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Study of the electrical / chemical manipulation of rat neuronal cultures in dual compartment devices on MEAs

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ABSTRACT

Development of controlled in-vitro neural network models has long been a major challenge for neuroscience research. In-vitro models may provide good experimental access and better understanding of brain diseases and their treatment options, e.g. the motor pathways affected in Parkinson's disease (PD).

Although some motor symptoms of Parkinson's disease are being treated with Deep Brain Stimulation (DBS) for more than a decade, progress in understanding the mode of action of DBS is slow. *In-vivo* access to the relevant neuronal networks is severely restricted, and organotypic brain slices are unable to capture enough of the three-dimensional pathways that are the substrate of PD's motor symptoms.

On the other hand, in-vitro neural network models are promising tools for gaining insight into many chronic brain diseases and their treatment. In an attempt to build an in-vitro neural network model, the DBS group at Philips Research led by Prof. Michel Decré has demonstrated a dual compartment neurofluidic system with inter-connected micro-channels to connect neurons from their respective compartments on a planar Micro-Electrodes Array (MEA) (1). Cell culture protocols of dissociated rat cortical neurons in such devices, axonal growth through micro-channels, and analyses of functional connectivity between compartments through electrophysiological studies were demonstrated in their earlier work (2).

In this present work, electrical and chemical neuromodulation of cells in the dual-compartment device is demonstrated. With the objective to further demonstrate functional connectivity across the micro-channels a detailed study on the influence of electrical / chemical neuromodulation is presented.

In the case of electrical stimulation, spreading of electrical artifacts in the cell culture medium between the compartments is clearly evident in our earlier studies. Various configurations of stimulating electrodes and their influence on artifact spreading in the compartments are demonstrated. With the final goal of studying different populations of neurons i.e. cortical and thalamic, a more specific stimulation focusing on a smaller area which affects only one compartment was investigated. A bipolar configuration used together with 4 external grounded sites connected to the neural basal medium was chosen as the most optimal configuration and has been studied in this report.

The effects of this new configuration on cortico-cortico devices were also explored by the evoked response elicited in the cells. It is shown that this new configuration is focused on a smaller area than the unipolar stimulation at the same frequency and amplitude reported in literature (3).

In further confirm the earlier work on fluidic isolation between the compartments, an extensive study on the influence of medium diffusion from one compartment to the other compartment with time was analyzed with spectrophotometric technique and the results presented in this work.

Additionally, the influence of synaptic blocker in one compartment and the influence on the network propagation when Tetrodotoxin (TTX) was added in compartment with electrical stimulation are presented.

SOMMARIO

Lo sviluppo di modelli di reti neurali in ambienti controllati in-vitro e' stata a lungo una delle maggiori sfide nel campo delle ricerche neuroscientifiche. Modelli in-vitro forniscono un buon accesso sperimentale per una migliore comprensione delle malattie neurodegenerative e del loro trattamento, quali ad esempio le vie motorie affette dal morbo di Parkinson.

Nonostante alcuni disagi motori nel morbo di Parkinson siano stati trattati per più di una decade attraverso la Deep Brain Stimulation (DBS), i progressi nella comprensione delle modalità di azione della DBS sono lenti. L'accesso in-vivo alle reti neurali coinvolte dallo stesso morbo è duramente compromesso, mentre le slices cerebrali organotipiche sono incapaci di catturare a sufficienza la tridimensionalità delle vie neurali coinvolte nel substrato dei sintomi motori nel Parkinson.

D'altro canto, i modelli di reti neurali in-vitro sono strumenti molto promettenti per l'indagine di diverse malattie cerebrali croniche e del loro trattamento. Con l'intento di costruire un modello di rete neurale in-vitro, il DBS group in Philips Research guidato dal prof. Michel Decré ha dimostrato che un sistema neurofluidico a doppio compartimento con micro-canali interconnettenti è in grado di porre in comunicazione neuroni dalle loro rispettive camere su una matrice planare di microelettrodi (MEA) (1). Protocolli di coltura di cellule corticali di ratto, crescita assonale nei micro-canali e analisi della connettività funzionale tra compartimenti attraverso studi elettrofisiologici sono stati dimostrati in uno studio precedente all'interno dello stesso gruppo (2).

Una volta raggiunta una certa confidenza negli studi delle cellule corticali di ratto, si rivela interessante l'applicazione degli stessi studi su diverse popolazioni di neuroni. Nello specifico, prendendo ispirazione dalle vie neuronali coinvolte nei meccanismi di generazione del morbo di Parkinson, ci si prefigge lo scopo di analizzare le dinamiche delle comunicazioni sinaptiche tra popolazioni diverse di cellule quali corticali, talamiche, il corpo striato e i segmenti interni del globus pallidus (4).

Perciò all'interno di questo progetto si inizia l'estrazione di cellule provenienti dal talamo. Individuate all'interno degli atlanti del cervello di ratto embrionale al 18° giorno di gestazione, si è cercato di eseguire la raccolta di tali neuroni dalle parti interessate. Questo, permetterà uno studio delle dinamiche oltre che delle sole cellule talamiche, anche un ipotetico collegamento con cellule corticali nello stesso piatto.

Andando ad investigare il tipo di stimolazione che si può eseguire su cellule talamiche, è inoltre possibile vederne gli effetti su cellule corticali per un più approfondito studio delle tecniche DBS. Mentre, molti studi di stimolazione elettrica neuronale corticale sono stati effettuati in-vivo e in-vitro e lo stato attuale della ricerca su questo tipo di colture corticali ha raggiunto una comprensione tale per cui i parametri di stimolazione elettrica sono già stati definiti (3), interessanti sviluppi sono possibili.

Nel presente lavoro, la neuro modulazione elettrica e chimica di cellule neurali è spiegata. Con l'obiettivo finale di dimostrare ulteriormente la connettività funzionale attraverso i micro-canali, uno studio dettagliato sull'influenza della neuro modulazione elettrica / chimica è presentato. In particolare si vuole cercare di determinare l'effetto di una eccitazione di una delle popolazioni sull'altro compartimento, e determinare così non solo la connessione in termini di correlazione tra i due compartimenti, ma anche e soprattutto il tipo di influenza che una popolazione di neuroni ha su un'altra, come ad esempio potrebbe essere tra talamiche e corticali. Prima di arrivare ad uno studio di questo tipo, è necessario osservare gli effetti di una stimolazione elettrica e/o chimica sui MEA ed eventualmente sui dispositivi applicatisi.

Nel caso di stimolazione elettrica, la diffusione di artefatti elettrici e di un certo tipo di risposta neuronale evocata nei due compartimenti è evidente nei nostri studi precedenti. Con lo scopo finale di studiare diverse popolazioni di neuroni, quali corticali e talamiche, una più specifica stimolazione focalizzata su una superficie minore in grado di influenzare solo uno dei compartimenti è stata studiata in questo progetto. Ciò viene utile quando, come è stato osservato, il tipo di stimolazione fornito alle colture corticali non si adatta alle colture subcorticali / talamiche che sono state estratte e che oltremodo ne danneggia la specifica attività spontanea.

In conseguenza di ciò, varie configurazioni di elettrodi stimolati o posti a massa e della loro influenza sulla diffusione degli artefatti tra i compartimenti sono state sperimentate. Un nuovo tipo di configurazione bipolare diversa dalla vecchia unipolare utilizzata congiuntamente a 4 punti di massa distanti dalla stimolazione collegati al medium basale neurale è stata scelta come configurazione ottimale ed i suoi effetti sono stati documentati in questo report. L'effetto di questa nuova configurazione su dispositivi con cellule corticali nei due compartimenti è stato esplorato attraverso la risposta evocata neuronale. In particolare, è mostrato che questa nuova configurazione risulta essere molto più focalizzata rispetto alla stimolazione monopolare data alla stessa frequenza e ampiezza che è stata ampiamente utilizzata in letteratura.

I risultati presentati in questo report documentano che una stimolazione con configurazione bipolare risulta focalizzata nell'area appena intorno gli elettrodi stimolati. Tuttavia, si potrebbe obiettare che nel corso della stessa stimolazione bipolare, gli elettrodi che non mostrano nessuna risposta potrebbero essere non attivi oppure poco responsitivi. Per questo, sono stati effettuati alcuni esperimenti in cui unitamente ad una stimolazione bipolare si è effettuare lo stesso giorno dell'esperimento oppure qualche giorno dopo, una stimolazione unipolare che ha dimostrato ancora una volta una propagazione della corrente elettrica su tutto il piatto.

Per quanto riguarda le stimolazioni di tipo chimico, è necessario documentare un isolamento chimico inter-compartmentale, per far sì che l'effetto di un determinato componente influisca solo sul comportamento di una delle due camere.

In questo progetto si è accertata una ulteriore conferma del precedente studio sull'isolamento fluidico tra compartimenti attraverso uno studio estensivo sull'influenza della diffusione di medium da un compartimento all'altro rispetto al tempo analizzando le soluzioni dei due compartimenti con tecniche spettrofotometriche. I risultati sono presentati in questo lavoro.

Un secondo approccio è stato anche utilizzato basato sull'influenza sull'attività spontanea dei neuroni di alcuni composti conosciuti come la Tetrodotossina (TTX) oppure un composto in grado di bloccare reversibilmente le connessioni sinaptiche tra neuroni. In particolare si è dimostrato in questo progetto e in studi precedenti (1) che l'influenza dei due composti chimici applicati ad una delle due camere, non influenza l'attività sinaptica dell'altro compartimento. Oltre a ciò, dato il particolare effetto del secondo composto (il Bloccatore di Sinapsi) interessanti studi sull'attività genuina delle cellule può essere studiato. Infatti, mentre normalmente le colture corticali sono pesantemente influenzate da fenomeni di bursts, interrompendo la comunicazione tra cellule la sincronia sparisce portando i neuroni a perdere la precedente attività di burst in favore di più isolate attività di spiking. Nei nostri studi si sono verificati anche episodi di inibizione tra neuroni, per cui nel momento di esposizione a TTX o Bloccatore Sinaptico di una popolazione, alcuni elettrodi del compartimento adiacente hanno registrato un aumento di attività.

In più, date le caratteristiche di reversibilità della tossina TTX, è stata possibile eseguire una prova della focalizzazione della stimolazione elettrica fornendo al contempo una inibizione di tipo chimico associato ad una stimolazione di tipo elettrico. Nel caso in cui si registri una risposta evocata alla stimolazione

elettrica nel compartimento non chimicamente alterato, la propagazione elettrica si verifica; d'altra parte nel caso in cui precedentemente si registra una risposta evocata e successivamente attraverso l'uso di TTX si dimostra la non presenza della stessa risposta nei due compartimenti, significa che la stimolazione rimane focalizzata in uno dei compartimenti e che una possibile propagazione sinaptica avviene.

Altri esperimenti con composti chimici sono stati effettuati. In particolare si è cercato un composto con effetto stimolante su popolazioni talamiche. Il composto che è stato testato in questo progetto è l'acetilcolina. Mentre da una parte la letteratura si dimostra sorpassata e poco applicabile a colture dissociate di cellule neuronali di ratto, i nostri esperimenti non hanno evidenziato nessuna particolare caratteristica comune, per cui non è stato possibile trarne utili conclusioni. Si raccomanda perciò uno studio più approfondito sia dell'effetto dell'acetilcolina sia alla ricerca di un composto in grado di eccitare le cellule neuronali.

In conclusione, gli studi sulle cellule neuronali basati sui MEA risultano essere interessanti e promettenti. Ulteriori progetti che stanno interessando l'argomento riguardano lo sviluppo di MEA 3D capaci di catturare la tridimensionalità di colture di cellule neuronali sparse, ma anche di slices e di intere sezioni di cervello. Caratteristiche di tridimensionalità risulterebbero essere di particolare aiuto non solo in fase di registrazione, ma anche di stimolazione fornendo così una più appropriata localizzazione. Su questa linea, alcuni gruppi di ricerca si stanno inoltre interessando alla Brain Machine Interface in senso largo, in cui cercano di collegare cellule neuronali su MEA a dispositivi robotici in grado di interagire con la realtà, fornendo così un "corpo" al cervello simulato dalle colture neuronali.

1 INTRODUCTION

Many efforts have been made to unveil the intricate structure of the brain, which is still an object of many studies. The increasing number of neuroscientists and the raising interest in the field make neuroscience a promising field that will surely affect our lives in the near future.

Studies about the interactions between neural cells would empower basic research on brain mechanisms and may contribute further to diagnosis and therapy of several neuropathologies related to dysfunctions of neural physiological activity like epilepsy, depression or Parkinson's disease.

Electrical stimulation has been often applied to nervous system. Remarkable therapeutic benefits have been provided however its underlying principles and mechanisms are still not clear. DBS technique applies these concepts to stimulate a part of the brain by a medical device implanted able to reversibly change neuronal activity in a controlled manner.

Computational models as well as in-vivo and in-vitro studies of biological neurons are being doing to discover the underlying principles of this technique.

While computational studies seem to be promising, they have to rely on biological experiments. In-vivo electrical stimulation experiments are objectively difficult because of the number neurons that have to be controlled with a limited number of recording sites. Moreover, ethical barriers regarding acute brain studies and the difficultness in controlling all the variables make them inapplicable.

In-vitro studies on MEAs eliminate many of those variables reducing the number of analyzed cells and provide also an ideal experimental environment. Additionally, a sufficient grade of complexity is still maintained so that functional activity and structural properties typical of in-vivo neural networks on the scale of thousands of neurons are still observable. This complexity is high enough to study learning, memory and distributed information processing in biological neuronal networks that can finally refer to more general brain mechanisms. The complexity of natural neural networks is such that without control of the neuron positions and connectivity, doubts that can be raised in their understanding. This has led many to investigate methods for guiding neural cell adhesion and growth. While MEAs normally record from a large group of neurons and from their synaptic connections adhered to the surface of the glass, a different approach consists of imprisoning neurons in vicinity of a electrode, as in (5). This approach constrains single neurons in a cage in which only one electrode can record the signal from. This is an optimal solution to have a one-to-one correspondence between neuron and electrode. However, the random nature of the biological networks, the relatively low total number of neurons trapped in cages and the technology that is available make this latter approach still not feasible.

MEAs also provide the structure to electrically stimulate the neurons. Stimulation and recording are made possible by the same array of electrodes providing so an excellent background for neuronal studies. Groups have been also applied MEAs to create an interface between living neural tissue and artificial constructs, making for instance a bi-directional connection between cultured neurons and external devices, like robots (6).

Several studies have already been done regarding the response of cortical neuronal cells to electrical stimulation (3), and how the response can be modulated (7). Good knowledge has been achieved with these studies, although there is still a lack in the studies of other populations of neurons i.e. the ones involved in the pathway of the Parkinson's disease extracted from thalamus, striatum and the internal segment of the globus pallidus(4). A study on these cells would provide useful data on how the cells behave when cultured in communities, and above all how they interact between different populations.

In Minimally HealthCare Department within Philips Research, Eindhoven, The Netherlands, a helpful dual-compartmental neurofluidic PDMS device has been created for this latter purpose. Each PDMS device stays on a MEA, dividing the array of 60 electrodes between the two chambers, 30 electrodes per chamber. This allows culturing a neuronal population inside each compartment. Spontaneous activity of the neuronal cells inside the compartments can be recorded from DIV 4 up to DIV 21(1). Moreover, PDMS is able to put into communication the two neuronal populations in the two compartments trough the small micro-channels that avoid the passage of the neuronal bodies (8). Moreover, the correlation between the signals recorded from the two neural populations demonstrated that the two compartments influence each other (2).

Unipolar electrical stimulation has been observed to spread in the whole device. However, to better make use of the PDMS features, it's advisable to stimulate only the cells in one of the two compartments and observe the effect on the cells of the other synaptically communicating compartment. We have been studying ways to improve the focus of the electrical stimuli by doing propagation experiments and we discovered bipolar configuration used in association with 4 grounded sites at the edges of the device to be the best situation. It was then applied to the neuronal cultures.

