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Por medio de la presente le comunico a usted que he revisado el documento académico final cuyo título es **Establishment of analytical methods for 3D neural cell structures**, respecto a su forma y contenido, el cual es presentado por los alumnos del Programa de Evaluación Final de la Universidad de Monterrey:

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Cordialmente,

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Profesor

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c.c.p. Dra. Martha Salomé López De la Fuente / Directora del Departamento de Ingeniería

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ABBREVIATIONS

APS- Active Pixel Sensor

bFGF - Basic Fibroblast Growth Factor

BO - Brain organoids

CO - Cortical Organoids

CMOS - Complementary Metal-Oxide Semiconductor

DDW - Double Deionized Water

HBSS - Hank's Balanced Salt Solution

hESC - Human Embryonic Stem Cells

NSC - Neural Stem Cells

NS - Neurospheres

SFSCM - Serum Free Slice Culture Medium

SSSCM - Serum Supplemented Slice Culture Medium

LIST OF FIGURES

Figure 1. General illustration of a neuron..... 13

Figure 2. Membrane potential phases 14

Figure 3. HD-MEA chip, prime model 15

Figure 4. BioCAM X equipment..... 16

Figure 5. 3D structures and 2D structures..... 18

Figure 6. NS on HD-MEAs electrode array..... 19

Figure 7.Types of organoids..... 21

Figure 8. General strategy illustration 28

Figure 9. Vibratome Leica VT1200S..... 32

Figure 10. Parts of the vibratome..... 37

Figure 11. Berner B-[MaxPro]²-130..... 40

Figure 12. Microscope Nikon Eclipse LV150N 44

Figure 13. Microscope Camera DS-Ri2 44
Figure 14. Chip Placement 45
Figure 15. BrainWave X electrode array layout 47
Figure 16. BrainWave X electrode array layout and microscope image 48
Figure 17. NS samples growth onto electrode array (75d) 52
Figure 18. Selected electrodes in BrainWave X layout overlapped with a micrograph 53
Figure 19. Signals from selected electrodes in BrainWave X 54
Figure 20. Negative signals from NS in Dr. Cell. 55
Figure 21. Positive signal from NS in Dr. Cell 56
Figure 22. Organoid slices 80 days old 58
Figure 23. Organoid slices 40 days old 58
Figure 24. CO slice after 50 days of slicing 59
Figure 25. CO slice on electrode array with anchor on top 61
Figure 26. CO slice after removing the anchor 62
Figure 27. CO slice recorded signals 63
Figure 28. CO recording analyzed on Dr Cell. 64
Figure 29. CO recorded analyzed on Dr Cell: only spike 65
Figure 30. Optimization of high amount of data 66
Figure 31. Illustration of the cell on top of a measuring electrode, positive signal. 68
Figure 32. Illustration of the cell on top of a measuring electrode, negative signal. 69
Figure 33. Measurement of COs presented by UCSD 71

LIST OF TABLES

Table 1. Comparison between 3D and 2D structures 18
Table 2. Parameters for incubation process 29
Table 3. Preparation of 50 ml of NB+ medium 30
Table 4. Growth factors added to NB+ medium 31

Table 5. Preparation of 50 ml of serum supplemented slice culture medium 34

Table 6. Preparation of 50 ml of serum free supplemented slice culture medium 34

Table 7. Vibratome settings for slicing of COs 36

Establishment of analytical methods for 3D neural cell structures

INDEX

1. SUMMARY 10

2. BACKGROUND 11

2.1 Electrophysiology 12

2.1.1 Electrophysiology of neurons 12

2.1.2 Measurement of electrophysiology in neurons with HD-MEA chip 14

2.2 Cell culture 16

2.2.1 3D cell culture 17

2.2.2 Comparative between 2D and 3D cell culture 18

2.3 Neurospheres 19

2.4 Organoids 20

2.4.1 Brain Organoids 21

2.4.2 Cortical Organoids 22

2.5 Relevant research with cortical organoids 23

2.5.1 State of art 23

2.6 BioMEMS lab 24

2.6.1 Study of 3D structures at BioMEMS lab 25

3. JUSTIFICATION 25

4. HYPOTHESIS 26

5. OBJECTIVES 26

5.1. General objective 26

5.2. Particular objectives 27

6. METHODOLOGY 27

6.1 General Strategy 27

6.2 Procedures prior to sample positioning on the HD-MEA chip 29

6.2.1 Neurospheres 29

6.2.1.1 Origin 29

6.2.1.2 Storing conditions and handling (NSC) 29

6.2.1.3 Preparation of Neurospheres 30

6.2.1.4 Incubation 30

6.2.1.5 Medium specifications 30

6.2.2 Cortical organoids 31

6.2.2.1 Origin 31

6.2.2.2 Storing conditions and handling 31

6.2.2.3 Slicing protocol 31

6.2.2.4 Incubation 37

6.2.2.5 Medium specifications 38

6.2.2.6 Control stainings 38

6.3 Sample positioning on chip procedures 40

6.3.1 HD-MEA chip preparation 40

6.3.2 Sample positioning on the HD-MEA chip 42

6.3.2.1 Neurospheres 42

6.3.2.2 Cortical Organoid Slices 42

6.4 Microscopy and imaging 43

6.5 Electrophysiology measurements 46

6.6 Sample disposal 50

7. RESULTS 51

7.1 Neurospheres 51
7.1.1 Sample positioning on HD-MEA chips 51
7.1.2 Electrophysiological recordings 52
7.1.3 Electrophysiological analysis 54
7.2 Cortical Organoids 56
7.2.1 Slicing Protocol 56
7.2.1.1 Life expectancy of CO after slicing 59
7.2.2 Sample positioning on HD-MEA chips 60
7.2.3 Electrophysiological recordings 62
7.2.4 Electrophysiological analysis 63
7.3 Optimization of high amount of data 66
8. DISCUSSION 67
8.1 Signal spike positive and negative outcome 67
8.2 Anchor use 70
8.3 CO signals 71
9. REFERENCES 73

1. SUMMARY

The study of the human brain has been a topic of interest since the early beginnings of medicine, understanding how the brain functions has been a task that many scientists have addressed. Nowadays these studies can be performed inside a laboratory with cell models under a controlled environment that can be altered depending on the topic of interest.

The present research focuses on the use of 3D neural cell structures as resembling models of neuronal connectivity, to do so, during this study we employed cell models using Neurospheres (NS) and Cortical Organoids (CO) derived from human embryonic stem cells.

The shape of these structures is spherical, which presents a problem specially when dealing with relatively big structures. The spherical shape produces necrosis (i.e. the neurons die) in the center of the structure due to the lack of enough nutrients. This necrosis depends on the size of the structure, NS do not grow enough for this to be an issue. Conversely, COs are bigger and therefore, in order to avoid necrosis COs must be sliced in order to culture them for longer periods of time, and provide samples that could be studied in parallel with the NS.

Then, the cultured NS or CO can be studied with a High Density Microelectrode Array chip (HD-MEA) in which the samples will be placed, this chip is inserted into an electrophysiological recording hardware called BioCAM X

which can detect spikes and present them on a PC for further analysis on a software called BrainWave X.

This analysis could later be corroborated with Dr Cell, a program developed at the BioMEMS lab that can improve the analysis by the use of filters, thresholds, synchrony tests and spike bursts detection.

2. BACKGROUND

In 1791 Luigi Galvani proposed that electrical impulses were important for the nervous system, while analyzing how a frog's muscles contracted when a nerve encountered an electric current. In 1932 the concept of synapses was presented by Sir Charles Sherrington and Edgar Adrian; they were able to show how the neurons are connected by electrical and chemical signaling. (Queensland Brain Institute, 2019; Bellis M, 2019).

These discoveries were the beginning of neuroscience. Nowadays these studies are possible in controlled environments due to development of techniques and procedures which make possible to stimulate, record and analyze brain signals.

Culture systems have made progress in research areas in neuroscience. The use of culture systems has been of interest for scientists because it allows them to study complex multicellular systems in a controlled environment, the

present research focuses on the development of procedures to analyze the electrophysiology on 3D neural cell structures.

2.1 Electrophysiology

Electrophysiology is a branch of neuroscience which is responsible for the study of electrical activity in living neurons and the molecular and cellular processes that produce their signals, it is a major tool for the understanding of brain function (Carter M. & Shieh J., 2010).

2.1.1 Electrophysiology of neurons

It is possible to make electrophysiological measurements in neurons by two methods, intracellular and extracellular. The intracellular method measures the potential difference in between an electrode inside the neuron and a reference electrode which is located in the surrounding medium. On a similar case the extracellular method measures the potential difference between a reference electrode in the medium but in this case the other electrode is only touching the outer part of the cell (Bear et al, 2016).

These methods measure the action potential, which is the sequence of ionic movements across the neuronal membrane across the sodium and potassium channels. The neuronal membrane at its resting state has a higher concentration of potassium ions inside the cell than the outside, on the other hand, for the sodium ions is the opposite, a higher concentration is outside the cell than the inside, this is achieved thanks to the sodium-potassium pump, the

membrane potential at this point is approximately -70mv which means it is polarized (Bear et al, 2016).

When the neuron receives a strong stimulus through the dendrites that make the membrane potential rise to -55mV then an action potential is triggered in the axon hillock. The sodium channels open in the membrane, since the concentration of sodium ions is higher in the outside they rush into the cell, causing the membrane to depolarize, this is the rising phase. The membrane potential has an overshoot because of the relative permeability in the membrane towards sodium (Bear et al, 2016).

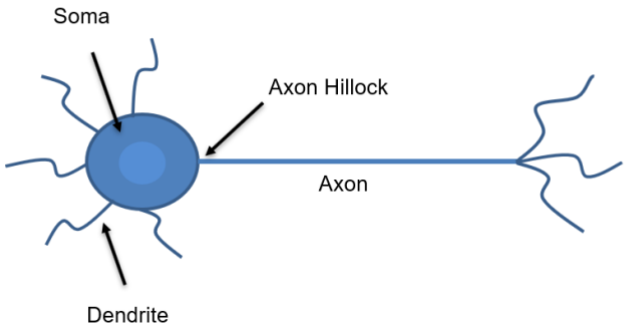

Figure 1. General illustration of a neuron

Neuron illustration with the parts relevant for the development of the present research, soma, dendrite, axon, and axon hillock.

Once the sodium channels close, the potassium channels open, the concentration of potassium is higher in the inside of the membrane, so the ions travel to the outer part, this causes the membrane to repolarize, this is the falling phase. Since the potassium channels close more slowly there is an undershoot making the membrane more negative before reaching to the resting state (Bear et al, 2016).

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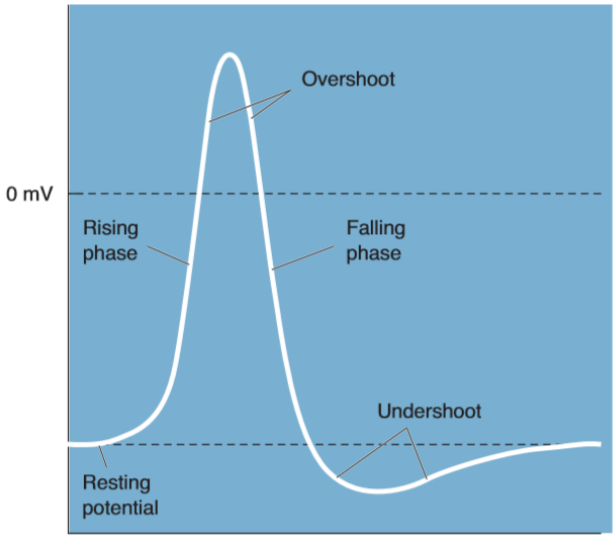

Figure 2. Membrane potential phases

Representation of the membrane potential phases with a 0-basis axis defining the different positive and negative states of the signal. [Figure] Adapted from: (Bear et al, 2016) Neuroscience exploring the brain.

2.1.2 Measurement of electrophysiology in neurons with HD-MEA chip

The electrophysiology measurements for this project were performed with a High-Density Microelectrode Array chip (HD-MEA). This chip uses Complementary Metal-Oxide Semiconductor (CMOS) and Active Pixel Sensor Technology (APS), by replacing the light-sensitive element with a metallic electrode into each unit element (pixel) and integrating amplification with filter stages, the chip is able to collect high resolution data in a higher magnitude than conventional systems (3Brain, 2018).

