and their one-to-one interaction is well consolidated, not enough is known with respect to the structure and function of neuronal networks in the human cerebral cortex [15].

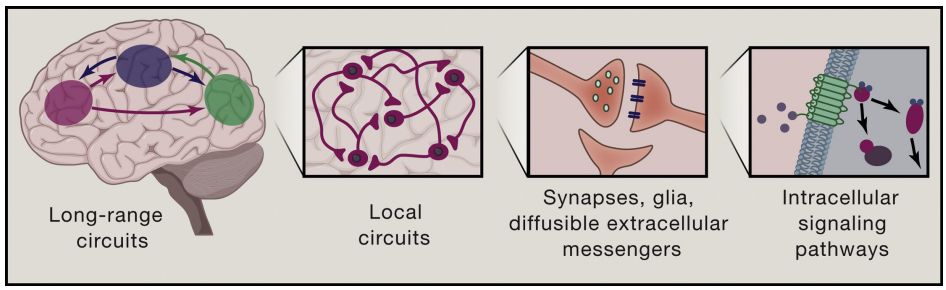

Figure 1.3: Brain multiscale [15]

1.2 Neuron electrophysiology studies

One of the main purposes of neuroscience is to understand the link between the functional connectivity-map of neuronal circuits and their physiological or pathological functions [10].

Hence, neuron electrophysiology – the study of electrical properties of neuronal cells is extremely important in order to comprehend not only how the brain works but beyond that, to elucidate mechanisms of neurological disorders, to learn how they affect electrical activity patterns, also the potential applications or effects of pharmaceutical drugs.

In order to investigate that, *in vitro* and/or *in vivo* studies are performed. Both methods have their limitations. For *in vivo* studies, the acquired information can be affected by the surrounded areas that are not under control. While *in vitro* techniques demand to be designed to exhibit a comparable behavior with respect to *in vivo* models.

In vitro neuron networks can be obtained through the extraction of a neural tissue slice; in this way the network has already developed and it is treated to be maintained alive *in vitro* at least for the time necessary for the realization of experiments. Main issues with this approach are the disturbances caused by dead neurons at the boundaries and the 'under sampling' because the slices seized do not represent the same network complexity.

A second method for *in vitro* studies is the use of dissociated neuron cultures from embryonic mouse. In such manner, the cells grow and develop neuron networks *in vitro* similar to the ones *in vivo* if cultivated in a suitable and controlled environment to sustain their viability.

The cells require an environment that resembles the physiological one, which assures proper nutrients, adequate temperature and pH.

1.3 Multi-electrode arrays (MEA)

The study of *in vitro* neuronal networks needs suitable technologies to interface with the model itself. For this reason, the method of investigation needs to be carefully chosen in order not to cause disturbances on the cell culture and not to interfere with its growth.

Nowadays, the gold standard for measuring *in vitro* extracellular electrical activity from neuronal networks are Multi-electrode arrays (MEA). They consist of a device where multiple microelectrodes (few tens of micrometers) are arranged onto a transparent glass or on metal thin film substrate through which it is possible to record neural signals.

Different number of electrodes and types of arrays can be used depending on each application. The electrodes are biocompatible, usually made of titanium nitride (TiN), with a low impedance that enhances signal to noise ratio. The electrode leads are fabricated in titanium (Ti) or indium tin oxide (ITO) and their surface is coated with biocompatible insulators such as silicon nitride. These insulators, coated with molecules that promote cell adhesion such as polylysine favor neuronal coupling to the device surface.

Therefore, cell lines or primary cell preparations can be cultivated directly on the MEA constituting a powerful experimental tool. Cells are plated internal to the glass ring in the core of chip, which encloses the culture medium [Figure 1.4].

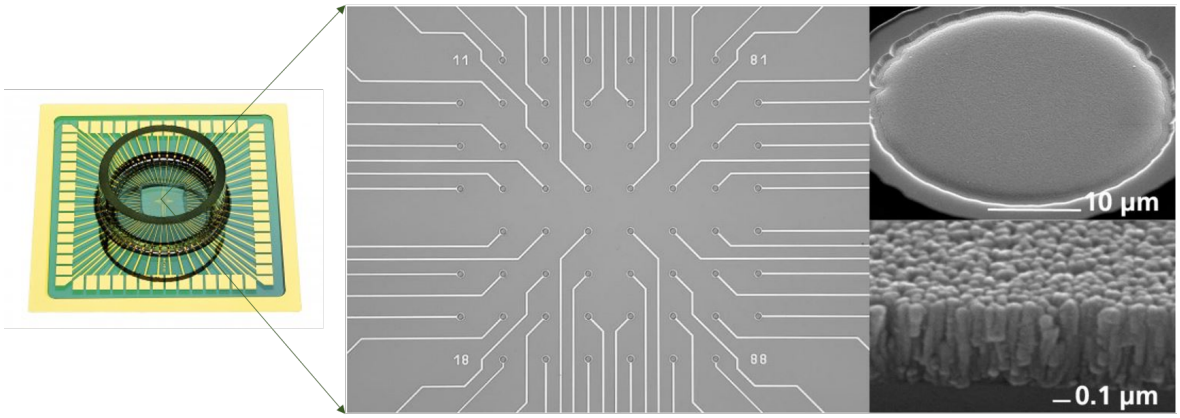

Figure 1.4: MEA chip overview, showing electrodes layout and planar electrodes microscopic images

Hence, neurons are directly connected to the microelectrodes by a neuro-electronic junction [Figure 1.5], and their extracellular signals can be recorded simultaneously and in a non-invasive way.

Extracellular potentials reveal changes in current or potentials of neuron group cells enclosing a microelectrode, covering it or in its proximity (50 - 70 µm) [13]. Signals coming from these neurons (action potentials) are superimposed and micro transducers sense the resulting electrical activity. Recorded signals are amplified and sent to the data acquisition system.

The characteristics of the detected signal can rely on the spatial distribution of cells surrounding the electrode, on the electrode physical aspects, the culture medium, and the data acquisition chain [13].

Amplitudes of extracellular action potentials can vary between 30 to 400 µV peak-to-peak [2], depending on the type of biological preparation and the maturity of the cell culture [2]. The frequency content ranges from 300 Hz to 5 kHz. Background noises (around 5 - 20 µV peak-to-peak) can be thermal, depending on the electrode impedance, and biological, with respect to electrical activity in the proximity of the micro transducers [13].

Moreover, MEA-chips can electrically stimulate the electrodes to send signals to the sample, this is done by applying voltage or current pulses to the microelectrodes, inducing a current flow through the surface [21]. They generate a transient voltage able to polarize the cells membrane and influence their electrical activity. Electrodes voltage must be low in order to prevent damage to the electrodes or cells, which also justifies the importance of a low electrode impedance.

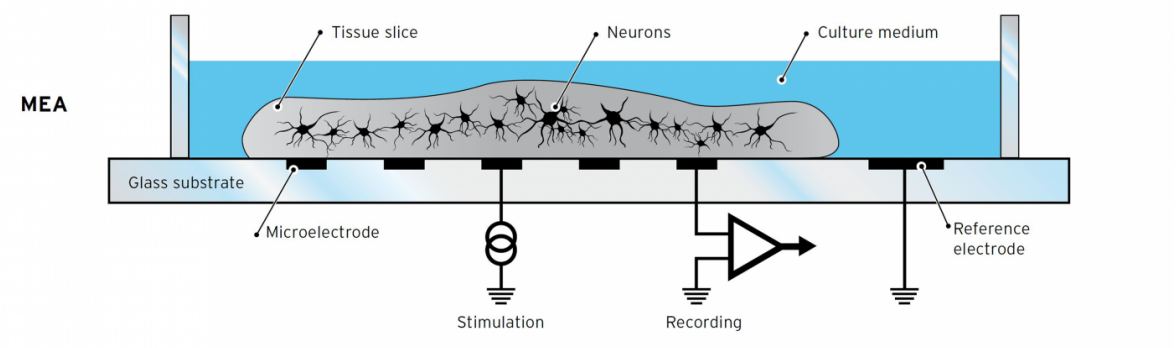

Figure 1.5: MEA substrate-integrated schematic layout [21]

In particular, MEA technology allows long-term (weeks, even months) recordings from a large population of neurons if they are integrated with a setup controlling environmental conditions and it has been proven valuable to characterize the neuron networks dynamics of different biological preparations, in both time and space. Additionally, this system also permits flexibility to modify and readapt experimental setups.

Main drawbacks of MEA are related to a strong attenuation of the signal, low quality of electrical coupling, spatial resolution and to an impossibility to gather information about the underlying mechanisms that triggered individual neuron activity [10].

Even though spike sorting computer algorithms permit waveform analysis from multiple electrodes, making feasible to distinguish activity of single neurons, it is still not possible to discern which event generated it. Electrodes are unable to perceive subthreshold synaptic potentials, that mediate significant signaling between neurons. Thus, neurons that do not fire cannot be identified – those are called dark neurons.

Regarding multi-electrodes spatial resolution, a higher number and density of electrodes (high density MEAs) enhances detailed information gained by extracellular recordings and allows signals to be sent over long distances. However, a higher spatial resolution lowers signal to noise ratio and increases computational costs.

Extracellular signals obtained are weak in strength, especially when compared to intracellular recordings. Moreover, quality of the signal is affected by the electrical coupling that depends upon electrode geometry and impedance. Lower the electrode impedance, better the signal to noise ratio. In contrast, reducing electrode size to improve spatial resolution, cause an increase in impedance. Enlarging only the electrode surface of electrodes is used to counterbalance this limitation.

On the contrary, intracellular recordings by sharp or patch electrodes present a good electrical coupling with the cell, being able to 'read' subthreshold synaptic potentials. Those characteristics allow measurements of ionic currents, a correspondence between morphology and function of a neuron and establish a correlation with the signal source.

However, intracellular techniques can only record signals from a few neurons at the same time, are complex to perform and invasive to cell, because electrodes are inserted inside the cell or in its membrane interfering with the cells and even causing their death. Intracellular recordings duration is limited by mechanical and biophysical instabilities and therefore, show to be not suitable for long-term acquisitions.

Hence, an ideal device for neuron signals recording would integrate the advantageous characteristics of both intracellular and extracellular recordings in order to understand the importance of single neurons to the network behavior. This system would be able to: simultaneous record and stimulate hundreds of neurons individually; maintain electric coupling with neurons for long time periods; keep track of transmembrane potentials amplitude range -80 mV - +30 mV), subthreshold synaptic potentials (amplitude in the range of ±0.5 – 10 mV), membrane oscillations (between ±5 mV and frequencies 1 - 50 Hz) and action potentials (around 100mV)[10].

So far, nanostructured electrodes appear as a promising solution as their dimensions are compatible with the neuron size, making signal recordings possible for individual neurons and more, they present a better electrical coupling due to their shape such that cells engulf the electrodes[16]. However, this technology still needs further improvement.

1.4 Importance of environmental conditions

Long-term neuron electrophysiology studies using multi-electrode arrays are only feasible if the cells plated on MEA chip (culture dish) are maintained inside an incubator which provides the correct environmental conditions for they growth such as sterility, appropriate temperature, pH (5 % relative carbon dioxide concentration), osmotic pressure, and high relative humidity to prevent evaporation of cell culture medium.

In general, every time cell recordings are necessary the culture is taken out of the incubator to be placed on a standard experimental setup [Figure 1.6] to have their signals recorded by the data acquisition system. Even though the mentioned standard setup is equipped with a heating plate, capable to provide adequate temperature, the other essential parameters for cell maintenance are not under control which makes uninterruptedly recording over extended periods impracticable. MEA long term recordings are usually limited, carried out over recurrent periods of time.

When neuron cell cultures are temporally out of the incubator, not only for recording but also for imaging and medium exchange, they undergo abrupt environmental changes. There are transitory oscillations in pH, due to a lower concentration of carbon dioxide in air, in temperature, and osmolarity, that can alter neurons viability and electrophysiological activity.

In addition, there is the risk of contamination of the cultures through air and also of mechanical stress as result of manual handling[13].

With altered environmental conditions there is the probability of obtaining inaccurate data on the recordings, that does not reflect the culture electrophysiological state. This problem affects MEA experiments reproducibility and analogy between distinct neuron cultures under the same experiment.

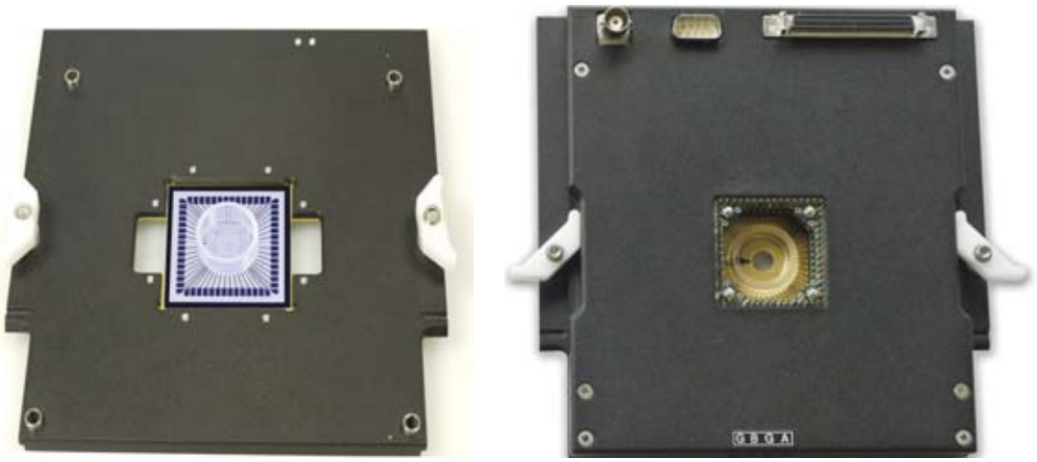

Figure 1.6: MEA standard experimental setup, MEA1060 amplifier (Multichannel systems, GmbH)

The previously presented issues reinforce the necessity of a reliable system, able to maintain neuronal cell cultures in a suitable and stable environment, allowing change of culture medium, pharmacological experiments and supporting imaging with the slightest interference to the surroundings, while recording data uninterruptedly during long time periods.

Many applications would benefit from long term monitoring of neuronal activity such as investigating neuronal plasticity; network development, especially for the following of human pluripotent stem cells neuron differentiation and maturation; drug screening, making possible to study the onset of a pathological mechanism.

1.5 Aim of the thesis

The aim of the work described in this thesis dissertation was to be able to perform long-term and parallel electrophysiological recordings on an experimental environmental chamber based on Multielectrode arrays (MEA) developed in previous work [14].

Objectives of this thesis consisted of:

(i) Improvement of algorithms used in data analysis;

(ii) Characterization of heating and electrical properties of an Indium Tin Oxide (ITO) heater and improvement of the chamber anti-condense system, using the ITO panel;

(iii) Validation of the updated environmental control system;

(iv) Validation of the setup performance in recording continuously the electrophysiological activity from dissociated hippocampal neuron cultures, using 2 MEA in parallel, for multiple days.

The results demonstrated the capability of the system to record long-term activity from in vitro neuronal cultures, thanks to the control of environmental conditions that were kept within physiological values throughout the experiments. This paves the way to the use of the setup not only to record well-known neuronal network activity (e.g. the hippocampal one), but also to monitor the growth and maturation of pluripotent neuron-like stem cells and the long-term effects of pharmacological treatments on those networks.

The work was conducted at the Experimental Imaging Center (CIS) Laboratory of San Raffaele Hospital.

Chapter 2

Material and Methods

2.1 Description of the Culture Chamber

The culture chamber used in this work was previously developed by Giulia Regalia [13] and later improved in previous works [17] [20]. The aim of this device is to perform long-term recordings of neuronal cultures (plated on multielectrode arrays chips) in a controlled environment, also allowing the change of culture medium and parallel recordings. In order to accomplish this goal, this chamber is integrated with a control unit, a commercial heating water bath, a humidifier, a gas cylinder, anti-condensation solution, environmental sensors, and a data acquisition system.

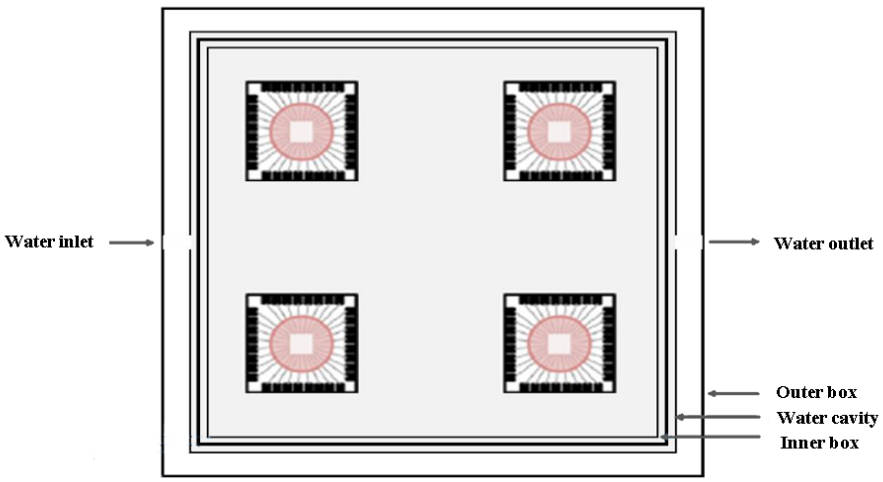

Figure 2.1: Top view of chamber lower part

Table 2.1: Chamber dimensions

	BxWxH (mm)	Thickness (mm)
Inner box	190x190x30	5
Outer box	240x240x45	15
Cover	240x240x10	10

This chamber is made of PMMA (Poly-methyl-methacrylate), material chosen because of its good optical transparency and thermal insulating characteristics. It includes an inner and an outer box with a sealed cavity filled with water between them and a top cover plate [Figure 2.1], with detailed measurements showed in Table 2.1.

On the bottom of the internal box, polylactide (PLA) supports (50 x 50 mm) are placed to allocate four MEA chips [Figure 2.1].

The cover plate contains:

- airtight openings – air inlet and air outlet;
- cylinder tubes with silicone membranes, covered with Parafilm for syringe insertion to exchange medium or to perform pharmacological experiments with minimum interference to the cells' environment and sterility;
- gaps to place the sensors and connectors to the MEA acquisition boards;
- openings for inverted microscope objectives insertion beneath each MEA chip.

To assure the sealing of the lid and the upper surface of the lower boxes a silicone membrane (3 mm thick) is placed between these parts; the final closure is done by eight small clamps located in the external walls of the chamber. The chamber is simple to be cleaned and it can be sterilized with Ultra Violet rays or Ethylene Oxide.

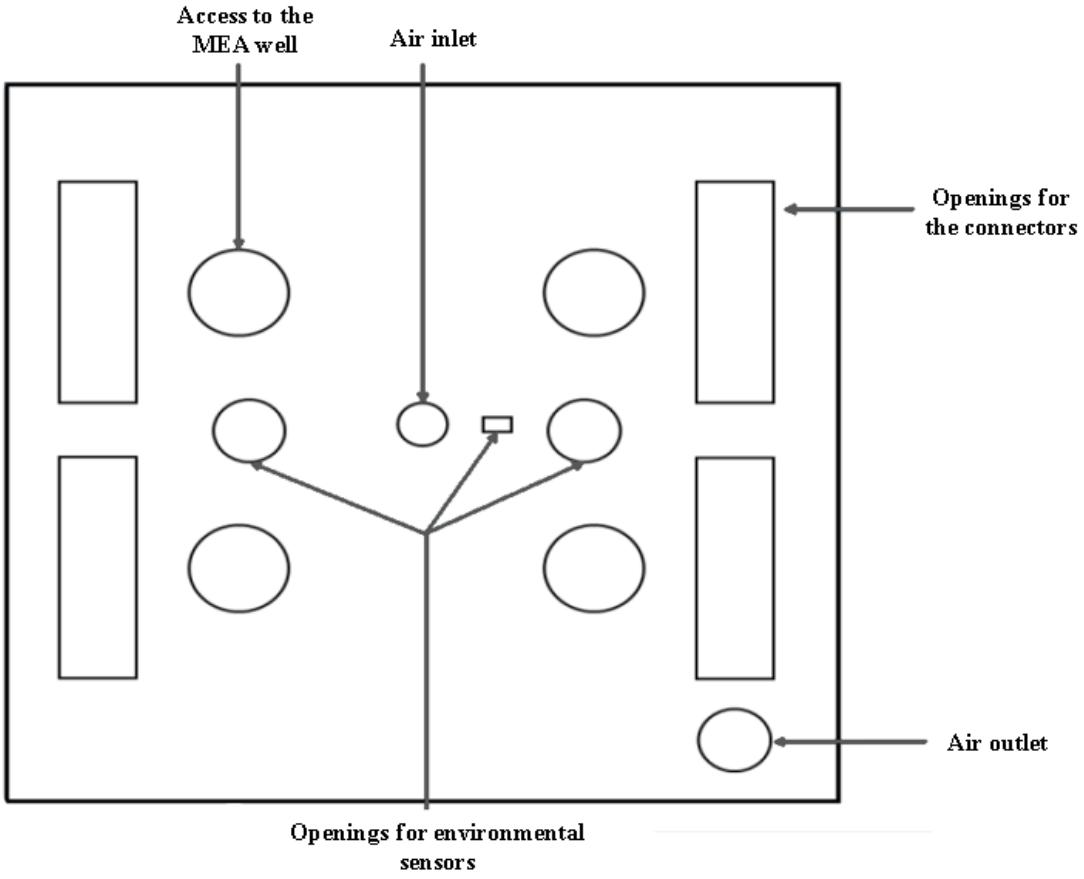

Figure 2.2: Top view of chamber upper part

2.2 Description of the Environmental Control System

In order to maintain the viability of cells, especially during long-term recordings certain environmental conditions are strictly necessary. Those conditions are: temperature in the range of 36-38 °C (physiological range); relative humidity higher than 90 %, to avoid evaporation of cell culture medium; air percentage of carbon dioxide (CO_2) between 4.5-5 % to maintain the pH in the physiological range of 7.2-7.5[13].

Therefore, chamber settings must be adjusted to those environmental parameters, which requires a control unit (Arduino Due board), sensors and other equipment, as showed in Figure 2.3 below.