Chemical agents can also alter the behavior of the neuronal cells in one of the two compartments. TTX is known to be an effective inhibitor of the spontaneous activity, while recently a substance able to impede synaptic communication has been used to study the difference between early and late response (9). However, a more interesting study about neuronal activity excitatory solutions would enhance the study on the relation between the two different populations. This has brought our group to study the effect of Acetylcholine on cortical and subcortical/thalamic cells.

To make use of all the features the dual-compartmental system can provide, a preliminary study about chemical isolation between the two chambers has been done (1). We enhanced that research, using two different approaches: a study on the effective leakage of the PDMS by a spectrophotometer and by the study of the changes in the spontaneous activity when a synaptic blocker is inserted in the other compartment.

Demonstrated the chemical isolation between compartments, TTX could be safely put in one compartment without affecting the other. The effect of TTX in association with an electrical stimulation both in the same compartment was finally the best proof to show the focus of the stimulation on only one compartment.

2 BACKGROUND

2.1 MEA TECHNOLOGY

Traditionally the electrical properties of neurons have been investigated using conventional electrodes, such as glass micropipettes, in a way that neurophysiologists could create a detailed picture about the single cell properties. Nevertheless, investigating the neurons and the CNS in-vivo can be difficult due to the huge amount of cells to analyze, to the time required for the electrode placement and to the mechanical damage of the cells over long periods of time caused by the insertion of electrodes.

The mammalian neurons have also been studied in vitro in the form of dissociated monolayer cultures for several decades. Such cultures retain many morphological, pharmacological, and electrical properties of natural neural networks in vivo (10) and allow much more detailed observation and manipulation than intact brains, at the molecular, cellular, and network levels(11)(12)(13)(14)(15)(16).

Micro Electrode Arrays (MEAs) are a valuable tool for electrophysiological measurements as they are non-invasive and allow monitoring of the neurons' electrophysiological activity over a long period. Extracellular MEAs were chosen over intracellular electrodes for multiple reasons. Intracellular electrodes change a cell's physiology by perforation and by the introduction of the patch pipette solution, ultimately leading to cell death within at most a few hours. Now, it is widely recognized that MEA technique is a valuable method for the investigation of functional activity and structural properties of "in vitro" neuronal preparations (acute brain slices, dissociated cultures, organotypic cultures). MEA technology and the related culture methods allow an easier combination of electrophysiology with imaging (Ca\Na imaging) too and bring together a huge variety of possible applications. Additionally, recording the same neuron at multiple sites is difficult intracellularly but simple, robust, and nondestructive with MEAs.

The first work using the MEA technology started in 1972 (17) and a milestone was reached when recordings obtained from cultures of dissociated neurons were first reported by Pine et al., in 1980 (18).

MEAs are composed of an array of microelectrodes which are able to detect local variations of the electrical potential that are created by the movement of ions through the protein channels of cells in their immediate vicinity, targeting of several sites in parallel for extracellular recording and stimulation (19). These devices, mostly fabricated by standard photolithographic techniques from the semiconductor industry, consist of a glass substrate where a matrix of typically 60 thin film micro-electrodes is embedded. All the electrodes are then linked to a recording system that can provide both electrical recording and stimulation functions.

In this way they enable long-term monitoring of the electrophysiological activity of neuronal cell cultures, both dispersed cultures and slices, and provide a unique window to observe spatio-temporal patterns of activity in intact two-dimensional layers of neurons (20).

MEA technology has been used to investigate the behavior of different types of cells, like cardiac cells, retinal cells and neural cells. Various groups have worked with different types of neuronal cell cultures but most of them have focused their attention on cortical (21)(22).

As different applications require different solutions, the market leader (Multi Channel System, Reutlingen, Germany) provides several geometries: Planar, 3D, Thin, Flexible MEAs, etc.,(19).

The characteristic dimension of a microelectrode ranges from 10 to 50 μm. As a result, a single electrode may record signals from several neurons. The standard planar configuration we used for this work

consisted of 30 µm diameter electrodes with 200 µm of distance among them (See Figure 1). A spike sorting algorithm has been developed to detect and differentiate spikes generated by the neurons in the MEAs.

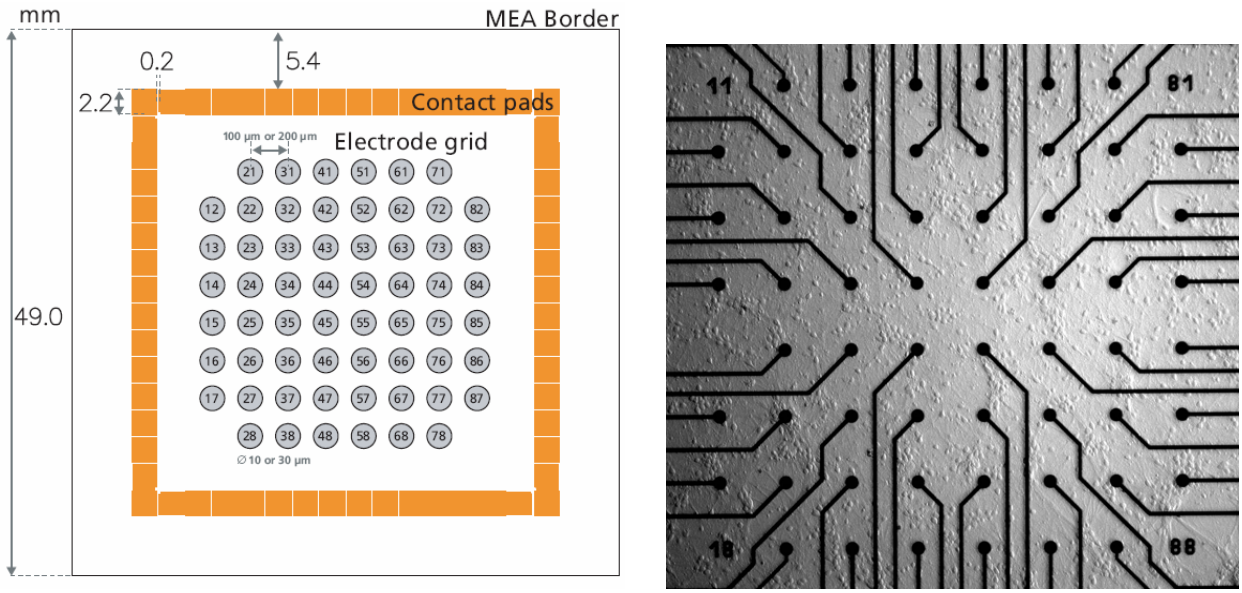


Figure 1: A_ Model of the MEA array with electrodes number in the 8x8 grid; B_ A real photo of one device.

The numbering of MEA electrodes in the 8x8 grid follows the standard numbering scheme for square grids: the first digit is the column number, and the second digit is the row number. For example, electrode 23 is positioned in the third row of the second column. These numbers are the same numbers that are used as channel numbers in the MCRack program (19). Usually electrode 15 (the one that is missing in Figure 1B) is used as internal ground, due to its bigger shape and to the further position in the array. The glass substrates are coated with dielectric Silicon-nitride (Si_3N_4) to provide electrical isolation for the connection traces while leaving the recording sites open.

Since Si_3N_4 insulating surface is highly hydrophobic, an oxygen plasma treatment is necessary in order to obtain a hydrophilic surface. The insulator layer must be treated with molecules that promote cell adhesion to obtain healthy long-term neuronal cultures. The most widely used molecules are poly-lysine, laminin and polyethyleneimine (PEI) (20). Due to its strong adhesive properties, PEI is often used in dissociated culture studies (23) and in the experiments conducted by our group since 2006 (24).

The functional characteristics of the MEAs allow long-term recordings of spontaneous and stimulated neuronal network activity. However the complexity of natural neural networks is such that without control of the neuron positions and connectivity, many doubts can be raised about their understanding. This has led many groups to investigate methods for guiding neural cell adhesion and growth. When biological coatings are patterned, the controlled cell growth at least temporarily yields patterned cultures which could ultimately be useful for studying structure/function relationships in neuronal networks.

Different techniques using different adhesion promoters/inhibitors substrates can be found in literature (25) (26). Usually, ordered growth recedes after several days in vitro, preventing long-term studies of designed neural networks.

A different approach consists of imprisoning neurons in the immediate vicinity of the electrode by physical means. This concept has led to the creation of the so-called “Neurochip” (5). In this device,

neurons are physically imprisoned inside micro-cages on top of electrodes obtained by micro-machining of silicon wafers. The major drawback of the produced devices was that neurons tended to escape from the wells that are supposed to keep them in close contact with the electrodes (20). The displacement of neurons cultured on planar surfaces is due to strong pulling forces that the neurites exercise on the soma. The problem was overcome when Pine et al. introduced the "neurocage" approach, using surface micromachined parylene cages (27). This approach constrains single neurons in a cage in which signals are recorded by only one electrode. This is an optimal solution to avoid neuronal migration that is typical during the first week of culture and to have a one-to-one correspondence between neurons and electrodes. On the other hand, compromising the intrinsic random nature of the neural networks and the relatively low total number of neurons trapped in cages remain severe limitations.

Because of these, some investigators have left culture control behind, and opted for random, dispersed cultures with spontaneous activity networks, combined with globally distributed electrodes that "sample" population activity by recording neurons' activity within the population (28).

The functional characteristics of the MEAs permit mid- to long- term recordings of both spontaneous and evoked neuronal network activity patterns and of their spatio-temporal evolution. Under this perspective, large neuronal ensembles coupled to MEAs represent an interesting intermediate level (from in-vitro single cells to in-vivo studies) for investigating information processing and dynamics in neuronal systems under controlled condition and by means of applications of external stimuli and/or physical constraint(29)(30).

Our configuration goes one step further into differentiation along those lines: we investigated neuronal cultures placed inside micro-fluidic structures, inspired from the literature (31), directing the organization of the neural network in a physical barrier through which only the neurites can pass. Explication about the particular configuration we used is discussed in the paragraph 2.3.

2.2 CORTICAL CELLS STUDIES ON MEAS

MEA technology allowed investigating the behavior of different types of cells. Several groups have worked with different types of neuronal cells cultures from spinal cord (32) and hippocampus (33) and above all cortex (21)(22).

In vitro cortical networks are spontaneously active (34). The firing rate changes during development are strictly related to the age of the network. The most prominent feature of the electrical activity of high-density dissociated cortical cultures is their propensity for synchronized bursting (35)(36)(37)(38). The cells in these cultures begin firing after ~4 days in vitro (DIV) and soon thereafter synchronize their activity globally across the culture(21). This synchronization takes the form of intense bursts of activity that typically contain a large number of spikes at many channels, densely packed together in time. The time between individual spikes in a burst, is generally of the order of several milliseconds. The burst itself has a duration ranging from hundreds of milliseconds to seconds and they recur several times per minute(39). Bursting persists for the lifetime of the culture, although the fully synchronized bursts of young cultures are gradually replaced by more spatially localized bursts in maturity. Globally synchronized bursting is an extremely robust phenomenon. Suppressing it using pharmacological agents like glutamate receptor blockers also abolishes most or all other spontaneous electrical activity.

Bursting phase lasts only for days or, at most, weeks. The persistence into maturity of bursting in culture may then be interpreted as a sign that cultures are arrested in their development (40). A hypothesis has been raised about the persistence of global bursts in dissociated cortical cultures that could be a result of deafferentation (21). Deafferentation has two effects. As first, the lack of (thalamo-cortical) input might lead to increased strength of connections within the network. Infact, Turrigiano (41) showed that blocking

the inputs to cortical neurons using TTX during development significantly increased the strength of excitatory connections. As second, the lack of structured input from sensory receptors and the presence of strong excitatory connections put the network in a highly unstable state in which positive feedback between excitatory cells can easily lead to synchronized bursts of activity (42).

Substituting sensory input with multi-electrode stimulation (43) could have the same effect as an elevated tonic firing rate while putting thalamic cells communicating with cortical cells could provide the necessary thalamocortical inputs that therefore should reduce the predominance of global bursts, favoring more locally differentiated neuronal activity.

In order to put in communication different populations of neurons i.e. cortical and thalamic cells, we needed a special device able to offer a physical structure that can keep the two populations divided. A description of this special PDMS device is provided in the next paragraph.

2.3 MULTI-COMPARTMENTAL PDMS ON MEA

2.3.1 PDMS PROPERTIES

Following Campenot's pioneering work (44), microsystem technologies have been used to initiate in-vitro studies of separated neuronal populations, either for chemical studies in both closed (31) and open (45)(46) compartments, or for extracellular recordings of electrical activity in open compartments (47).

A closed-compartment neurofluidic device with micro-channels connecting two compartments has been presented previously (1). The PDMS device design has been inspired by the work of Taylor and Rhee (48).

The polydimethylsiloxane (PDMS) is a silicone-type elastomer that is poured onto a hard master pattern to create castings that can then be peeled off the master and placed on a rigid substrate (typically glass or MEA for recording). PDMS was chosen because it answers the criterion of biocompatibility, is transparent, highly permeable to O_2 and CO_2, cheap and easy to mould. All these attributes make it very attractive for cell culture Microsystems. The 3-mm-thick PDMS devices used in this project, have 2 microfluidic compartments ('B' and 'C' in Figure 2) of 100µm height and 8mm length interconnected with micro-channels of 10µm height, 3µm width and 150µm length that are spaced at regular intervals of 60 µm(31). The micro-channels prevent the movement of cells between compartments. The devices were fabricated by conventional soft lithographic molding techniques that have been already described (1). Four 6-mm-diameter reservoir holes ('H' in Figure 2) were drilled in the fabricated devices using laser technique.

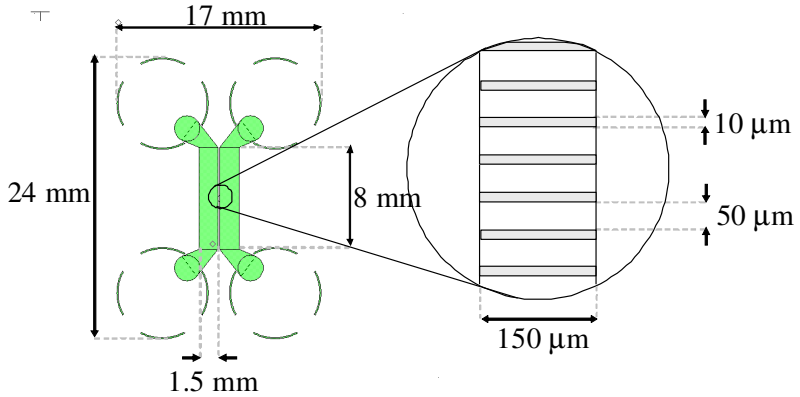


Figure 2: Schematic layout of dual compartment device design used for neuronal cell culture; A- Reservoir (diameter=6mm), B and C – Dual compartments (width=1.5mm, height=0.1mm, length=8mm), D – Micro-channels (width=10µm, height=3 µm)

In Figure 3 a photo of the same device with a blue food coloring substance inside to highlight the shape of the micro-channels.