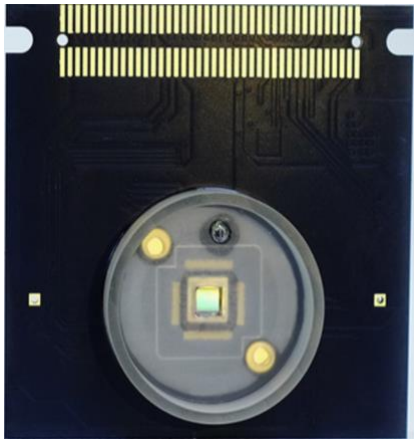

Figure 3. HD-MEA chip, prime model

Primer model of the HD-MEA chip which contains 4096 electrodes and a medium well of 2ml. [Figure] Adapted from: https://www.3brain.com/biocamx.html

The HD-MEA chip model prime has an arrangement of 64x64 electrodes, making a total of 4096, they have a square form and measure 21 µm on each side, they have a gap of 42 µm between them. The sampling frequency in the full electrode array is 18 kHz, this means that it makes a measurement every 0.0555 milliseconds, which makes it appropriate for the measurement of action potential since the process from beginning to end last about 2 ms (3Brain, 2018; Bear et al, 2016).

BioCAM X is the system which makes the recordings from the HD-MEA electrodes. The chip is inserted into the system and with the software from the BioCAM X it's possible to measure, display, record and analyze the data obtained from the sample in the chip. This system has a plate to control the temperature in the chip chamber and the reference electrode for the measurements.

Figure 4. BioCAM X equipment

Equipment used for the measurement of signals from the HD-MEA chips. [Figure] Adapted from: www.3brain.com

Once the sample is placed on the electrode array with its corresponding medium, measurements are possible with the use of two electrodes, one is a measuring electrode which is located near the cell and the other one is a reference electrode located at a distant area from the sample, with this system the ionic movement can be measured.

2.2 Cell culture

The procedure of growing cells in a controlled environment is called cell culture, it requires specific care and conditions like regulated temperature, gas, and medium. There are three categories of cells in cultures: fibroblastic, epithelial like, lymphoblast like. The difference between them is the cells' morphology (Thermofisher Scientifics, 2016). In this project the cell cultures correspond to the epithelial like category, as they have a polygonal and elongated shape.

2.2.1 3D cell culture

Normally the cell culture used for different studies is called monolayer or 2D, meaning that the cells grow onto the culture flask and it becomes a plane of cells connected and interacting only in a 2D environment. This type of cell culture is the base of studies as they have a high reproducibility rate, are well established and are inexpensive when maintained in proper conditions that elongate the life expectancy (Joseph et al, 2018).

2D cell cultures have been a part of diverse studies as they have a lot of beneficial factors that make them the most used type of cell culture, however, the biggest con is that they can only imitate the human tissue to one extent as they can only communicate in a 2D plane.

Cells in human tissue interact in a 3D structure, which makes the 2D cell cultures non-accurate as they grow on a flat surface and the interaction between cells is limited. For this reason, the use of 3D cell cultures has begun to be widely researched as it provides a more accurate model for study, in 3D models cells are able to interact in all directions giving a better simulation of conditions of a living organism.

2.2.2 Comparative between 2D and 3D cell culture

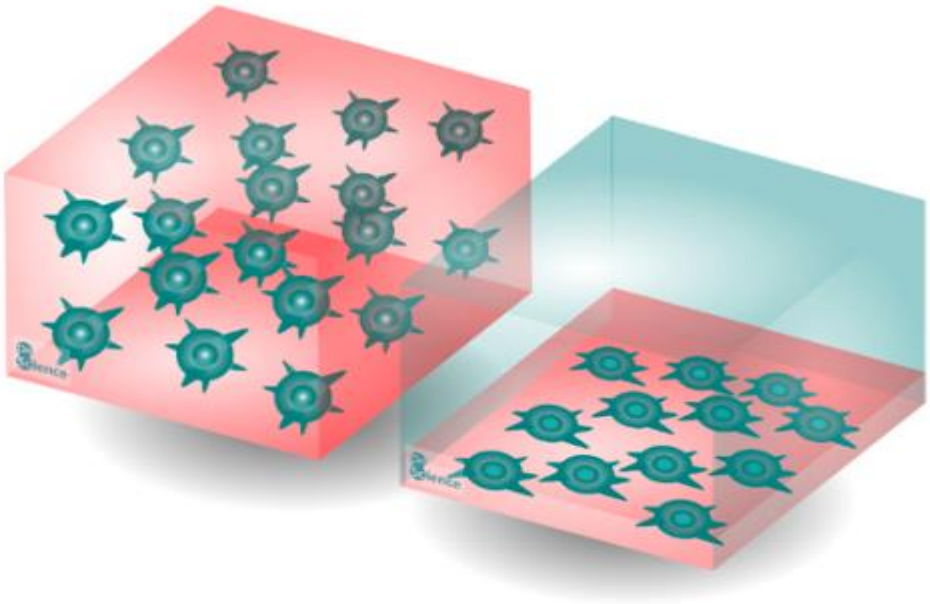

Figure 5. 3D structures and 2D structures

Comparison of the overall form of 3D and 2D structures. [Figure] Cultivos celulares 3D vs Cultivos celulares 2D (2018)Adapted from: onscience.es

Table 1. Comparison between 3D and 2D structures

Characteristic	**3D structures**	**2D structures**
Morphology	Cells grow in a 3D environment	Cell grow on a flat surface
Shape	Multilayer culture	Monolayer culture
Communication between cells	Cells communicate in all directions imitating with more precision the in vivo behavior of cells.	Cells communication is limited to a 2D plane.
Nutrient absorption	Cells don't absorb enough nutrients in the core of the structure.	Cell have a proper absorption of nutrients as all the layer receives the same amount of medium.
Cost	More expensive and time consuming, with noncommercial medium.	Inexpensive with commercial medium available.

(Joseph et al, 2018; Kolenda et al, 2016)

As table 1 shows, there are many benefits of using 3D structures as they are more accurate to in vivo cellular behavior, nevertheless, there are still some factors that are considered difficult to work with, for example, the nutrient absorption and the overall maintenance of 3D structures, this project seeks to implement methods to handle and study 3D neural cell structures at BioMEMS lab, there are different types of 3D cell structures, the ones used in the lab are NS and COs.

2.3 Neurospheres

Neurospheres (NS) are clusters of heterogeneous cells, these cells can be neural stem cells, neural progenitor cells, neurons, astrocytes or oligodendrocytes. Neural stem cells are capable of self-renewing and can differentiate up to three neural lineages, astrocytes, neurons and oligodendrocytes (Narayan et al, 2016).

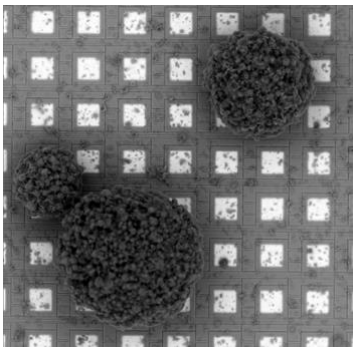

Figure 6. NS on HD-MEAs electrode array

3 days old NS on top of the electrode array, photograph taken on the first day of insertion, largest NS covers only 9 electrodes from the 4096.

In this cell culture neural stem cells (NSCs) are placed in serum-free medium with growth factors, such as epidermal growth factor or Basic Fibroblastic Growth Factor (bFGF). The NSCs will begin to proliferate and form small clusters. After approximately seven days of culture, depending on the cell source, the NS typical size is 100 to 200 µm in diameter and have 10,000 to 100,000 cells approximately. NSCs can be induced to differentiate in the presence of low serum medium and specific growth factors (Stemcell Technologies, 2015). The NS employed at BioMEMS lab contain only neurons, because the current research focus only requires the analysis of these specific cells.

2.4 Organoids

Organoids are three dimensional structures produced by cell cultures that emulate the architecture of a tissue or organ.

They are derived from stem cells which can divide and produce different types of cells; when producing a right environment for the organoids they can organize into tiny structures that resemble miniature organs composed of many cell types. Their size may vary from 0.55 microns, which is the average thickness of a human hair, up to 5 millimeters (Barbuzano, 2017).

It is considered that several organ models can be created with these methods, however, so far, the types of organoids that have been produced

resemble the brain, kidney, lung, intestine, stomach, liver and mammary glands (Barbuzano, 2017).

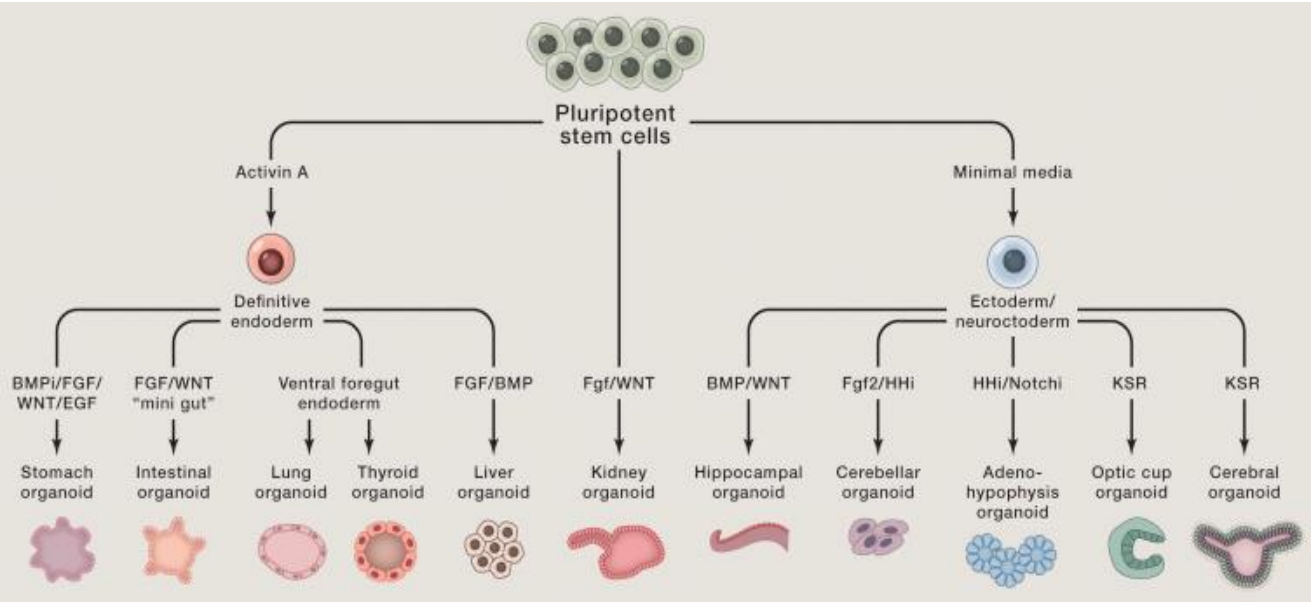

Figure 7.Types of organoids

Types of organoids produced so far and their basic development steps. Adapted from "Modeling develop and disease with organoids" by Clevers, H. (2016) Cell Press. Volume 165, Issue 7.

This project focuses on the use of 3D structures to study the resembling electrophysiology of 3D neural cell networks, for this purpose the use of brain organoids shall be explained in further detail.

2.4.1 Brain Organoids

Brain organoids (BO) are also known as neural or cerebral organoids, they are 3D cell structures, which are derived from human pluripotent stem cells and can recapitulate the organization in the development of the brain, turning them into a miniature brain that provides a model to study neurological development and disorders (Stemcell Technologies, 2017).

BO can be categorized by different factors, one of them is by the protocol with which they have been created, self-patterned BO are the ones that develop autonomously, which means that they do not have addition of external signaling molecules, the BO that require the addition of this signaling are called pre-patterned. (Heide et al, 2018).

The result of the self-patterned protocols is a whole-brain organoid as it develops different regions by self-organizing in the growth Matrigel. On the contrary brain-region-specific organoids are induced with small molecules to have a certain region characteristics. (Heide et al, 2018). The organoids used for this project are COs, which means they correspond to the pre-patterned creation protocol.

2.4.2 Cortical Organoids

Cortical organoids (CO) resemble the cerebral cortex, they are the most common used organoids for research since this area is the most evolutionarily expanded region of human brain and often the one affected in many neurological disorders (Qian et al, 2019).

The size of a cortical CO may vary in between 2 to 4 mm in diameter, they have a spherical-like shape, their size is considered large in comparison to other 3D structures, however in terms of comparison with the cerebral cortex these COs are a miniature version, the reason being that CO lack vascularization and circulation which makes the core suffer necrosis for the lack of nutrients and

oxygen, thus growing them to larger sizes will provide a bigger necrotic area in the core (Qian et al, 2019).

Different protocols have been developed for the CO creation, and the most frequent question is whether these structures are mature enough to produce significant signals that resemble the behavior of neurons in the brain.

It has been shown that excitatory and inhibitory neurons in CO are functional, but not as mature as adult neurons, there have been extensive studies to confirm this functionality in CO created by different protocols, some of them shall be explained in further detail in a comparison of the state of art (Qian et al, 2019).