Temperature is regulated according to three mechanisms. The first one is through the heating of the water that circulates in the cavity between inner and outer boxes using a commercial pump (E306, Lauda GmbH), equipped with a Pt100 probe (Tb) and a heater (Hb) [Figure 2.4]. Then, warmed air from a temperature-controlled humidifier flows into the

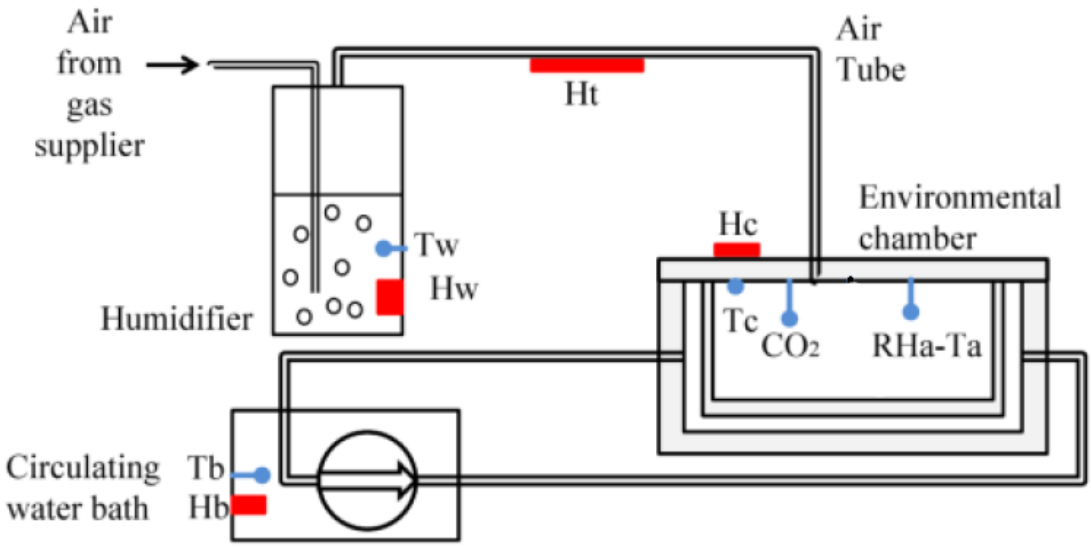

Figure 2.3: Overview of environmental control

chamber. Finally, an Indium tin oxide surface heater (Hc) is placed externally on the top cover plate, regulated by a power supply in order to avoid condensation of water, protecting the electronic devices against oxidation and corrosion.

Indium Tin Oxide (ITO), a semiconductor material, is coated with PolyEthylene terephTte (PET) constituting a thin, transparent, and conductive film. When powered, it provides heat[7]. An ITO film (surface resistivity 60 Ω/sq, ITO coating 130 nm thick, Sigma Aldrich) with 220 x 200 mm was used and cut according to the configurations of the chamber and applied on the top of it. The connections to the power supply were done by means of bus bars (lead wires fixed using copper tape) on each side of the heater [Figure 2.6].

This last heating solution provided an improvement with respect to previous works, where a Nickel-Chrome wire was used to heat the chamber surface, while temperature readings were performed by a Pt100 probe integrated to a proportional-integral-derivative (PID) controller[17]. Changes were made because the reported system was not completely able to prevent condensation and its effectivity was dependent on the wire distribution on the surface.

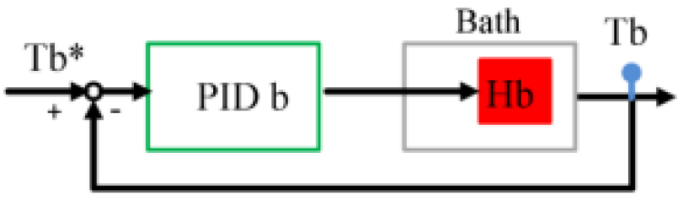

Figure 2.4: Temperature control of water in the chamber cavity [14]

Relative humidity (RH) is controlled by a humidifying and heating module. A 50 L / 150 bar cylinder flows a mixture of gas (5 % CO_2, 20 % O_2, 75 % N_2) into a flow meter (Platon NG series, range: 60:600 ml/min) to first select the flow rate able to trigger humidification and avoid possible gas losses. The gas mixture then flows to a rigid tube that goes to the interior of a glass bottle filled with 450 ml of sterile water.

The bottle is covered with a nitrile rubber sheath, to improve thermal insulation. Inside the bottle, there is a Nickel-Chrome heater (Hw) regulated by a PID controller and a glass filter candle, which delivers the air flow to the chamber through a thermally insulated silicone tube heated with a bounded Nickel–Chrome wire (Ht). The humidifier water temperature is measured by an immersion Pt100 thermal resistance (Tw) which provides feedback to the heater inside the bottle.

A digital humidity and temperature sensor (SHT75, Sensirion Inc) is used to measure internal chamber temperature and RH.

Relative humidity value depends on the temperature's measurements of the circulating water bath (Tb), of the humidifier (Tw) and of the chamber surface (Tc); it also depends on the power delivered to the air tube heater (Ht) [Figure 2.5].

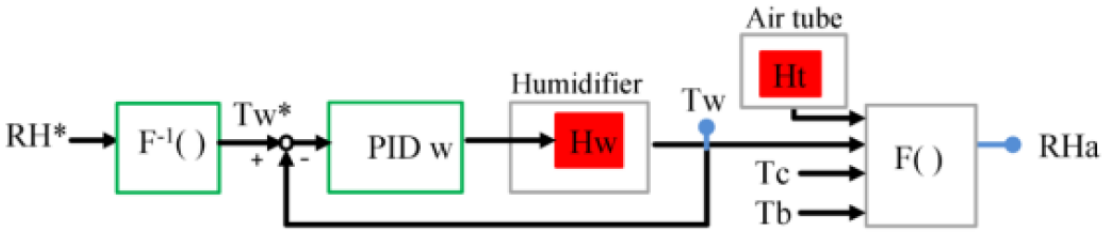

Figure 2.5: Relative humidity control – F() is an empirical relationship between RHa and Tb, Tc, Tw and Ht [14]

Regarding the carbon dioxide concentration, an appropriate gas mixture (5 % CO_2, 20 % O_2, 75 % N_2) is supplied, as mentioned before. CO_2 percentage in the chamber environment is monitored by a digital infra-red CO_2 sensor (COZIR Probe, Gas Sensing Solutions Ltd).

The control unit comprises a microcontroller (Arduino DUE board) and custom printed circuit boards (PCBs). Temperature, RH and CO_2 sensors signals are read by the microcontroller, which drives the outputs. The environmental parameters are displayed in real time on Serial Monitor (Arduino software IDE) in a computer. They are: chamber temperature [°C]; relative humidity [%]; CO_2 concentration [%]; air humidifier temperature [°C]; heater PID power status (255 ON— 0 OFF); humidifier set point (255 ON— 0 OFF)[20] .

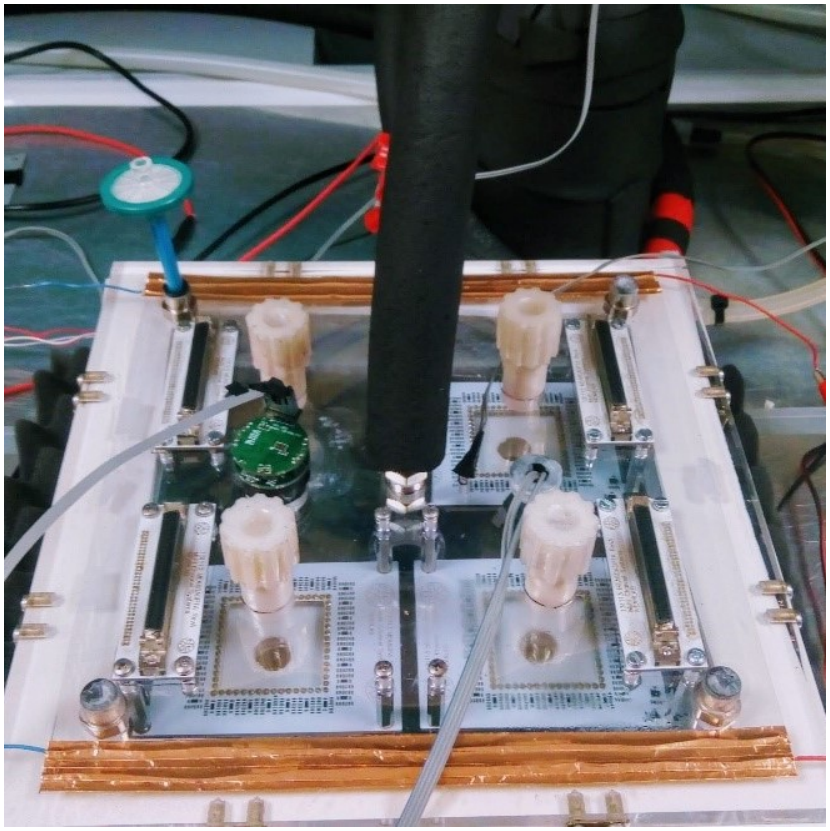

Figure 2.6: Top view of environmental chamber including: ITO heater and environmental sensors

2.3 Experimentations with Indium Tin Oxide heater

Indium tin oxide heater (ITO) coated with polyethylene terephthalate (PET) was tested with diverse sample sizes (3 cm x 3 cm, 6 cm x 6 cm, 20 cm x 22 cm), voltages and different configurations of lead wires and copper tape placing using a power supply. Temperature was measured using a PCE-313 A (PCE) sensor, resistances and voltages were measured using a multimeter.

Those tests were performed in order to understand the material behavior and the voltage required for our application.

2.4 Description of the Data Acquisition System

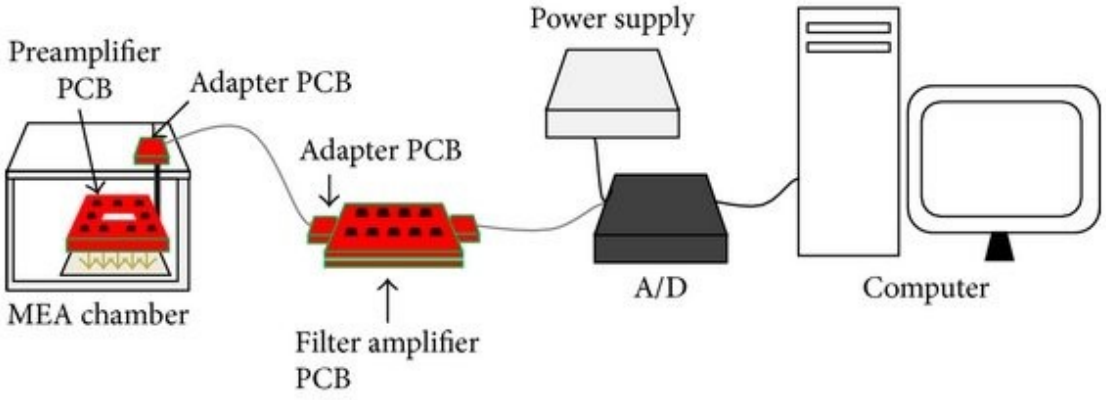

Figure 2.7: Data Acquisition Setup

Electrodes signal coming from 60-channel MEA (multielectrode array) chip are read first by a pre-amplifier board. An array of connectors links the pre-amplifier to an external adapter placed on the chamber top plate. The external adapter is then connected to a filter amplifier board which conveys the signal to a data acquisition and analog/digital converter board USB-ME64/128 (Multi Channel Systems, GmbH) that subsequently sends the signal to a computer to be monitored/recorded using the MCRack software (MCS GmbH) [Figure 2.7].

Each pre-amplifier board is located inside the chamber above MEA chip support and fixed to the internal part of the cover plate. It contains 60 gold spring pins including one reference pin to attach to MEA chip contact pads. There are pre-amplifier boards with different

Table 2.2: Characteristics of pre-amplification boards

Board	Gain	Bandwidth
56-10-4K6 II	46	10 Hz - 4.6 kHz
92-10-4K8 IV	92	10 Hz - 4.8 kHz

gains and frequency ranges as showed in Table 2.2.

Pre-amplifier and filter-amplifier are connected through a 68-pin shielded cable (MCS GmbH) compatible with pre-amplifier output connector and filter amplifier input connector.

Filter amplifiers are either custom or commercial ones from Multi Channel Systems (MCS GmbH), with different specifications as showed in Table 2.3.

Varied pre-amplifiers and filter-amplifiers specifications give versatility to our system, allowing it to be configurated according to our needs.

Filter amplifier output is connected to the USB-ME-64/128-System (64 or 128 channels, 16 bit, ($\pm$ 4 V, sampling frequency up to 50 kHz/channel, MCS GmbH) using a second cable. This system acquires and digitize analog input signals that are then transmitted to the computer via universal serial bus (high speed USB) and recorded/monitored by the MCRack software[9].

An external power supply PS40W ($\pm$ 7 V, 42 W, MCS GmbH) is plugged to the data acquisition system USB-ME-64/128 in order to power the pre-amplifiers and filter-amplifiers connected to it [12].

The MEA chip used in this work is a standard 60MEA200/30iR-Ti (MCS GmbH), with a glass ring and 60 TiN (titanium nitride) electrodes, an internal reference electrode, contact pads and tracks opaque also in TiN and SiN (silicon nitride) isolator. These electrodes are aligned in an 8 x 8 grid, have a diameter of 30 µm and interelectrode space of 200 µm [19].

Table 2.3: Characteristics of filter-amplification boards

Board	Gain
Filter Amplifier MCS FA60/64	12
Filter Amplifier Custom FA	20

MEA electrodes are not symmetrically arranged, so the MEA chip has to be positioned with the reference pin aligned to the one of the pre-amplifier[19]. MEA chip and its layout are showed in Figure 2.8.

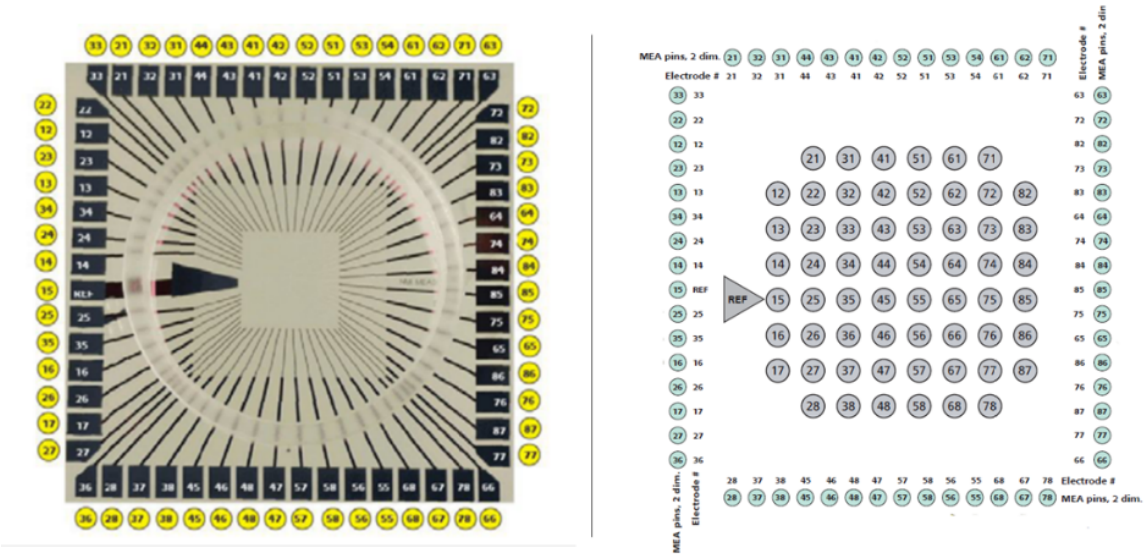

Figure 2.8: MEA chip and MEA layout

2.5 Description of the standard reference acquisition system

The MEA benchmark acquisition system described below Figure 2.9 is used in our work in order to validate the custom experimental setup by means of short-term recordings.

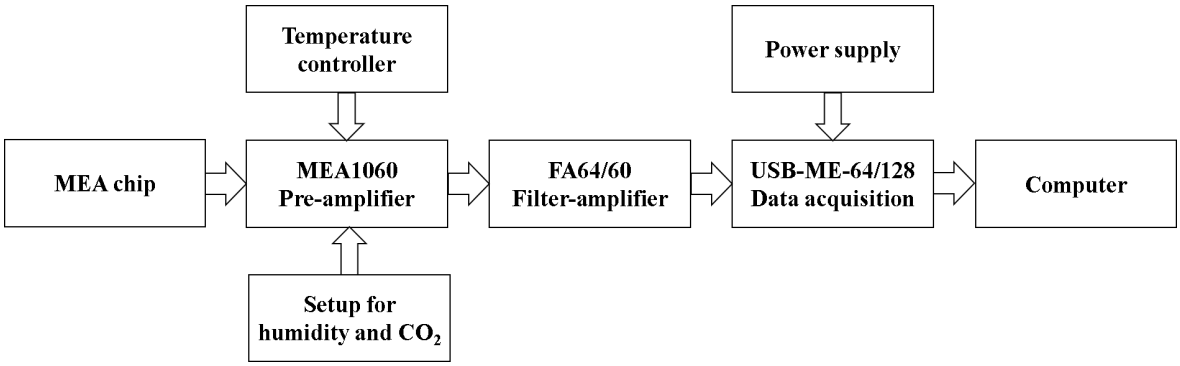

Figure 2.9: Benchmark acquisition system

The main differences between the commercial setup (MCS, GmbH) Figure 2.9 and our custom setup Figure 2.7 is the use of a MEA1060 amplifier (MCS, GmbH) and the environmental control.

The MEA1060 (gain 55) supports one MEA chip and contains a heating plate that it is connected to a thermal PID controller TC02 (MCS, GmbH), which maintains the temperature at 37 °C. As this system is heated from the bottom, it is not the most suitable solution to perform a refined control of the cell culture temperature.

In order to prevent the evaporation of the cell culture medium, a sealed bottle with water that receives CO_2 flow from a cylinder is used (gas mixture 5 % CO_2, 20 % O_2, 75 % N_2). A rigid plastic lid with a hole for tubing is placed on top of the MEA1060 amplifier and makes possible to pipe the gas through the bottle. However, with this solution it is not possible to monitor or adjust relative humidity values.

Another approach is the use of lids (polytetrafluoroethylene) covered with a FEP membrane (fluorinated ethylene-propylene, 12.5 µm thick) developed by [1]. This a transparent hydrophobic semipermeable membrane, it allows the diffusion of gases (O_2, CO_2), avoiding evaporation. Thus, it reduces the need of a humidified environment and limits the risk of contamination.

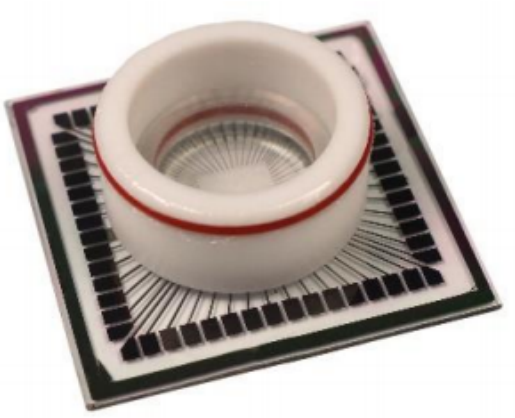

Figure 2.10: MEA chip with membrane cover

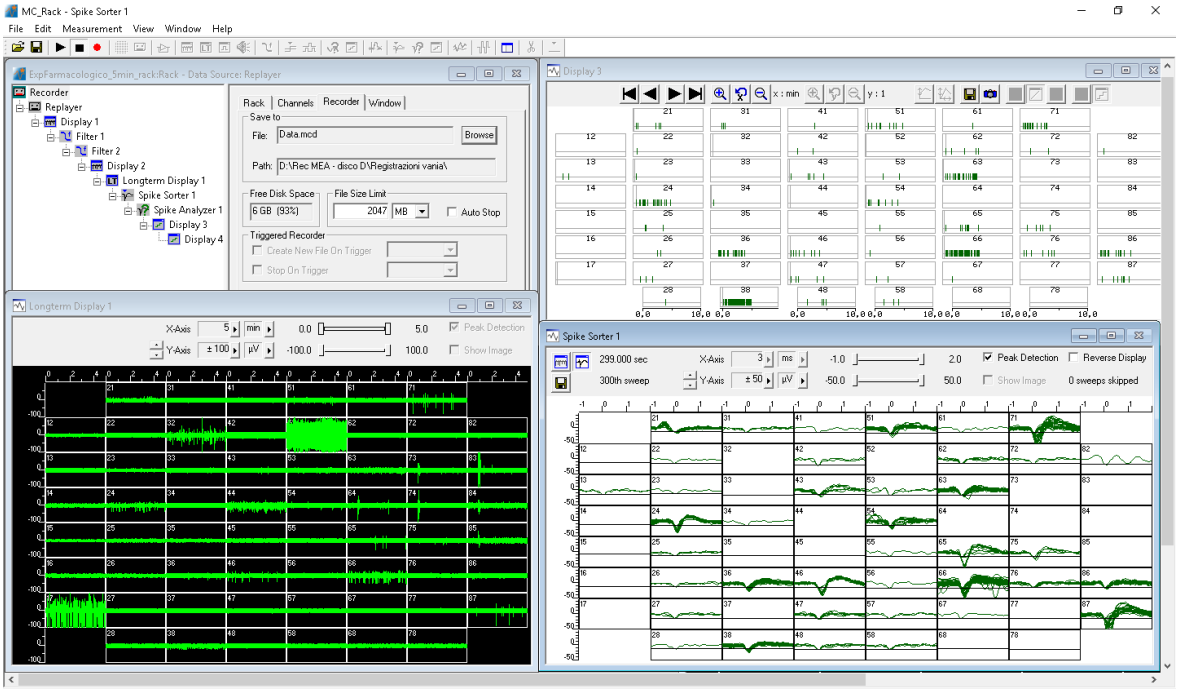

Figure 2.11: MC_Rack software: General view

2.6 Description of the software MCRack

MCRack software is used to real time monitor, record and analyze the data coming from MEA chip and, when integrated with the USB-ME-64/128-Systems hardware, it constitutes the data acquisition system.