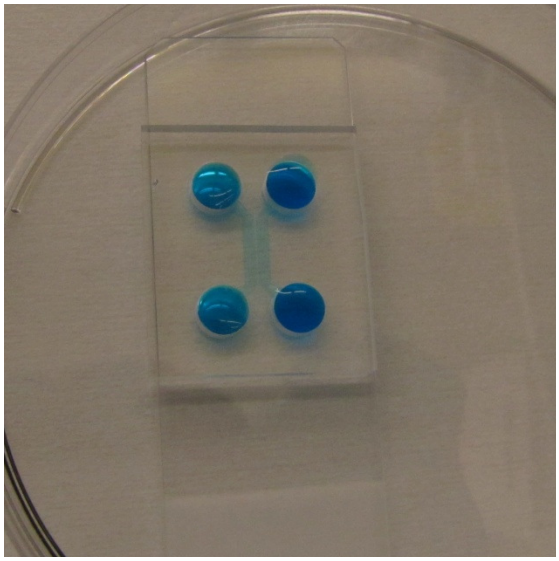


Figure 3: Photo of a PDMS filled with a blue food coloring substance to highlight the shape of the micro-channels

The cleaning protocol for the PDMS devices consists of: leaving the devices overnight in an ultrasonic bath to clean the surface, removing micro particles with a scotch tape and storing them in 70% ethanol until the day of cell culture. Each PDMS device was then rinsed in sterile De-ionized (DI) water for 3x times and sterilized in a dry oven at 120° C for 15 min and placed on cover-slips with the micro-channels facing downwards for oxygen-plasma treatment. PDMS is hydrophobic. However the chambers have to be hydrophilic to be able to fill them with medium for culturing. This is done by oxidizing with oxygen plasma treatment for 60 seconds, at 0.5 mBar and 200W (TePla Semi Auto 300 Plasma Processor). This treatment renders the microfluidic compartment and the micro-channels hydrophilic, while preserving hydrophobicity of the contact surface, thereby preventing leakage.

The PDMS devices were then aligned and reversibly bonded onto planar Microelectrode Arrays (MEAs). Prior to the placement of the PDMS devices, MEAs were sterilized in a vacuum oven, coated overnight with a solution of Polyethylenimine (PEI) (Sigma-Aldrich, stock solution at 50% w/v in water, Ca. No.P3143) at a concentration of 40 µg/ml to provide cell adhesion and rinsed thoroughly in sterile water (tissue culture grade, GIBCO, Invitrogen, Cat. No. 15230).

Thanks to properties of the PDMS, the devices have shown interesting characteristic that make them suitable for these applications. Microfluidic-based cell studies present many advantages when compared to conventional in vitro techniques or mini culture systems: they have the ability of precisely controlling the environment around the cells, the requirement for solvents, reagents and cells are small, liquid transport is faster, design versatile (49). Fluid flow in micro-channels exhibits several characteristics, the most important of which is laminar flow. This is very important for our application since thanks to this property we can uniformly deposit the cells into the microchambers assuring a uniform density of cells along the microchambers (1). With turbulent flow we would have non uniform plating. Indeed the cells are plated from one of the reservoirs on one side of the chambers and then the fluid is drawn by capillarity into the chambers.

2.3.2 APPLICATION OF TTX

To ensure the biological origin of the recorded activity within the compartments, experiments were performed using Tetrodotoxin (TTX), to silence the spontaneous activity and results were reported elsewhere(1). TTX has shown suppression of network activity within a compartment and subsequent recovery of network activity through successive wash cycles.

As demonstrated, after the addition of TTX to one compartment the spontaneous activity in the cells of the same compartment completely disappears while in other compartment the activity persists as shown in Figure 4.

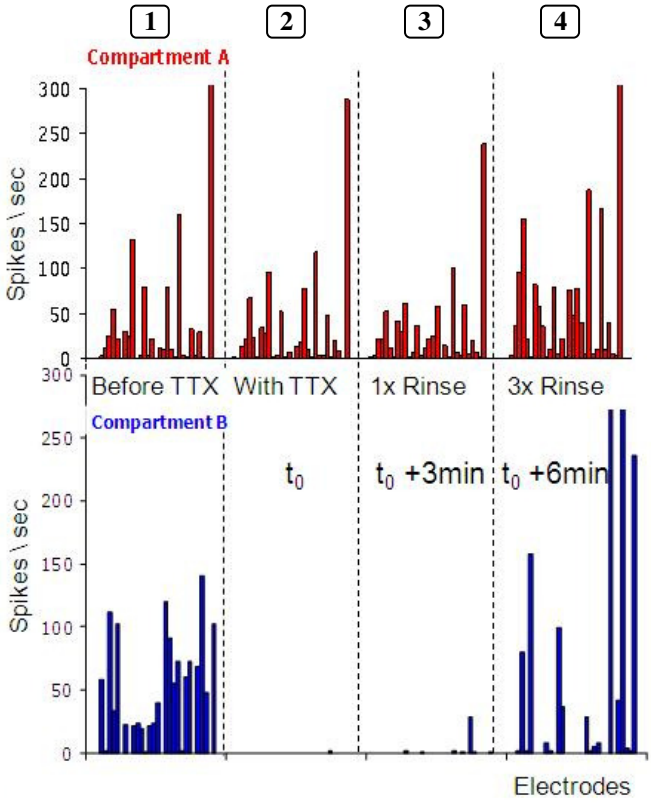


Figure 4: Network activity in the dual-compartment device (Compartment A in red and Compartment B in Blue);

Top: Spike rate analysis in compartment A (not chemically altered): 1) before addition of TTX; 2) during the application of TTX in compartment B; 3) after 3 minutes and 1 rinsing, 4) after 6 minutes.

Bottom: Spike rate analysis in compartment B (altered by TTX injection): 1) before addition of TTX; 2) Suppression of network activity with the addition of TTX ; 3) after 3 minutes and 1 rinsing, 4) Recovery of spikes after three washout cycles.

This demonstrates that there is a genuine spontaneous network activity in the cells contained in the compartments.

Given the small size of the channels (width = 10 µm and height = 3 µm) connecting both the compartments, it is estimated that it would take several hours for the diffusion of molecule from one compartment to the other (31). It is possible to safely add TTX to one compartment and be sure that it wouldn't spread to the adjacent compartment during the course of the experiment. This highlights the physical and fluidic separation between the two compartments.

2.4 COMMUNICATION BETWEEN THE TWO COMPARTMENTS

2.4.1 GROWING NEURITES THROUGH THE MICRO-CHANNELS

Long term neuronal cell culture viability in the device and neurites' growth through the micro-channels connecting the compartments has been demonstrated experimentally (1) (31).

Visual observation of the culture over the developmental period indicates neurite growth across the compartment from ~DIV 3. Neurites were observed to cross-over to the adjacent compartment through the micro-channels along the whole length of the compartment. Phase contrast imaging of cell bodies isolated within a compartment and neurites crossing over to the adjacent compartment confirmed the physical containment effected by the micro-channels (Figure 5A). It shows extensive dendritic and axonal arborization within the compartment of origin, as well as individual neurites crossing over to the other compartment through the micro-channels. To further substantiate structural trans-compartment connectivity by axonal crossover to the adjacent compartment, transfection of individual neurons with eGFP (green fluorescent protein) and DsRed was performed (Figure 5B) and reported earlier(1).

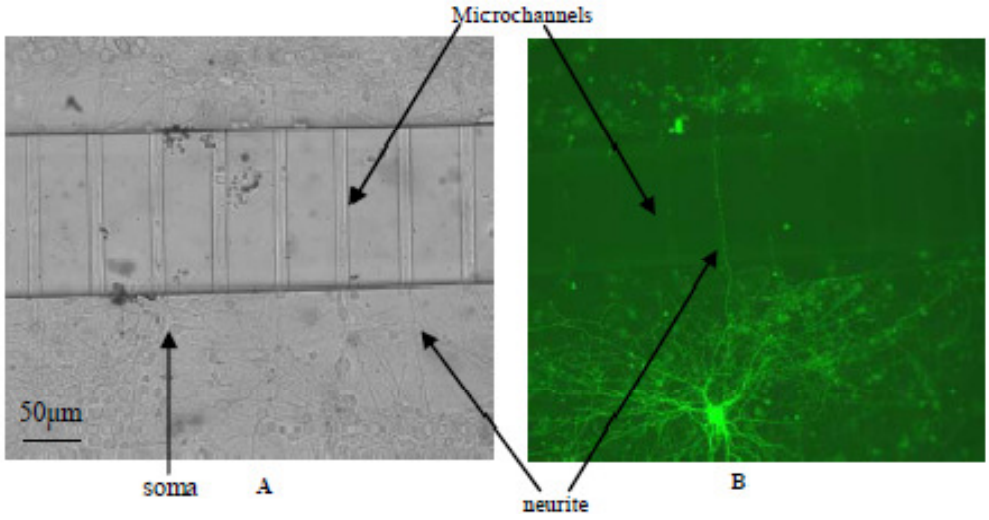


Figure 5: Neurite growth through micro-channels. A: Phase contrast image of neurites grown across the micro-channels; B: Transfection imaging of a neurite grown across the micro-channels connecting the compartments (Source (8)).

2.4.2 INTER-COMPARTMENTAL SYNAPTIC CONNECTION

Along with physical connections through the neurites, information exchange within the two compartments was shown.

Synaptic connection in form of correlation between the spikes and the bursts occurred in cortical cells in the 2 compartments was shown in the previous works within the same group in Philips Research by Davide Ciliberti (2).

Cross-Correlation, by definition, the average probability of observing a spike in a target channel before or after a spike in a reference channel, is calculated taking as reference the time-stamp of a detected spike in an electrode and correlating it with the time-stamp of the immediate next spike in the second electrode. This is done for all the spikes that occur in the first electrode. The different time-stamps are then summed and normalized to one, in order to have a probability of 100% if the second spike occurs always with the same delay referred to the first spike. An example of Cross-correlation graph is shown in Figure 6 where

the peak is centered in 0ms and has a value of 0.27 out of 1, the probability of correlation between the spikes of the two electrodes.

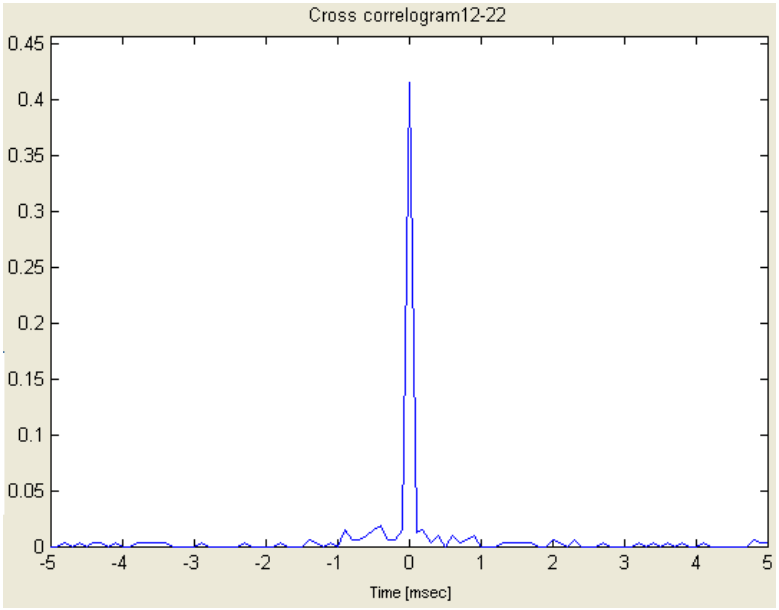


Figure 6: example of correlation between electrodes 12-22; [values: x-axis -5:+5 ms; y-axis probability of correlation]

To estimate the Cross-correlation, a value C_{peak} was calculated as the integral of the area around the peak, taking as window a definite number of bins dependent from a τ that in most of the cases is 1 bin.

A Mean Cross-Correlation Graph has been used, defined as the correlation graph of one reference electrode with a target electrode, putting together the graphs of the whole device. Two Mean Cross-Correlation Graphs have been extracted: Intra-compartmental (between one electrode with all the electrodes in the same compartment) and Inter-compartmental (between one electrode with electrodes in the other compartment). Moreover, in order to have a reference, another graph has been extracted with the correlation between one electrode and a shuffle peak-train as the other compartment (Shuffle-correlation).

The objective was to show that there is a correlation between the two compartments with a comparison with the shuffle-correlation and to prove that the nearest column is more correlated to the other compartment than the furthest.

Collecting the data of the electrodes in one column (symmetrically from the center of the PDMS) with the electrodes of the selected area, a cross-correlation was calculated in two passages:

1. Selection of one of the statistical parameters like Average, Median, Max, 95th percentile and application to the group of C_{peak} of the selected area, in order to obtain a value that correspond to the original electrode

2. In order to collect all the column data symmetrically (so as 4 and 5, 3 and 6, 2 and 7, 1 and 8) another statistical parameter like median, average, max has been used

A Wilcoxon signed rank test has been used. It can be used as an alternative to the paired Student's t-test when the population cannot be assumed to be normally distributed. Null hypothesis: no differences in the inter-compartmental correlation between real and shuffled data.

It proves with a p-value of 2.9305×10^{-004} that the level of the inter-compartmental correlation is significant: the compartments have a functional connection, besides the structural one.

A Friedman Test has also been used. It is useful to detect differences in treatments across multiple test attempts. Null hypothesis: no variations of the inter-compartmental correlation in the 4 regions.

The significance of the statistic that is the probability of having the same treatment is represented by a p-value of 2×10^{-3}. It has been demonstrated that the closest neurons to the micro-channels communicate with the opposite compartment more than all the other (more distant) ones.

3 MATERIALS AND METHODS

3.1 HARVESTING NEURAL CELLS

Harvesting neural cells is a critical phase of the project; in our experiments, we have used cells taken from 2 different parts on the brain: cortical and sub-cortical / thalamic regions.

The extraction of cortical cells is a procedure well known is the MEA research community, i.e. Potter's group at Georgia University (50). The whole procedure was inspired by the work described in (34). In our experiments primary cultures of embryonic cortical neurons of rats are used due to their potential of proliferation compared to post-natal cells.

The procedure to isolate the cells has been renewed during the project.

A schema of the phases followed with the differences between the two configurations is shown in Table 1:

Table 1: Phases during harvesting of rat neural cells

Phase	Previous protocol	New protocol	Comment
1	An 18 days pregnant Wistar rat is euthanized (95% of O_2 is slowly replaced by 100% of CO_2 for reducing the stress of the animal, UNO euthanasia unit).		As per the approved protocols for the care and use of lab animals in the Netherlands
2	The abdominal skin is cut paying attention not to damage internal organs and the uterus containing the embryos is removed, placed on ice and put under laminar flow hood.		
3	The fetuses are decapitated, the cerebra is extracted and placed in DMEM (Dulbecco's Modified Eagle Medium, GIBCO Invitrogen).		
4	The cortices, isolated from the basal ganglia and the hippocampus, are cut in small pieces (~0.2 mm), inserted in 15 ml of Trypsine/EDTA solution at 0.125% (GIBCO, Invitrogen)	The cortices, isolated from the basal ganglia and the hippocampus, are left as they are inserted in 15 ml of Trypsine/EDTA solution at 0.125% (GIBCO, Invitrogen) in HBSS with Ca/Mg	The isolation took longer than expected, so they survive better when not disintegrated.
5	The cells are incubated (CO-170, Innova incubator, New Brunswick Scientific) for 45 minutes to allow the enzymatic digestion.	The cells are incubated (CO-170, Innova incubator, New Brunswick Scientific) for 25 minutes to allow the enzymatic digestion.	In the previous protocol we added more cortices to the Trypsin.

6	The Trypsine/EDTA solution is aspirated and a 12.5% solution of Soy Bean Trypsine inhibitor (GIBCO, Invitrogen, Ca. No. 17075-029) in DMEM is added into the tube.	The Trypsine/EDTA solution is aspirated and DNase is added into the tube.	The Trypsin could cleave DNase.
7	Add Dnase after Trypsin inhibitor	Stop Trypsin with 10% FCS in neuro basal	In the previous protocol we avoided using FCS for the cortical culture.
8	The pieces are triturated with a sterile Pasteur-pipette until no aggregates are visible anymore.		
9	The tube is filled with 10 ml of DMEM and spun in a centrifuge (Heraeus Multifuge 3 S-R) for 5 minutes, at 1000 rpm, at 20º C.		
10	The supernatant is removed, the tube is flicked in order to loosen the pellet and the cells are gently resuspended in Neurobasal Medium (GIBCO, Invitrogen, Ca. No. 21103), supplemented by 1% fresh stable L-Glutamine (GlutaMAX™ 100x, GIBCO, Invitrogen, Ca. No. 35050-038), 1% Penicillin-Streptomycin Solution (GIBCO, Invitrogen, Ca. No. 15140-122) and 2% B27 Supplement™ (GIBCO, Invitrogen, 17504-044)) and 10nM Triiodo thyronine.		B27 supplement is a specific trophic complement for CNS neurons that contains the hormones, growth factors and antioxidants necessary to guarantee long-term viability of CNS neurons in serum-free cultures (51). Glia growth was not suppressed.
11	Count cells in dilution	Count cells in cell concentration	An average of 70 millions of primary cortical cells per culture was produced.