2.5 Relevant research with cortical organoids

2.5.1 State of art

Several investigations have been performed in the last three years in order to analyze 3D structures maturation and ability to produce proper signals. These early investigations started with the use of forebrain spheroids generated from human pluripotent stem cells, which resemble dorsal or ventral forebrain to recapitulate the saltatory migration of interneurons similar to migration in fetal brain (Birney et al, 2017). More recently, the use of cerebral organoids has been a key feature in analysis, with protocols that use time-lapse imaging, immunostaining, and single-cell RNA sequencing to interpret cell migration (Bershteyn et al, 2017; Yakoub et al 2019). Even some investigations use a

slicing protocol for BO, in order to prevent necrosis, these studies introduce the use of agarose as an insertion block for the slicing of BOs (Lancaster et al, 2017; Giandomenico et al, 2019). In order to improve neuronal survival and axon outgrowth, some researchers have adapted an air-liquid interface culture to BO that have been previously sliced (Giandomenico et al, 2019).

2.6 BioMEMS lab

BioMEMS lab is a s1 level lab located in the Technical University of Applied Sciences of Aschaffenburg, as the name states the lab's focus are microelectromechanical systems. The head of the BioMEMS lab is Prof. Dr. Christiane Thielemann, whose area of expertise is electrical engineering, biosensors and electrophysiology studies. The lab team consists of post-doctorate, doctorate and master students and a handful of bachelor students.

The laboratory has many ongoing investigations focused on topics like cell-based sensors, 3D cell culture, neuro bio signal processing, radiation induced effects on cells and bioprinting. The fields of cell-based sensors, 3D cell culture and neuro bio signal processing are of great importance to the present research as it revolves around the main objective which is to identify if the 3D neural structures handled at the lab produce mature signals.

2.6.1 Study of 3D structures at BioMEMS lab

BioMEMS lab has three different types of 3D structures, which are used for several research topics. Currently, there are researches using cardiac spheroids, neural spheroids and COs.

The studies done with spheroids in general include the analysis of effects of electromagnetic radiation on the electrophysiological properties of neural and cardiac cell networks (Mayer et al, 2018). Neural spheroids, also known as NS, have also been used to study the effects of a drug called bicuculline which is an antagonist of the GABBA receptor.

On the other hand, the use of COs at BioMEMS lab is quite recent as protocols for the use of these structures are still being established, nevertheless, the purpose of using them is related to the experiments mentioned above but on a more complex neural structure.

3. JUSTIFICATION

Studies regarding neuronal behavior are extensive and there has come a need to develop models that resemble this architecture in a controlled environment. The use of 3D structures was proposed as an alternative to develop these models and study different factors like development, drug screening and model diseases.

These models need to be verified, to do so, it is important to determine if the 3D structure is mature enough, meaning that they can produce signals. The signals are measured with the HD-MEA chip, which has an electrode array in a 2D plane that has the ability to measure resulting signals from the 3D structure interaction and determine if the differentiation protocols that provided these structures have been successful and the samples are ideal for further measuring and analyzing with 3D technologies.

4. HYPOTHESIS

It is possible to measure electrophysiological activity of 3D neural cell structures such as NS and COs with HD-MEA chips.

5. OBJECTIVES

5.1. General objective

To develop a protocol for measuring the electrophysiology displayed by 3D neural cell structures i.e. NS and COs.

5.2. Particular objectives

1. To establish a protocol for cutting organoids which provides slices with viable characteristics for electrophysiology measurements in HD-MEA chips.

2. To produce proper adhesion of the samples to the HD-MEA chips.

3. To record electrophysiological signals and analyze them using Dr. Cell, developed by BioMEMS lab.

4. To propose a solution for optimizing the analysis of high amount of data on Dr. Cell.

6. METHODOLOGY

6.1 General Strategy

The general strategy consists on the development of different protocols and methods to measure 3D cell structures in HD-MEA chips that allow the analysis of electrophysiological activity. The procedure is illustrated in Figure 8, starting with the slicing protocol for the COs, which provides several samples obtained from a single CO. This is required in order to prevent organoid necrosis and optimize nutrient supply of the sample; this procedure is not necessary for the NS as their type of structure is smaller therefore it does not present necrosis.

Before placing either the CO slices or NS, the HD-MEA chips need to be prepared with PEI at least one day in advance to ensure adhesion. Once the samples are over the electrode array of the HD-MEA chip, photographs can be taken with the microscope to verify where the sample is placed and how it grows onto the chip, this indicates that the attachment of the 3D structures to the chip has been successful providing a viable culture for electrophysiology measurements.

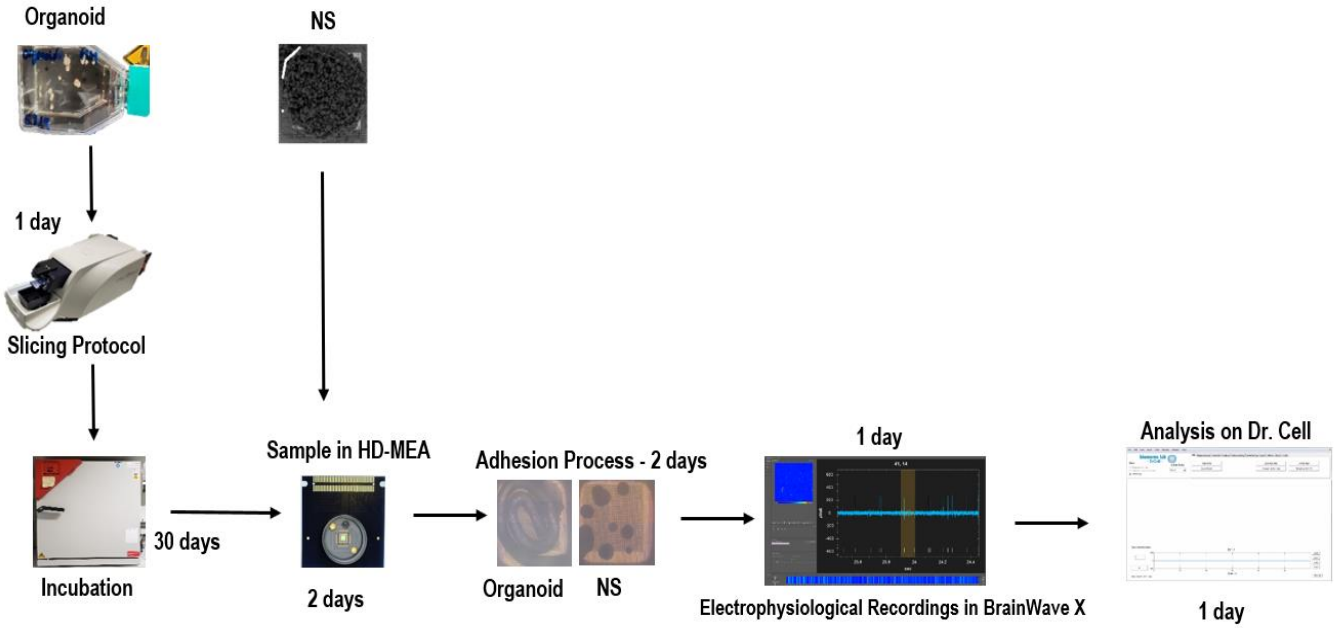

Figure 8. General strategy illustration

Representing the different steps to be followed in the project depending the 3D structure used. Total amount of time per CO organoid sample: 38 days in the case of recording signals in the first try. Total amount of time per NS sample: 6 days in the case of recording signals in the first try.

6.2 Procedures prior to sample positioning on the HD-MEA chip

6.2.1 Neurospheres

6.2.1.1 Origin

The differentiation of human embryonic stem cells (hESC) to neural stem cells (NSC) takes place at GSI Helmholtz Center for Heavy Ion Research of the Technical University of Darmstadt. Samples arrive at BioMEMS lab packed in a polystyrene box and are placed in the incubator after arrival.

6.2.1.2 Storing conditions and handling (NSC)

The NSC are contained in a cell culture dish (35 mm diameter) with neural induction medium (ThermoFisher Scientific), this medium is changed every second day.

Table 2. Parameters for incubation process

Parameters	
Temperature	37º C
Gas concentrations	CO_2 5% and air 95% (N_2 79% and O_2 21%)
Relative humidity	5%

6.2.1.3 Preparation of Neurospheres

When NS are needed for experiments the NSC are induced to create NS. To do so, the NSC are placed in ultra-low attachment cell culture flask to prevent attachment of the cells to the flask and be able to form NS, the medium used is NS-B27 and the following growth factors are added in every media change, bFGF, Dorsomorphin and Noggin.

6.2.1.4 Incubation

The NS are stored in the incubator with the same parameters as the NSC, the change of medium is done once a week for the NS inside the cell culture flask for 3 weeks or until there are no more NS. If there are NS placed in a HD-MEA chip this medium must be changed every 2 days,

6.2.1.5 Medium specifications

The medium is prepared every week without the growth factors (bFGF, Dorsomorphin and Noggin), these are added just before the medium is changed so they can be stable (fresh). The medium is prepared in the laminar flow cabinet.

Table 3. Preparation of 50 ml of NB+ medium

Concentration of reagents added	
Neurobasal plus medium	48.5 ml
Glutamax	0.5 ml
B27 plus supplement	1 ml
PEN/strep	500µl

Before changing the medium, growth factors are added, for every ml of medium.

Table 4. Growth factors added to NB+ medium

Concentration of reagents added	
Noggin	0.6 µl / ml
Dorsomorphin	1 µl / ml
bFGF	2 µl / ml

6.2.2 Cortical organoids

6.2.2.1 Origin

The COs used in this research come from GSI Helmholtz Center for Heavy Ion Research of the Technical University of Darmstadt. Samples arrive to BioMEMS lab packed in a low attachment flask inside of a polystyrene box.

6.2.2.2 Storing conditions and handling

The CO are contained in a low attachment culture flask 25cm^2 with nutrition medium elaborated by BioMEMS lab. Every 4 days the COs shall be transferred to a new flask with new nutrition medium. The incubation conditions are the ones presented on Table 2.

6.2.2.3 Slicing protocol

The slicing protocol is a methodical process which is required for the optimum analysis of organoids. Through this method COs are sliced to an

adequate thickness that ensures nutrient supply to avoid necrosis. The thickness selection also affects the ability to define structures in the microscope, with a thin slice the structures do not overlap each other.

This slicing process requires the use of a vibratome shown in Figure 9, an instrument used for the slicing of materials with a vibrating blade, the one used for this project is a Leica VT1200 S.

Figure 9. Vibratome Leica VT1200S

Vibratome used for the slicing protocol presented in this project, it does not include any attachments and it is used as presented. Adapted from: Leica Biosystems. Instructions for use VT1200/VT1200S vibrating blade microtome.

The slice protocol used for this project is adapted from Daza et al, 2007 modified after Giandomenico et al, 2019.

The required elements for the slicing protocol are listed below in order of required use:

- Laboratory precision scale
- Microwave

- Water bath set at 45 ºC
- Falcon tubes (15 and 50 ml)
- Sterile petri dish (35 mm)
- 100 - 1000 µl pipette and corresponding tips
- Sterile custom-made peel away embedding mold
- Paper tissue
- Sterile laboratory spatula kit
- Sterile small pincers
- Ice
- Polystyrene box
- Vibratome
- Super glue
- Sterile razor blades
- Sterile soft paint brush
- Six-well plates with Millicell-CM cell culture inserts (Millipore, CN PICM0RG50)

Reagents:

- Low melting agarose (Sigma, CN A9414)
- Cell culture medium, serum free, prewarmed to 37ºC
- Cell culture medium, serum supplemented, prewarmed to 37ºC
- Hanks Balanced Salt Solution without Ca2+ and Mg2+ (HBSS) (Thermo Fisher Scientific, CN14175095)

Preliminary processes:

a) Twenty-four hours before slicing:

1. Prepare 50 ml of serum supplemented slice culture medium and place it in a refrigerator.

Table 5. Preparation of 50 ml of serum supplemented slice culture medium (SSSCM)

Concentration of reagents in SSSCM	
Modified Eagles's medium: DMEM (Invitrogen, CN 10566016)	44 ml
Fetal calf medium 10% FBS	5 ml
50% (w/v) glucose	0.5 ml
PEN/Strep	0.5 ml

2. Prepare 50 ml of serum free slice culture medium and place it in a refrigerator.

Table 6. Preparation of 50 ml of serum free supplemented slice culture medium (SFSCM)

Concentration of reagents in SFSCM	
Neurobasal medium (Invitrogen, CN 21103049)	47.5 ml
B27 supplement (Invitrogen, 17504044)	1 ml
50% (w/v) glucose	0.5 ml
(v/v) glutamax	0.5 ml
PEN/Strep	0.5 ml

b) One hour before slicing, prepare 50 ml of 3% low melting agarose in HBSS.