In this software it is possible to add virtual tools that work independently from each other. In this work the frequently used tools are: a Butterworth 2^{nd} order pass-band filter with an infinite impulsive response (IIR) 200 Hz - 3 kHz, long-term display and the spike sorter.

Implementation of the desired band-pass filter is possible due to the use of two 2^{nd} order Butterworth filters: a high-pass with 200 Hz cut-off frequency and a low-pass with a 3 kHz cut-off frequency.

The *Long-term display* is used for monitoring electrodes signal continuously over a long time period, the time scale is defined by the user from 1 second up to 60 minutes. This tool shows the channels in the layout according to the channel map and can also be configured to show electrode raw data or filtered data in our case[9]. From this window, it is possible to identify whether there are disturbances in the signal [Figure 2.12 I, II] or whether the electrodes' signal is being read properly.

Figure 2.12: MC_Rack software: Longterm display, I and II are examples of channels with signal disturbances

The *Spike Sorter* tool allows the detection and extraction of spikes from the raw or filtered data by their shape or threshold. Spike data from each channel is overlaid and showed in the spike sorter window [Figure 2.14].

As spikes can have different waveforms and rhythm depending on the neurons they are derived from – usually from multiple neurons, it is important to separate single unit spikes.

The slope method recognizes the shape of a spike, which means the amplitude and the slope.

The threshold method identifies a spike when the signal crosses a determined value (µV) that can either be defined by the user or automatically defined by the software. Each channel can also have its own threshold defined separately. It also possible for the user to adjust that by dragging the threshold line in the spike sorter tool display.

Still in the spike detection configuration, the Negative Slope option is selected, which means that the fall of the curve is considered instead of the rise.

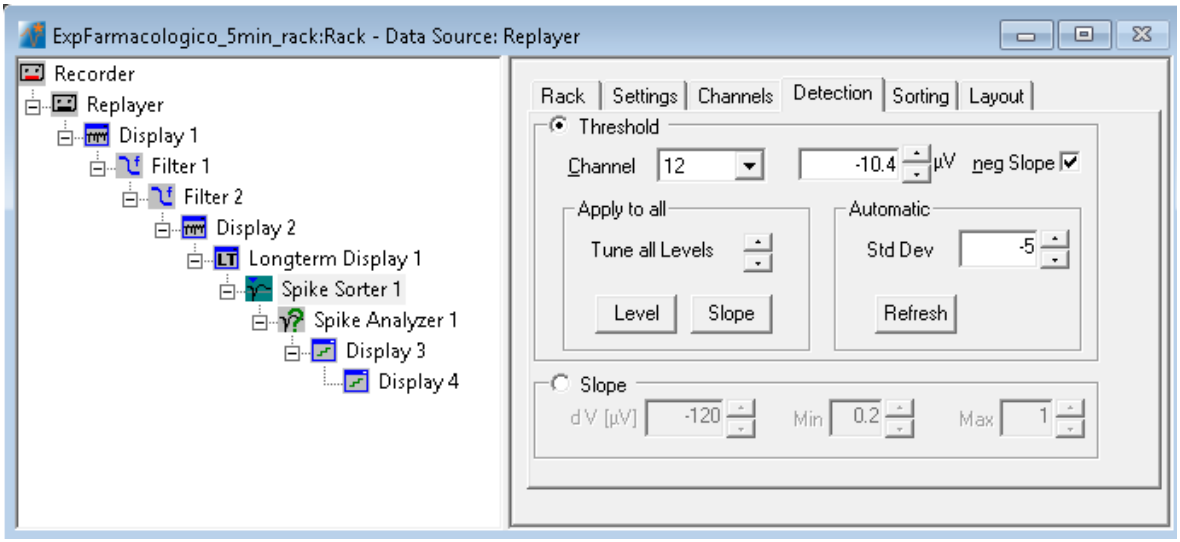

Figure 2.13: MC_Rack software: Spike Sorter configuration

The Automatic feature automatically assigns an individual detection level to each channel. The standard deviation of the noise of each data trace, calculated in the first-time interval of 500 ms, is used to estimate its signal threshold [9].

It is possible to set the factor by which the standard deviation is multiplied to set the detection threshold. Values between -5 and -7 are the most used in our work.

Waveforms that are recognized as spikes by either of the two detection methods, are extracted from the raw data. The time length before and after the spike detection event can be chosen by the user [9].

In the example below [Figure 2.14, I and III] represent predominantly noise, while II and IV could represent spiking activity with noise superimposed. Channels that present excessive disturbances and/or present waveforms that cannot be identified as spikes are removed from the recording [Figure 2.15].

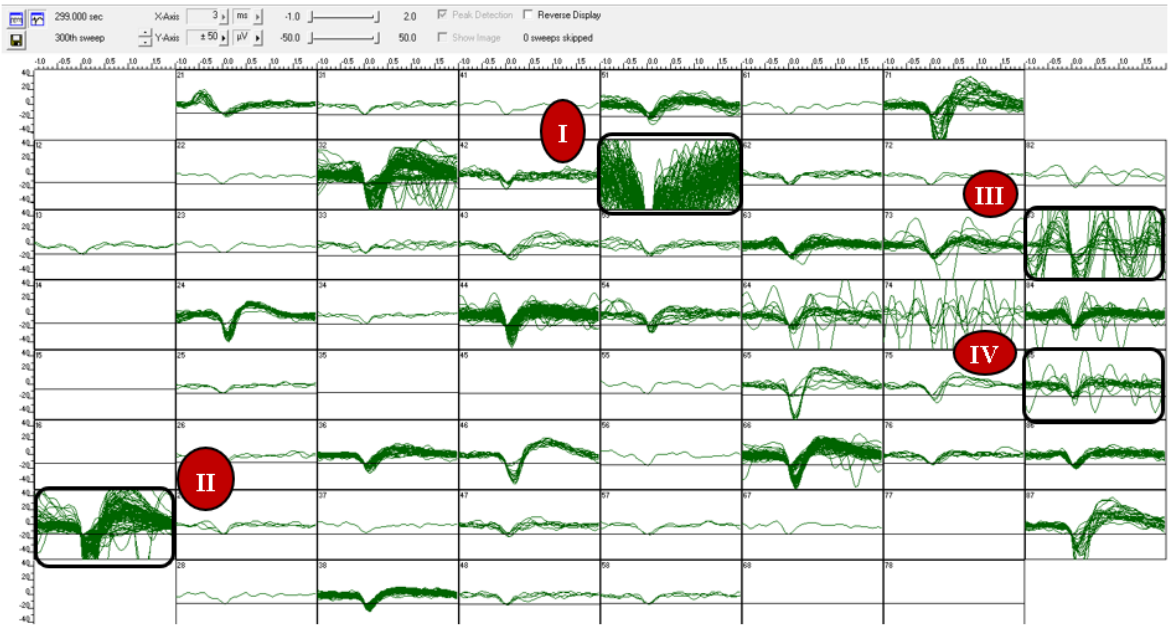

Figure 2.14: MC_Rack software: Spike sorter window

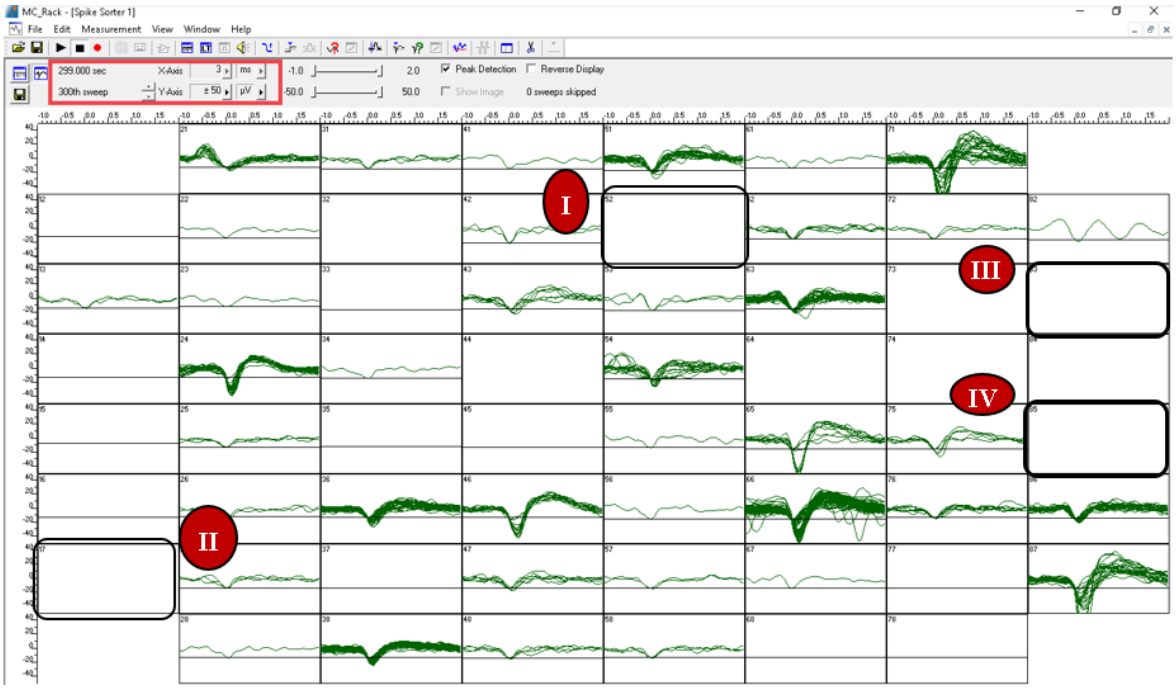

Figure 2.15: MC_Rack software: Spike sorter window after channels selection

The *Spike sorter* tool also allows the extraction of only time stamps. The time stamps window displays the time instants in which the spikes occur as single data points. It does not provide information about the spikes' waveform. Thus, we are able to access features like spike rate, spike bursts occurrence and spatial distribution of spikes. Data from this window can be exported to one single data file or it is also possible to export one file per channel.

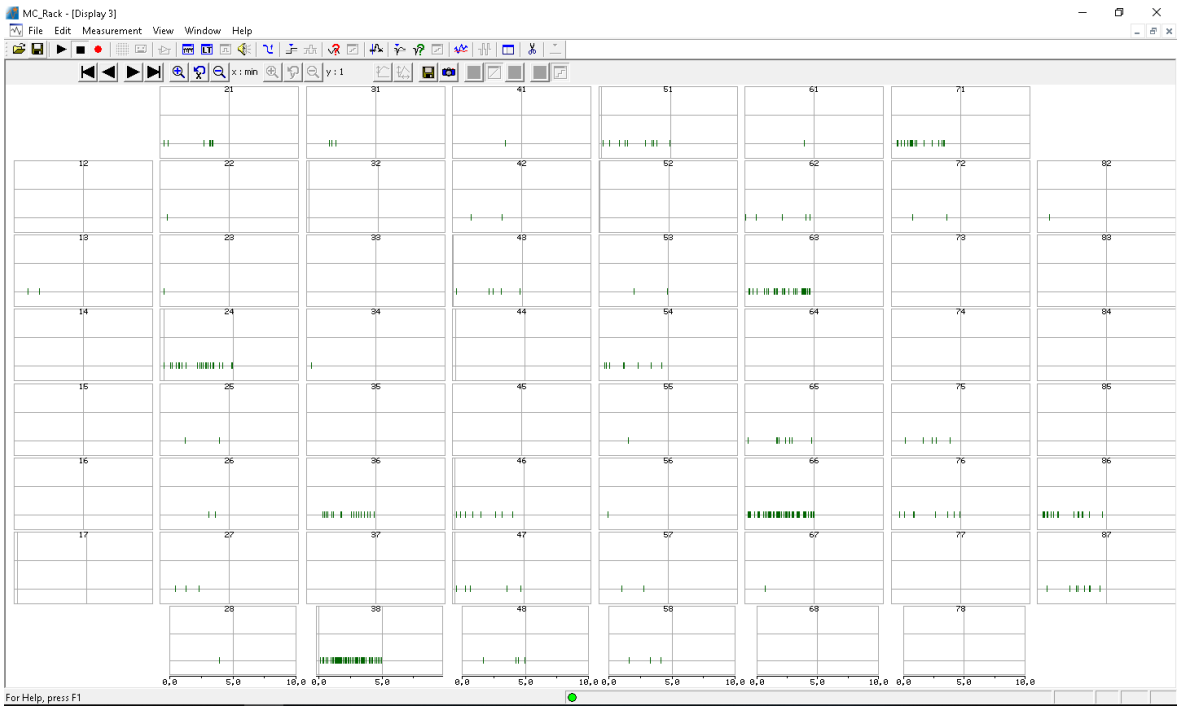

Figure 2.16: MC_Rack software: Time stamps window

2.7 Description of data analysis algorithms

The first part of this project consisted in the revision of the Matlab algorithms used for signal processing. There are two methods to perform the data analysis, one is considering all the valid channels selected on MCRack, obtained from a file .dat (Time Stamps window), and calculating the network parameters. The second method permits the analysis of a single channel, that is first extracted from a .mcd file, filtered and converted into a .mat file and then processed by algorithms that allow the proper calculation of signal to noise ratio (SNR) and power spectral density (PSD) for each selected channel (individually).

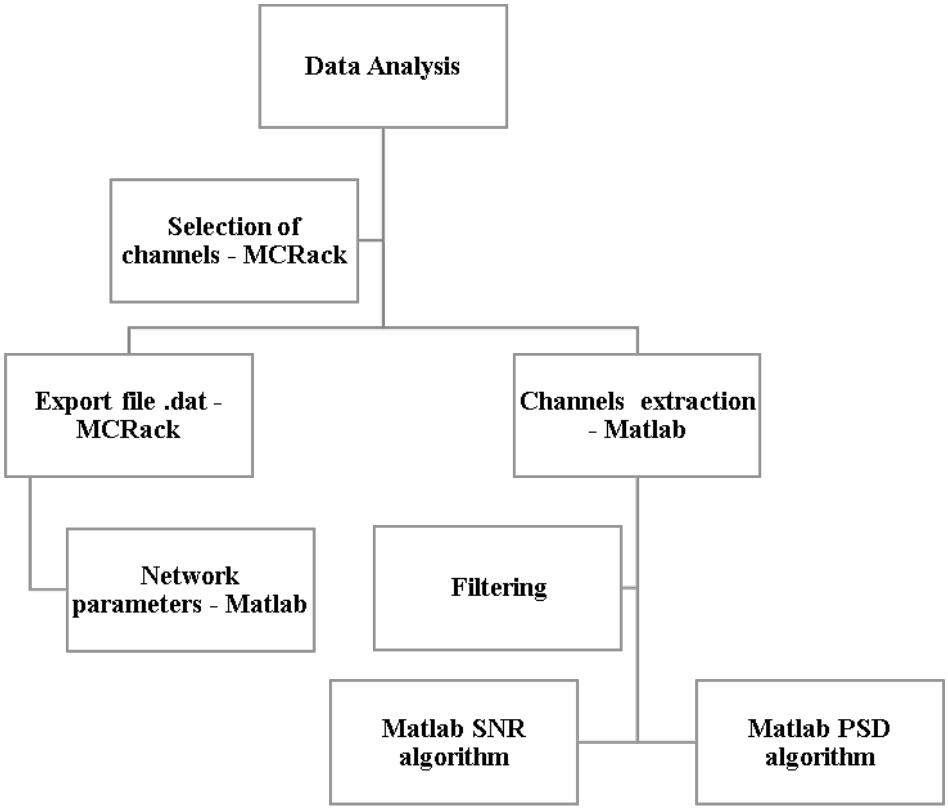

Figure 2.17: Simplified description of the data analysis process

The data obtained from .dat file are previously filtered by a second order pass-band Butterworth filter (200 Hz – 3 kHz) internal to the MCRack software. Further, this file is an input for a Matlab algorithm which determines spike, burst and network burst parameters, returning this information as output along with a raster plot (spatial-temporal distribution of spiking activity, time x electrodes), showed in Figure 2.18.

The parameters extracted with this algorithm are:

- the number of active channels – an active channel is considered a channel that presents more than 10 spikes in 5 minutes recording[5];
- the total number of spikes;
- the average spikes number, considering only the active channels.

A burst is defined as having 3 as the minimum number of spikes, with minimum inter burst interval of 100ms and with minimum intra burst interval of 100 ms[3]. The considered parameters are:

- the average number of bursts per channel, considering only those channels that have at least 2 bursts [4];
- average duration of the burst (s);
- average number of spikes per burst;
- percentage of spikes in the burst compared to the total spike number;
- inter and intra burst average interval (s).

Regarding network bursts, when bursts are synchronized, the computed parameters are[2]:

- the average duration of network burst (s);
- average number of channels involved in the network burst, compared with the number of active channels;
- average number of spikes involved in network burst.

Although the calculation of parameters is performed using data from the time stamps, when analyzing single channels, we preferred to implement a more efficient filter – a pass-band Butterworth filter of 4^{th} order (298 Hz – 3006 Hz) with a finite impulse response (FIR) using the *designfilt* function of Matlab together with the *filtfilt* function to obtain a zero-phase distortion in the signal.

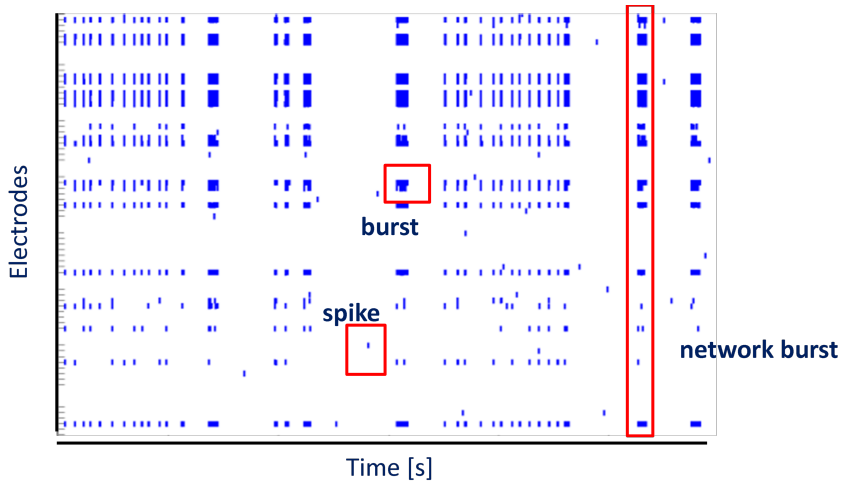

Figure 2.18: Raster plot

SNR and PSD algorithms were also reviewed, and a few modifications were made with respect to previous works.

In a simplified way, the SNR algorithm detects when a spike occurs and then obtains its features. It identifies when a sample exceeds the threshold and delimitates a 3 ms window (1ms before the event and 2 ms after) in the signal that is defined as a spike.

The previously developed SNR algorithm receives as an input the raw data of a single channel obtained from MCRack, the sampling frequency, the refractory period and what we defined as standard deviation factor (SDF), which is a factor that multiplies the noise estimation in order to calculate the threshold (Th) for the spike detection.

It returns as output the noise estimation in the first 500 ms of the signal, the time instants when a spike was detected, the signal to noise ratio (SNR), a matrix that contains the spikes waveform, the mean of value, the amplitude ratio, spikes amplitude and spike width according to this matrix, in which the calculations are explained below. It also plots the spike's waveform over time and the superimposition of the spike's waveform in a 3 ms window.

$$Noise\_estimation = \sqrt{\frac{1}{n-1} \cdot \sum_{1}^{n} (y-\mu)^2} \text{ , where } \mu \text{ is the mean of y.} \tag{2.1}$$

$$Th = -SDF \cdot Noise\_estimation \tag{2.2}$$

$$SNR = 20 \log \left(\frac{\overline{Amplitude_{spikes}}}{6.Noise_{estimation}} \right) \tag{2.3}$$

$$Amplitude\ ratio = \left| \frac{max\_val}{min\_val} \right| \tag{2.4}$$

$$Amplitude\ spikes = max\_val - min\_val \tag{2.5}$$

$$Spike\ width = |pos\_min - pos\_max| \tag{2.6}$$

Min val and *max val* correspond respectively, to the minimum and maximum values obtained from the matrix that contains the spikes amplitude. *Pos min* and *pos max* correspond to time instant where *min val* and *max val* occurred.

The hypothesis of white noise was excluded, Kolmogorov-Smirnov and Chi-square goodness-of-fit tests were applied to the first 500 ms of our signal to evaluate if the noise derives from a normal distribution.

The algorithm PSD calculates the signal power spectral density in terms of $\mu V^2\,Hz^{-1}$.

It receives as input the file .mat - raw data from a single channel, sampling frequency, and the interval of frequency in which the PSD should be computed. The number of samples of the signal is re-divided in 10ms intervals and then the peak to peak values, root mean square deviation and also the root mean square of the noise band in this interval are calculated and averaged for the entire signal. The distribution of power per unit frequency is calculated using the Welch's method.

RMS values of broadband, spikes band and band without electric hum are computed using the integral of the signal power spectral density. This algorithm gives as output all of the values mentioned, the signal plot and the power spectral density plot.

2.8 Validation of SNR algorithm with MEA Signal Generator

The 60MEA-signal generator (MCS, GmbH) device simulates different biological signals being useful for the testing of the setup and algorithms without the need of using biological signals.

MEA-SG allows the selection of waveforms through the DIP switch (category) and control button (waveform from a category). The waveform selected in this work was hippocampal neurons spikes, this configuration is composed of a sequence of 3 spikes that repeats itself along the recording [11].

Data acquisition was performed without any environmental control and using pre-amplifiers 46II, 92IV [Table 2.2] and MEA1060; filter-amplification boards FA60 and custom FA. MCRack software was used for the recordings.

Due to the presence of a higher noise level between spikes occurrence, a modification of

the signal to noise ratio (SNR) algorithm was performed, specifically to manipulate signals from the MEA-SG in order to detect spikes properly. Instead of calculating the standard deviation in the first 500 ms of the signal, this parameter is calculated using the inter-spike interval. With this alteration in the code, it was also possible to validate filter and PSD algorithms.