The second population of cells we were trying to collect thalamic cells. Dividing the central body of the little brains from the cortex, the midbrain part is excluded removing also the bottom part that is the hypothalamus. The thalamic part can be easily recognized as in the atlases in Figure 7 and Figure 8.

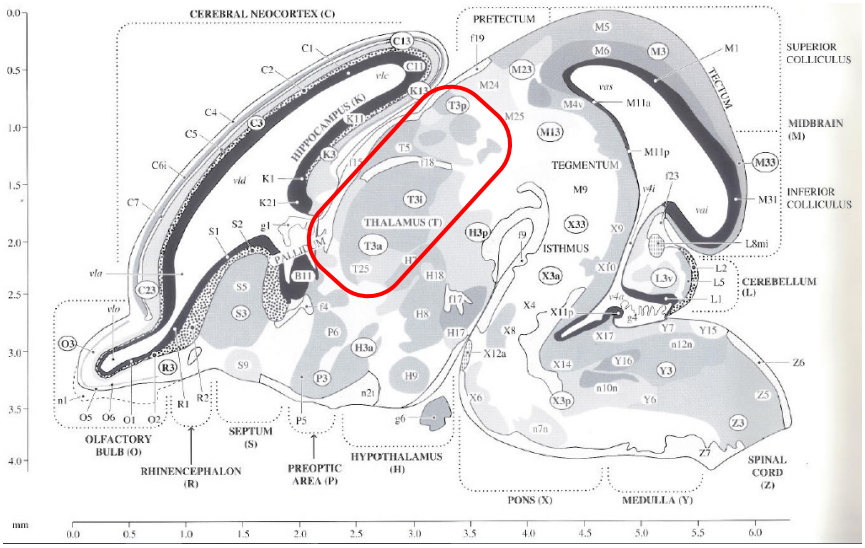


Figure 7: Atlas of the sagittal view of a 18 day embryonic rat

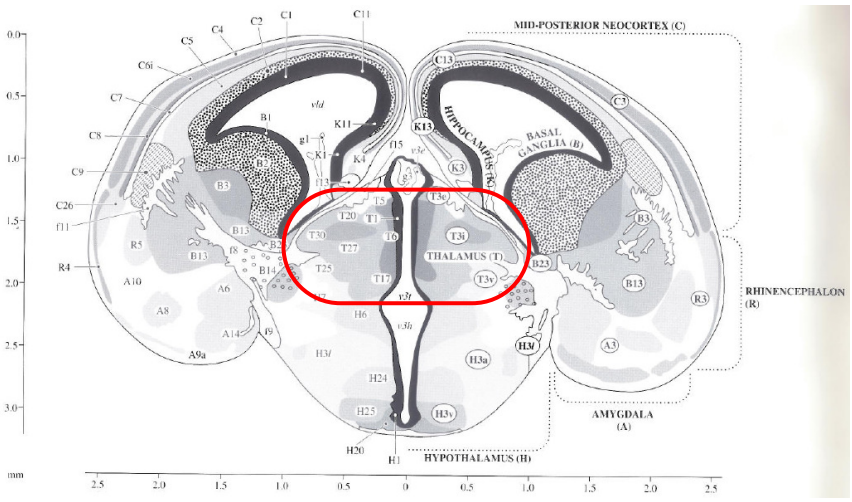


Figure 8: Atlas of the coronal view of a 18 day embryonic rat

Then, the procedure made for the cortical cells has been done also for the thalamic ones.

The only difference in maintaining the thalamic cells is the presence of some serum in the culture medium (3%FBS+1%HS) for at least DIV5 in culture. Then a diminution of the dosage during the following days (2%FBS+0.5%HS) is done finally eliminating it after DIV8-9 to avoid the glia overgrowth during the long-term culture (more than DIV15). This detail was inspired by (52)

An average of 3 millions of cells per culture was so obtained. Due to the small size of the embryonic rat brain and to the fact that there are no evident separations between regions in the brain, it is difficult to confirm that we could obtain thalamic cells successfully and hence, we will refer to the cells we have harvested in this case as sub-cortical/thalamic cells.

The harvested cells are then ready to be plated in the devices: the Ring MEAs, the dual compartment MEAs and the coverslips that we used as controls.

In the dual compartment devices the cell platting is performed with a high resolution pipette (Eppendorf, Reference, 0.5-10 µl, accuracy 0.1 µl). The micro-tip is visually inserted into the reservoir at the compartment entry. The plating occurred by injecting the cell suspension from one reservoir into the inlet of each compartment. The cultured cells were plated on PEI coated MEAs substrates at a surface concentration of ~ 2 x 10^5 cells/cm^2 and ~ 1 x 10^5 cells/cm^2.

Having collected two different populations of cells, several devices were prepared:

- Ring-MEA devices with only cortical cells (we referred to those devices with ‘Ring Cx devices’)
- Ring-MEA devices with only sub-cortical/thalamic cells (we referred to those devices with ‘Ring Th devices’)
- Close dual-compartment devices with cortical cells in both compartments (we referred to those devices with ‘Cx-Cx devices’)
- Close dual-compartment devices with cortical cells in compartment A and sub-cortical cells in compartment B (we referred to those devices with ‘Cx-Th devices’)

The devices were then incubated in a humidified incubator (CO-170, Innova incubator, New Brunswick Scientific) at 37° C supplied with 5% CO2.

Every 2-3 days the medium in the close compartment devices was changed. We used a serum-free Neurobasal medium, supplemented with B27 and glutamax. Under the aforementioned conditions we were able to record stable electrophysiological signals over 4-6 weeks period.

3.2 RECORDING SET-UP

To provide an indication of the health and functional connectivity of cells in culture visual observations at the microscope and recordings of the spontaneous activity of the neural network were done. Moreover the recordings at the first stage were used to select the most active electrodes that subsequently will be stimulated.

The materials we used for our recording system are mainly based on the MEA60-Inv-System-Standard (Multi channel System, Reutlingen, DE).

3.2.1 MULTI-CHANNEL-SYSTEM

Extracellular signals were recorded at 10 kHz using a MEA60 system (MultiChannel Systems, Reutlingen, Germany). This acquisition system (Multi Channel System) is composed of MEAs electrodes (matrix of 60 electrodes), the MEA1060-Inv-Standard amplifier (gain 1200x), the MC Card that acquires and digitalizes the signal up to 50 kHz, a temperature control (TC-01) set to 37ºC, a stimulus generator (STG 2000) and a data acquisition computer with installed software MCRack (software for visualization, recording and on-line analysis). According to literature (21) and to experimental analysis, we decided to acquire the signal at a sampling rate of 10 kHz or 25 kHz. This is a good compromise between the computational effort to store and record the data and the ability to detect all the spikes the cells produces. Moreover, the Shannon sampling theorem is fulfilled.

Regarding the bandwidth, we recorded the signal at the maximum bandwidth provided by MCRack (i.e. 10 Hz up to 3000 Hz). Then, according to literature (21) we applied a High-Pass software filter at a cut-off frequency of 200 Hz. The system (MEA and amplifier) is under a bio-safety laminar flow hood under sterile conditions to reduce the probability of infections.

A spike sorter algorithm already implemented (1), allowed us to visually see the spikes in each electrode while recording.

Devices were checked several times during their activity. The cells were observed to be electrically active by DIV 10. Depending on the frequency we used to record the cultures for 300 or 600 seconds, waiting some minutes in order for the culture to adapt to the new environment and to absorb the eventual mechanical perturbation.

Spikes detection has been done off-line using a threshold-based algorithm.

For spike detection and other off-line analysis we used a software tool SpikeManager (53) developed by Prof. S. Martinoia, which includes several tools developed in MatLab (The Mathworks, Natick, MA) for the analysis of electrophysiological data.

3.2.2 NEUROLIDS

Given the particular configuration we used (dual compartmented PDMS with a total medium volume of the reservoirs being 60 µl) and long-time taken for recordings and stimulations of the same device (up to ~2.5 hours), particular care was taken to prevent excessive evaporation during the recording sessions. A sterile recording box was with 90% relative humidity and 5% CO_2 supply was used (1).

"Neurolids" were made to prevent evaporation of the medium contained in the reservoir and to provide the device with grounding electrodes necessary for electrical stimulation/recording of network activity. A neurolid is a rectangular base of glass glued together with another glass frame that takes the shape of a cover of a box. Normally 2 little holes of 0.5 mm were made by laser at a distance of 5 mm from the upper edge where 2 reference electrodes were inserted.

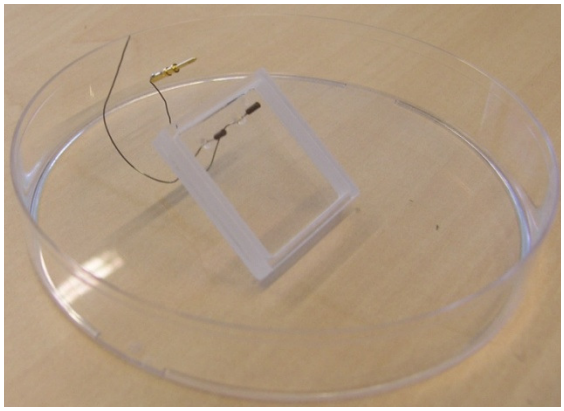


Figure 9: Neurolid2: neurolid with 2 electrodes

As the PDMS prevents the medium to physically reach the big ground reference electrode provided internally in the MEA, we had to find a way to connect the culture with a ground. The neurolid was so created to prevent evaporation of the entire medium contained in the device, but also to connect the medium of the device with a ground area.

Therefore, the neurolid was put on the PDMS during recordings and stimulations where the two electrodes in the neurolid were put physically in touch with the medium.

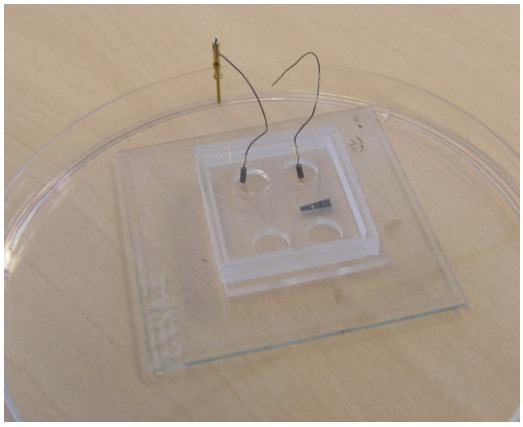


Figure 10: Neurolid2 put on the PDMS – 1 grounded electrode per each compartment

Doing some experiments described in chapter 0, a need of a different configuration was envisioned. We needed a symmetric configuration that would have 4 different grounded electrodes in the all the 4 reservoirs of the device.

So, 2 more holes have been added to the neurolid symmetrically built opposite to the first two holes as we show in Figure 11 and . They have been done respecting the shape of the 4 reservoirs in the device.

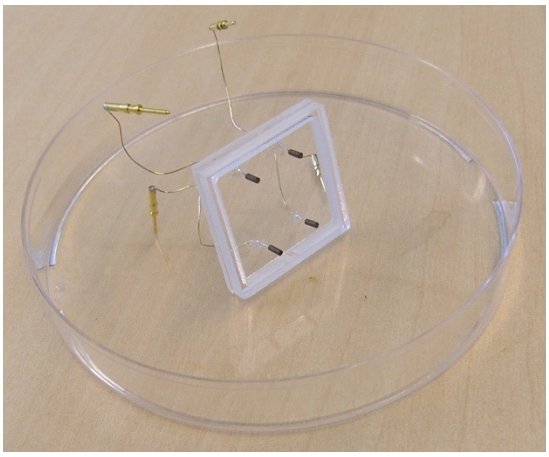


Figure 11: Neurolid4: neurolid with 4 electrodes

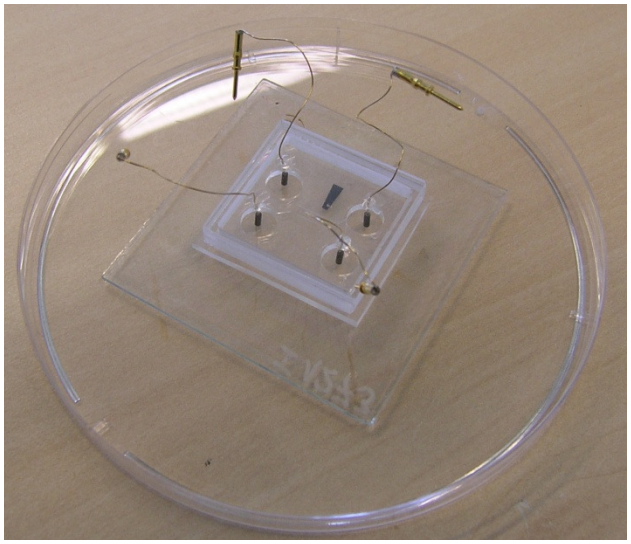


Figure 12: Neurolid4 put on the PDMS – 2 grounded electrode per each compartment (1 per reservoir)

The next figure shows the three layers of the configuration used, starting from the top:

- Neurolid4
- PDMS
- MEA

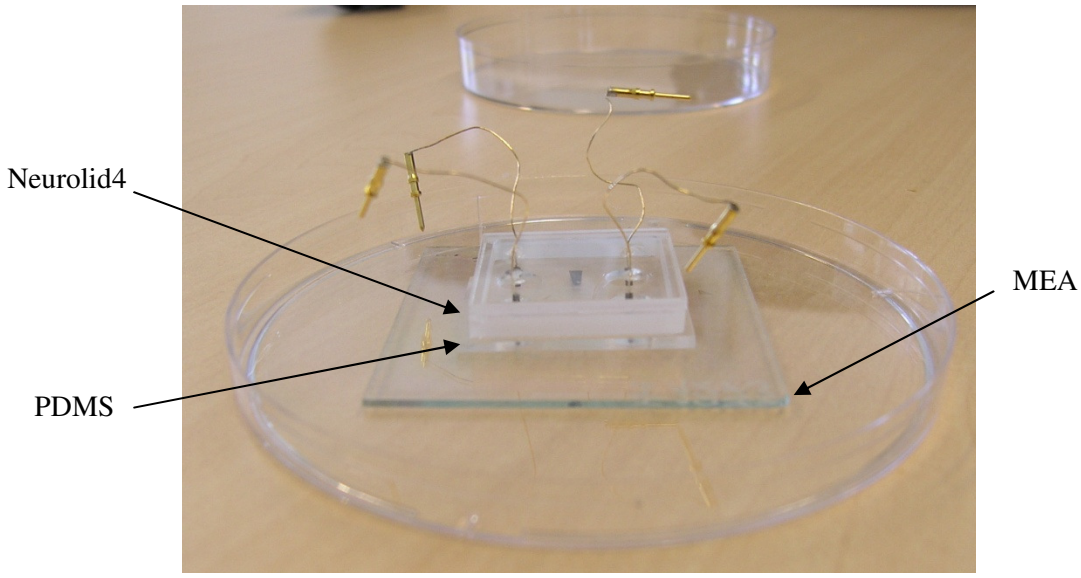


Figure 13: The complete scheme highlighting the three layers

3.3 ELECTRICAL STIMULATION

Electrical stimulation has become a know procedure in the area of study of neural cells cultured on MEAs. It has been observed that given the right stimulation, the cells respond giving a signal back. Experiments have been done to find the right stimulations the cells need, as shown for example in (3).