1. Add 1.5 g of low melting agarose to 50 ml of HBSS and stir.
2. Microwave the solution to a boil until it’s clear.
3. Place in 45ºC water bath.
4. Prepare petri dish with HBSS surrounded by ice inside a polystyrene box.

c) 45 minutes before slicing:

1. Prepare the six-well plate with Millicell-CM cell culture inserts with SSSCM and incubate at 37 ºC.
2. Transfer SFSCM from the refrigerator to a 37 ºC incubator.
3. Transfer the petri dish with HBSS below the laminar flow cabinet.
4. Collect mature CO with a pipette and wash away the remaining nutrition medium in the petri dish with HBSS.
5. Transfer CO to the custom-made embedding mold.
6. Absorb as much HBSS as possible from the surface of the organoid with a paper tissue.
7. Add low melting agarose in liquid state to the mold.
8. With the use of the laboratory spatula kit move the organoid inside of the agarose so the remaining HBSS blends in.

9. Place the mold inside a new petri dish and incubate at 37 ºC for 10 minutes.
10. Remove the petri dish from the incubator and place it in the polystyrene box for 10 minutes.
11. Prepare vibratome area with sterile elements such as razor blades, paintbrush and laboratory spatula kit.

Slicing processes:

1. Collect agarose solidified block and glue it onto the specimen plate of the vibratome.
2. Insert the specimen plate inside the buffer tray.
3. Adjust settings such as speed, amplitude and thickness of the slice.

Table 7. Vibratome settings for slicing of COs

Vibratome settings	
Thickness	300 µm
Amplitude	1.5 mm
Speed	0.8 mm/s

4. Fill the buffer tray with ice-cold HBSS.
5. Collect the plates with Millicell-CM inserts and SSSCM from the incubator.
6. Start slicing process and in between slices remove each slice with the spatula kit and soft paint brush into Millicell-CM inserts in the plates.

Figure 10. Parts of the vibratome

Parts of the vibratome used for the slicing protocol, the ice tray is filled with ice right before slicing, the buffer tray is filled with HBSS and the specimen tray is where the agarose block is glued. Adapted from: Leica Biosystems. Instructions for use VT1200/VT1200S vibrating blade microtome.

Post slicing procedures:

1. Incubate slices in six-well plate for 1 hour at 37ºC.
2. Change SSSCM to SFSCM.
3. Maintain cultures in SFSCM in incubation with daily medium changes.

6.2.2.4 Incubation

After the slicing protocol the slices of COs must be incubated for 30 days in order to finish their differentiation process and be fit for their positioning on HD-MEA chips. During these 30 days they require daily medium changes of SFSCM in order to provide enough nutrients.

The incubation process requires to maintain the slices in the Millicell CM inserts as these provides and air liquid culture, meaning that the inserts function as a semipermeable membrane that makes the slices be in contact with the medium on one surface only.

6.2.2.5 Medium specifications

The incubation medium is the SFSCM which contains a neurobasal solution with different nutrients and antibiotic to prevent infection.

The medium change process requires to transfer the six-well plate from the incubator to the laminar flow cabinet and withdraw the old medium.

Afterwards new SFSCM is added into the wells with the use of a pipette and different tips per well, this process must be made without adding the medium directly to the inserts as the liquid must go under it in order to maintain the air liquid culture.

6.2.2.6 Control stainings

To ensure that the slices are receiving enough nutrients, control stainings shall be made as a way to determine if the cells are alive, the staining method requires the following elements:

Reagents:

SFSCM

Cell tracker

DAPI

One and a half hour before microscopy:

1. Prepare staining medium mixing 2 ml of SFSCM, 6 µl of cell tracker and 3 µl of DAPI.
2. Transfer the desired slice from the Millicell CM insert to an empty well or petri dish.
3. Add the staining medium to the container where the slice was placed.
4. Incubate for 45 minutes.
5. Transfer container to the laminar flow cabinet and remove the staining medium.
6. Add 2 ml of fresh SFSCM and incubate for 10 min.
7. Transfer container to the laminar flow cabinet and remove the SFSCM.
8. Repeat step 6 and 7 for another 2 times.
9. Remove most of the medium in order to maintain the slice at a defined position.

Microscopy:

1. Turn on the microscope system and adjust the temperature control to 37ºC.
2. Clean all surfaces with ethanol 70%.
3. Insert the container into the microscope chamber.
4. Adjust sample into the ray of light and find the middle part.
5. Adjust program settings and start capturing images

6.3 Sample positioning on chip procedures

6.3.1 HD-MEA chip preparation

The HD-MEA chip must be sterilized before deposing the NS or CO slices. Depending on the sample used, a different coating is selected for proper attachment. The chip must be handled with latex gloves and gripped by the sides. The contact pads should never be touched.

This protocol requires the use of a laminar flow cabinet to provide a sterile environment, the one used for this project is a Berner B-[MaxPro]²-130 shown in Figure 11.

Figure 11. Berner B-[MaxPro]²-130.

Laminar flow cabinet used at BioMEMS lab. [Figure] Adapted from: https://www.berner-safety.de/media/3587/gb_12_0569_a.pdf

In order to prepare the HD-MEA chip, the following materials are required:

- Double deionized water (DDW)
- Ethanol 96%
- Ethanol 70%
- Microliter pipette

- Sterile petri dish
- Paper tissue
- Poly-ethylenimine, 1:500 dissolved in borate buffer (PEI - Sigma 482595)

Preliminary processes:

a) Twenty-four hours before deposing samples in HD-MEA chip

1. Spray the outside of the sterile petri dish with ethanol 70% before placing it in the laminar flow cabinet.
2. Use a paper tissue soaked with ethanol 96% to clean the external part of the chamber from the HD-MEA chip.
3. Place the chip inside the laminar flow cabinet.
    - *Note: From this moment the chip will remain inside the petri dish to maintain the sterile environment.*
4. Fill the chips' chamber with ethanol 70% for twenty minutes with the microliter pipette.
5. Remove the ethanol with the microliter pipette and rinse the chips' chamber with DDW 4 times.
6. Let the chip dry inside the laminar flow cabinet with the petri dish halfway open for proper ventilation.

b) Coating the electrode array

1. Fill the chamber of the chip with PEI using the microliter pipette.

2. Place the chip inside the incubator for one hour.
3. Remove the PEI and rinse with DDW water 4 times with the microliter pipette.
4. Let dry the chip inside the laminar flow cabinet with the petri dish halfway open for proper ventilation overnight.

6.3.2 Sample positioning on the HD-MEA chip

6.3.2.1 Neurospheres

1. With the microliter pipette take 5-7 NS.
    - *Note: Adjust the microliter pipette to 200 µL to avoid taking out too much medium from the culture dish.*
2. Gently take out the medium with NS in the electrode array of the chip.
3. Place the chip inside the incubator for 10 minutes.
4. Fill the chamber of the chip with 2 ml of NS/B27 medium.
    - *Note: The medium is previously prepared and preheated in a water bath at 37 ºC.*

6.3.2.2 Cortical Organoid Slices

1. Transfer the selected slice of organoid from the Millicell CM insert to a petri dish.
2. Remove the surrounding agarose with the use of a laboratory spatula kid and small pincers.
3. Place the organoid slice on the electrode array of the chip.

4. Place a custom-made anchor on top of the slice to prevent it from floating in the medium and ensure attachment onto the electrode array.
    - *Note: The anchor was made from stainless steel to prevent corrosion on the electrode array. Placing the anchor must be done carefully to prevent damage on the electrodes.*
5. Gently fill the chamber of the chip with 2 ml of SFSCM.
6. Incubate for 3 days with daily medium changes.
7. Remove the anchor and continue incubation process.

6.4 Microscopy and imaging

Microscopy is necessary in this research as it gives information about where the sample is positioned and how it grows during incubation, this also ensures that the signals obtained in later protocols correspond to the positioning of the 3D structures on the HD-MEA chip.

For this procedure, the microscope used is a Nikon Eclipse LV150N combined with a DS-Ri2 digital sight microscope camera. These devices allow capturing digital images from the sample of interest shown in the figures 12 and 13.

Figure 12. Microscope Nikon Eclipse LV150N

[Figure] Adapted from: Nikon Instruments Inc.

Figure 13. Microscope Camera DS-Ri2

[Figure] Adapted from: Nikon Instruments Inc.

In order to make an image of the sample, the following materials are required:

- Ethanol 70%
- Paper tissue

Preliminary processes:

a) Adjusting microscope software settings:

1. Select the 10x optical lens.
2. Select the option “Scan large image” in order to adjust advanced setting.
3. Set the image scan from left to right
4. Set frames to 3x4, this setting will cover the entire electrode array.

b) Acquiring photographs

1. Spray a paper tissue with ethanol 70% to clean the stage of the microscope that will be in contact with the petri dish.
2. The chip must be aligned as shown in Figure 14, if not, the chip must be placed correctly under the laminar flow cabinet.
    - *Note: When the image is being taken the lenses from the microscope might push the petri dish if is not placed correctly.*

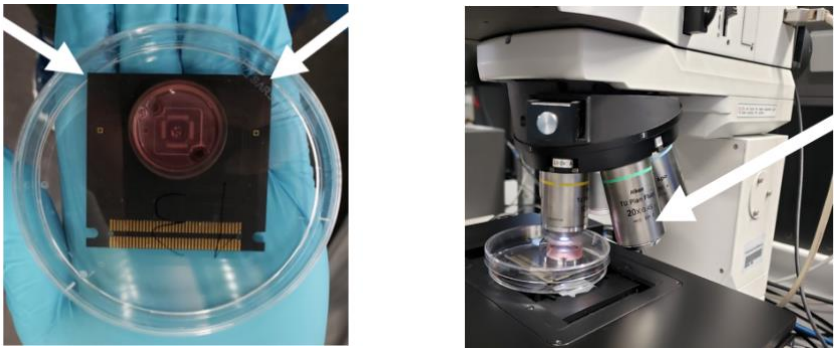

Figure 14. Chip Placement

Correct placement of HD-MEA chip on the petri dish and microscope Nikon Eclipse LV150N

3. After placing the petri dish in the stage of the microscope, focus the upper left corner of the electrode array to start the image acquisition.

6.5 Electrophysiology measurements

To record electrophysiological signals from the HD-MEA a MEA system is needed. In this device the HD-MEA chip is inserted in order to get the electrophysiological measurements. The MEA system used in this project is the BioCAM X which in conjunction with a specialized software makes it possible to measure and record electrophysiological signals. The BioCAM X has temperature control for the samples and a recording frequency of 18 kHz.

The software used to record the signals is called BrainWave X. This software has a layout that displays a square that is composed of the 4096 electrodes in the HD-MEA chip, the arrangement of the electrode array is shown in Figure 15, each electrode changes color depending on the voltage signal detected.

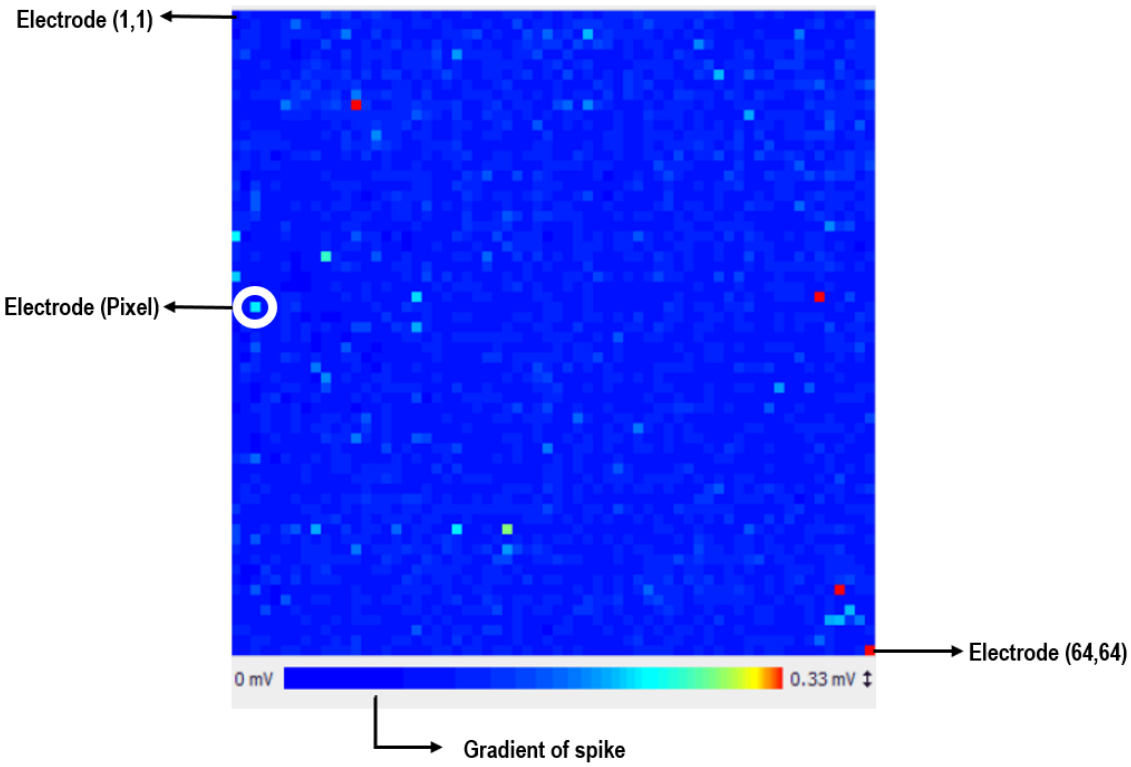

Figure 15. BrainWave X electrode array layout

Electrode array displayed on BrainWave X, the top left corner corresponds to the electrode (1,1) and the bottom left one corresponds to (64,64). The gradient of spike goes from a dark blue which corresponds to 0 mV up to red which corresponds to 0.33 mV.