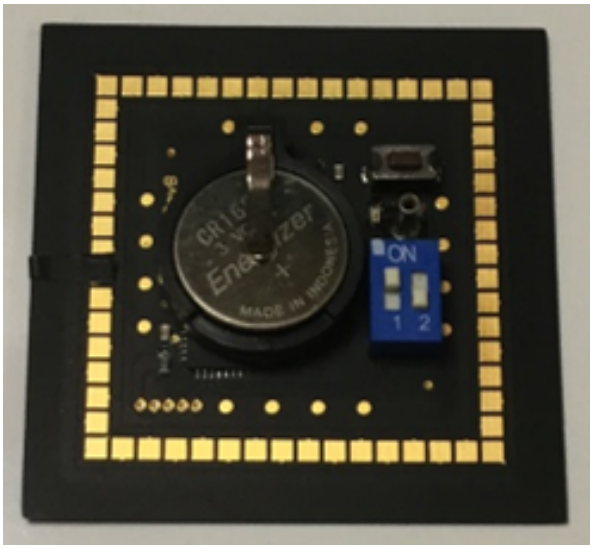

Figure 2.19: Signal Generator

2.9 Validation of Environmental Control Chamber

A 2-hour parallel recording inside the environmental chamber was performed using two MEA chips filled with phosphate buffered saline (PBS) solution. PBS is a buffer frequently used in biological applications, because it mimics the pH, osmolarity, and ion concentrations of the human body [18]. The aim of this acquisition was to assure the stability of environmental parameters (temperature, relative humidity, and CO_2) and in addition to guarantee the absence of water condensation.

Materials used were: environmental chamber – including a FEP membrane described in used to protect CO_2 sensor against humidity; pre-amplifier boards 46II and 92IV – specified in Table 2; filter amplification boards FA64 and custom FA – specified in Table 3; USB-ME-128 system – it allows the connection of two data acquisition chains; two MEA-chips of identification 26004 and 20145.

MEA chip 26004 was positioned aligned with the pre-amplifier board 46II, which was combined with the FA64 board, resulting in a total gain of 920.

MEA chip 20145 was positioned aligned with the pre-amplifier board 92IV, which was combined with the custom FA board, resulting in a total gain of 1104.

MCRack software was used for data monitoring and recording.

2.10 Validation of Environmental Control Chamber with Hippocampal Neuronal Cultures

2.10.1 Cleaning of the chamber system

Materials used: 70 % Ethanol solution, sterile water (S.A.L.F. S.p.A), bleach solution 10 ml/l.

Ethanol solution is used to clean the whole chamber, especially the inner part, including tubes for medium exchange and metal parts. Culture chamber is dried in a UV hood overnight. The bleach solution is added to the water-bath in order to flow it through the entire fluidic system. Sterile water is used to fill- up the humidifier bottle.

2.10.2 Preparation of MEA chip

Materials used: Sterile chips, filtered polylysine 2 mg/ml, filtered plating medium, sterile PBS, sterile water, petri dishes 60 mm and 90 mm.

MEA chips are submitted to a plasma treatment previously to the beginning of preparation. This treatment is done in order to increase hydrophilicity of the chip surface. The MEA chips are dry heated at 110 °C for 2 hours, together with MEA lids with FEP membranes.

The preparation of MEA chip starts 2 days before the dissection of cells from rat hippocampus.

In the first day, chips are placed on top of the small petri dish lid and inside the 90 mm petri dish, filled with sterile water. On each chip, 1ml of plating medium is added and then chips are let on incubator for 1 hour. After that, chips are washed 2 times with sterile PBS, polylysine solution is added and then chips stayed overnight in the incubator.

In the second day, polylysine is removed using a pipette and each chip is washed more 3 times with sterile PBS solution. Plating medium is added again and the MEA chips return to the incubator. After one hour the chips are ready to be used.

2.10.3 Preparation of hippocampal cultures

After MEA chips preparation, they were sent to another laboratory for the cells to be plated on it. These cells are from hippocampal neurons of mouse prenatal. Animal handling has been performed in accordance with San Raffaele Scientific Institute guidelines and with an approved IACUC protocol number 694.

2.10.4 Data acquisition

Data were acquired from 2 MEA chips, identified as 26955 (MEA1) and 26957 (MEA2). MEA lids with FEP membranes were used to cover the chips.

First of all, 10 minutes recordings were performed on the benchmark setup in order to ensure the viability of cells. Thus, MEA chips were placed inside the environmental chamber with the same settings as described in the PBS acquisition and maintained in there during 4 days for recordings.

MEA chip 26955 data was acquired using pre-amplifier board 46II and FA64 board, resulting in a total gain of 920.

MEA chip 26957 data was acquired using the pre-amplifier board 92IV and custom FA board, resulting in a total gain of 1104.

MCRack software was used for data monitoring and recording.

Additionally, on the last day of recordings a pharmacological experiment was conducted administering the neurotoxin TTX (Tetrodotoxin) to the MEA1 chip.

Chapter 3

Results

3.1 Experimentations with Indium Tin Oxide heater

As explained in chapter 2, preliminary tests were performed to characterize the Indium Tin Oxide heater (ITO) and evaluate the feasibility of using it in our application.

Measurements obtained from small samples (3 x 3 cm and 6 x 6 cm) were important to understand material behavior although they showed to be not very repeatable and results were not conclusive about what would be a better configuration.

Furthermore, material showed to be sensitive to abrupt changes in voltage, surface damage and bending, altering its resistance. ITO resistance also showed to increase after creating an opening in the film.

Table 3.1: Values obtained varying voltage applied to the 21 x 22 cm sample: configuration 1 – one layer of copper tape, configuration 2 – two layers of copper tape

	Configuration 1			Configuration 2		
Voltage	I [A]	R [Ω]	T_{max} [°C]	I [A]	R [Ω]	T_{max} [°C]
24	0.34	70	33.4	0.37	65	35.0
30	-	-	-	0.45	67	41.0

Another important aspect is that the surface heater temperature is not uniform. Temperature was measured in different points of the samples and their center appeared to be the one with higher temperature. This evidence influenced the choice of the required voltage for our application. It is necessary to highlight that the desired heater temperature should be at least 37 °C in order to avoid water condensation.

Tests performed with larger samples were more adequate. Results obtained varying number of copper tape layers and voltage applied to the sample 21 x 22 cm are presented in Table 3.1.

It was possible to observe from data in Table 3.1 that the use of configuration 2 lead to a decrease in resistance and an increase in the maximum temperature. This effect was also noticed in one of the samples from initial tests.

Important to mention that resistance values obtained with a multimeter were not fully repeatable and stable. Therefore, resistance was also indirectly measured by varying voltage supplied to the heater and applying Ohm's law with the correspondent current values. In accordance with previously mentioned experimental results and with the literature [], the final configuration for use of the ITO panel was chosen: use of two superimposed layers of copper tape; length of lead wires equal to the larger side of the rectangle; voltage required around 30 V.

ITO resistance values after cutting of the material (in order to fit the chamber configurations) were measured with different voltages in different days.

From Table 3.2 it was observed that the resistance values had slightly variances. Moreover, when comparing resistance values from Table 3.1 and Table 3.2 at 30 V, an increase of 30 Ω was perceived.

Table 3.2: ITO resistance measurements varying applied voltage after material cutting

Voltage	I [A]	R [Ω]
18	0.20	90
20	0.23	87
25	0.28	89
30	0.31	97

3.2 Validation of Environmental Culture Chamber

During the 2-hours acquisition with MEA-chips filled with PBS, environmental parameters inside the chamber were maintained in an acceptable range Table 3.3 for more than 1 hour, when CO_2 started to malfunction presenting a discrepancy in its readings. Therefore, the acquisition had to stop in order to re-calibrate the sensor.

Thermal readings [Figure 3.1] were in the range from 35 - 39 °C, demonstrating that the ITO heater was capable to contribute to the heating of the surface (voltage applied 30 V, current 0.31 A) as the temperature of the circulating water bath was set in the beginning of the acquisition at 32 °C. Since the surface heater is not feedback controlled, we chose to regulate the temperature of the water bath instead, that had to be reduced in 1.5 °C in order to maintain chamber temperature at more tolerable values. Water condensation was not observed during the acquisition.

Relative humidity percentage values varied from 83 % to 90 %. Before the beginning of this experiment, there was a difficulty to raise system humidity values. This problem was bypassed adding small petri dishes filled with water inside the chamber.

CO_2 measurements were in the range of 4 - 6 %, presenting some altered values after the first hour of acquisition. Thus, CO_2 was re-calibrated (fresh-air calibration) in order for it to return to its normal condition. FEP membrane showed to not affect CO_2 measures.

Table 3.3: Environmental parameters values during 1-hour acquisition inside the chamber, including mean, standard deviation, minimum and maximum values

Environmental parameters	Mean	SD	Min	Max
T [°C]	37.31	1.26	35.31	39.11
RH [%]	68.72	2.01	83.67	90.34
CO_2	5.32	0.58	4.01	6.30

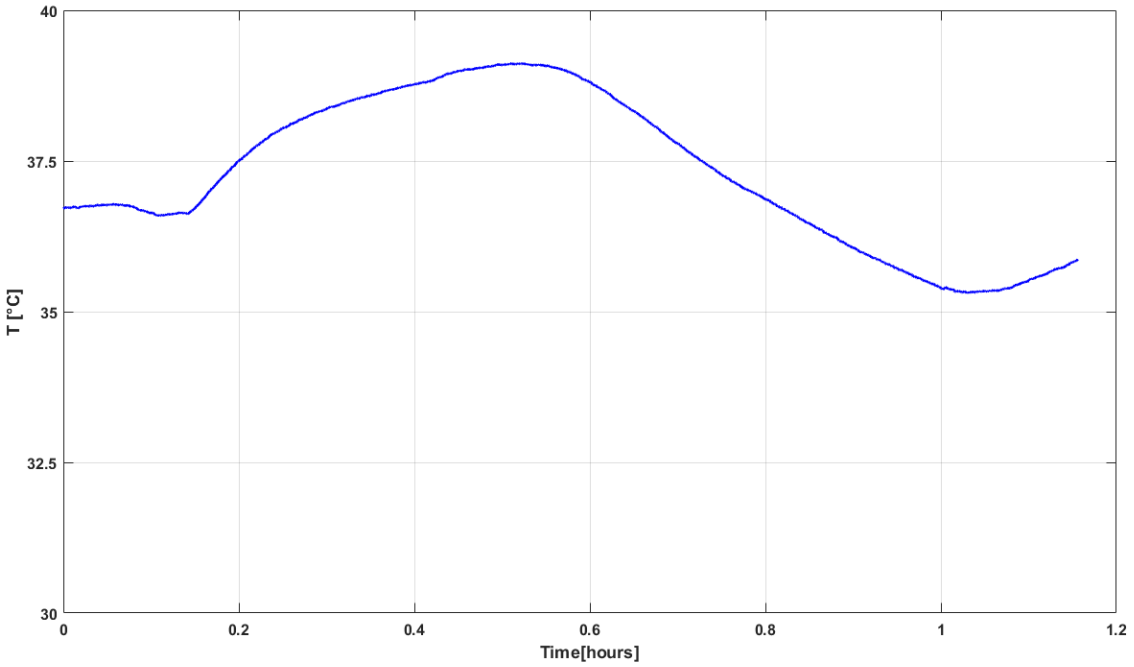

Figure 3.1: Temperature values inside the chamber during 1-hour acquisition

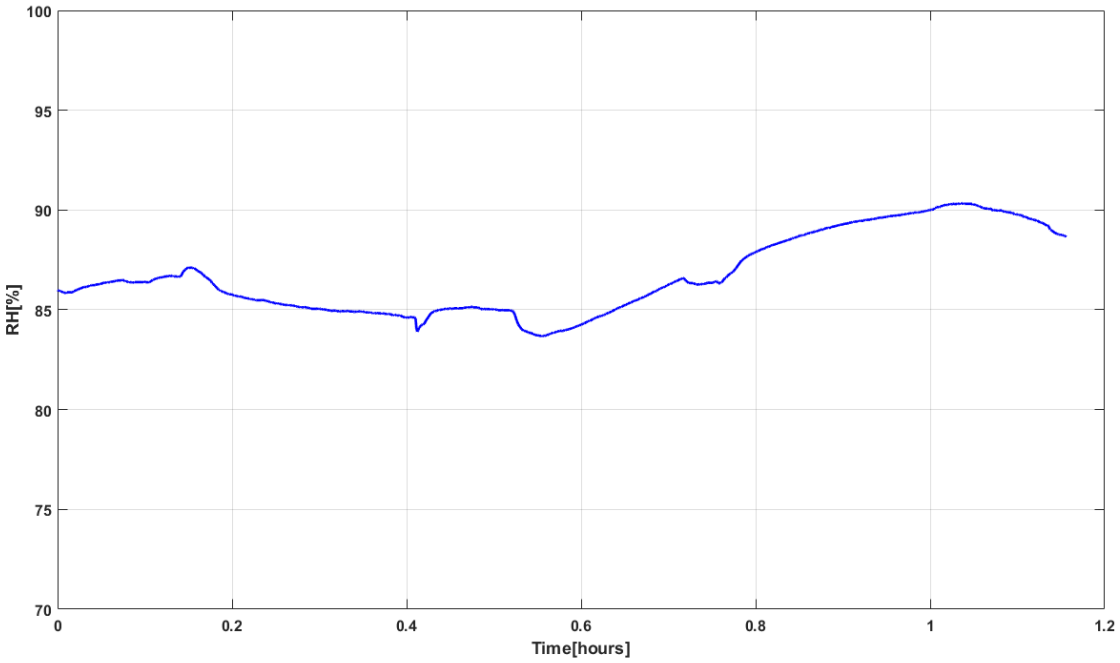

Figure 3.2: Relative humidity values in percentage inside the chamber during 1-hour acquisition

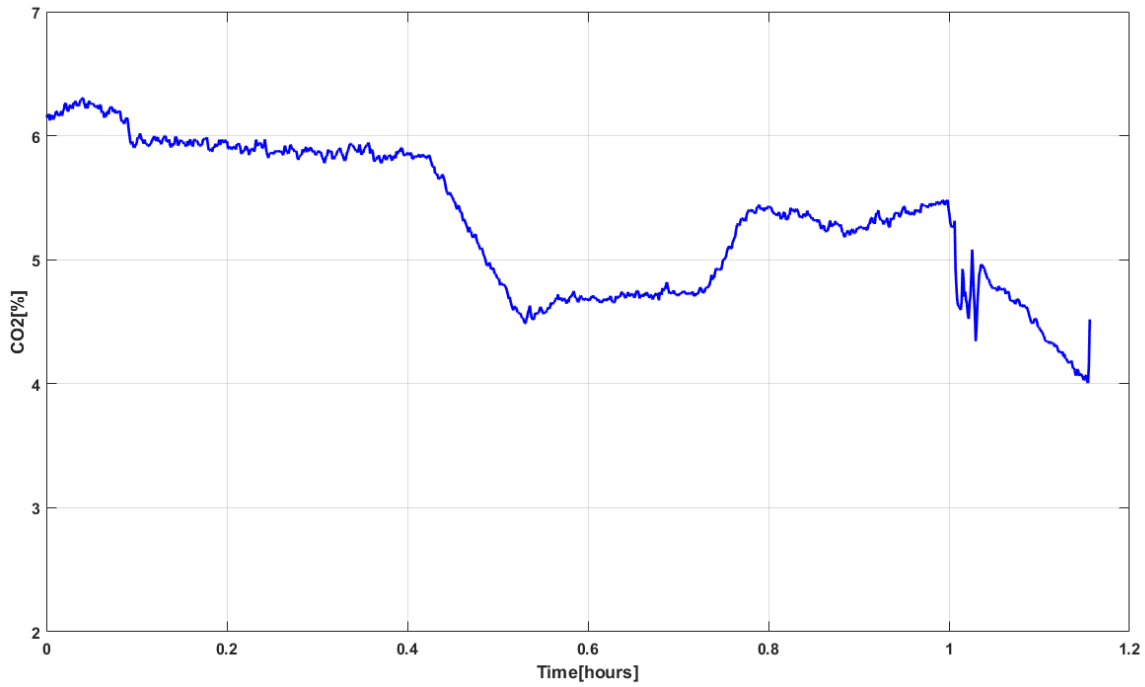

Figure 3.3: Carbon dioxide partial pressure in percentage inside the chamber during 1-hour acquisition

From the 2 hours data acquisition using MCRack software, 9 files of 5 minutes recordings were obtained (1 file each 15 minutes). Noise voltage observed was ±100 µV, although there was the presence of intermittent noise (false spikes), more frequent on data from 20145 chip. Choosing -7 as standard deviation on the software, this noise was not interpreted as spikes. Data from MCRack longterm window from the first and last 5 minutes of acquisition (after 120 minutes) are presented in Figure 3.4 and Figure 3.5.

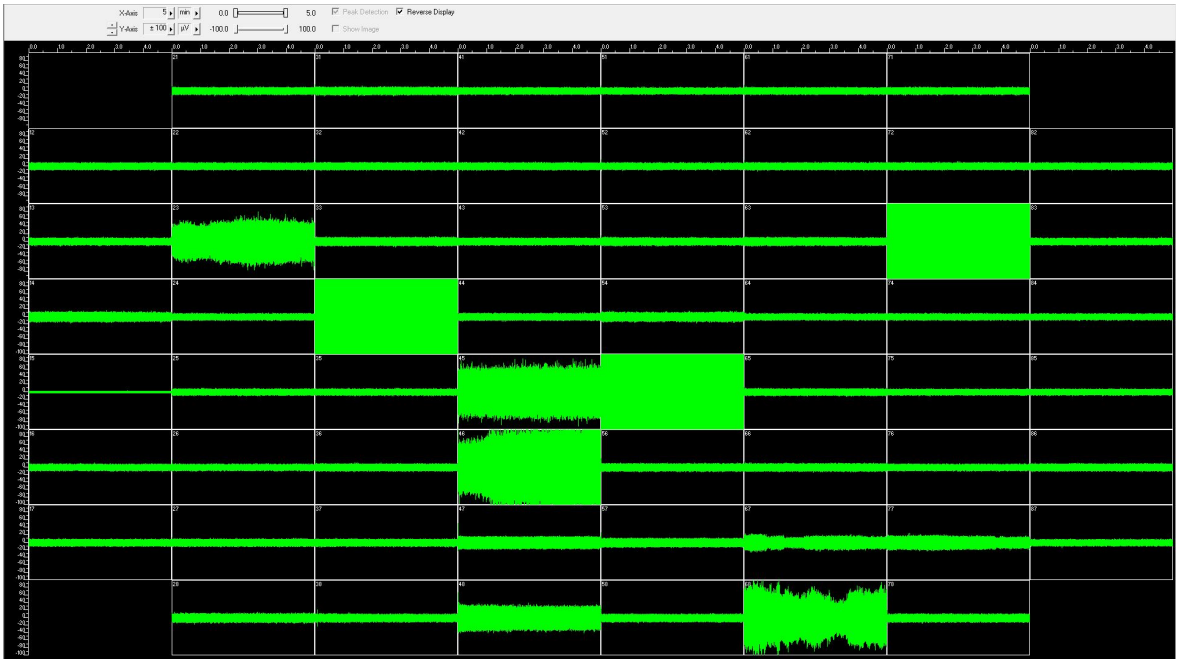

Figure 3.4: Longterm window, ± 100 μV - Data from chip 26004 in the first 5 minutes of acquisition

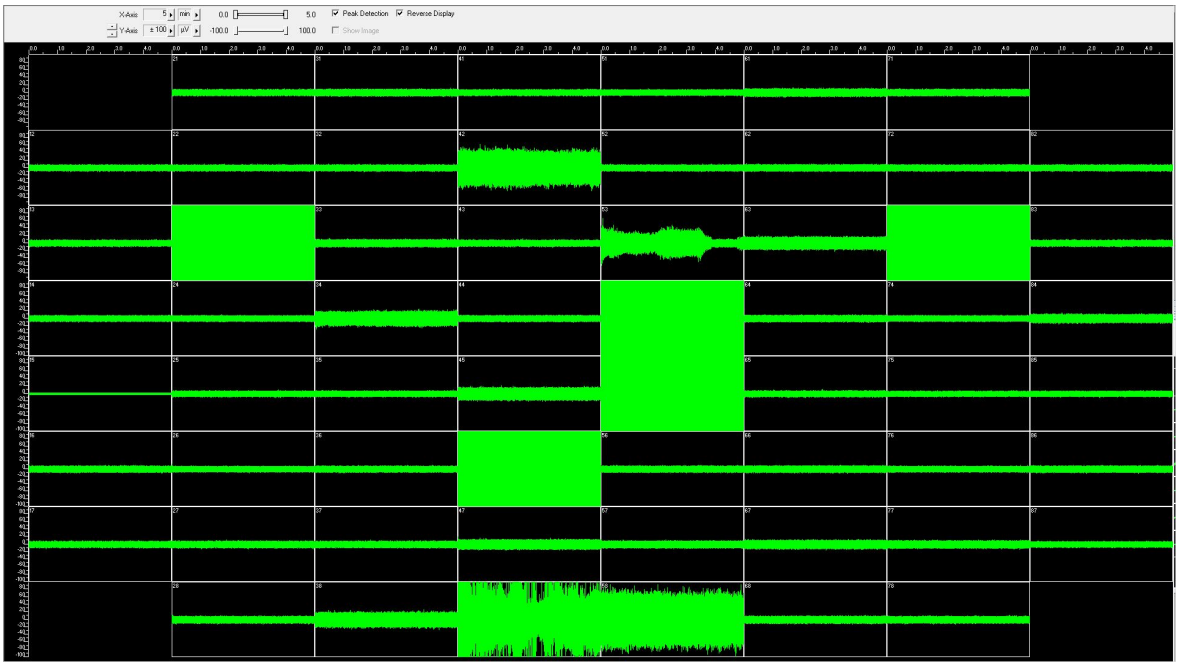

Figure 3.5: Longterm window, ± 100 μV - Data from chip 26004 in the last 5 minutes of acquisition

3.3 Validation of Environmental Control Chamber with Hippocampal Neuronal Cultures

Electrical activity recordings of dissociated hippocampal neurons cultures started in the 8th DIV (day in vitro). Results obtained from MEA standard setup and environmental chamber setup are presented.