Extracellular voltage stimulation used for testing the network evoked response was delivered from specific electrodes of the MEA.

The recording protocol we used was always composed by a first phase of spontaneous activity recording, a second phase of electrical stimulation followed, ending finally with a 2nd recording of spontaneous activity useful to compare the effect of the stimulation of the spontaneous spiking of the cells.

The stimulation we used for our experiments was:

- Test stimulus: The stimulus consisted of 50 biphasic pulses, 260 μs per phase, positive first, delivered at the frequency of 0.2Hz; the peak-to-peak amplitude was set at 1.52 V.

The train we used for the Test Stimulus was inspired by the work described in (3). It has been studied to provide significant evoked response in cortical cells.

Regarding the stimulation of sub-cortical / thalamic cells, we have been looking for the right stimuli to provide. Unfortunately, no publication was found regarding electrical stimulation of rat thalamic cells in dissociated cultures.

Electrical stimulations have been done with different modalities, as described below:

- Unipolar: one or more electrodes have been chosen to deliver the stimuli. The stimulation spreads through the medium and stops in the grounding electrodes that are in touch with the medium.

- Bipolar: the stimulation goes from a chosen electrode (positive pole) to another (negative pole). When the stimulation becomes negative the role is inverted so that the first electrode is now the negative pole and the second one is the positive one. The medium has been putted in touch with grounding electrodes.

Looking for a better focus of the stimulation that we couldn't reach with unipolar stimulation, we used a bipolar stimulus, inspired by (54). As explained in the article they made a study on the current spreading of three different configurations that can be applied to MEA devices. They in fact compared three electrode configurations as shown in Figure 14: unipolar, concentric bipolar and ground surface.

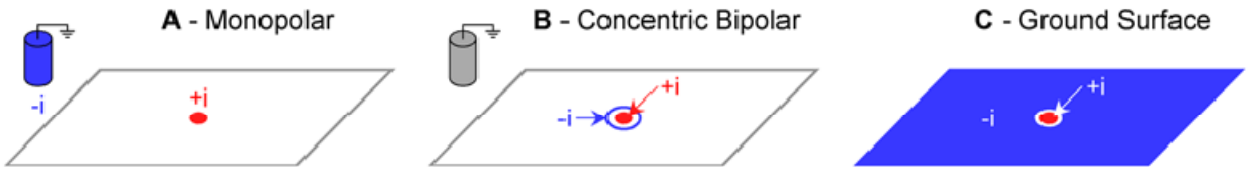


Figure 14: Three different configurations while stimulating electrically

They demonstrated that a bipolar stimulation is more focused than a unipolar one, when applied to a theoretical model of a straight fiber or cortical stellate cell passing 50 µm or 110µm respectively over the stimulated electrode.

The system in use can be configured to have a unipolar or a bipolar stimulation, based on the configuration used, as shown in Figure 15 and Figure 16.

Figure 15: Standard setup for unipolar stimulation

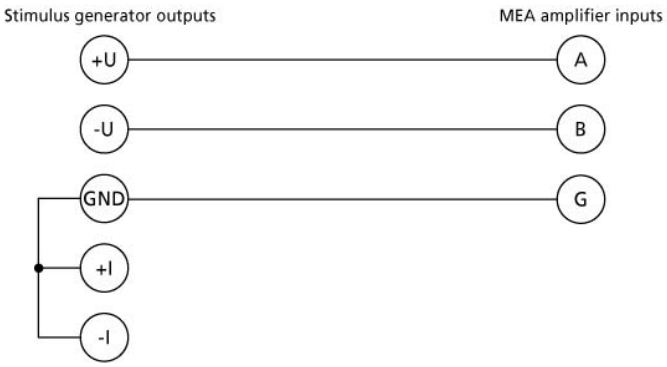


Figure 16: Standard setup for bipolar stimulation

Experiments with the two configurations will be shown in Paragraph 4.2.1, where we tested the propagation of the current in the whole device.

3.4 CHEMICAL COMPOUNDS

Further than electrical stimulation, chemical compound were used for the manipulation of neuronal cells in device.

We used for our experiments 3 chemical compounds: Acetylcholine, TTX and a solution that we called Synaptic Blocker. Those compounds will be described in the following paragraphs.

3.4.1 ACETYLCHOLINE

As documented in (55) and in (56), acetylcholine was observed to be an excitatory modulator of neuronal activity in mammalian cortex in literature.

The same substance applied to mammalian thalamus seems to have as well an exciting effect. In (57) it is mentioned that Acetylcholine at a concentration of 5mM applied to thalamic nucleus reticularis (nRt) neurons of guinea pigs slices in-vitro, inhibits single spike activity while mainly promoting bursts. In (58) it is shown the effect of the same compound on feline thalamic cells in-vivo to be mainly excitatory (80% of the cases) evident particularly in the ventro-basal thalamic part.

The suggested concentrations used are in the range of 10 to 30 µM. In our experiments we mostly used concentration of 20 µM in the neural medium (in private communications, Prof. S. Martinoia).

We've done experiments with this compound, as referred in paragraph 4.1.2.

3.4.2 TTX

Tetrodotoxin (TTX) is a potent neurotoxin. Tetrodotoxin blocks action potentials in nerves by binding to the pores of the voltage-gated, fast sodium channels in nerve cell membranes, essentially preventing any affected nerve cells from firing by blocking the channels used in the process(59). The effect is reversible, so that after the application of the cell culture medium, the original network activity recovers back again (3).

A fresh solution of 100nMolar of TTX were made in Neurobasal medium at 37 °C was prepared on the same day of the experiment.

The compound has been used in the previous work of the same project (1) as a proof of concept to show that the activity recorded is a genuine neuronal activity and it is neither background noise nor artifacts. Indeed when applied to one of the compartments it showed a considerable reduction in spontaneous network activity.

3.4.3 SYNAPTIC BLOCKER

Recently other compounds, more than TTX, have been used for the study of the behavior of the neurons. In literature we could find groups that are investigating ways to block the synaptic transmission that happen among neurons (3) (9).

They tried to block synaptically-evoked action potentials with fast synaptic receptor antagonists. In our case, we applied concentrations of 50 µM bicuculline methiodide (BMI), 100 µM 2-amino-5-

phosphonovaleric acid (APV), and 10 µM 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) (from Sigma) dissolved in culture medium, as mentioned in (9). These are antagonists of GABA-R, NMDA-R and AMPA-R, respectively.

The particular composition mentioned, has an inhibitory effect on synaptic channels, so that the communication between the neurons is prevented. The consequence is that only self firing neuron activity, while network effects such as bursts completely disappear(60).

3.4.4 PROTOCOL DURING CHEMICAL EXPERIMENTS

The protocol used while injecting the chemical substances mentioned was defined as follows:

As a control condition, the spiking activity was recorded for 5-10 minutes (depending on the sample frequency used) before adding the substance.

Next, cell culture medium in one of the compartment was completely removed by pipetting and replaced with an equivalent volume of the chemical solution. The effect of the substance was recorded, so recording spontaneous activity of the device for 5 minutes. After the experiment, the chemical compound was completely removed from the compartment and washed 3x times with with freshly prepared neurobasal medium. As it has been demonstrated, this procedure helps the cells to recover the original spiking activity (1). Five minutes recording was performed in these conditions.

Chemical compounds have been also used to prove certain characteristics of the electrical stimulation.

3.5 SOFTWARE FOR DATA ANALYSIS

3.5.1 MCS SOFTWARE

The software used for the recordings, MC_Rack version 3.4.0 is supplied by MultiChannel Systems, Reutlingen - Germany. The software provided us with a complete environment ready to record the signal coming from the MEA hardware. In the Figure 17, we can see a typical screenshot of the software with all the analysis instruments it provides.

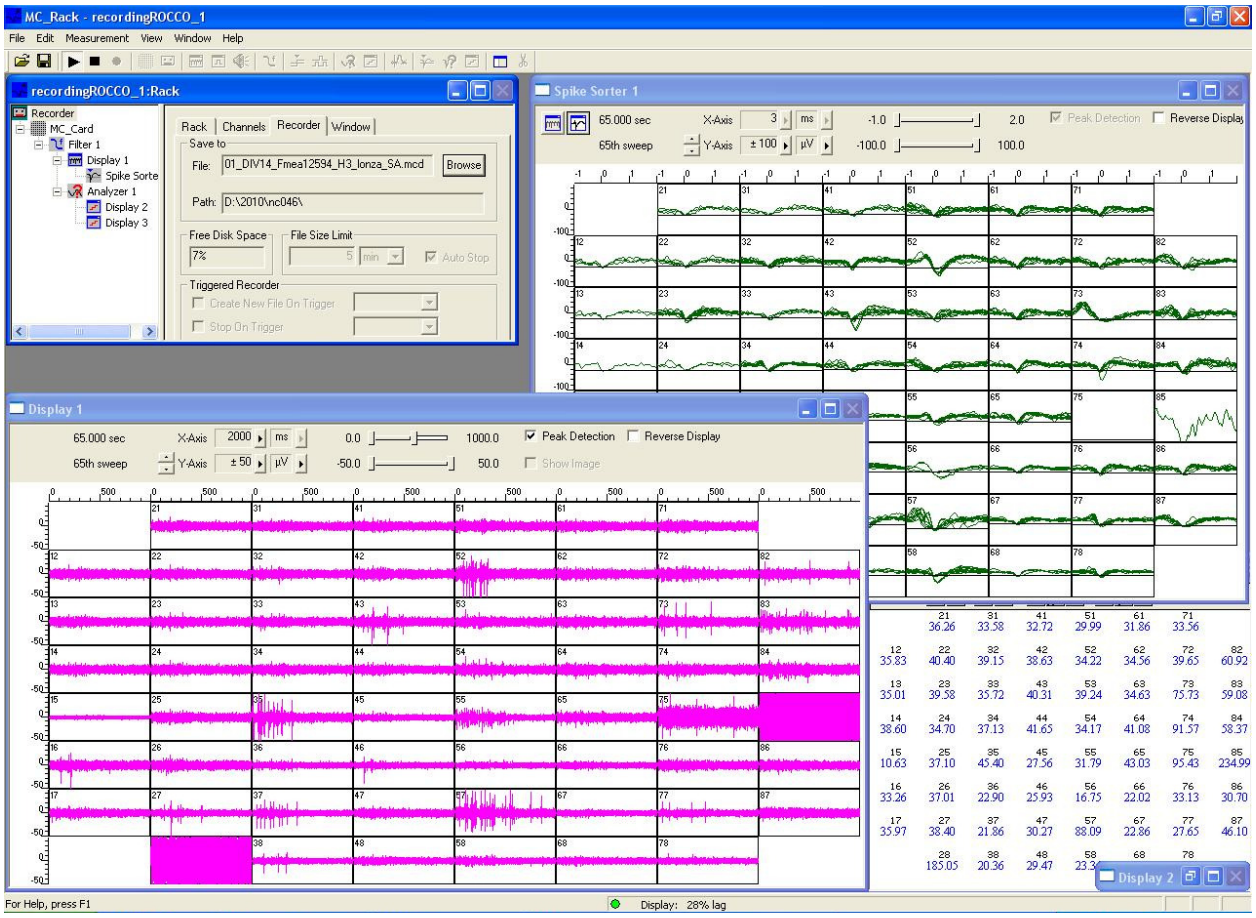


Figure 17: MCRack screenshot of an active culture; electrodes 28 and 85 are considered to be noisy

In the upper-left part we see the management center where it is possible to set all the details about the recording. New analysis tool can be also created to facilitate the acquisition and analysis of the signals. In the upper-right part we can see a real-time spike sorter able to recognize the different spikes pattern of the neurons while a recording is executed. This window is based on the work previously done in the same project(1).In the bottom-left part filtered (High-pass Butterworth 2nd order filter with a cutting frequency of 200Hz) recording signals are shown in a matrix based on the position of the electrode in the recording matrix. Finally in the bottom-right we find a matrix with a measure of the maximum peak to peak values per second per electrode. It was useful to determine analytically the activity power in an electrode.

To provide electrical stimulations to the devices we used another software provided by MCS MC_Stimulus 2.1.4 for STG 2008. It allowed us to set a stimulation pattern for the signal, choosing among the multiple possibilities of signal shape and duration. The stimulation protocol used in our experiments has been explained in the paragraph 3.3. A screenshot of the stimulation software is shown in Figure 15.

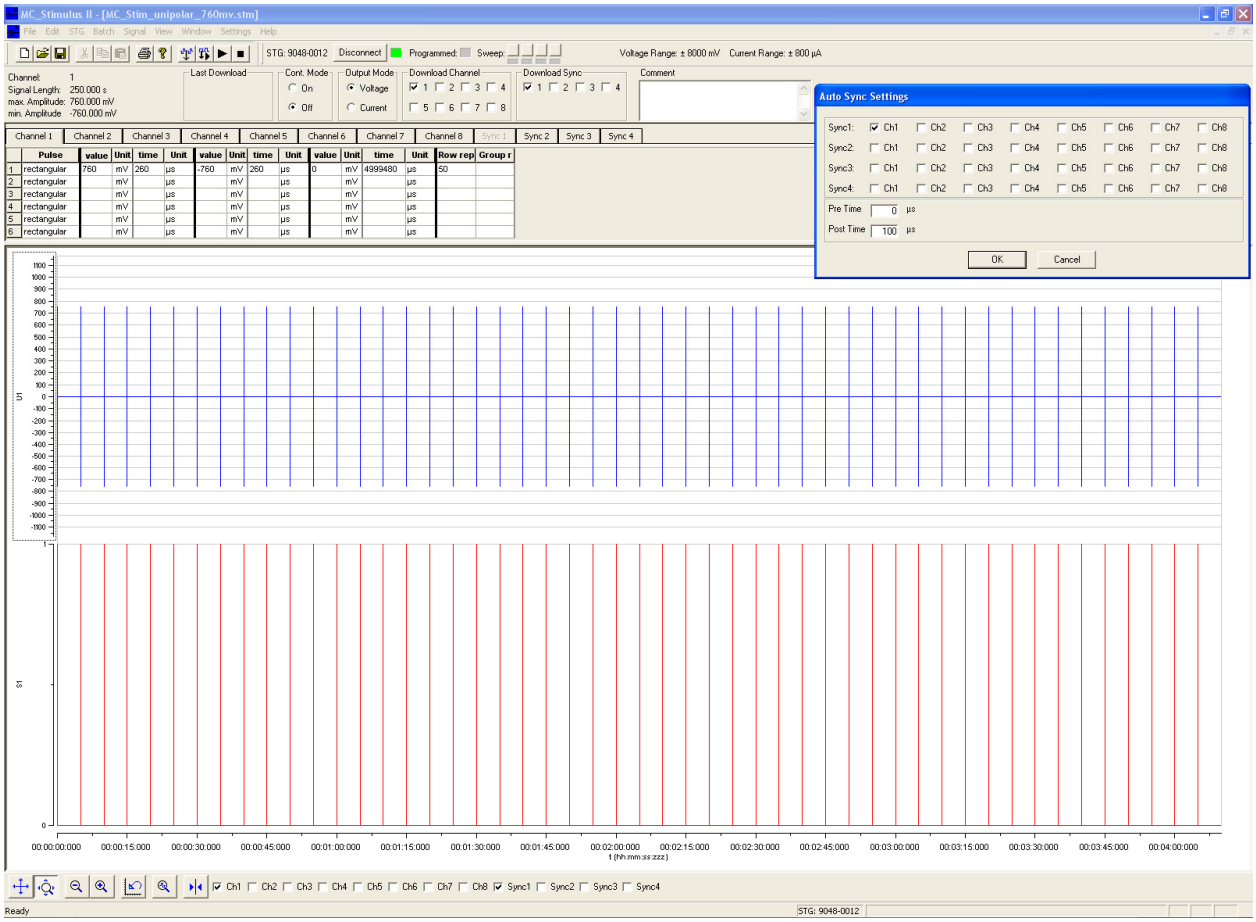


Figure 18: MCStimulus screenshot

MEA_Select 1.2.1 by MCS was used to set the type of stimulation (unipolar or bipolar) and the position of the stimulated electrodes. Moreover it is possible to choose which of the electrodes provided by the MEA is logically connected to ground, so plays as a ground during the stimulation. Figure 19 shows a screenshot of the software.