In addition to the photograph from the microscope, that provides an exact location of the sample it's possible to locate signals and differentiate them from noises or artifacts in the recording, for example in Figure 16 the electrodes with activity far from the NS are possibly noises or artifacts.

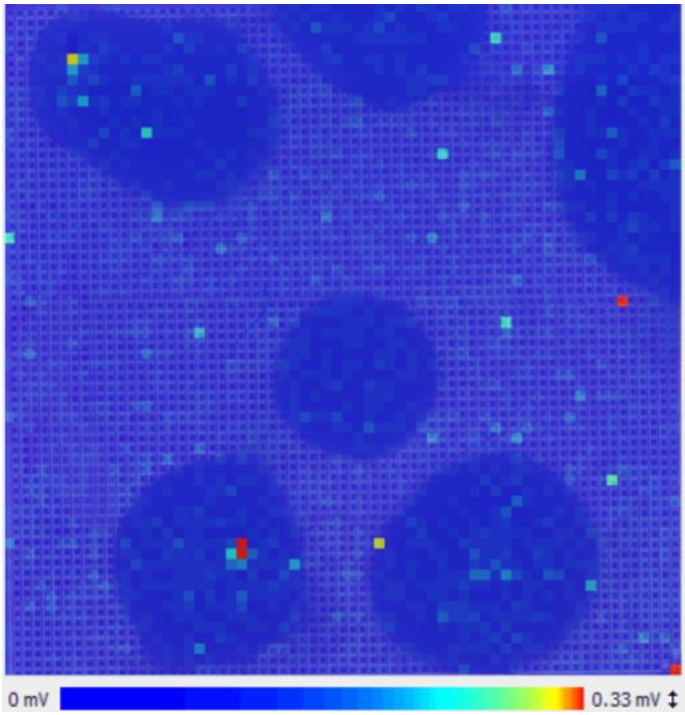

Figure 16. BrainWave X electrode array layout and microscope image

Superimposed images of electrode array and microscope image taken from a sample of NS. The image can confirm if the spike detected is in the surrounding area of the NS.

In order to record electrophysiological signals in the HD-MEA chip, the following materials are required:

- Ethanol 70%
- Paper tissue
- Silicone circular bases
- Aluminum lid

Preliminary Processes

a) Before inserting the chip in the module

1. The BioCAM X and the circular silicon supports must be cleaned with ethanol 70% before entering the laminar flow cabinet.
2. Place the supports in each corner of the BioCAM X.

    Note: This is used to absorb any kind of vibrations.
3. Open the BrainWave X software on the computer and set the temperature control to 37 ºC.

b) Placing the chip

1. Insert the chip in the slot and press the left button in the module to secure the chip.
2. Turn off the lights from the laminar flow cabinet and surrounding areas to avoid noise.
3. Once the chip is detected in the software, proceed to place the aluminum lid on top of the chip.
    a. Note: If the software doesn't detect the chip after 4 attempts, proceed to clean the slot with tools provided with the BioCAM X or the chip’s contact pads with ethanol 96%.
4. Start the program to collect the electrophysiological signals. It is recommended to wait between 3 or 5 minutes before start recording.

6.6 Sample disposal

After the experiments are over, the chip must be cleaned and stored to protect the recording are from dust.

In order to clean the HD-MEA chip, the following materials are required:

- Double Deionized Water (DDW)
- Microliter Pipette
- Terg-A-zyme (Alconox)
- Soft brush

Preliminary Processes:

1. With the microliter pipette remove the medium in the chip's chamber and the samples.

   Note: If there are sample remainings in the electrode array, a soft brush can be used to clean the surface.
2. Dispose the samples in an assigned container for s1 waste.
3. Fill the chips' chamber with Terg-A-zyme and wait for 1 hour.
4. Remove the Terg-A-zyme and rinse the chips' chamber with DDW 4 times.
5. Let the chip dry overnight and afterwards store in its case.

7. RESULTS

7.1 Neurospheres

7.1.1 Sample positioning on HD-MEA chips

The protocol for NS positioning on HD-MEA chips was adequate as it proved that the NS could stick to the electrode array surface and grow onto it. This growth was produced using the growth factors mentioned in the methodology. It is important to be able to grow the NS to the chips as this provides a wider area for possible spike detection. Figure 6 shows 3 days old NS on the electrode array, the NS cover about 9 electrodes from the array, this is the case for most NS placings as they have a small size while in the incubation flask.

Once the NS have been placed on the HD-MEA chip, the growth can be monitored through photographs taken every second day. This provides a time-lapse of growth onto the electrode array, Figure 17 shows an example of this process as the NS become larger every measurement and they even combine themselves with the surrounding NS.

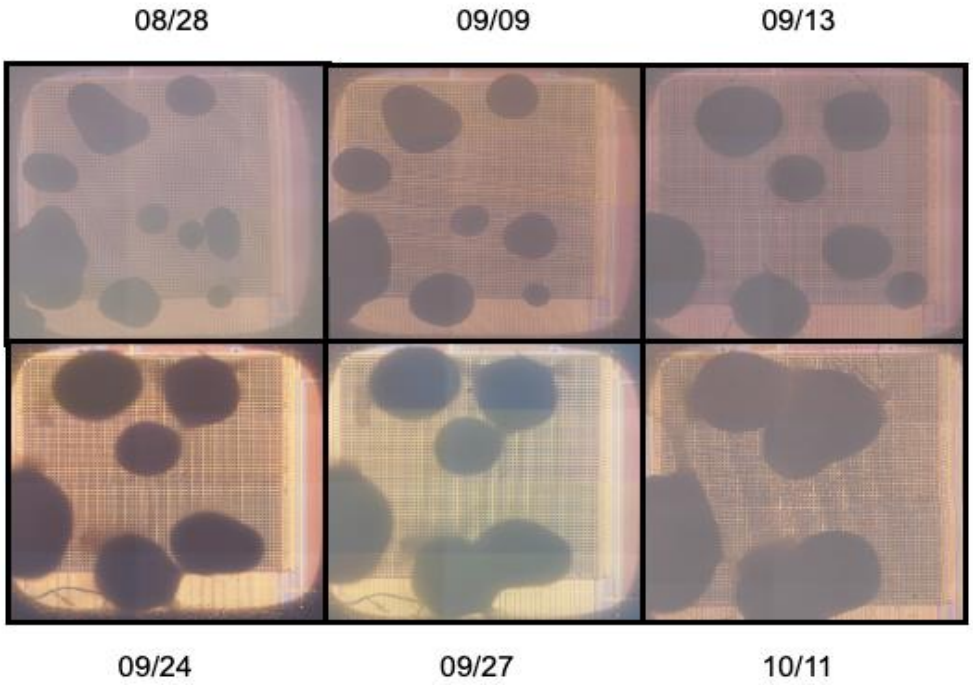

Figure 17. NS samples growth onto electrode array (75d)

Representative growth of NS onto the electrode array, pictures are normally taken every second day but for the purpose of illustrating these photographs have variable time in between.

7.1.2 Electrophysiological recordings

Previous to making electrophysiological measurements the NS were incubated for two days in the HD-MEA chip. After this process is finished, measurements were scheduled for once a week until the NS stopped showing signals.

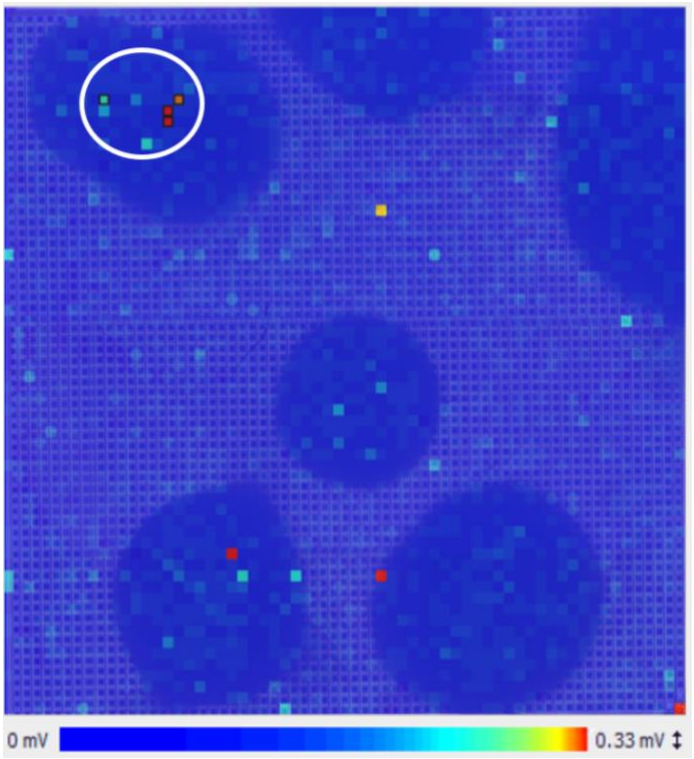

Figure 18. Selected electrodes in BrainWave X layout overlapped with a micrograph

Representative image of electrode selection on BrainWave X, the white circle has inside a few electrodes that light up but only the selected ones have a black outline.

Figure 18 presents activity in some electrodes below the NS, four electrodes were selected from this area to display the signals obtained from them.

Figure 19a shows the differential of potential measured at electrode 9,10 at 19.4 seconds, a possible spike burst can be seen due to the proximity of the spikes, this information can be obtained by analyzing the raw data in Dr. Cell. In a similar way, Figure 19b shows negative spikes at 19.2 seconds. Figures 19c and 19d present similar positive signals from 19.2 to 19.5 seconds.

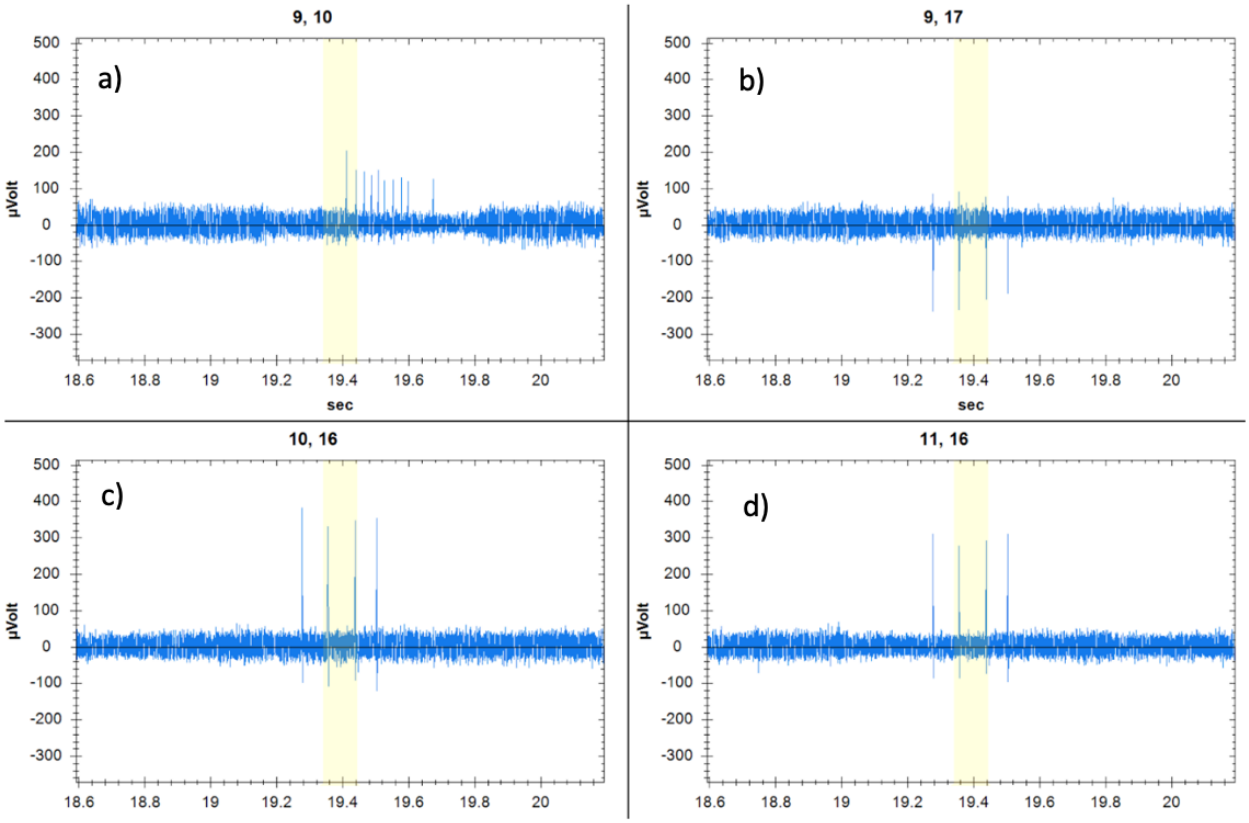

Figure 19. Signals from selected electrodes in BrainWave X

Signals detected from the electrodes selected in Figure 18, the variety in obtained signals can be shown as the electrodes show a difference in the intensity of the spike and even a negative signal.