3.3.1 Data acquisition with MEA standard setup

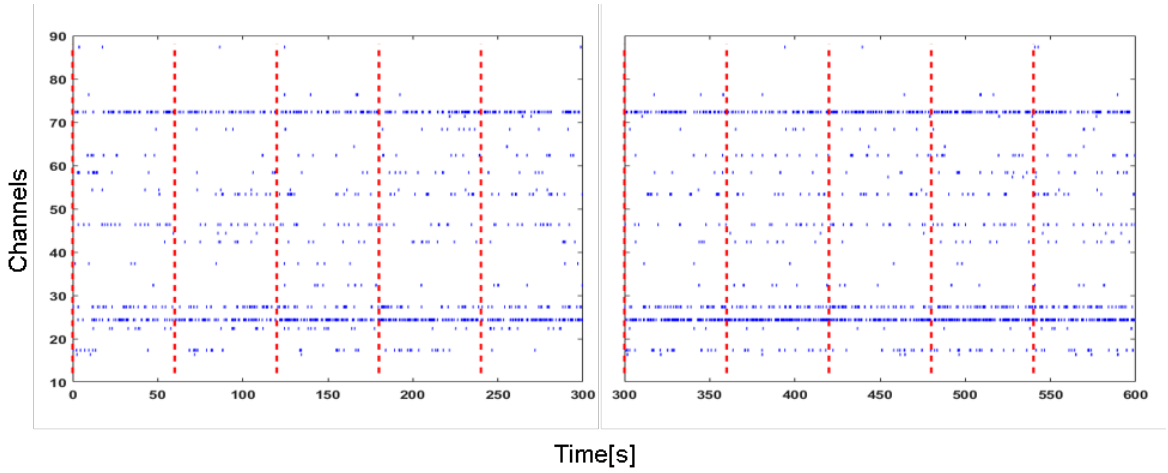

Figure 3.6: MEA1: Raster plots for 10 minutes acquisition using benchmark setup

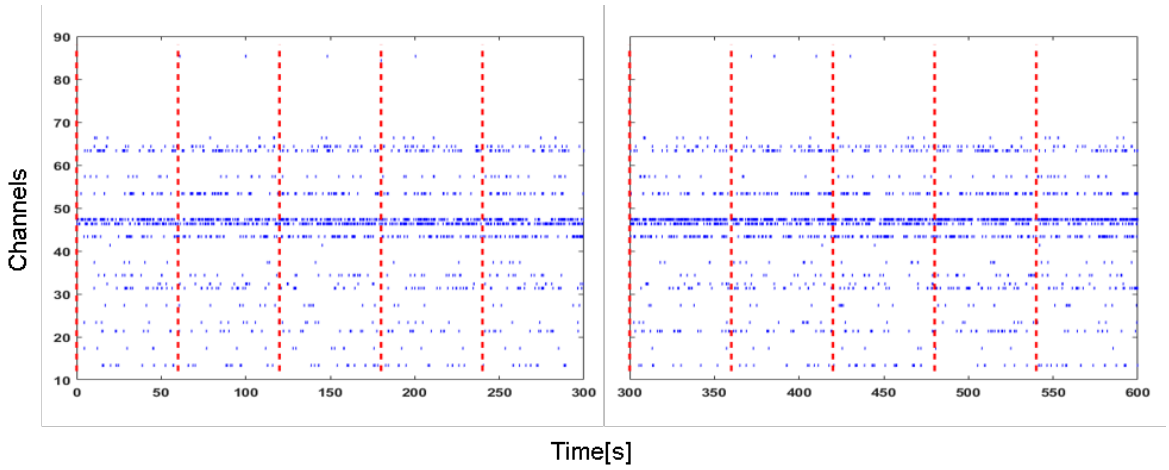

Figure 3.7: MEA2: Raster plots for 10 minutes acquisition using benchmark setup

Detected wave-forms from spike sorter window were obtained in order to assess the existence of spikes.

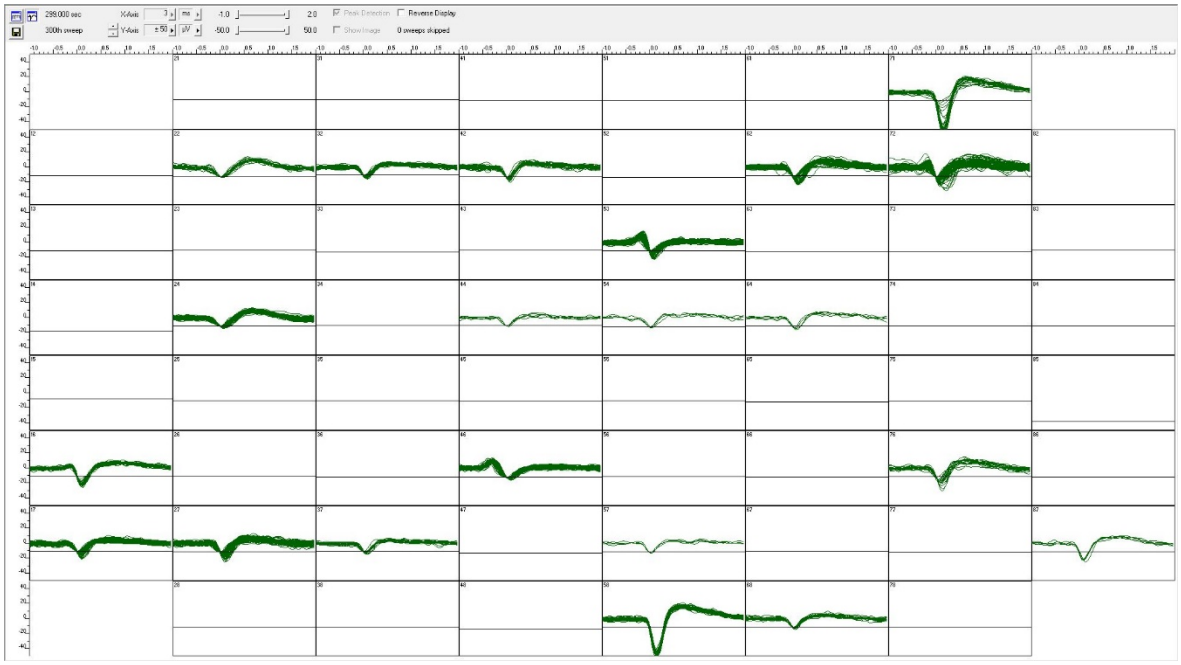

Figure 3.8: MEA1: Spike sorter window ± 50 µV, 7 SD, standard setup

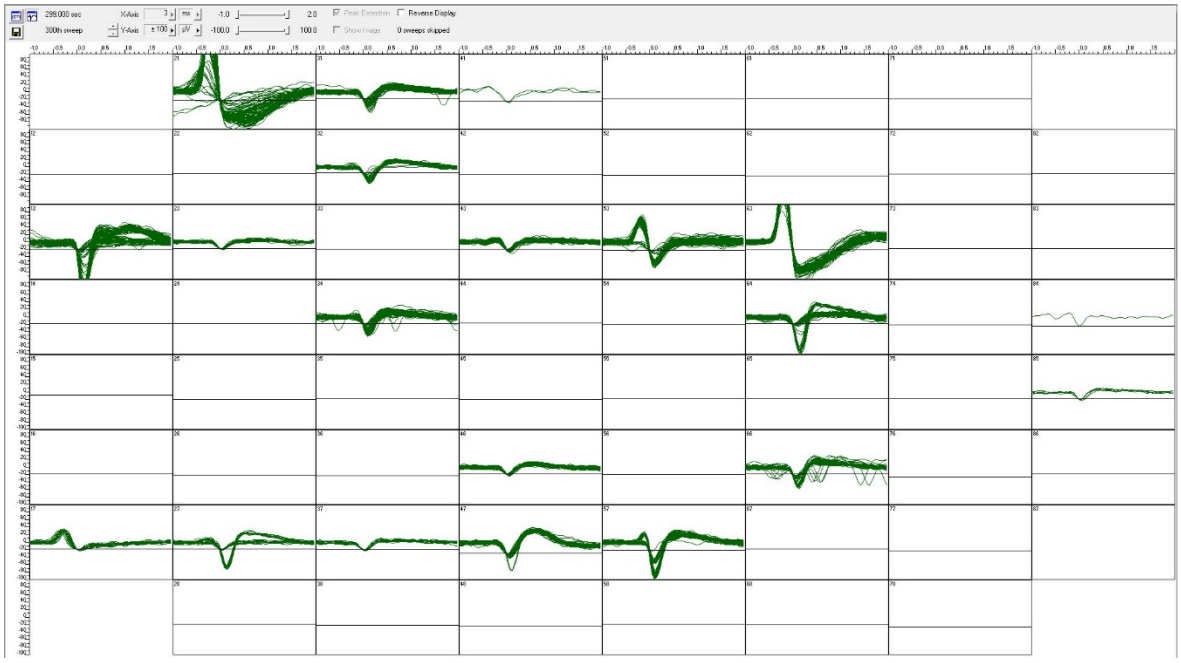

Figure 3.9: MEA2: Spike sorter window ± 100 µV, 7 SD, standard setup

3.3.2 Data acquisition with Environmental Control Chamber

It is important to mention the problems encountered during the experiment. In the first day of recordings some data was lost due to instability of the acquisition system. In the subsequent days, electrical coupling in MEA1 was maintained constant while in MEA2 there was an oscillation, leading to data loss in some channels. Channels that presented a considerable amount of noise or instability were removed from all files regarding the same chip, which affected the number of spikes observed in respect to the standard acquisition.

MEA1

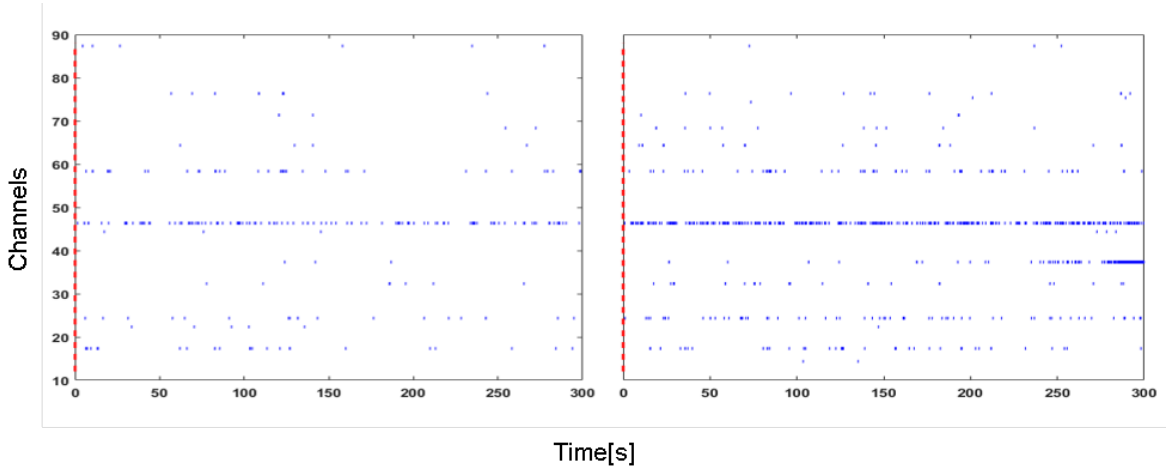

Figure 3.10: MEA1: Raster plots at the beginning and at the end of first hour of acquisition

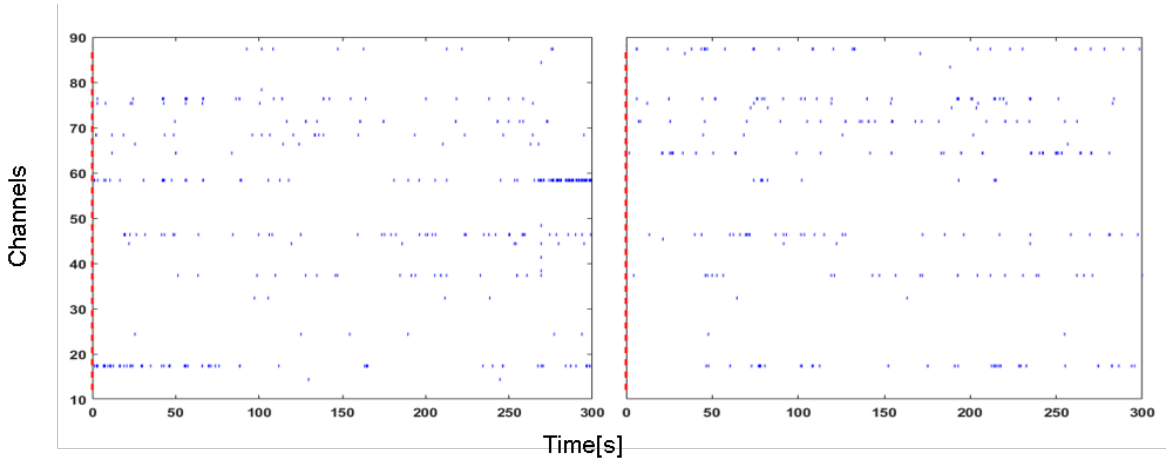

Figure 3.11: MEA1: Raster plots after 16 hours and 28 hours of acquisition inside the chamber

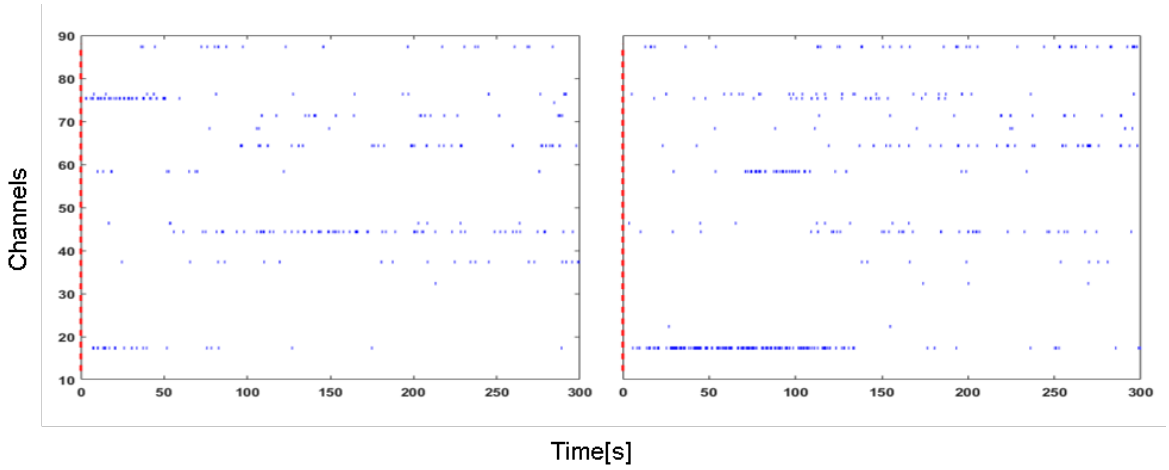

Figure 3.12: MEA1: Raster plots after 40 hours and 52 hours of acquisition inside the chamber

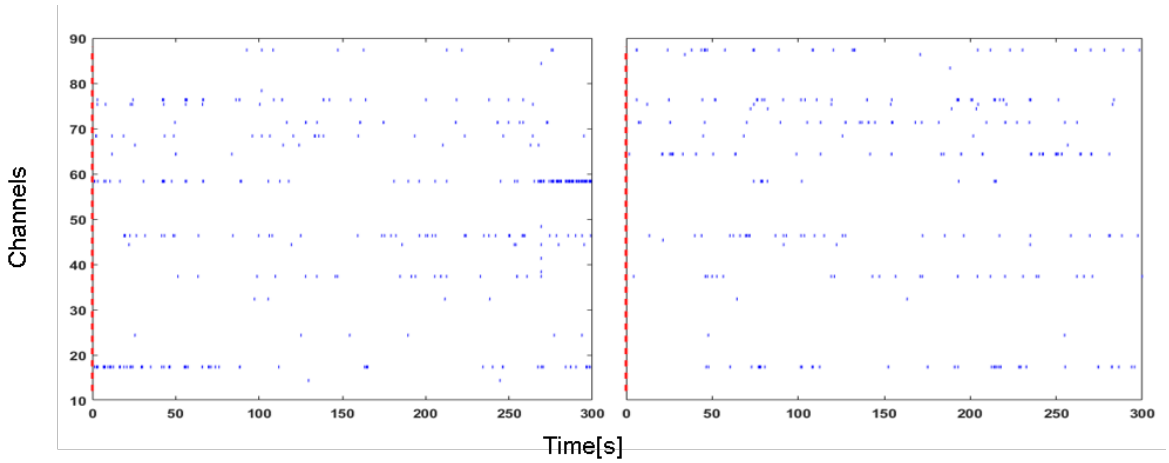

Figure 3.13: MEA1: Raster plots after 64 hours and 76 hours of acquisition inside the chamber

MEA2

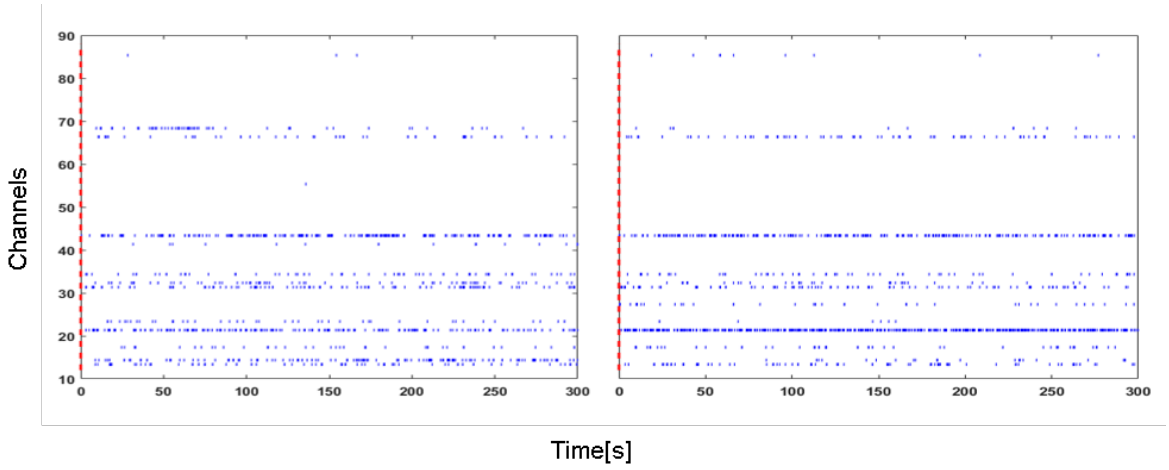

Figure 3.14: MEA2: Raster plots at the beginning and at the end of first hour of acquisition

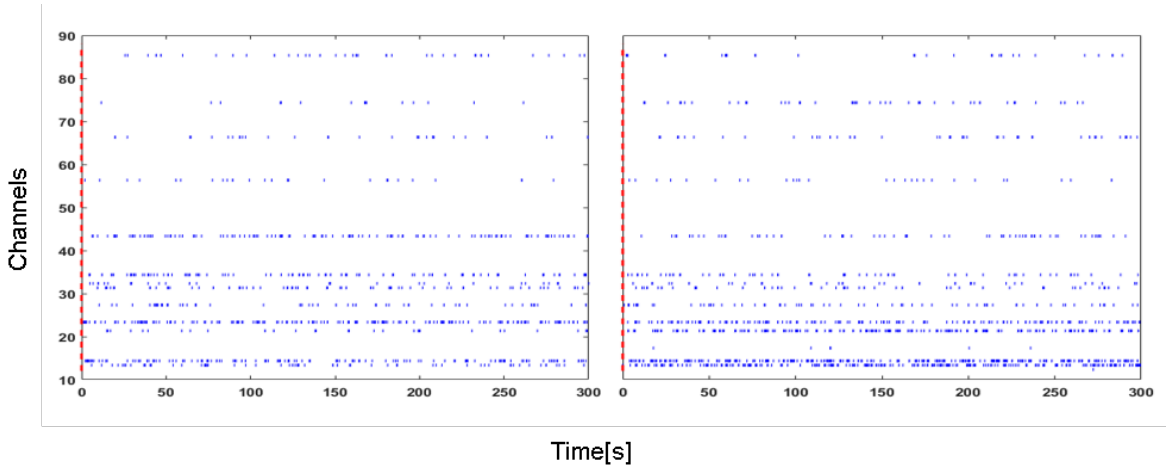

Figure 3.15: MEA2: Raster plots after 16 hours and 22 hours of acquisition inside the chamber

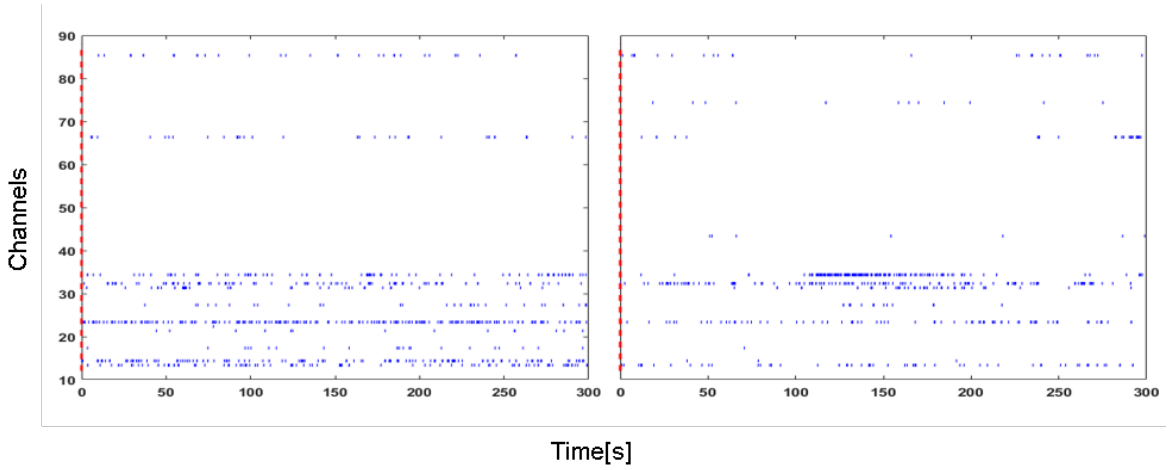

Figure 3.16: MEA2: Raster plots after 26 hours and 45 hours of acquisition inside the chamber

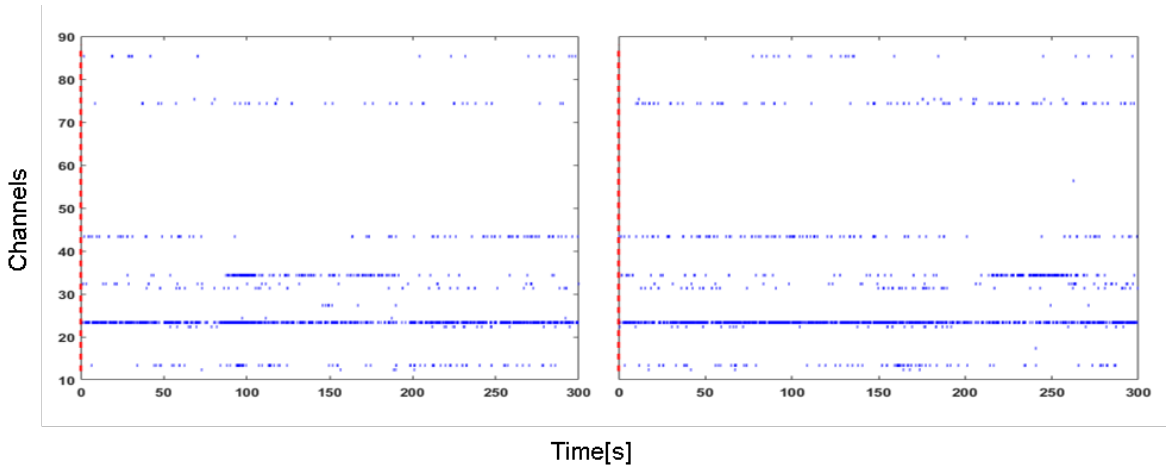

Figure 3.17: MEA2: Raster plots after 82 hours and 84 hours of acquisition inside the chamber

Spike sorter windows from both chips MEA1 and MEA2 in the first hour of acquisition are presented below.