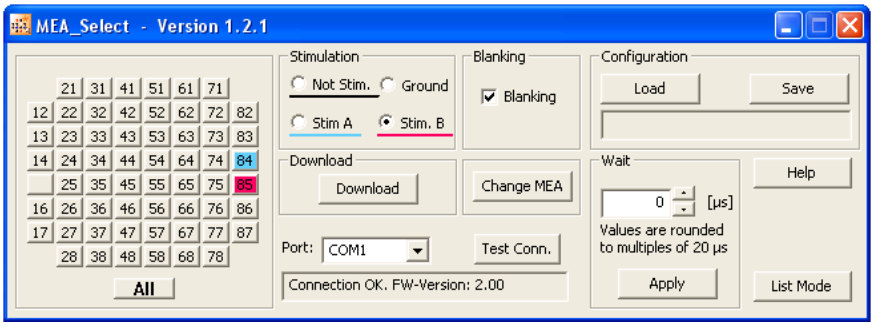


Figure 19: A MEA_Select screenshot

3.5.2 SPYCODE

In order to be able to analyze the data coming from MC_Rack software, we used the software SpyCode, an evolution of SpikeManager described in (53), which includes several tools developed in MatLab (The Mathworks, Natick, MA) for the analysis of electrophysiological data.

The first step in our analysis has been the conversion of the .mcd files (MC_Rack format) into .mat files. A recording of the timestamp of the stimulations were also stored.

A filter was then applied to the raw signal and, according to the literature (61), we apply a band pass Butterworth filter (200-3000Hz) in order to reject all the sources of low-frequency noise (i.e. 50-60 Hz noise and the local field potentials).

The third step regards the detection system of the events. We used an algorithm called Precise Timing Spike Detection (62). The PTSD algorithm considers consecutive portions of the signal and looks for the Relative Maximum/Minimum (RMM) whose peak-to-peak amplitude is above a defined differential threshold. The threshold, set independently for each channel, is computed according to the standard deviation of the noise of the signal. The algorithm requires three parameters: the threshold (defined by a standard deviation coefficient), the peak life time period (PLP), the refractory period (RP). The PLP and the RP are related to the duration of a spike and the minimum interval between two consecutive events. The algorithm computes the RMM of the raw data signal. When the RMM is a Minimum, the algorithm looks for the nearest Maximum within the PLP, and vice versa. If the difference between the two found RMM (differential value) overcomes the set threshold, the spike is identified and its timestamp stored.

The parameters we used for the PTSD were:

- Standard deviation coefficient: 8
- Peak life time period: 1.0 ms
- Refractory period: 1.0 ms
- Maximum stimulation frequency: 50 Hz

Once obtained the time stamps of the spikes, further analysis can be done.

A useful tool to analyze the spontaneous activity of a device is a Raster Plot. It shows a series of rows where each of them represents the activity of one electrode. A general view of a Raster Plot gives the idea of what is the general trend of the activity in every electrode. In Figure 20, a Raster Plot of a particularly active device is shown.

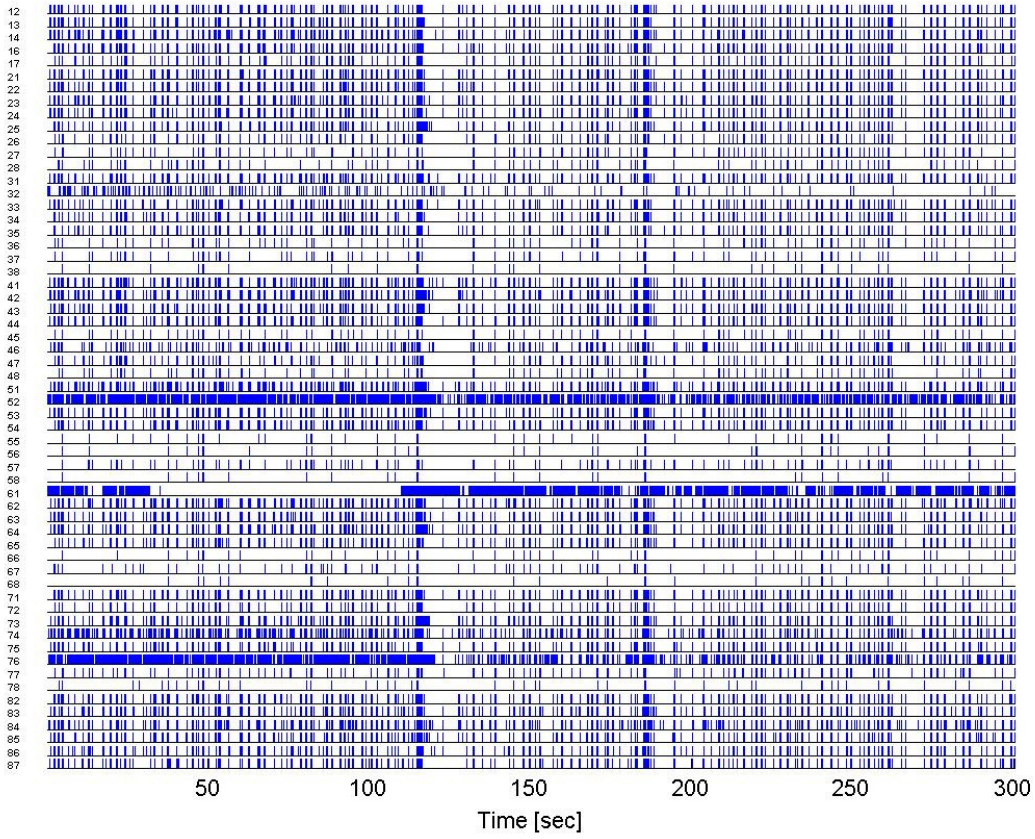


Figure 20: Raster Plot of an active device; y-axis represents the number of the electrode (Device #: 41_12549_24: Spontaneous activity)

Another analysis of spontaneous activity is the Average Firing Rate (AFR) parameter that counts the number of spikes per electrode in the whole recording and divides this number for the number of seconds the recording lasts. It is measured in spikes per second.

We developed a script that is able to provide the AFR per compartment for every experiment and to plot it together into a graph, showing the change of activity of the two different compartments. Moreover it counts the number of active electrodes in the culture, a parameter useful to determine the grade of global activity of the device. We characterized every device with the Number of Active Electrodes (NAE), based on the number of electrodes exhibiting an Average Firing Rate of at least 0.2 spikes per second, according to literature (7).

To study the response of the neurons to stimulation we used, the Post-Stimulus Time Histogram (PSTH) (63) in the SpyCode toolset that allows investigating the mean response of a network to stimulation. The PSTH expresses the probability of firing as a function of time after a stimulus. To construct a PSTH response plot, the culture is repeatedly stimulated. The delay of spikes occurring within a given window after stimulation is gathered and combined for all stimulations. The histogram of observed delays is the PSTH and expresses the mean response over all applied stimulations. We use a window size of 50 up to 400ms, and a resolution of 1ms in the construction of the histogram. We use the mean area of the PSTH, averaged over all active channels, as a single measure to quantify the response to stimulation. In Figure 46 the PSTH obtained from signals recorded from all the array electrodes, is shown. Samples from the responding microelectrodes and occurring in the 50 up to 400 ms-window after the stimulus were used to compute the PSTHs. The histograms are arranged over an 8x8 grid (the MEA layout).

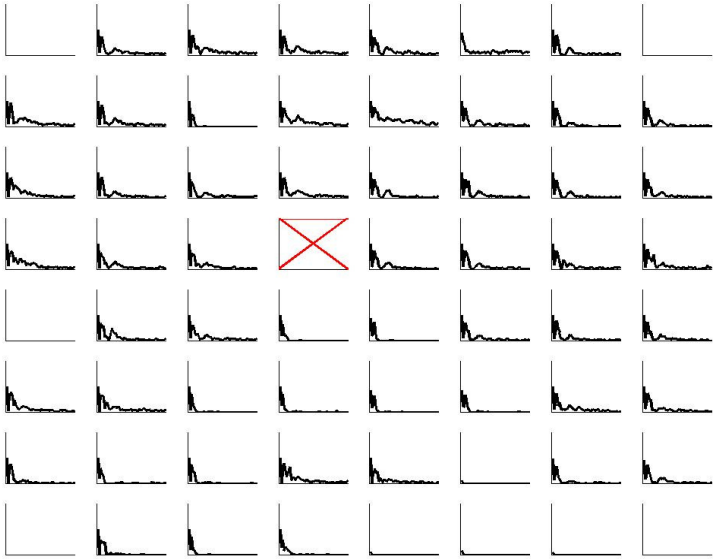


Figure 21: PSTH analysis of Unipolar electrical stimulation on a device on DIV 24 (Device #: 41_12549_24: Unipolar stimulation of electrode 44 (e44) (neurolid2))

4 EXPERIMENTS

Manipulation of dissociated cell culture by both electrical and chemical means is reported in this section. We have combined electrical stimulation with chemical neuro-modulators to influence the response of network activity. In the following section, many aspects of manipulation of network activity by external agents are reported.

4.1 CHEMICAL EXPERIMENTS

Chemical experiments relates to the experiments with special chemical compounds known for their effect on neural cells in culture. For details about those specific chemical compounds and about the protocol we've used, please refer to paragraph 3.4.

Acetylcholine experiments were initially done to show the effect of such a chemical compound on sub-cortical / thalamic and cortical cells.

Other well-known compounds were used to alter the activity of the cells. As the effect of TTX on rat neural cells is well known to be inhibitor of spontaneous activity (see paragraph 2.3.2), we've done experiments on our devices taking advantage of the particular dual-compartmented structure.

As third, we studied the effect of a chemical solution of multiple chemical synaptic communication blockers, able to prevent communication between neurons but still keeping the genuine own activity, as documented in (9).

4.1.1 INTERCOMPARTMENTAL CHEMICAL ISOLATION

In order to use chemical substances selectively in only one compartment of the dual compartment PDMS device, chemical isolation between compartments has to be proved. Application of TTX in one compartment and the selective manipulation of cells within the compartments has been reported earlier (1). In the article, due to the small size of the micro-channels and the passage of the neuritis through the micro-channels, it is believed that it would take several hours before the compound can pass through the micro-channels.

However, during the course of this project more detailed experiments have been done to demonstrate inter-compartmental chemical isolation.

Using a spectrophotometer, the presence of the neurotoxin TTX in neural medium or in water was analyzed. In Figure 22 a spectrophotometer graph of De-ionized (DI) water is presented. A spectrophotometer graph of a solution of TTX at a concentration of 100 nM in distilled water is shown in Figure 23. The two graphs show negligible change in the spectral response.

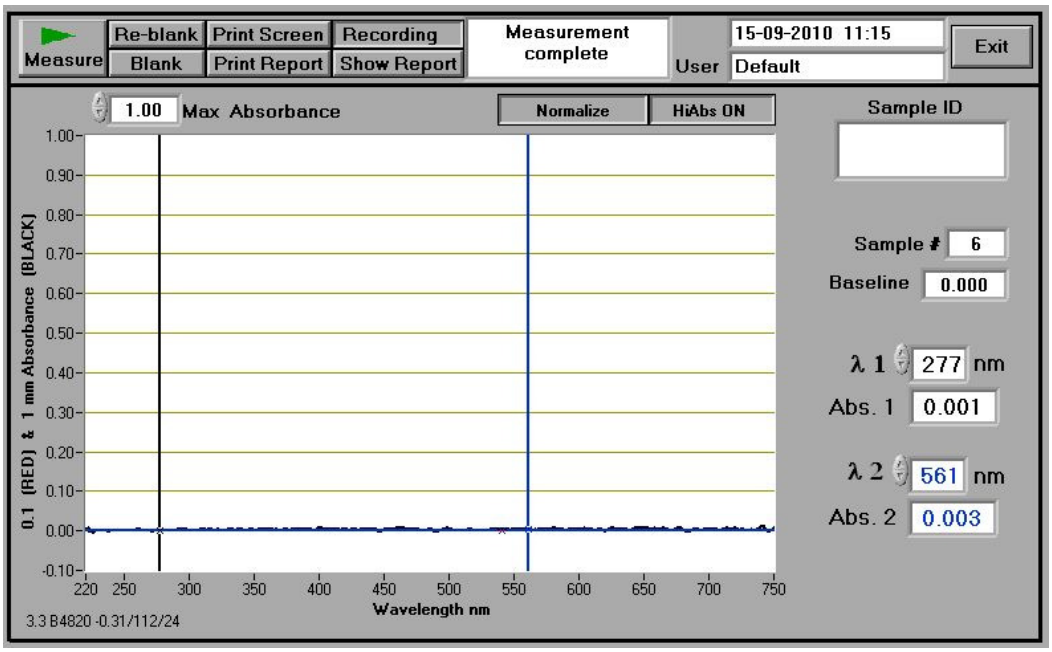


Figure 22: Spectrophotometer of DI water.

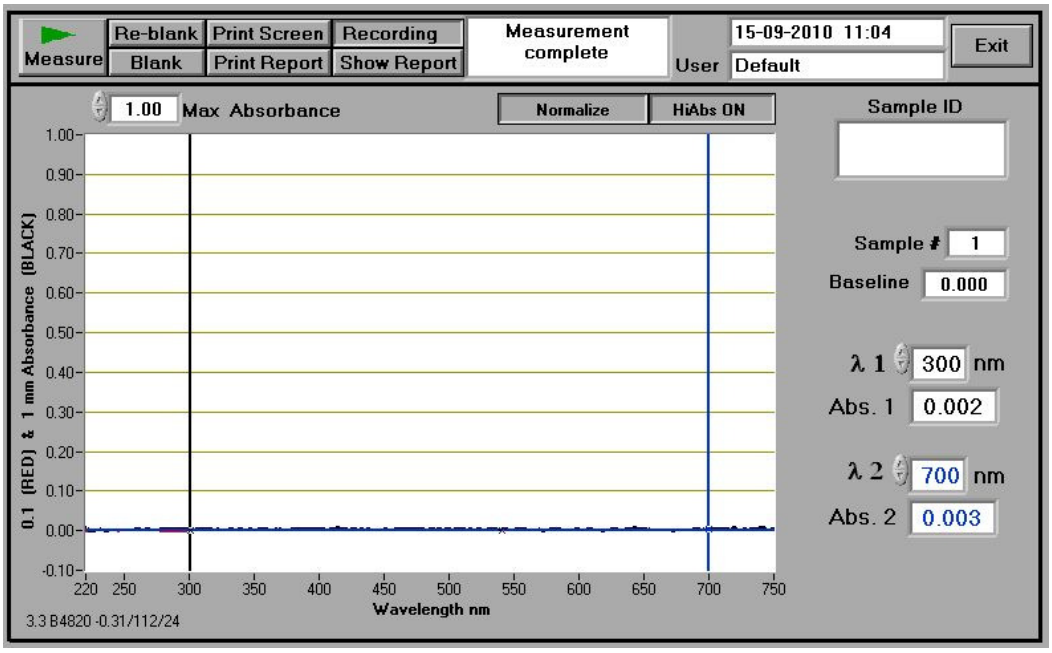


Figure 23: Spectrophotometer of DI water with a concentration of 100nM of TTX

The same measurement was done with neurobasal medium and neurobasal medium added with TTX at a concentration of 100nM. The two resulting graphs in Figure 24 and Figure 25 again show that is visually difficult to detect the presence of TTX, probably because of the very little concentration of TTX.