7.1.3 Electrophysiological analysis

Once the measurements were done in BrainWave X, the raw data files were uploaded to Dr. Cell to be analyzed. In this phase, filters and thresholds can be applied to improve the analysis, other options include the removal of artifacts, detection of spikes, burst and synchrony between all signals presented in the chip.

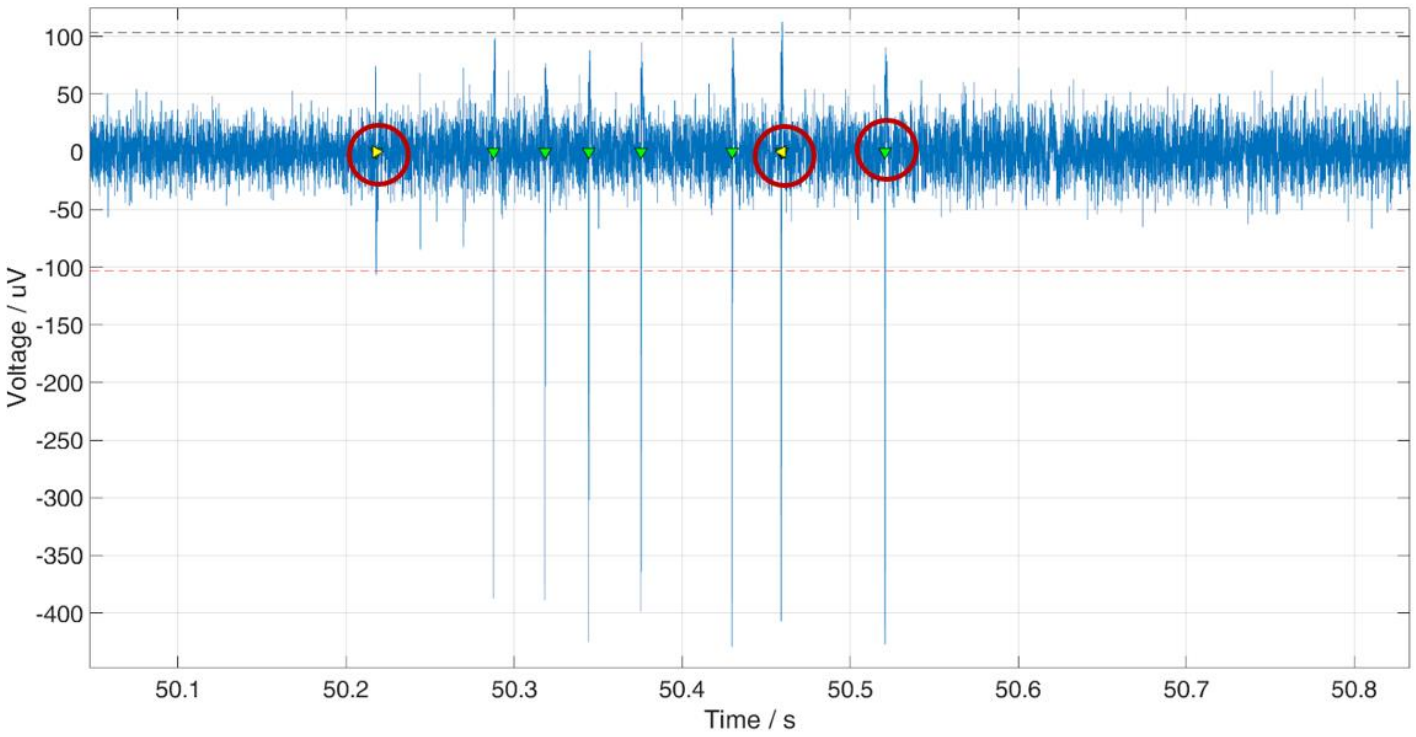

Figure 20. Negative signals from NS in Dr. Cell.

Analysis of recorded signal on Dr. Cell, yellow and green triangles indicate the beginning and end of a spike burst and the time when a spike surpasses the threshold, respectively.

Figure 20 displays a NS recording, on the x axis the voltage is shown in µV, the y axis shows the time in seconds. The horizontal dotted lines in colors red and black, represent the calculated threshold, an established minimum voltage required for identifying a spike and validating it as a signal.

The triangles located in position 0 of the y axis are important for the analysis, yellow triangles indicate the beginning and the end of a spike burst, this can vary depending on the selection made in the spike analysis. The green triangles indicate a valid signal once it recognizes that a spiked has surpassed

the threshold. Figure 21 presents a case were the spike surpasses the upper and lower threshold which gives two triangles in the same spike detection.

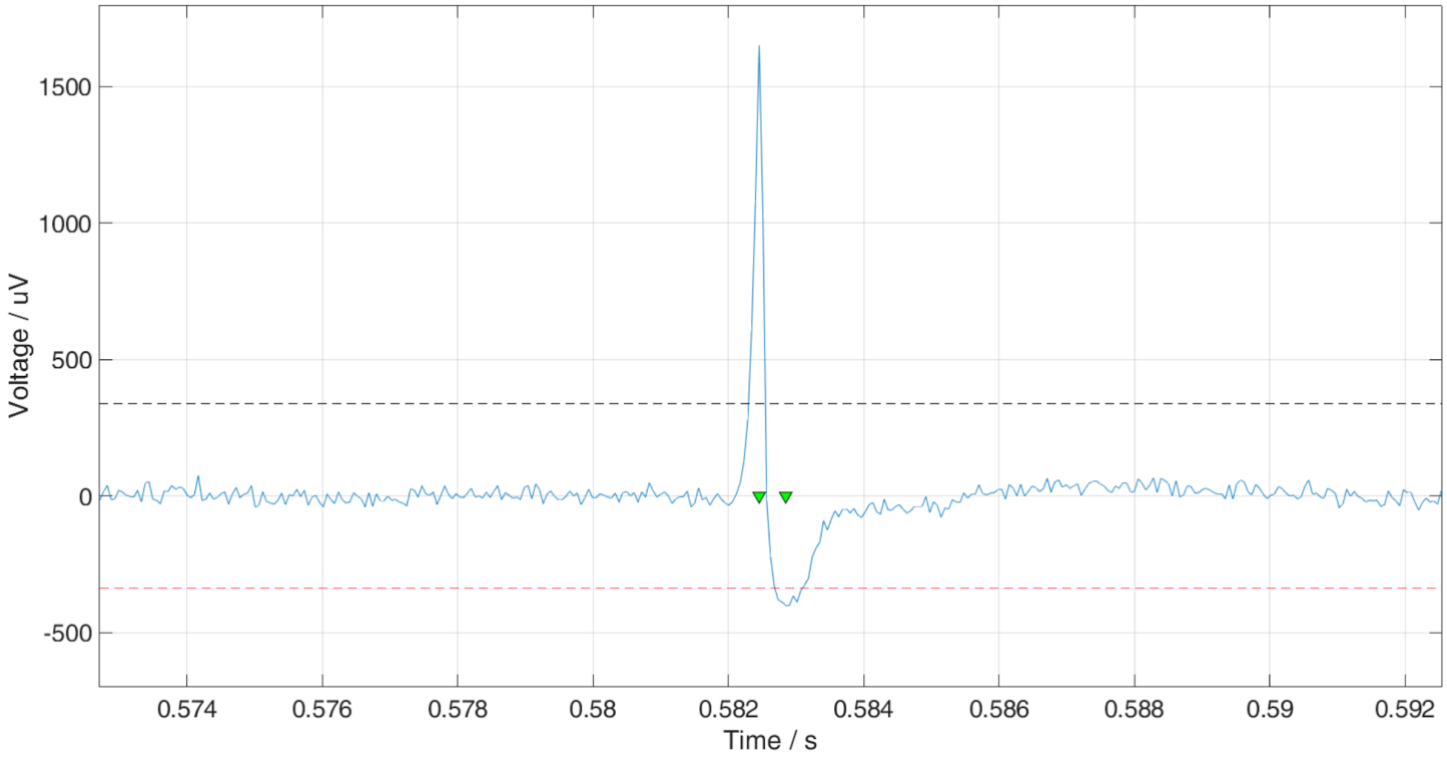

Figure 21. Positive signal from NS in Dr. Cell

Analysis of recorded signal on Dr. Cell, the representation of green triangles indicates in which point the spike surpasses the calculated threshold.

7.2 Cortical Organoids

7.2.1 Slicing Protocol

The slicing protocol has been successful with over 30 COs that have been cut into slices at BioMEMS lab. The protocol solved the main issue which was the attachment of the organoid to the agarose by removing medium from the surface

of the organoid and blending the remaining by flipping the organoid inside the agarose.

The shape of the organoid slice in the agarose may vary with some characteristics like age, density, and size. During the development of this project it was found that in order to have a circle shaped slice COs must have a high density and a minimum size of ~3mm; these characteristics can only be estimated by visual factors in the lab.

The slicing protocol also provided information about the ideal age for an organoid to be sliced as it was read in literature that this age was 60 days (Lancaster et al, 2017). The first COs sliced were 80 days old, which provided big circular structures but with holes in the middle due to core necrosis, these organoids attached well to the agarose and provided a large uniform slice but the remaining holes could not be fixed as the air liquid culture used in the lab does not provide growth factors to the CO slices, only nutrients which causes a slow development that does not have the ability to close down the holes in the 30 day culture period. Figure 22 shows slices of an 80 day CO. Note that in the central part of the slice there are two holes due to necrosis.

Figure 22. Organoid slices 80 days old

First batch of slices, the left image can show a few holes in the organoid, these are a result of the lack of nutrient supply in some parts of the COs. The right image has less holes in the overall structure, but it still has an opening in the bottom part.

The second batch of organoids was only 40 days old, this provided island like structures meaning they had variable shapes and were not a joint structure, but many small ones close by.

Figure 23. Organoid slices 40 days old

Second batch of organoid slices presents scattered bits of organoid in the agarose, this is not a bad result but not the best in terms of sample handling and insertion onto HD-MEA chips.

Therefore in this case the ideal age for slicing would be close to the 60 days as the literature recommends, but it has to be mentioned that in the case of having the two options shown above the older COs present better characteristics

for the purpose of this project as they give a larger shape which may result in a better positioning of the organoid on the HD-MEA chip.

7.2.1.1 Life expectancy of CO after slicing

In order to confirm that the organoids survive the slicing protocol and receive enough nutrients with the air liquid culture a staining protocol was implemented for them. This protocol was a control method performed on a sample because after staining the slice it becomes no longer viable.