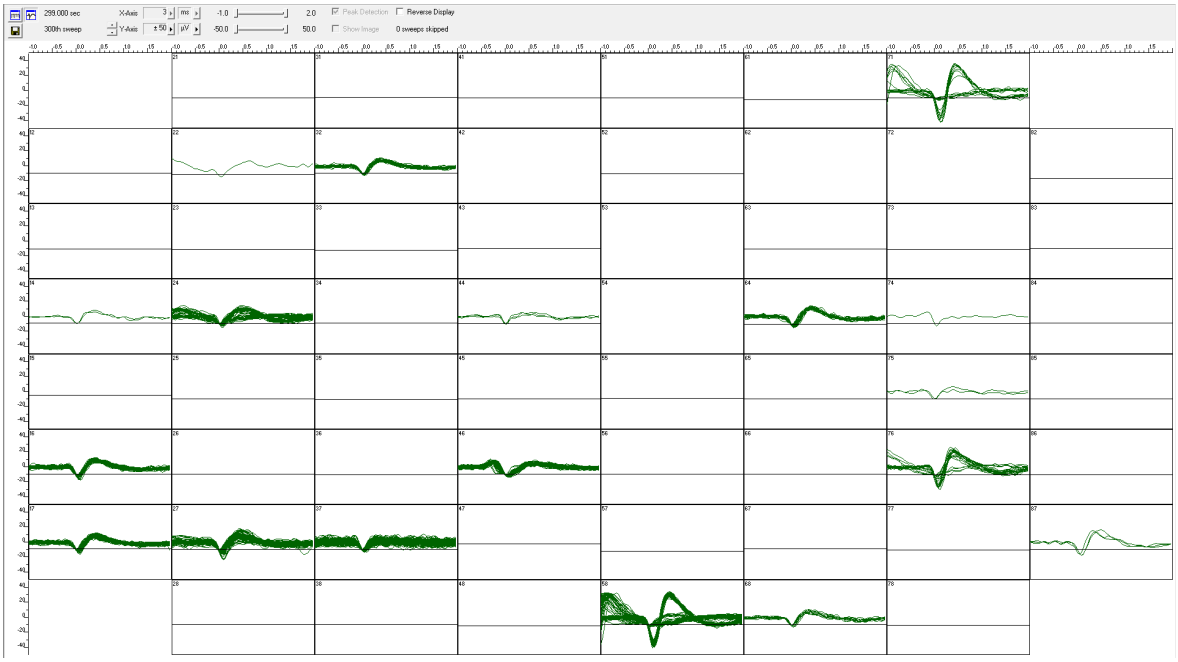

Figure 3.18: MEA1: Spike sorter window ± 50 µV, 7 SD, custom setup

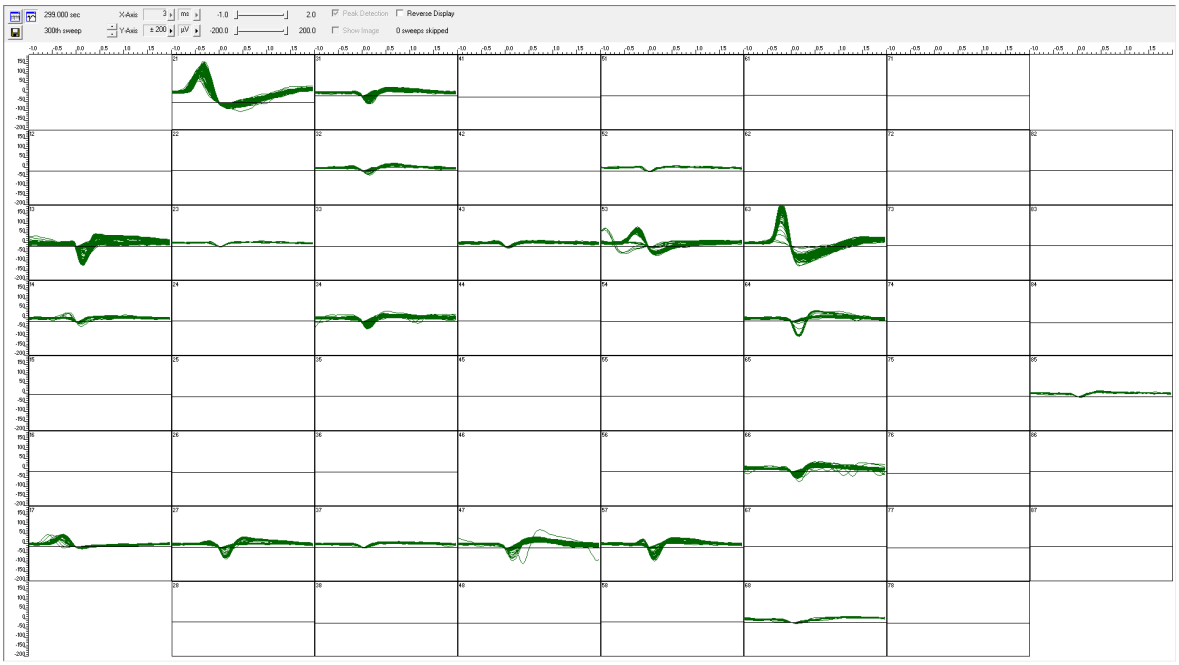

Figure 3.19: MEA2: Spike sorter window ± 200 µV, 7 SD, custom setup

3.3.3 Environmental control

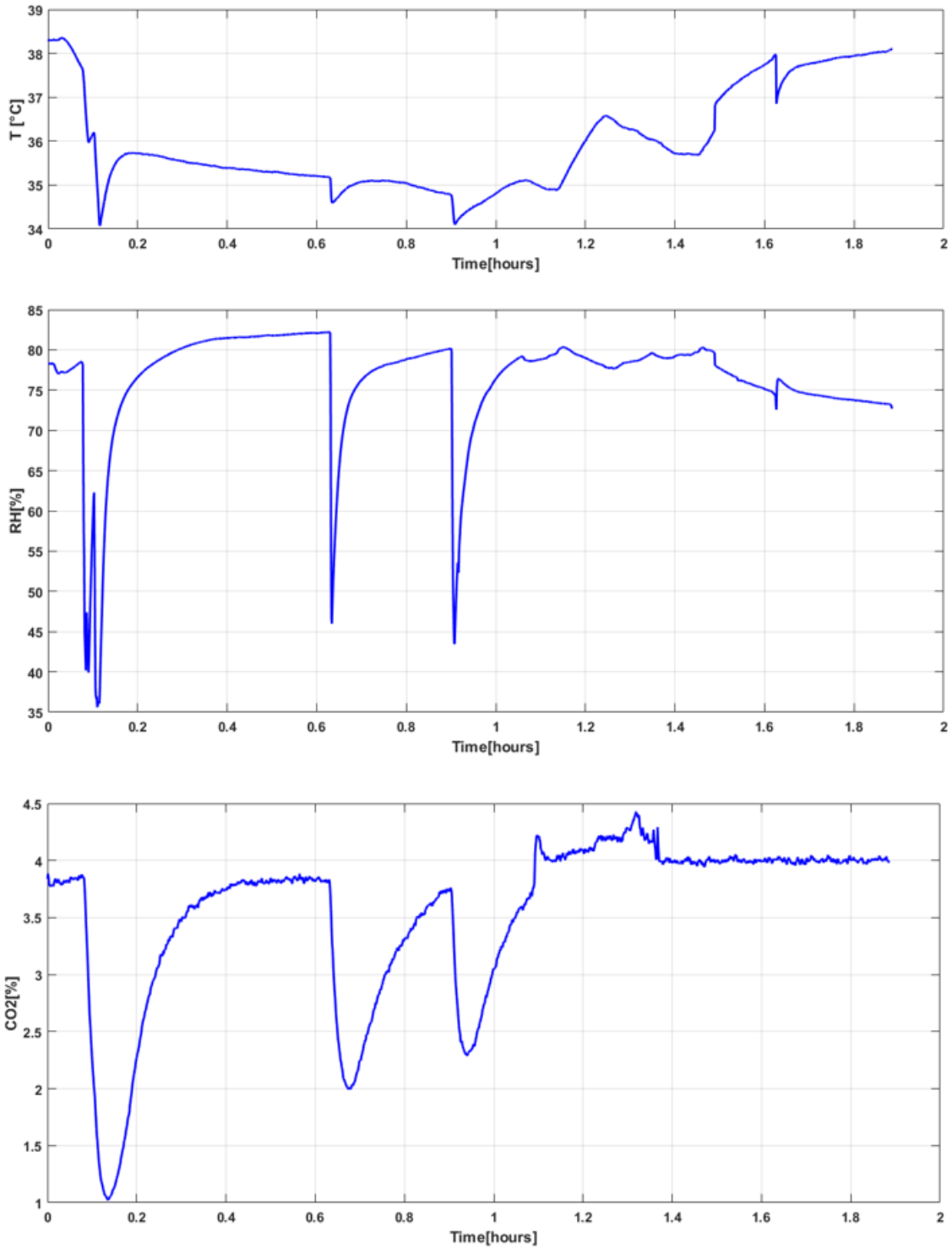

Figure 3.20: Performance of environmental chamber: temperature, relative humidity and CO_2 concentration values

Before the onset of the recordings with the neuron cultures, the performance of the system to stabilize its environmental parameters was observed, as can be seen from Figure 3.20. The chamber was opened in three moments: first due to PBS chips being removed (time instant between 0 - 0.2 hr; then second and third correspond respectively to the moments when MEA1 and MEA2 were inserted inside the chamber (time instants between 0.6 - 0.8 hr and 0.8 - 1 hr).

Environmental parameters measured after the start of the acquisitions are showed below. Due to data lost on Serial Monitor (Arduino), we could not follow the behavior of the system along the entire time of recordings. Although, parameters showed to be stable during most of the time.

Variances in temperature (around 1.5 °C) were noticed when electronic equipment was removed to fix instability problems of acquisition [Figure 3.21].

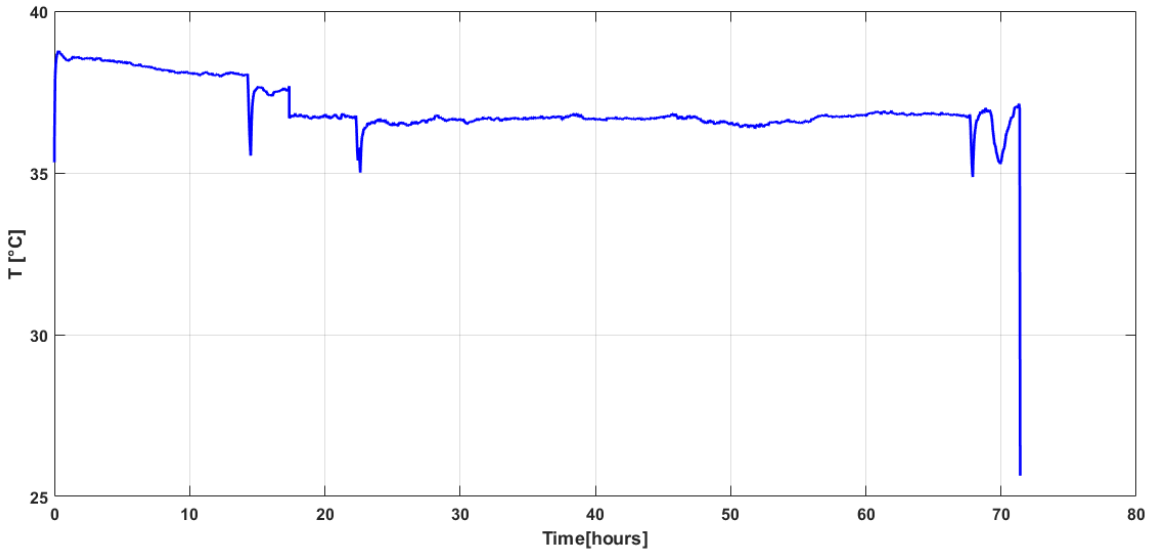

Figure 3.21: Environmental chamber: temperature values during acquisition

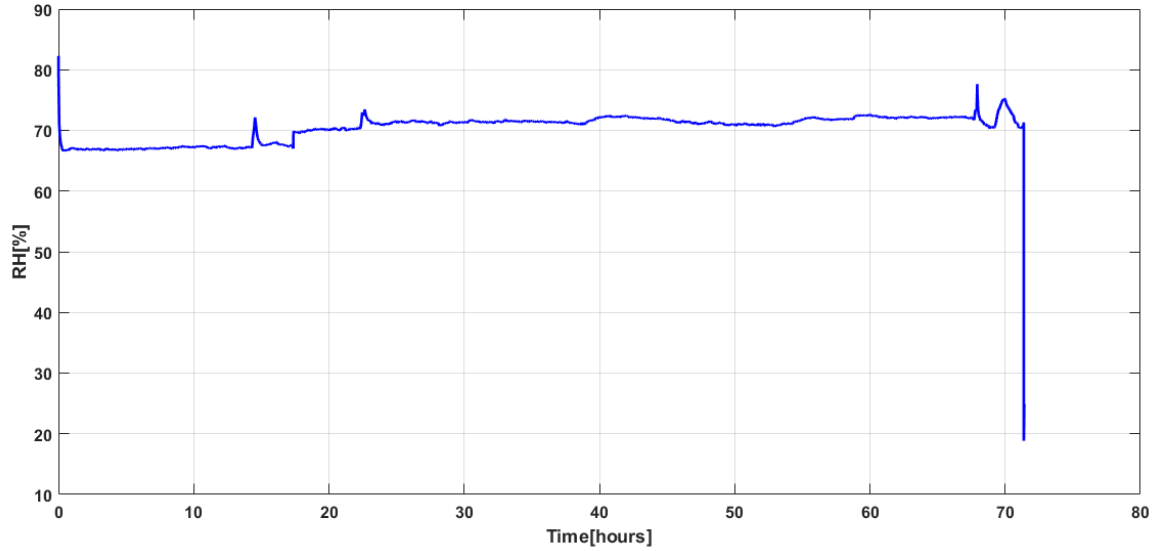

Figure 3.22: Environmental chamber: relative humidity [%] values during acquisition

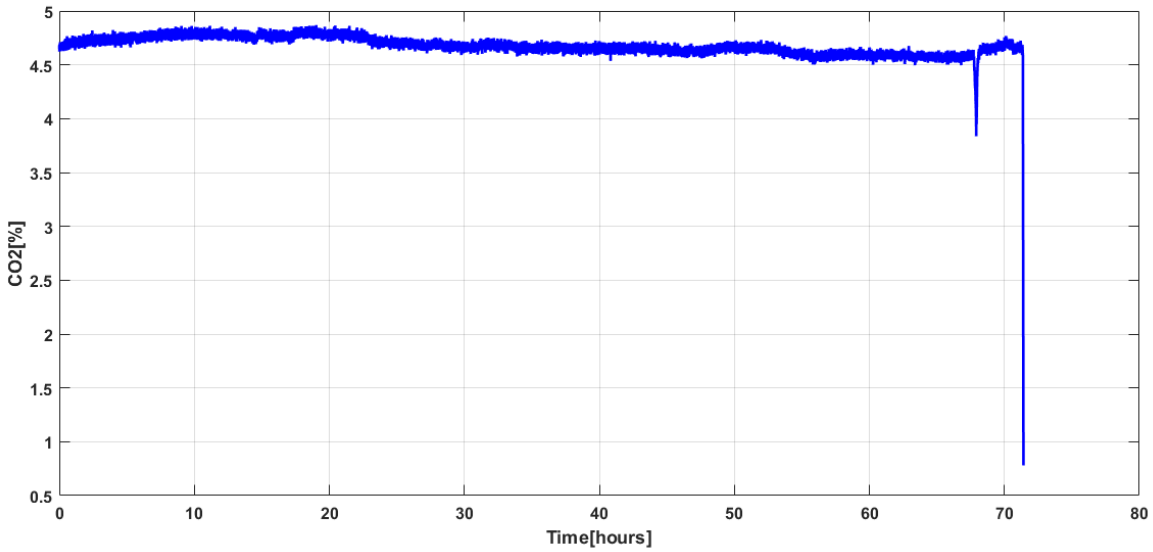

Figure 3.23: Environmental chamber: carbon dioxide partial pressure [%] during acquisition

From Table 3.4 presented below, it is possible to notice that in general, temperature and CO_2 were maintained in the desired range. However, relative humidity was found to be lower in respect to the set point required (at least 90 %), fact that was compensated by the use of the FEP membrane[1].

Table 3.4: Environmental parameters values during long-term acquisition inside the chamber, including mean, standard deviation, minimum and maximum values

Environmental parameters	Mean	SD	Min	Max
T [°C]	37.00	0.72	34.87	38.77
RH [%]	70.46	2.27	66.61	82.28
CO_2	4.68	0.10	3.84	4.87

3.3.4 Network parameters

Network parameters extracted for both chips MEA1 and MEA2 were: total number of spikes, mean number of bursts, burst duration and percentage of spikes in burst. Those values and the relationship between them for both chips MEA1 and MEA2 are showed.

From the results, it was possible to notice an increased spiking activity at the beginning of the acquisitions. This may be due to the abrupt changes in the environmental conditions when moving the MEA-chips from the standard setup to the custom one.