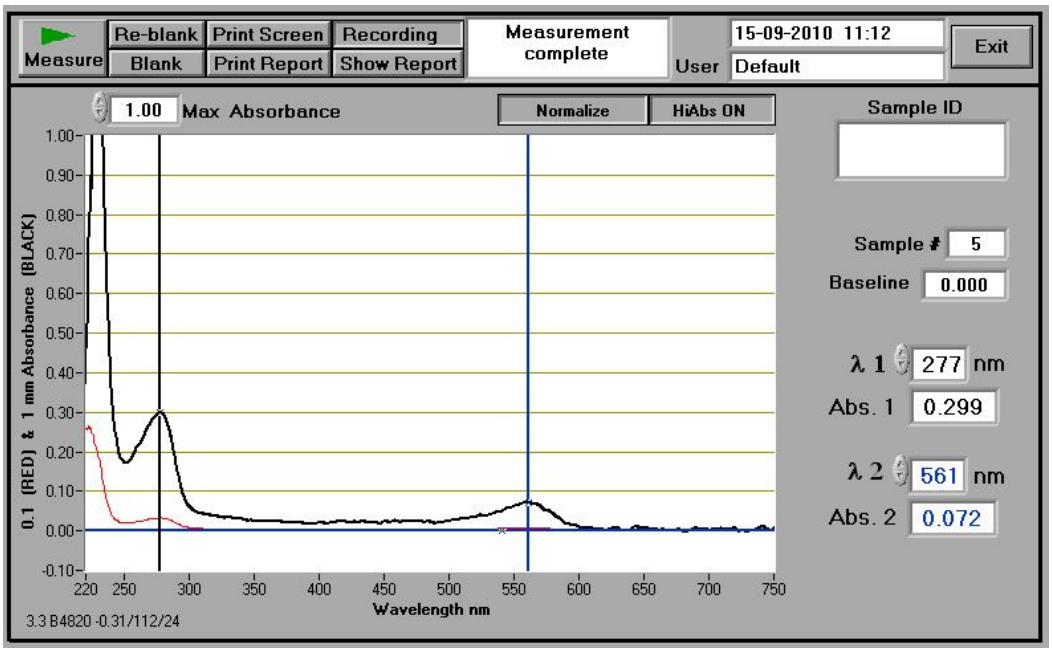


Figure 24: Spectrophotometer of neural medium

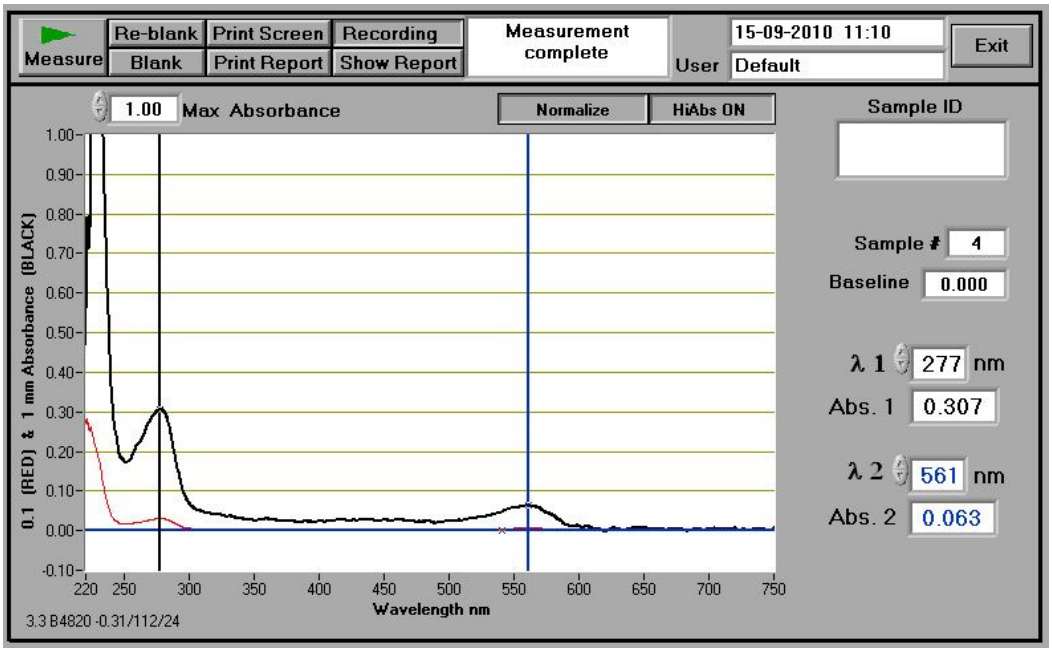


Figure 25: Spectrophotometer of neural medium with a concentration of 100nM of TTX

Giving the negligible difference between the graphs, a detection of low concentrations of TTX in the neurobasal medium wouldn't be visually possible.

Hence, a different compound was used to check if there any mixing of compounds due to diffusion between the two compartments can be detected. A food coloring material that has a strong blue coloration was analyzed with a spectrophotometer; The graph is shown in Figure 26.

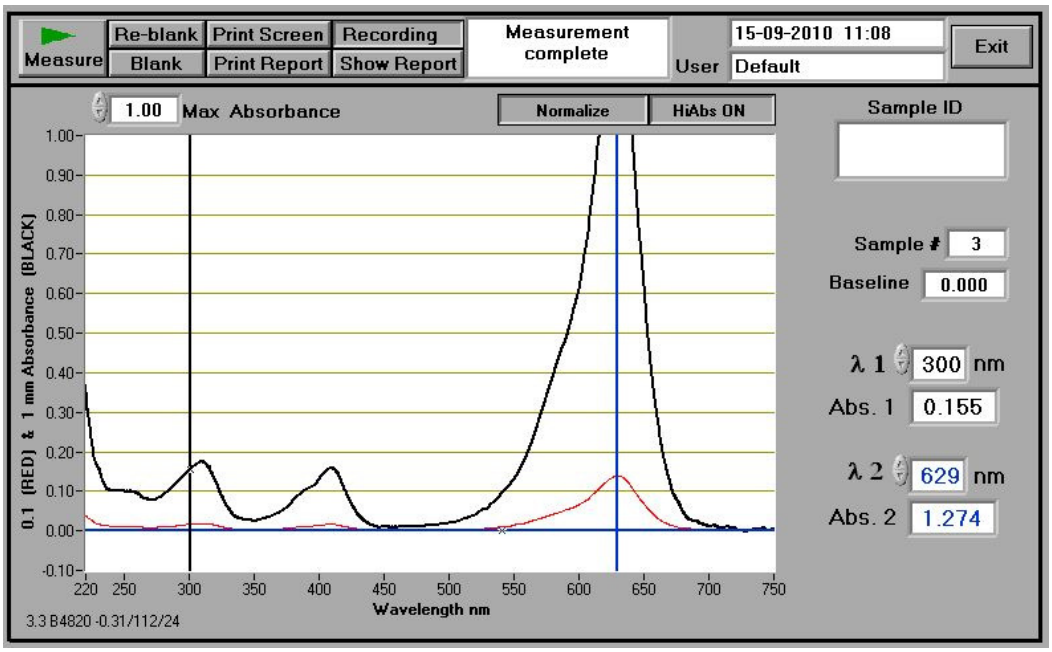


Figure 26: Spectrophotometer of the blue food additive

Evident peaks are distinguishable in the graph of the solution. Due to this, it can be used with DI water to verify the level of diffusion through the micro-channels. The DI water was added in one compartment and a blue food coloring compound in the other compartment in the same quantity. It is visually possible to see the shape of the compartment where the blue food additive, as shown in Figure 27.

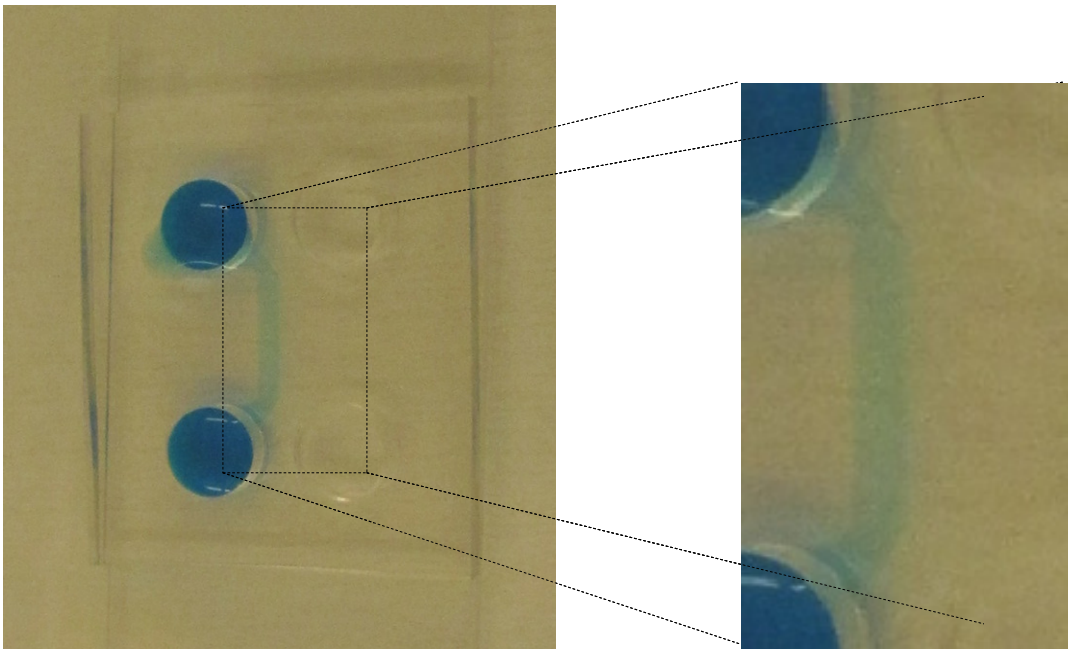


Figure 27: PDMS full with blue food additive in one compartment and water in the other

After 15 minutes, DI water was removed from the device and analyzed it in the spectrophotometer. The food coloring substance wasn't detected to diffuse into the other compartment, as shown in Figure 28.

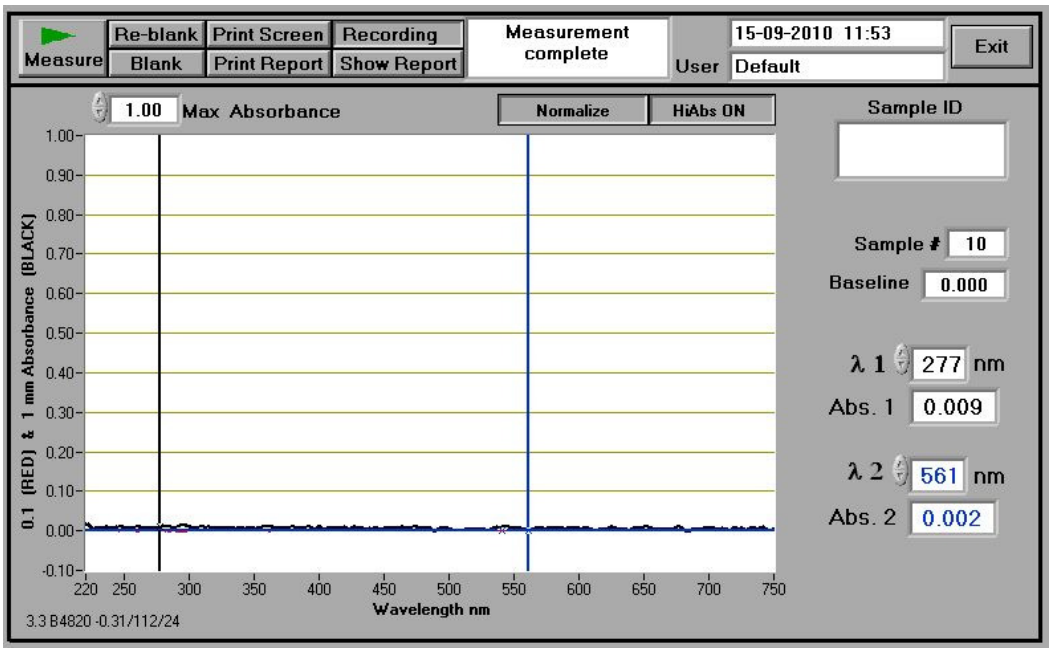


Figure 28: Spectrophotometer of H_2O after 15 minutes in compartment A in a PDMS where the compartment B contained a blue food additive, easily detectable by the spectrophotometer

Moreover, an exposition of 24 hours with the same configuration was done. The result is shown in Figure 29.

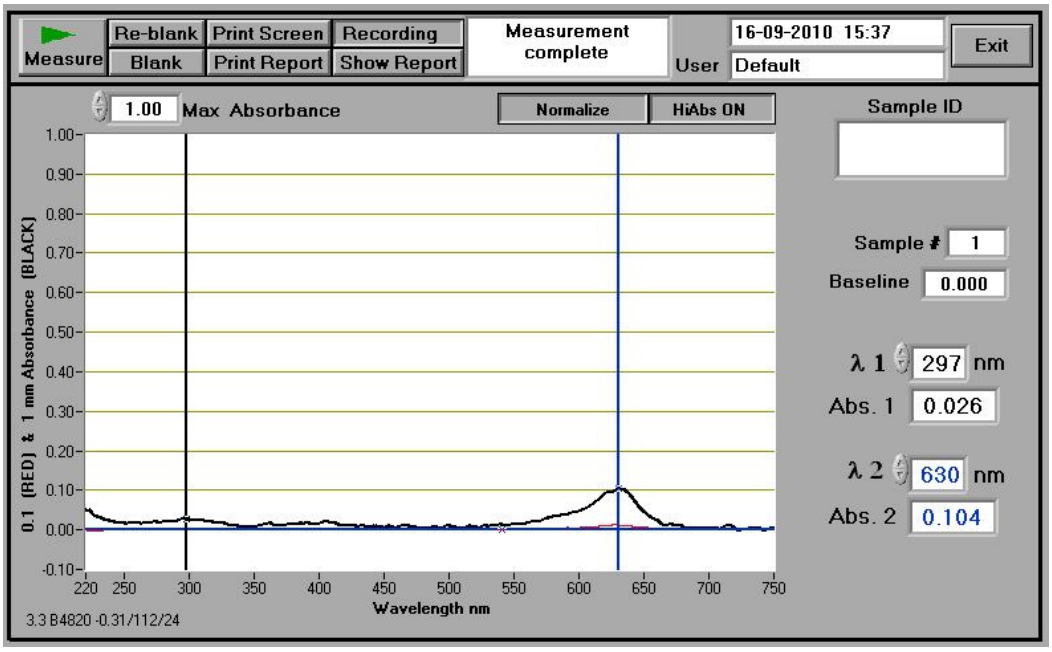


Figure 29: Spectrophotometer of H_2O after 24 hours in compartment A in a PDMS where the compartment B contained a blue food additive.

As a further proof the same experiment was done with water and neurobasal medium. DI water was added in one compartment while neurobasal medium was put in the adjacent compartment. After 8 minutes of exposure, the entire DI water was analyzed. The resulting graph is shown in Figure 30.