The staining protocol gave important information in life expectancy of the slices as it shows in Figure 24 that organoid slices survive at least 50 days with the proper care

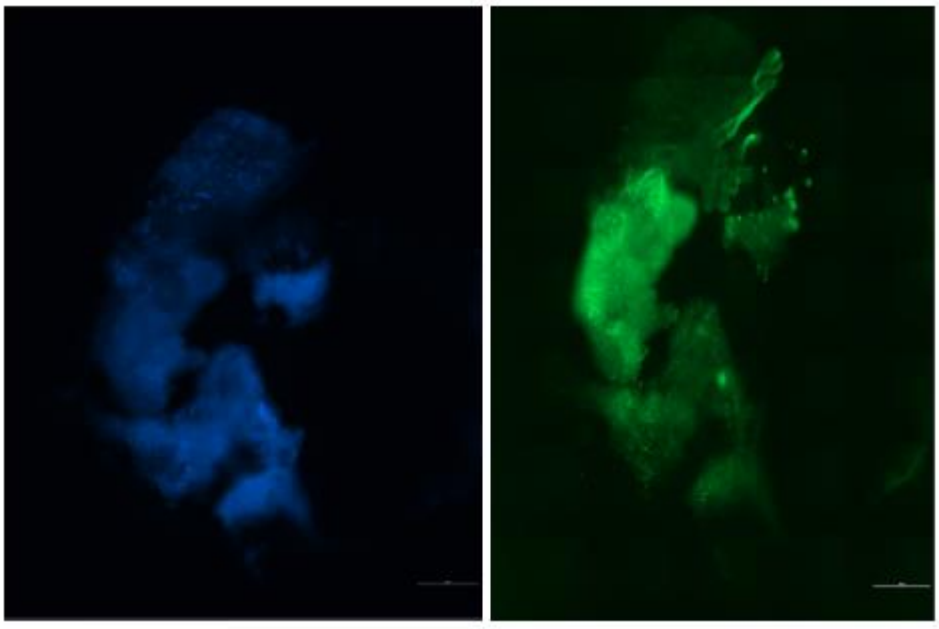

Figure 24. CO slice after 50 days of slicing

Longest living CO slices to be stained in the laboratory after 50 days of slicing, the stainings can show that although there are dead cells in the structure the alive ones prevail in this shot.

It has to be mentioned that COs are quite sensible and will not survive any contamination factors, as it was seen that, when they came in contact with bacteria, the development of contamination process was quite fast, these factors can be avoided by making sure that all of the equipment used to handle the slices is sterile.

7.2.2 Sample positioning on HD-MEA chips

This methodology presented a new approach as the use of anchors was never suggested before at BioMEMS lab, it was necessary as the thickness of the slice prevented it from sinking in the medium and touch the electrode array of the HD-MEA chip.

The protocol implemented was successful as the anchors gave the slice enough weight for it to be in touch with the electrodes, the PEI also ensures that the slice sticks and grows on the surface of the electrode array.

Figure 25 shows a CO slice which was around 80 days old when it was sliced, so the overall shape is circle like and covers the top right part of the electrode array.

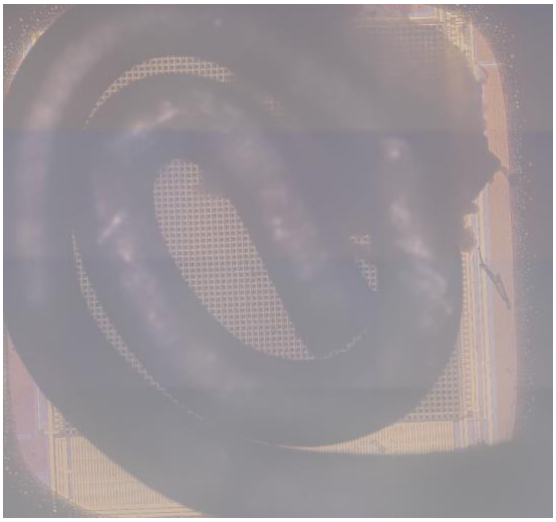

Figure 25. CO slice on electrode array with anchor on top

Inserted CO slice with stainless steel anchor lying on top, the slice can be seen to be a full surface on the top right corner of the electrode array. This slice corresponds to the first batch of COs.

Unfortunately, this slice was contaminated after 4 days of having the anchor on top of it which contributed to determine a lower waiting time for the adhesion of samples onto the HD-MEA chips and a proper sterilization process with the anchor being submerged for 5 minutes in ethanol instead of just spraying it.

Figure 26 presents a CO slice, in this case the CO was around 40 days old when it was sliced so the overall shape is island like and it covers different parts of the electrode array, for this process the micrograph was taken after the anchor was removed to decrease sample movement and help the adhesion process.

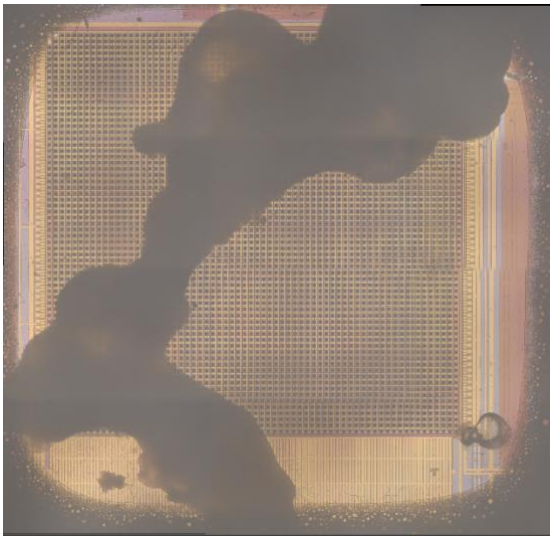

Figure 26. CO slice after removing the anchor

Inserted CO slice after stainless steel anchor removal, the slice can be seen to be more scattered as younger organoids tend to have less structure. The figure shows that even though the slice is not circle shape it still has several electrodes covered.

The end result was a defined protocol for positioning the slice and the anchor for 2 days to ensure adhesion.

7.2.3 Electrophysiological recordings

The electrophysiological recordings done where adequate as we recorded signals (voltages). As such, we recorded spikes with higher amplitude than the noise band. Plot shown in Figure 27 has signals of 200 µm.

Despite what is previously mentioned the capability of having measured changes in the differential of potential within a CO is a breakthrough because to the best of our knowledge, there are no previous recordings. This confirms that the established protocols are optimal to be repeated.

There is still a need to implement a control method for more samples in order to determine an expected CO signal or even analyze if the COs are in fact mature enough to produce significant signals.

Figure 27 shows representative signals acquired in recordings, it can be seen that the spike goes up above 200 µV, complying with some characteristics that determines if it is in fact a signal, one is that the signals goes above the noise band and the other one is that the spike happens in less than a 1.5 ms (Bear et al, 2016).

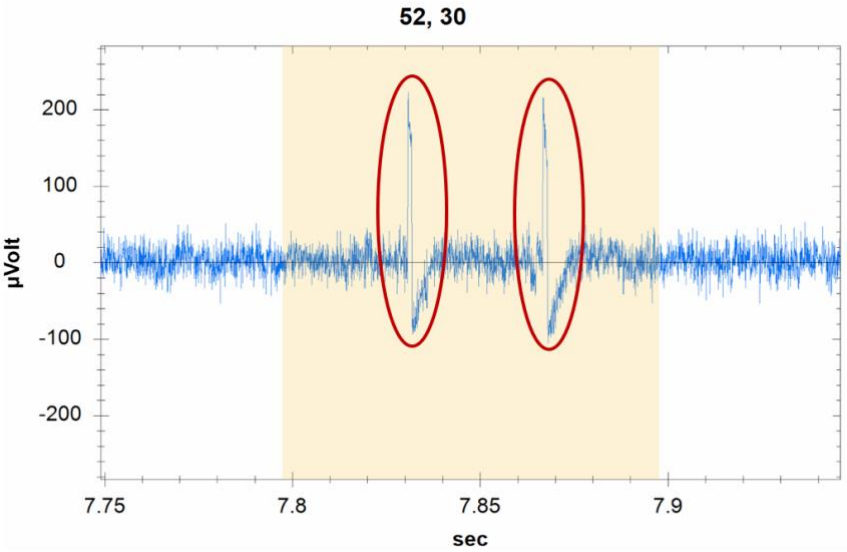

Figure 27. CO slice recorded signals

Recorded signals on electro 52,30 of 84 days old CO at the time of cutting and 124 days at the time of measurement.

7.2.4 Electrophysiological analysis

Figure 28 shows identified signals using Dr. Cell, here it can be seen that COs analysis is different from NS, and signals have some similar characteristics but overall different shapes.

Although the analysis is not ideal it can still be seen that the program validates some spikes since they surpass the calculated threshold, yellow triangles behave in an unfamiliar way since they are marking some spike bursts, it is unclear why this issue happens but since these have been the first CO recordings analyzed with the program it is an area of opportunity for the developers.

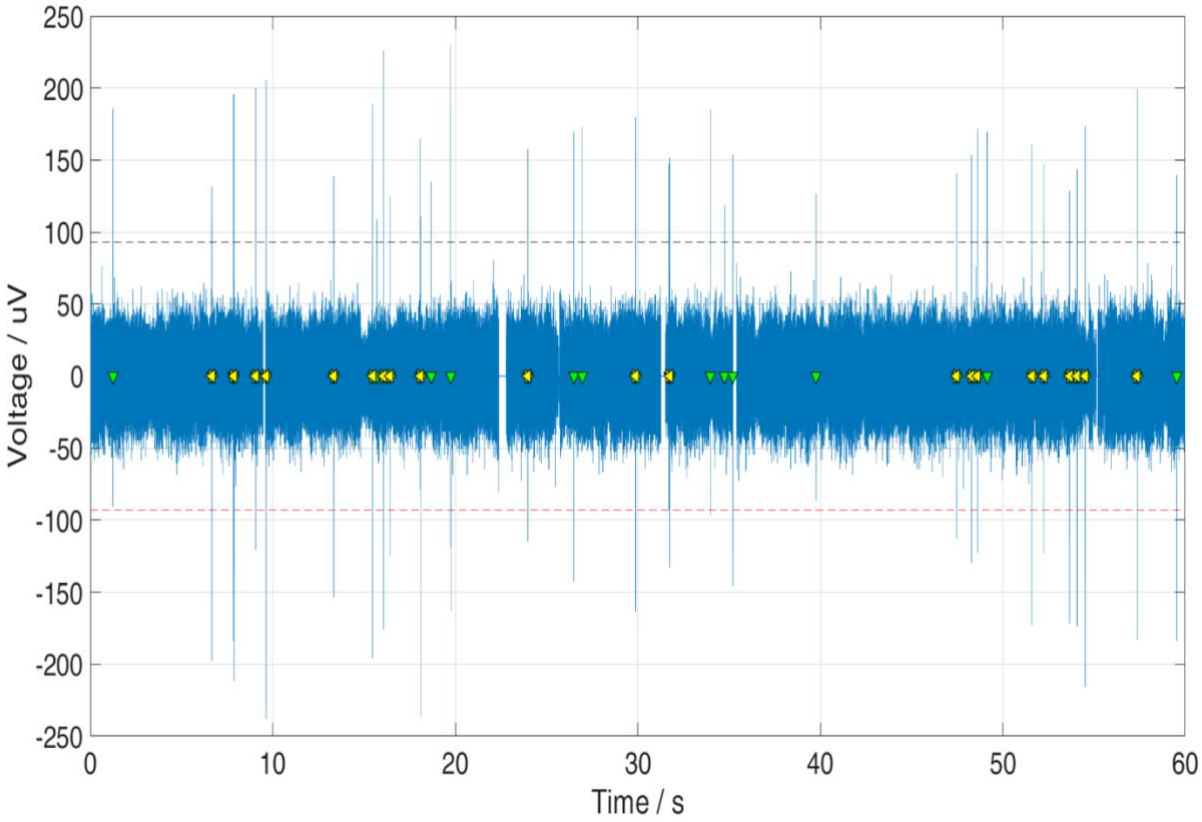

Figure 28. CO recording analyzed on Dr Cell.

Analyzed recording of 124 days old CO sample, the spike detention program seems to have difficulty in detecting the spike burst as it marks some that are not visible. There are several green triangles that indicate the surpassing threshold by spikes.

Figure 29 is a clear example of the behavior mentioned above as it was maximized to a point where only one spike can be seen in the graphic. This signal

shows two green triangles as the spike first surpasses the upper threshold and then falls just enough to surpass the lower one, resulting in a validated signal according to the program.