MEA1

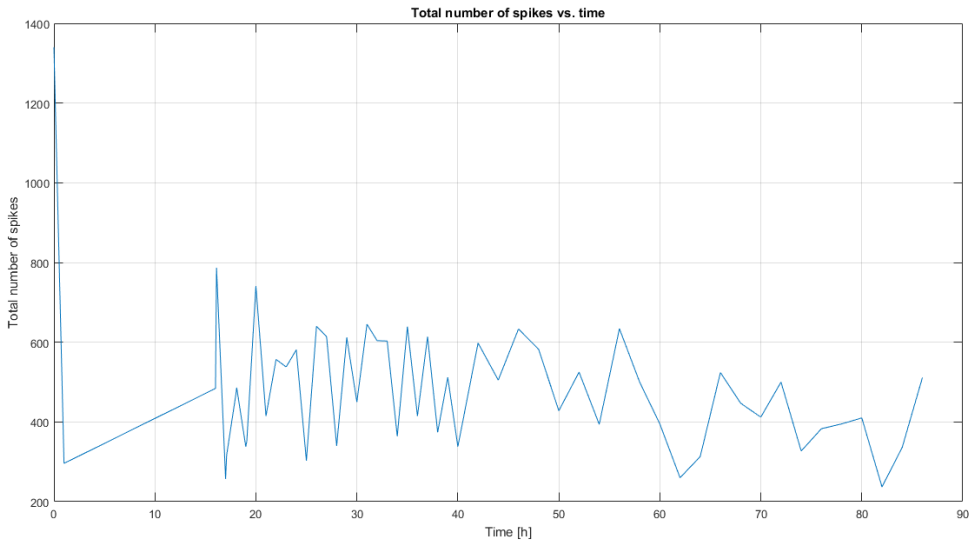

Figure 3.24: MEA1: Total number of spikes in time along the acquisition

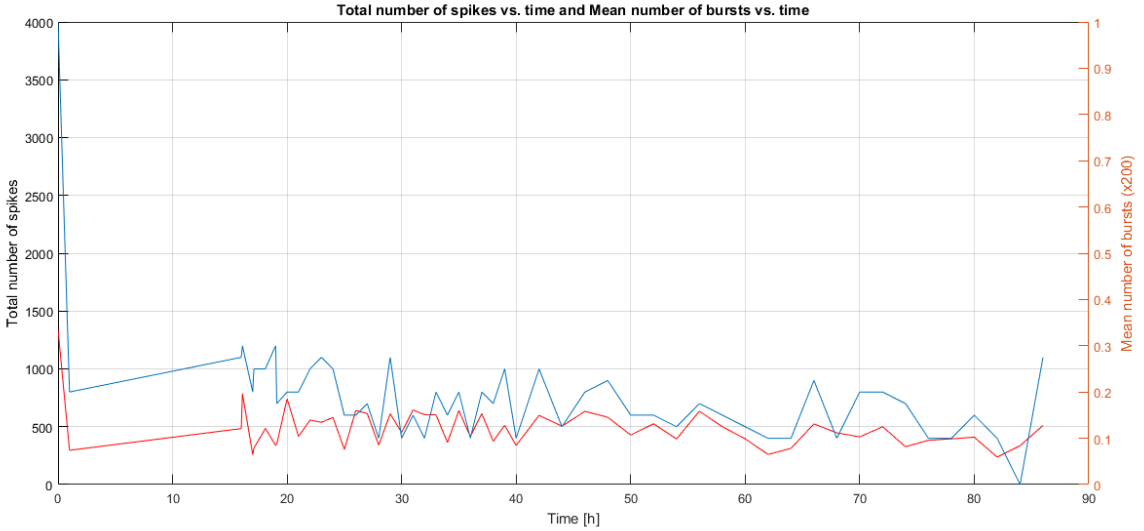

Figure 3.25: MEA1: Total number of spikes (in blue) and mean number of bursts (in red) in time

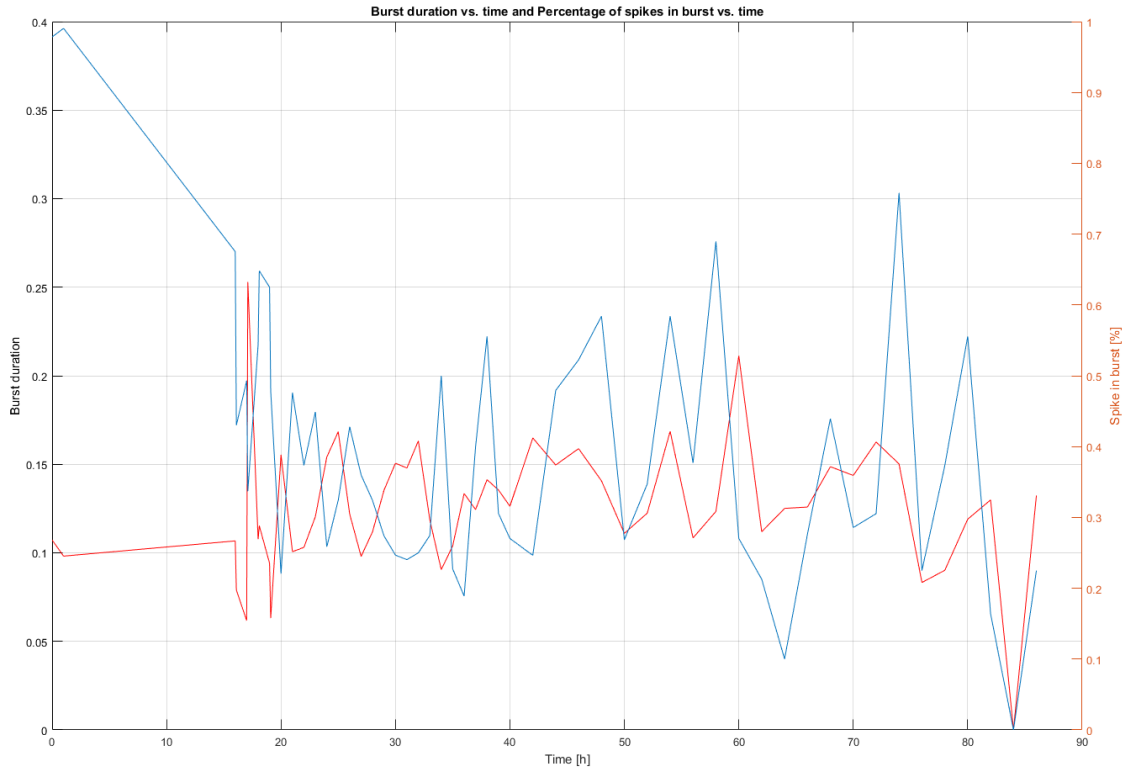

Figure 3.26: MEA1: Burst duration (in blue) and percentage of spikes in burst (in red) in time

MEA2

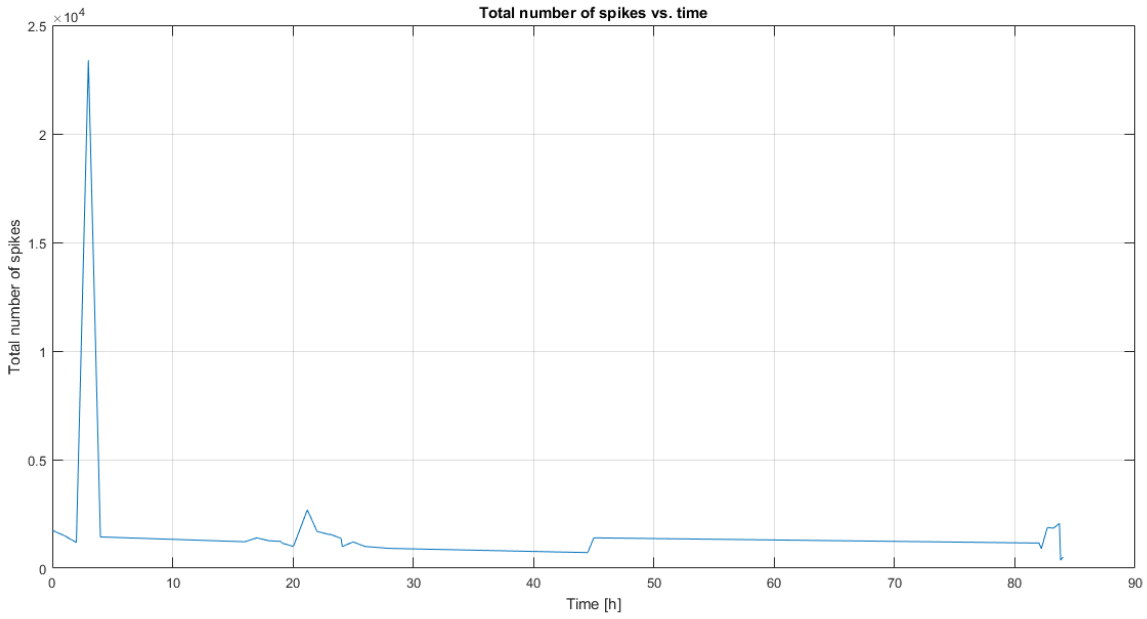

Figure 3.27: MEA2: Total number of spikes in time along the acquisition

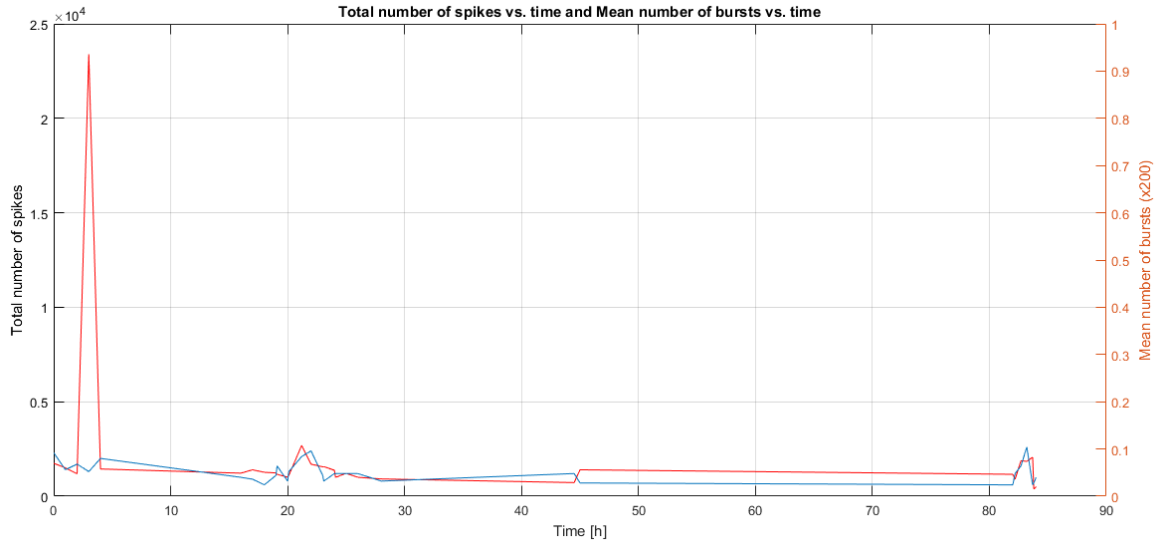

Figure 3.28: MEA2: Total number of spikes (in blue) and mean number of bursts (in red) in time

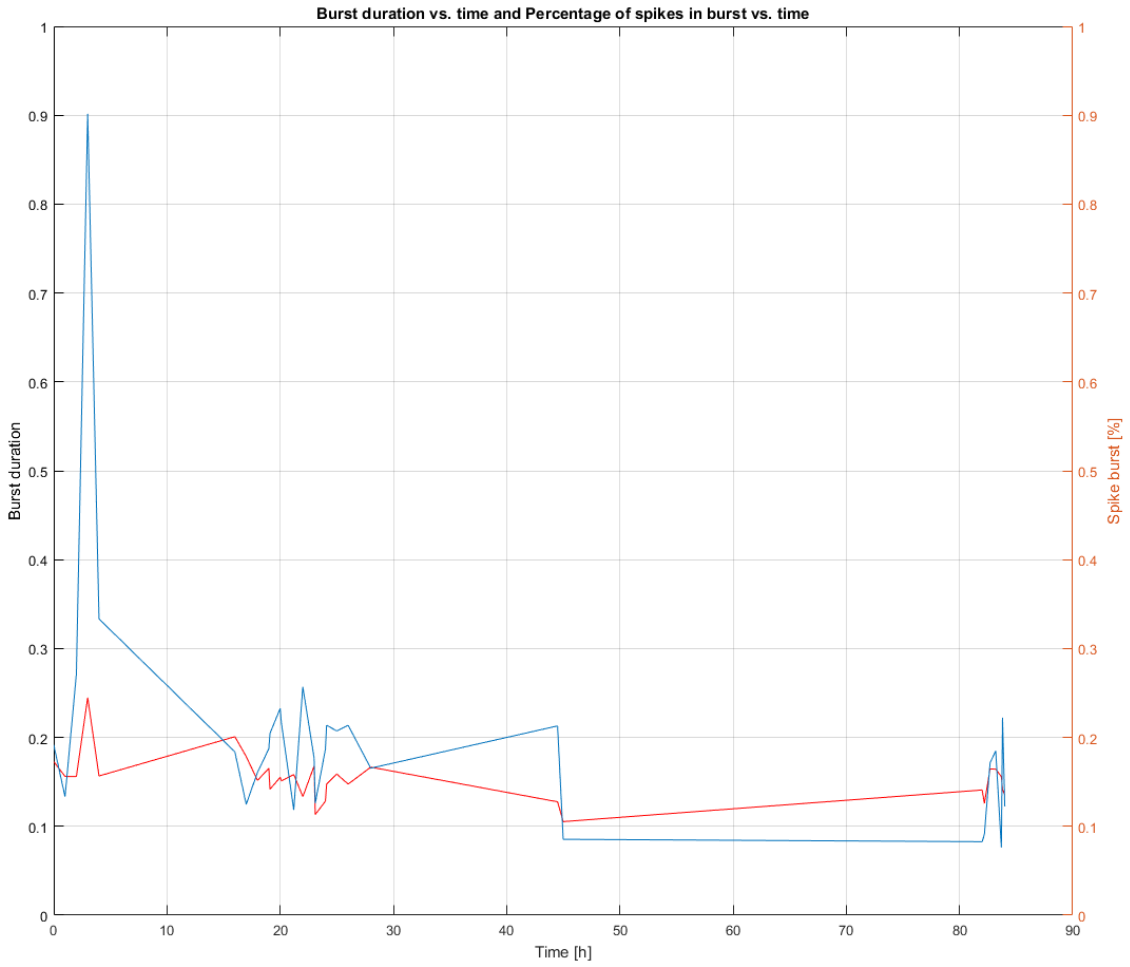

Figure 3.29: MEA2: Burst duration (in blue) and percentage of spikes in burst (in red) in time

3.3.5 Pharmacological experiment

Spiking activity after application of 100 nM of Tetrodotoxin (TTX) to MEA1 chip in three different time instants is showed in Figure 3.30. Recordings of 5-minutes were performed to follow the electrical activity along the experiment.

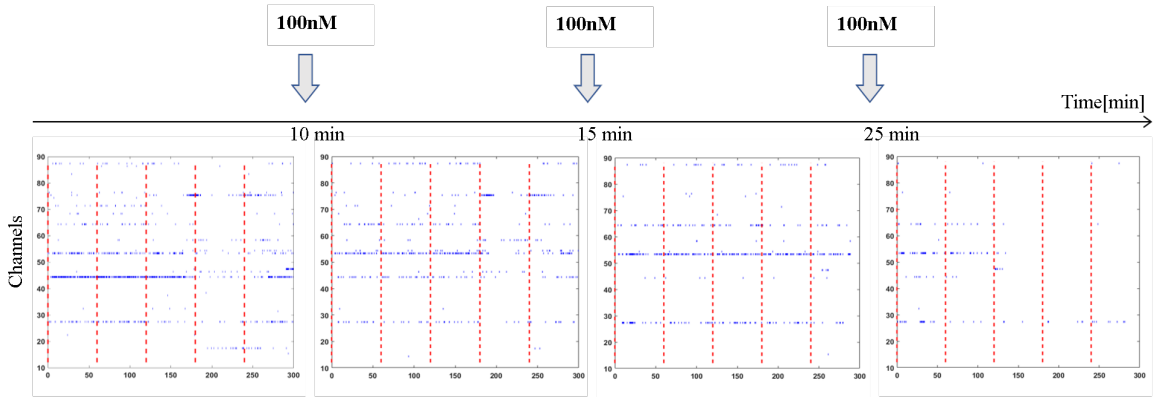

Figure 3.30: MEA1 Raster plots obtained before and after events of 100 nM Tetrodotoxin (TTX) administration

The decrease in the number of spikes in time is evidenced on the plot total number of spikes versus time showed in Figure 3.31.

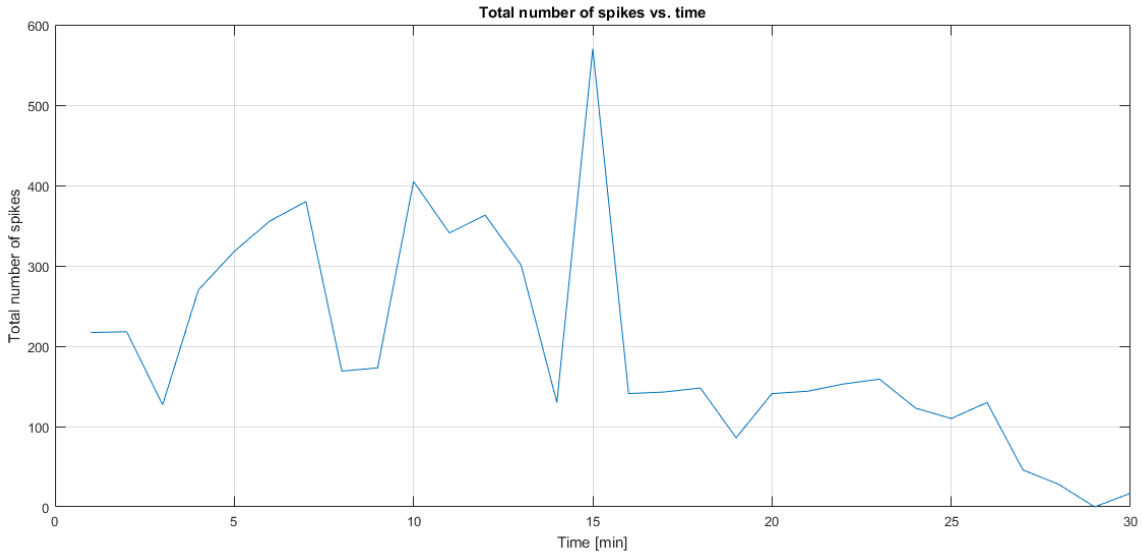

Figure 3.31: Total number of spikes during pharmacological experiment

3.3.6 Microscopy images

Microscopy images obtained after the acquisition are presented below. Using those images and MEA layout it is possible to correlate electrodes signal with their position in the chip.

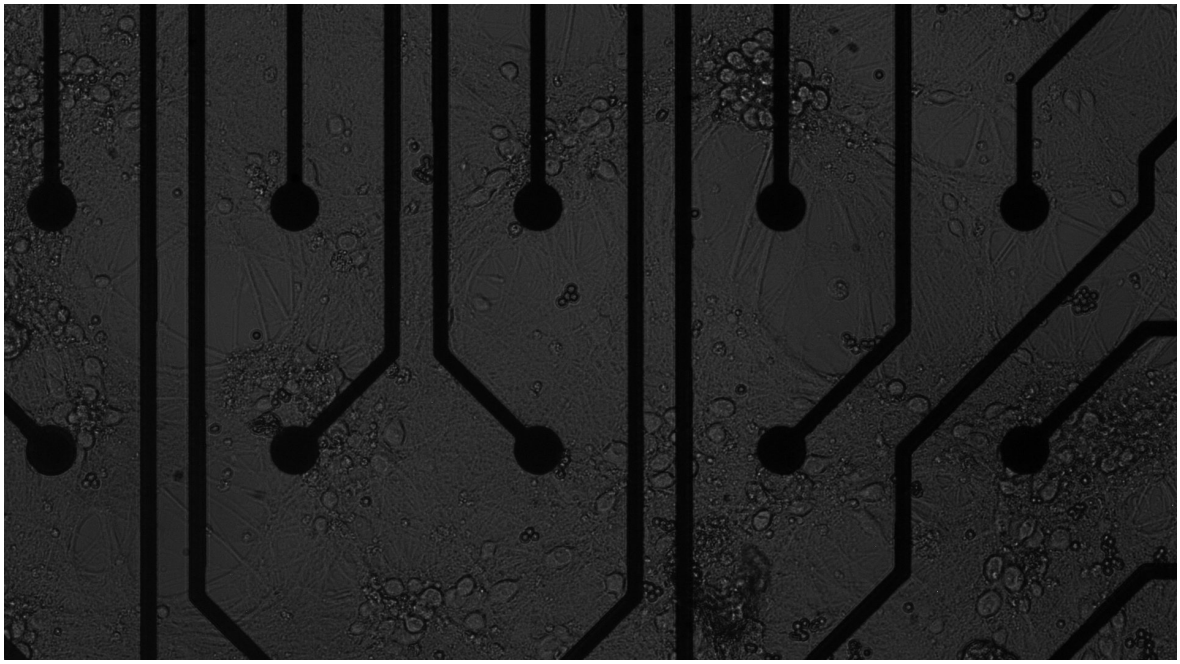

Figure 3.32: MEA1 10x magnification

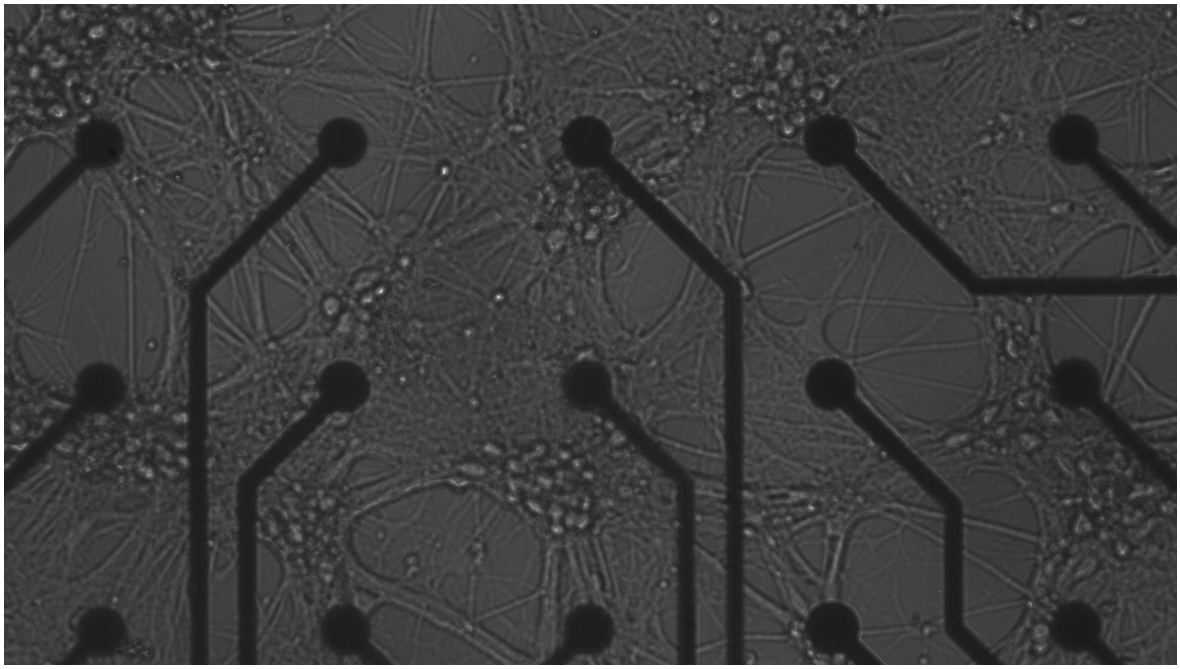

Figure 3.33: MEA2 10x magnification

3.3.7 Channels' analysis

Signal to noise ratio and power spectral density analysis were performed on signals (time stamps) obtained with the standard setup and the custom setup. Data from channel 58 of MEA1 and channels 13 and 63 of MEA2 at the beginning of the acquisitions was extracted and analyzed.