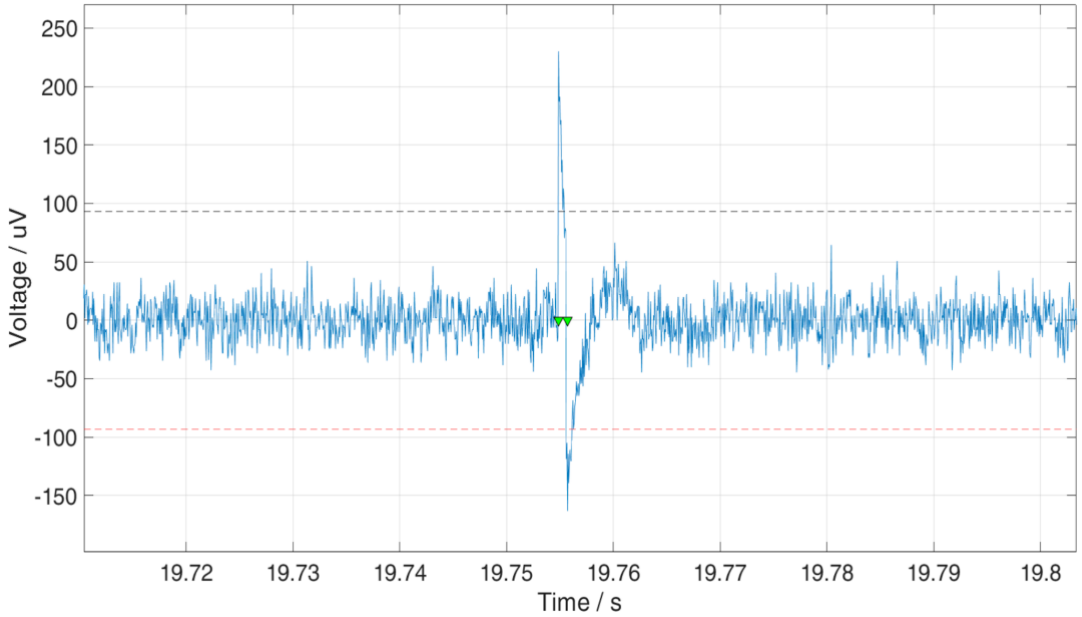

Figure 29. CO recorded analyzed on Dr Cell: only spike

124 days old CO recorded signal analyzed on Dr Cell to confirm that the threshold detection is correct.

7.3 Optimization of high amount of data

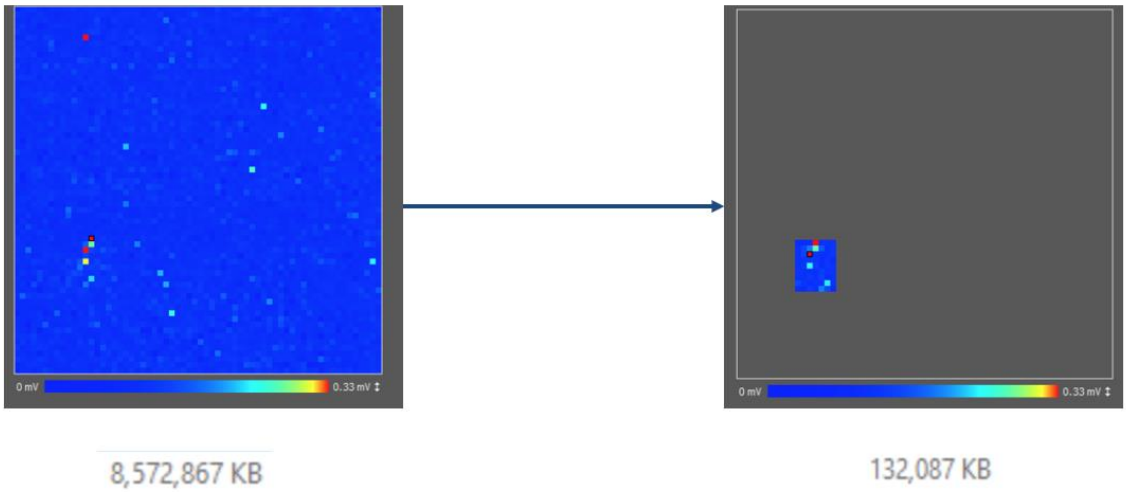

Figure 30. Optimization of high amount of data

Figure 30 shows two cases presented on the recording of electrophysiological activity on Brainwave X, this selection of desired electrodes decreases the size of the file and allows for more recordings to be made.

A special request by BioMEMS lab is that every student that works on a project related to Dr. Cell has to give an idea to solve the optimization of high amount of data obtained from recordings, this is a recurring issue as the data obtained from the electrophysiological recordings uses a lot of space and only computers with certain memory can withstand these procedures.

While developing the project an idea was proposed as it was visible that in some cases the samples do no cover most of the electrode array, meaning that there is information loading into the matrices of Dr. Cell that can be filtered. The recordings done in BrainWave X can be customized to a desired number of electrodes. In the case presented in Figure 30, the area of interest was selected according to the activity registered in that zone.

A 1-minute recording was made and comparing the size of files it can be seen that it decreases from 8.6 Gb to 133 Mb. This solution could be implemented in Dr. Cell program, so it disregards the non-selected electrodes and focuses only on the active ones.

This is only a proposed solution where the samples are positioned in one specific part of the electrode array, it could also be widened to cover the electrode in quadrants, so we have a larger coverage but without overworking the program.

8. DISCUSSION

8.1 Signal spike positive and negative outcome

During the development of the project one unknown factor came across in most of the measurements and it is the fact that the recordings presented positive and negative spikes. Accordingly, an extensive research was done to find a possible answer for this. A general answer is that the measurement outcome might change depending on the positions of the measuring electrode and the reference electrode, although how much more can we go into detail?

A computer designed model developed by Bestel, R. represents the activity happening in neuron at the time of the measurement, Figure 31 is a

simplified version of that model. So, the hypothesis is that the outcome signal can be affected by at least the following two causes.

Figure 31 presents a case incoming signal comes from a dendrite on the top part of the soma, this will cause the potassium channels on the opposite part of the soma to open, where the electrode is located, since the potassium concentration is higher on the inside, these ions start exiting the neuron, making it more positive in that specific part, this will result in the measurement electrode being more positive than the reference electrode in that moment, providing a positive spike.

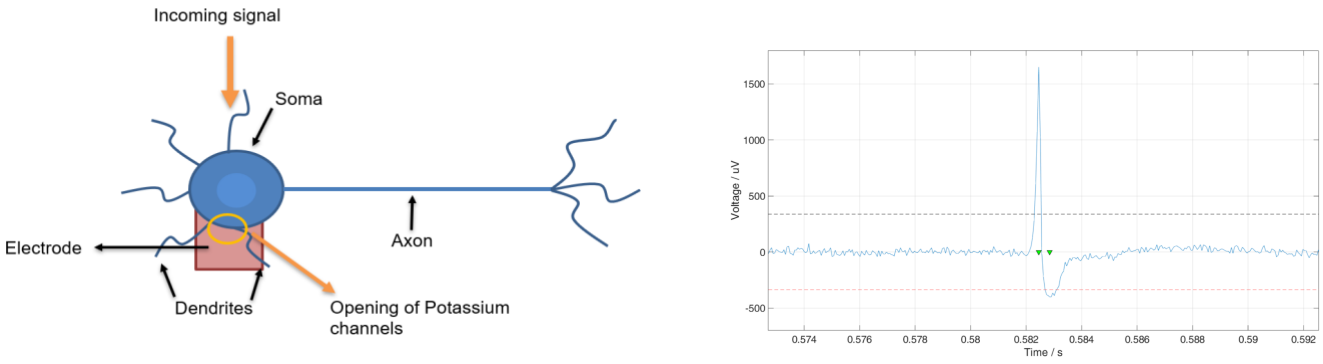

Figure 31. Illustration of the cell on top of a measuring electrode, positive signal.

Representation of cell on top of an electrode, the electrode is presented as the red square located in the lower part of the soma.

Figure 32 shows a different case where the measuring electrode is located on the axon, represented as the a square. In this case the action potential can start from an incoming signal of any dendrite. Once the action potential starts

traveling through the axon a series of sodium and potassium exchange cycles begin to happen.

So in the part of the axon where the electrode is located, the sodium channels open and sodium ions travel inside of the neuronal membrane making the medium in that specific part negative, this will result in the measurement electrode being more negative than the reference electrode in that moment, providing a negative spike.

After this the potassium channels open, throwing ions out through the membrane, making that specific part to slowly become more positive, this can be seen as the measurement electrode begins to be less negative than the reference one. At last this signal has an overshoot because the potassium channels close slowly producing the signal to go a little bit over the starting point.

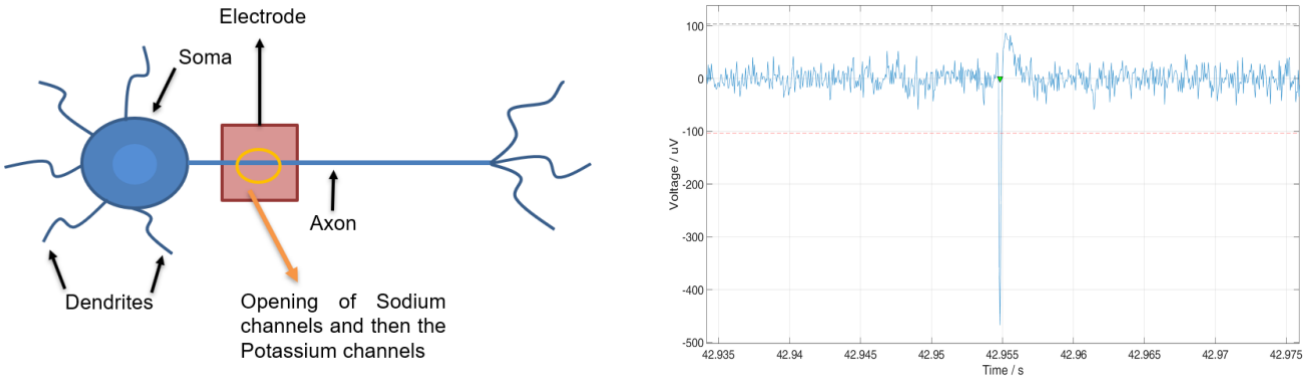

Figure 32. Illustration of the cell on top of a measuring electrode, negative signal.

Representation of cell on top of an electrode, the electrode is presented as the red square located only on the axon.

8.2 Anchor use

The overall use of an anchor in the developed protocol fulfills its purpose as it makes the sample sink and be in contact with the PEI on the electrode array ensuring its adhesion, but it is yet to be defined if this is the best method that could be applied for this necessity.

There are anchors already made for this purpose with different materials that could have been used in order to make the final product better, however all of the anchors at hand were too big to fit in the 9mm^2 space. Therefore, the solution was to make anchors with stainless steel that fit in this area, this material was chosen because it is the most common used one for these purposes.

It has been considered that maybe the use of such a hard material could damage the electrode array in the long term, which is why an area of opportunity in this field could be covering the anchor with a soft material like PDMS in the form of mesh, as putting a complete block on top of the sample could prevent the absorption of nutrients.

In order to be able to repeat these processes there needs to be an improvement in the anchor system so it is a safe protocol for the samples and the HD-MEA chips.

8.3 CO signals

The spikes detected in BrainWave X and by Dr. Cell have certain characteristics that can be directed towards it being a signal more than an artifact, these factors are: the intensity of the spike is higher than the noise band going almost to 230 µV, this spike also happens in less than 2 ms.

Unfortunately, there are no publications to date that present an expected signal from COs, so far, a preprinted version of a scientific article by Trujillo, C., associate project scientist in UCSD, addresses this issue.

With the use of MEA chips and a spike detection algorithm the article suggests that the CO network electrophysiological signatures correlate with human preterm neonatal EEG features. (Trujillo et al, 2019)

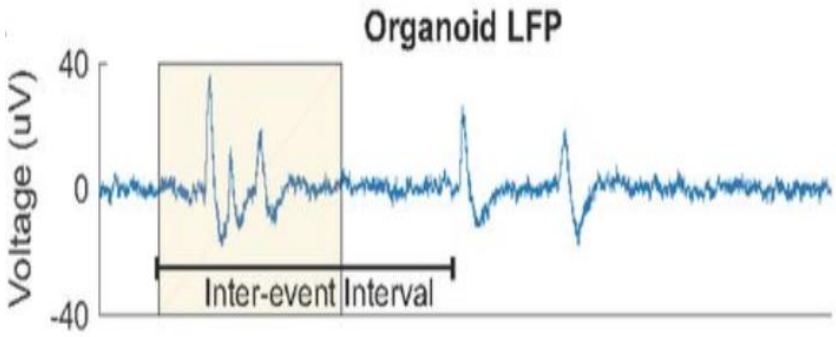

Figure 33. Measurement of COs presented by UCSD

LFP refers to the electric potential in the extracellular space around neurons recorded.

The signals presented in this article might have some characteristics that look similar to the ones described in the results section, which gives the impression that CO signals may look completely different from the NS signals.

The article closes by stating that functional equivalence between the COs and a full neonatal cortex is not claimed yet (Trujillo et al, 2019).

To provide an expected signal with accuracy there needs to be more comparative results, this could be achieved by building a database that can be used to identify several behaviors involving neurons and action potential in COs.

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Honor Pledge

We, Ismael Lozano Flores and Leticia Erandeny Duarte Reyna, hereby declare that we have done this final evaluation project with academic integrity.