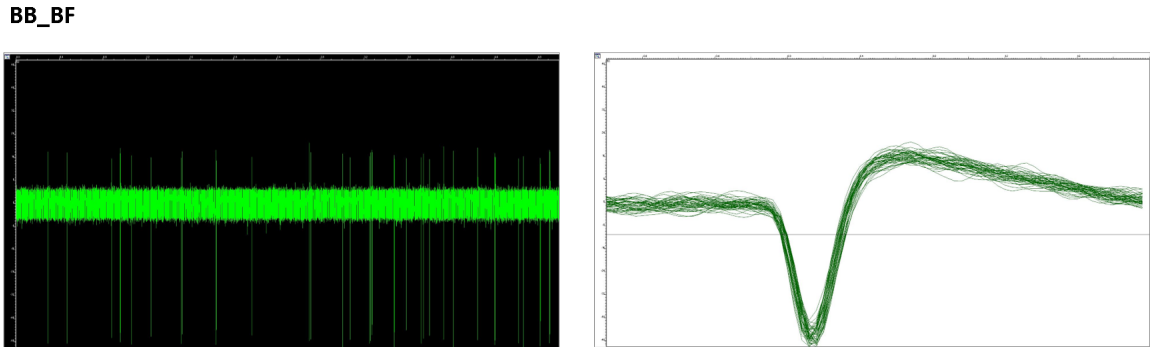

Figure 3.34: Channel 58: Longterm and spike sorter window ± 50 µV, standard setup

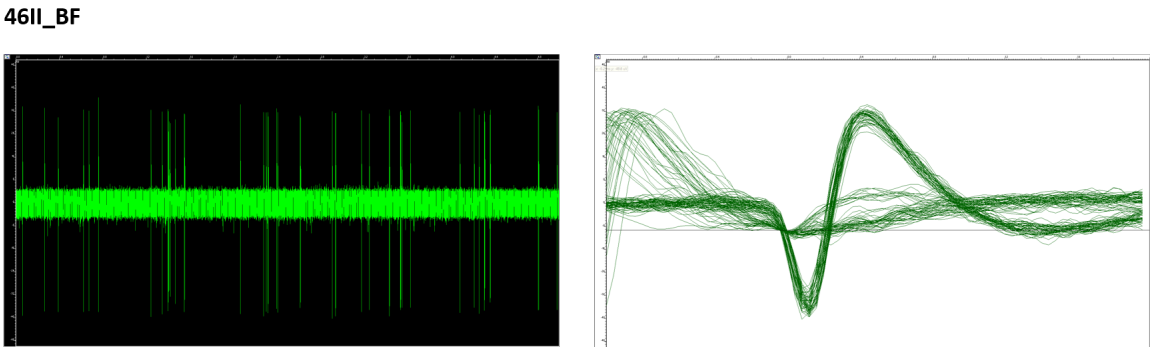

Figure 3.35: Channel 58: Longterm and spike sorter window ± 50 µV, custom setup

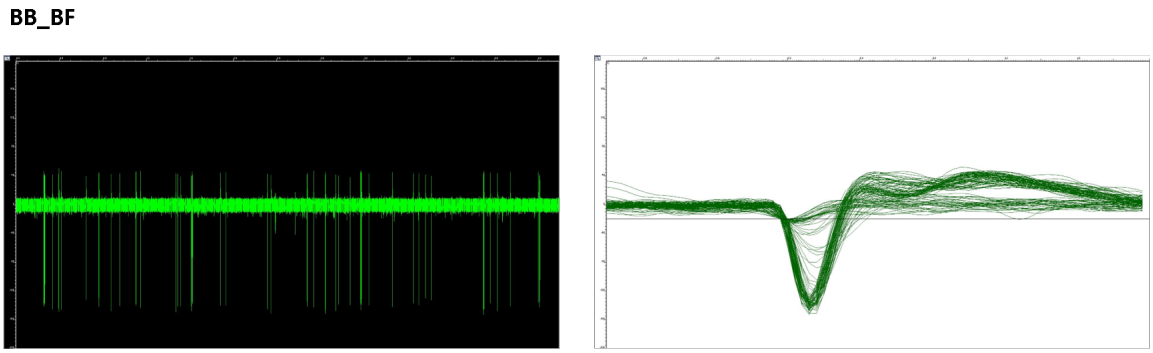

Figure 3.36: Channel 13: Longterm and spike sorter window ± 200 µV, standard setup

92IV_FF

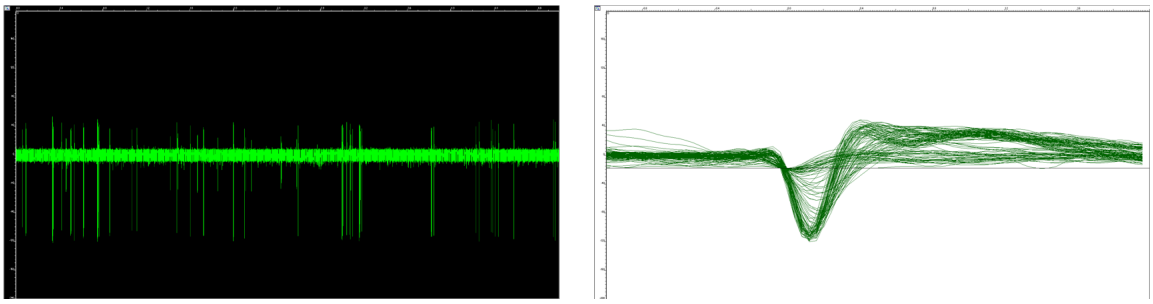

Figure 3.37: Channel 13: Longterm and spike sorter window ± 200 µV, custom setup

BB_BF

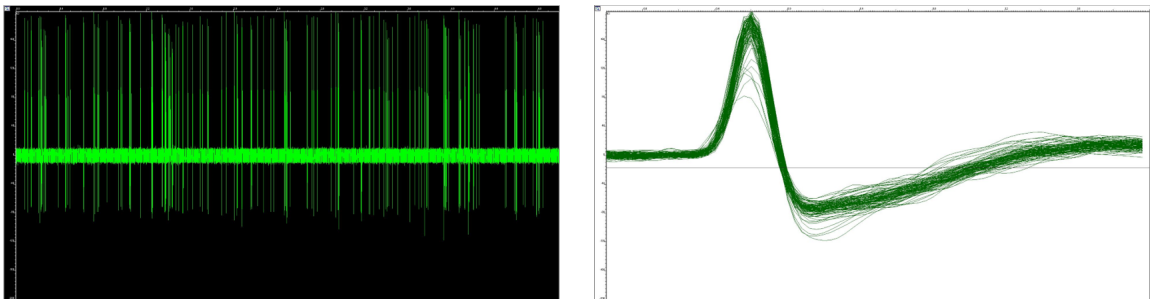

Figure 3.38: Channel 63: Longterm and spike sorter window ± 200 µV, standard setup

92IV_FF

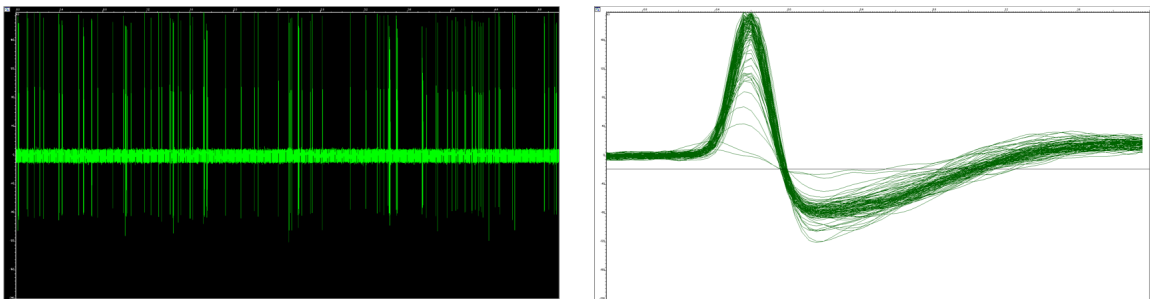

Figure 3.39: Channel 63: Longterm and spike sorter window ± 200 µV, custom setup

Signal to noise ratio analysis

Signal to noise ratio analysis was performed using data from the channels presented in the previous section at the beginning of the acquisitions. Signal waveforms reconstructed with Matlab seem to be coherent with the waveforms obtained with spike sorter. Although, on channel 63, as having a waveform with positive slope, algorithm was not able to properly reconstruct the signal.

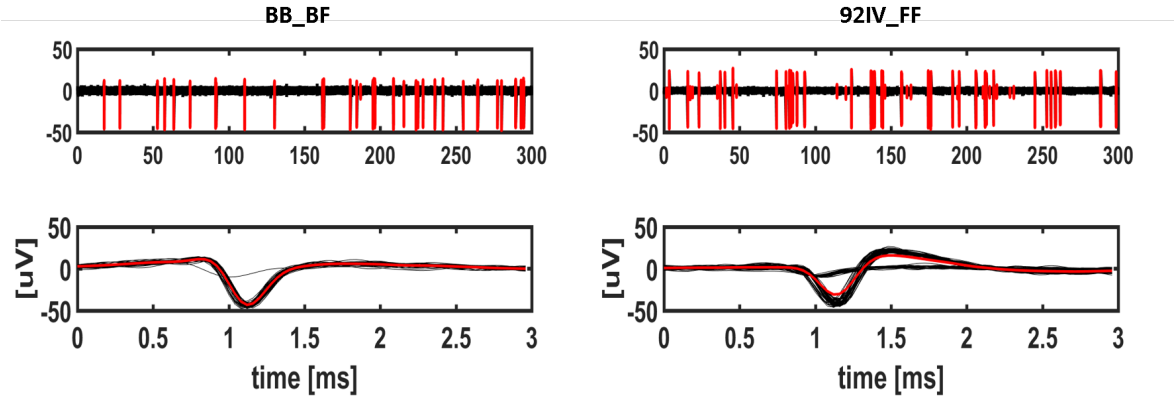

Figure 3.40: Channel 58: Signal to noise ratio, standard setup and 92IV-FF

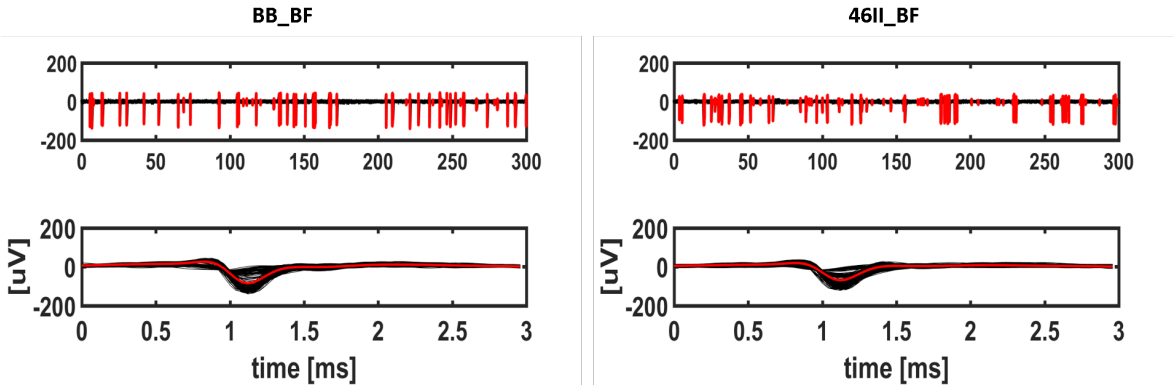

Figure 3.41: Channel 13: Signal to noise ratio, standard setup and 46II-BF

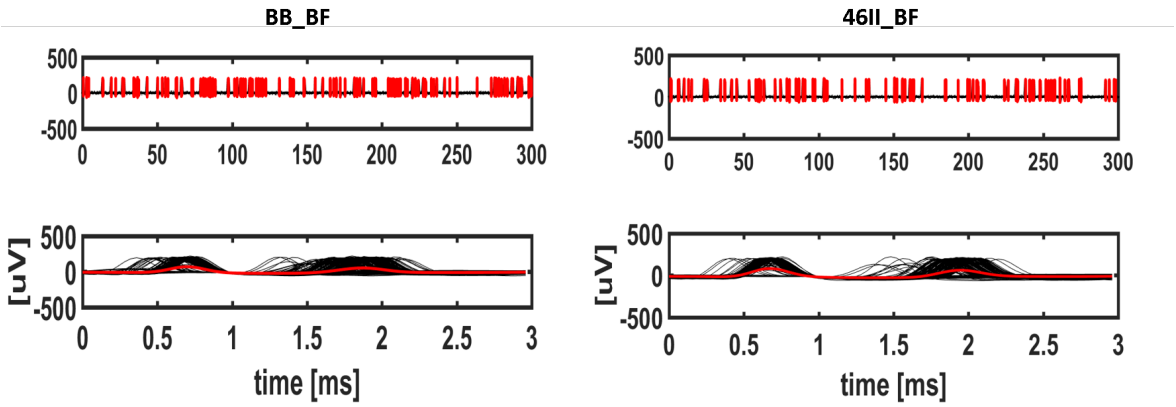

Figure 3.42: Channel 63: Signal to noise ratio, standard setup and 46II-BF

Power spectral density analysis

Frequency content obtained appear to be coherent with the frequency band of spikes (300 Hz – 3000 kHz). We noticed that channel 58 presents a difference in its frequency content when comparing standard and custom setup.

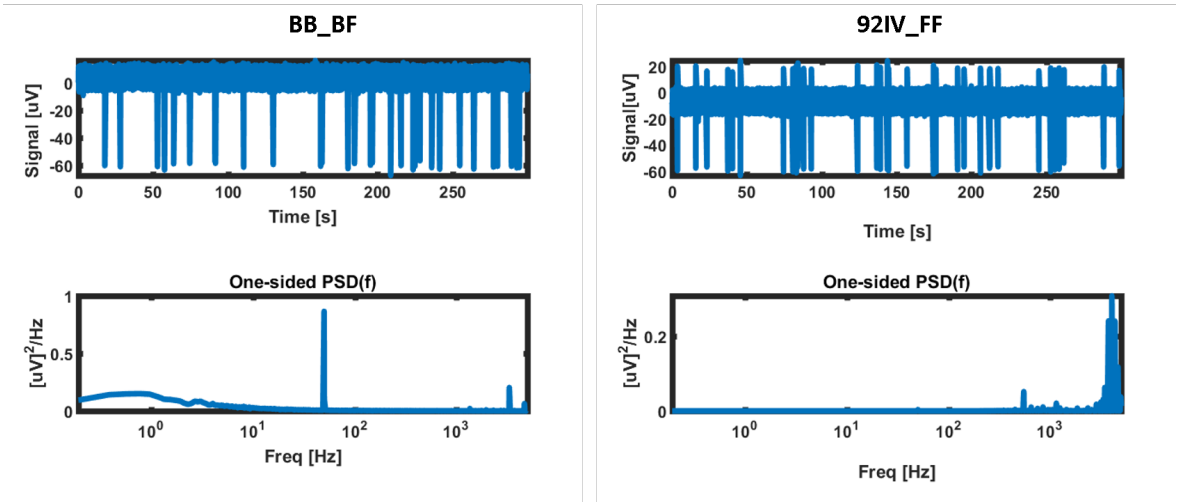

Figure 3.43: Channel 58: Power spectral density, standard setup and 92IV-FF

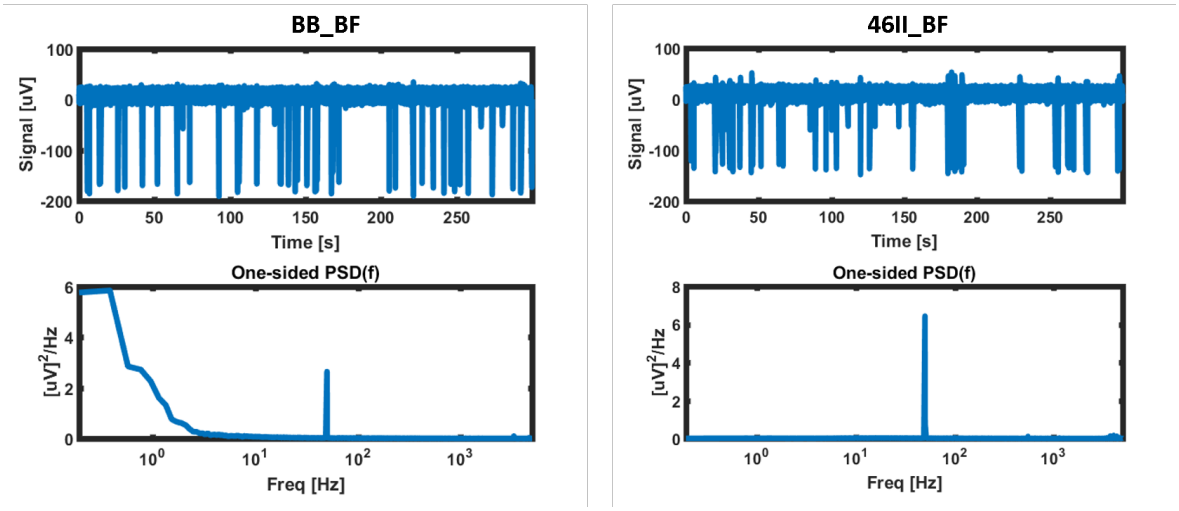

Figure 3.44: Channel 13: Power spectral density, standard setup and 46II-BF

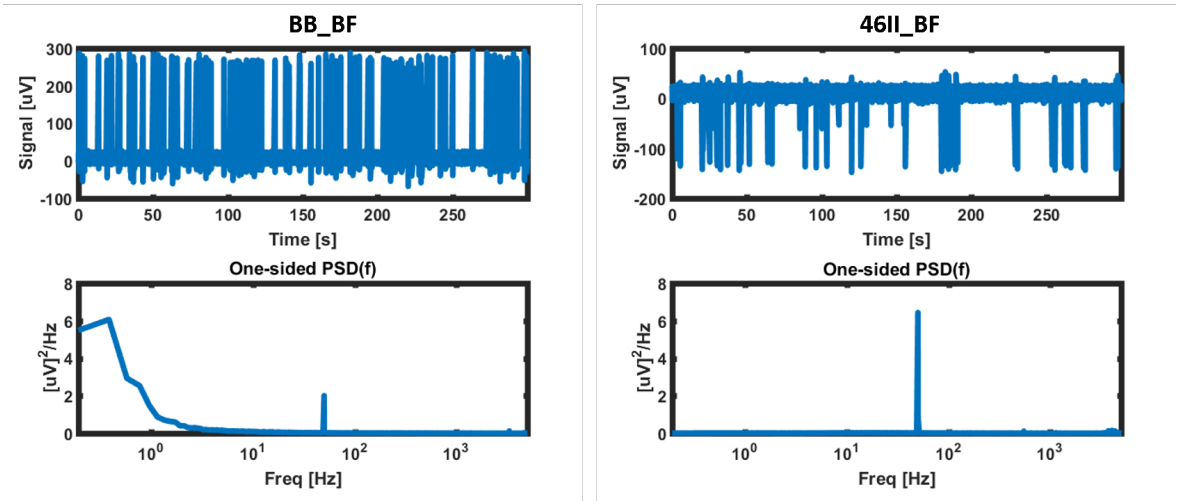

Figure 3.45: Channel 63: Power spectral density, standard setup and 46II-BF

Chapter 4

Discussions and Conclusion

The main purpose of this work was to validate the environmental chamber performance for long-term parallel acquisitions using hippocampal neuron cultures, with the prospect of employing it to study neuron-differentiated human pluripotent stem cells.

Therefore, to achieve this goal it was first necessary to improve the chamber anti-water condensation system. A study of the heating and electrical properties of an Indium Tin Oxide heater (ITO) was performed in order to access the feasibility of applying it to our environmental chamber. Material properties observed were non-uniform temperature along the surface, resistance values depending on bus bars and lead wires configuration and also on the presence of openings or cracks[7]. This study was important to delimit our application requirements, as voltage and the optimal arrangement of copper tape and wires. Established that, experiments were conducted with the ITO panel applied to the surface of the chamber. ITO heater was able to reach temperatures in the desired range (around 37°C) with a short response time and it was successful in retaining heat along the duration of the experiments with no water condensation.

Furthermore, improvements were made to the chamber sensor connections and a FEP membrane was used to cover the CO2 sensor in order to protect it when relative humidity values are high.

Afterwards, a 2-hours parallel acquisition using MEA-chips filled with PBS was carried out to validate the modifications applied to the environmental chamber. This step was crucial to understand the limitations of the system before the start of the experiment with the hippocampal neurons. Those limits comprehended: instability, presence of noise (fake spikes and electric hum 50 Hz/100Hz), determine a correct temperature for the water circulating bath and difficulty to raise relative humidity values. Even with these drawbacks, the environmental control obtained showed to be acceptable.

Regarding the acquisition of neuron cultures spontaneous activity, recordings with MEA standard setup were valuable to verify if the neurons presented electrical activity before placing MEA-chips inside the chamber.

Thus, experiment with hippocampal neurons was carried out in our setup along 4 days. Instability and a substantial amount of noise in the recordings was noticed in the first day. In the subsequent days, acquisition from one of the MEA-chips was instable, electrical signals from some channels vanished. This fact may be attributed to the loss of electrical coupling between chip contact pads and pre-amplifier. To temporally solve instability problems, it was necessary to interfere with the system, removing and placing again filter amplifier connectors, which was not an ideal situation.

In addition, it is important to highlight that acquisition problems interfered with data analysis, making difficult to follow electrical activity signals of the cells during all days of recordings.

Regarding the environmental conditions, parameters (temperature, relative humidity, and pH) were maintained within physiological ranges. Although relative humidity in our system was not the appropriate one, the FEP membrane used to cover the cell cultures helped to avoid evaporation of cell culture medium. This fact is in accordance with the literature [1], that reports the capability of the FEP membrane to preserve osmolarity in a 65[%] relative humidity incubator.

On the last day of recordings, the conduction of a pharmacological experiment confirmed the existence of spikes in our readings and microscopy-imaging enable us to verify the characteristics of the cells after the experiment.

In conclusion, although the environmental chamber setup still needs further improvements it demonstrated to be able to maintain viability of the cells during the multiple days of recordings, with potential for being a powerful instrument for long-term and parallel acquisitions for hippocampal neurons, neuron differentiated stem cells and ultimately to help understand and model disease mechanisms.

4.1 Future developments

Further developments for the anti-water condensation system would include use of printed bus bars instead of copper tape to provide more stability; understand precisely the non-uniform heating of ITO the surface in order to implement a feedback control system and also to comprehend if this non-uniformity would have an impact on the activity of the cells, maybe using infrared imaging[8].

Improvement of sensors calibration and reconditioning procedures is also important, as manner of standardize the measurement procedures.

Lastly, enhancement of shielding of the electronic circuits connected to the setup is crucial to provide more reliable system.

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