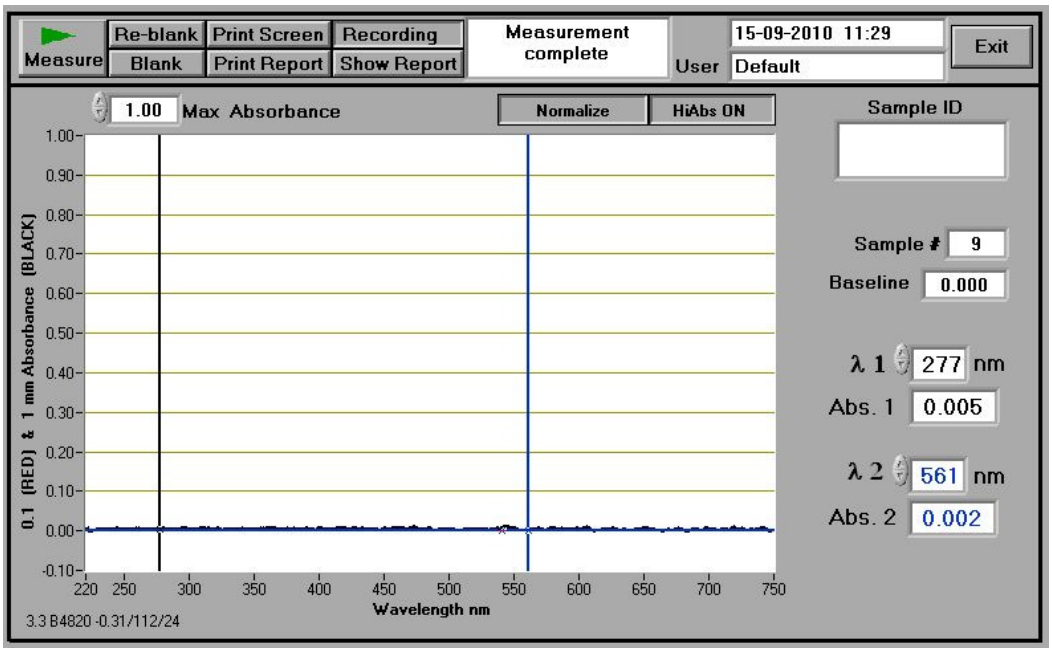


Figure 30: Spectrophotometer of H_2O after 8 minutes in compartment A in a PDMS where the compartment B contained a neural medium

Another experiment was done by adding neurobasal medium in compartment B while leaving the compartment A empty for 15 minutes. In this manner, the fluid is supposed to cross the micro-channels due to the principle of communicating vessels and the big difference in height of volumes between the two compartments. To collect all the fluid that might have crossed the micro-channels, DI water was added in the empty compartment A. Removing all the water after 15 minutes, it was analyzed. Result in Figure 31.

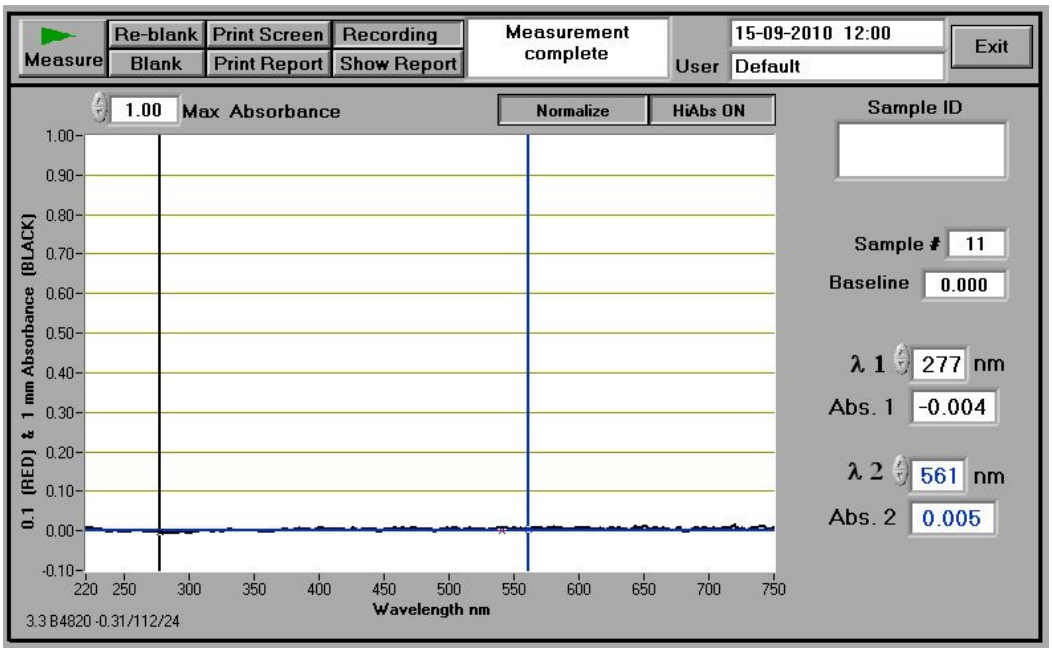


Figure 31: Spectrophotometer of H2O after 15 minutes in compartment A in a PDMS where the compartment B contained a neural medium. The neural medium was left before 15 minutes in compartment B with an empty compartment A

In one occasion an analysis with a leaking device was done (the PDMS didn't adhere perfectly to the MEA glass) and the presence of neurobasal medium in the other compartment with DI water was detected after 5 minutes of exposure. The graph is shown in Figure 32.

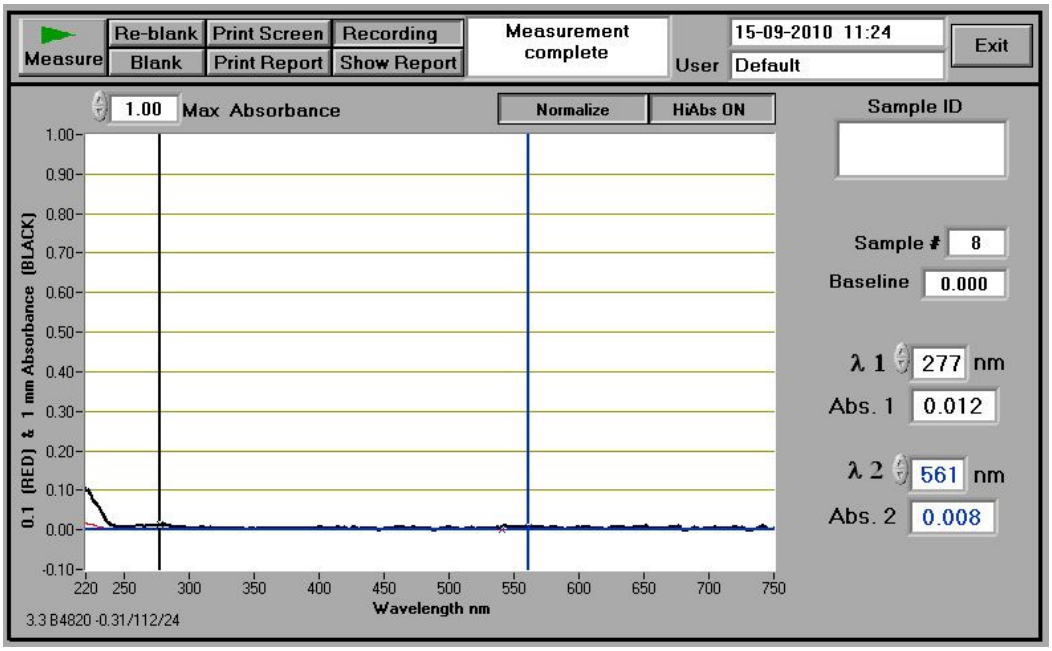


Figure 32: Spectrophotometer of H_2O after 5 minutes in compartment A in a leaking PDMS where the compartment B contained a neural medium

Based on these experiments, it was estimated that there might be no diffusion between the two compartments for the typical time of exposure of the substances during our normal recording procedure.

Other approaches are also possible to detect the presence of TTX. The mouse bioassay has historically been the most universally applied tool in monitor programs. However, it is mostly used for other kind of studies, like detection of the toxicity in marine waters.

Recently also another method has been presented by the use of HPCL (64), however having TTX dissolved in other substances i.e. neurobasal medium, it is difficult to know which peak is the one related to TTX.

A suggested approach useful to determine the concentration of substances in water after the exposure to the experiment with PDMS, would be the study of the spectrometer graphs of the same substance at different concentrations in DI water. Detecting different scales of concentrations from 1 to 1/10 or 1/100 of the same substance in water would highlight what the sensibility of the instrument we have is. To adapt to the diffusion of TTX, it would eventually be needed to be adapted with a coefficient of diffusion of TTX in water. We believe that a study in this direction may give good results.

4.1.2 ACETYLCHOLINE EXPERIMENTS

A mixture of acetylcholine and neural medium was used to make a comparison on the effect on cortical cells in respect to sub-cortical / thalamic cells. We prepared a culture of neural cells and platted them together in two different configurations: cortical-cortical devices (Cx-Cx) and cortical-subcortical/thalamic devices (Cx-Th).

Devices were monitored during different days-in vitro (DIV) and when we observed mature network behavior in a device with consistent number of active electrodes, chemical neuromodulation experiments were performed.

First we recorded spontaneous activity for 10 minutes at a frequency of 10 kHz. Then, the cell culture medium from compartment B was removed and replaced by Acetylcholine. The spontaneous activity was recorded again, in order to see the changes in network activity. In the next step, Acetylcholine (Ach) was removed from the compartment B and the compartment was rinsed 3x times with cell culture medium and the original cell culture medium was added back to the culture. The spontaneous network activity was recorded again for 10 minutes. Between the different phases of the experiment, we allowed at least 10 minutes of wait-time for the cells to adapt to the new conditions.

We have conducted our experiments on 4 Cx-Th devices and 3 Cx-Cx devices. Mean firing rate (MFR) of the spiking activity of the 7 devices are listed in table 2.

Table 2: Results of Acetylcholine experiments on Cx-Th and Cx-Cx devices. DIV=Day In Vitro; MFR=Mean Firing Rate; comp A/B=compartment A/B; NAE=Number of Active Electrodes (>0.2 spikes/second).

Device	DIV	Protocol phase	General MFR	MFR comp A	MFR comp B	NAE
321 (Cx-Th)	23	01 SA	1.72 (100%)	1.94 (100%)	1.5 (100%)	58
		02 30uM of Ach	0.55 (32%)	0.35 (18%)	0.75 (50%)	23
		03 SA	0.91 (53%)	0.91 (47%)	0.92 (61%)	31
12592 (Cx-Th)	24	01 SA	4.32 (100%)	5.64 (100%)	3.1 (100%)	47
		02 20uM of Ach	1.76 (41%)	2.47 (44%)	1.1 (35%)	34
		03 SA	1.8 (42%)	3.02 (54%)	0.89 (29%)	35
I1376 (Cx-Th)	24	01 SA	1.62 (100%)	1.16 (100%)	2.05 (100%)	30
		02 20uM of Ach	1.83 (113%)	1.23 (106%)	2.39 (117%)	29
		03 SA	3.43 (212%)	1.84 (159%)	4.9 (239%)	31
11155 (Cx-Th)	35	01 SA	0.69 (100%)	0.48 (100%)	0.87 (100%)	22
		02 20uM of Ach	0.8 (116%)	0.34 (71%)	1.22 (140%)	16
		03 SA	0.55 (80%)	0.46 (96%)	0.64 (74%)	7
14594 (Cx-Cx)	24	01 SA	5.85 (100%)	5.61 (100%)	6.07 (100%)	58
		02 20uM of Ach	3.99 (68%)	5.22 (93%)	2.88 (47%)	57
		03 SA	2.31 (39%)	3.31 (59%)	1.42 (23%)	57
14596 (Cx-Cx)	29	01 SA	1.66 (100%)	1.23 (100%)	2.04 (100%)	57
		02 20uM Ach	2.22 (134%)	0.97 (79%)	3.35 (164%)	51
		03 SA	1.38 (83%)	0.22 (18%)	2.42 (119%)	38
320 (Cx-Cx)	29	01 SA	0.17 (100%)	0.05 (100%)	0.28 (100%)	19
		02 20uM of Ach	0.13 (76%)	0.02 (40%)	0.23 (82%)	8
		03 SA	0.18 (106%)	0 (0%)	0.35 (125%)	6

The analyses are made on MFR and on NAE (Number of Active Electrodes).

Since every device has a different spiking activity at the beginning of the experiment, we took the initial averaged number of spikes per second (MFR) as 100%. The MFR of the next recording are based on that number.

Regarding the number of active electrodes (NAE), electrodes with more than 0.2 spikes / second were considered as active as explained in paragraph 3.5.

Considering the experiments on Cx-Cx and on Cx-Th devices separately (as in Table 2), network response due to Acetylcholine is not conclusive and further study on the influence of Acetylcholine at different concentration is necessary. As in Figure 33, the effect due to Acetylcholine is not conclusive.

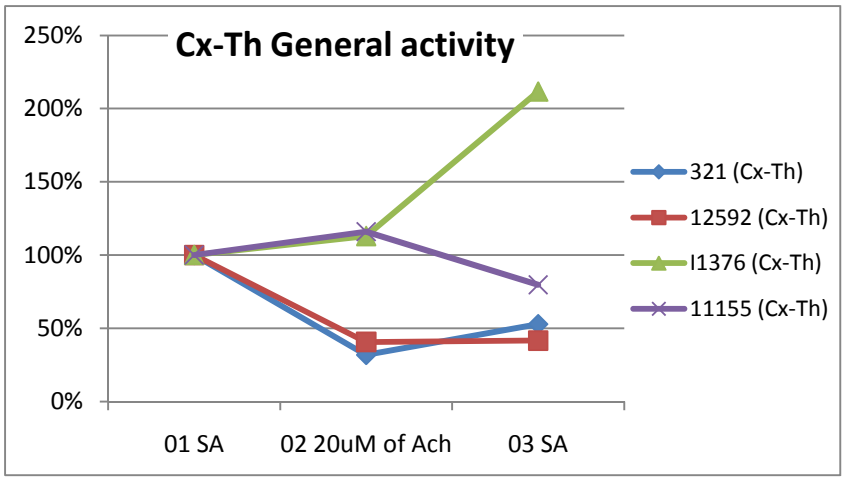


Figure 33: MFR of Cx-Th devices before-during-after the application of Acetylcholine

And analyzing the behavior of the compartment B alone, as shown in Figure 34, the response of cells to Ach is not repeatable under different conditions tested.

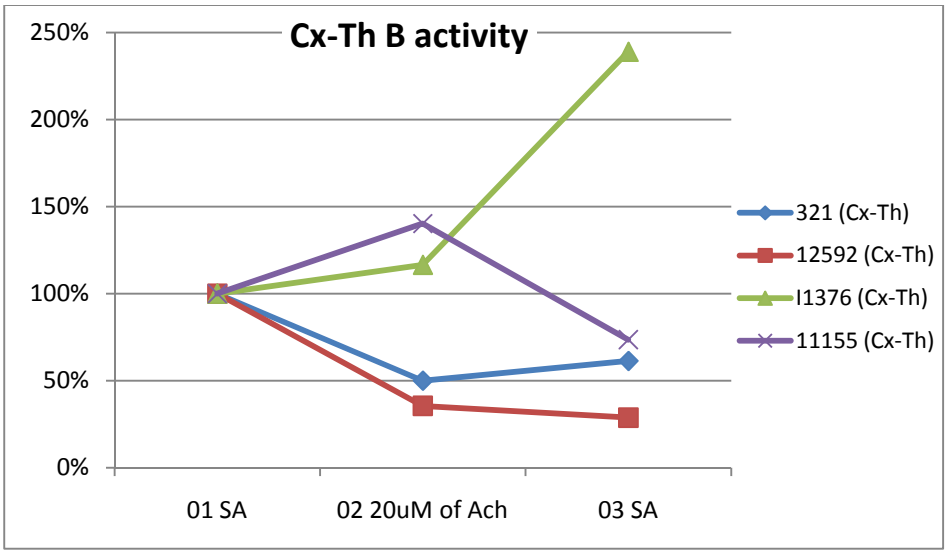


Figure 34: MFR of only compartment B of Cx-Th devices before-during-after the application of Acetylcholine

The devices show different behavior during the injection of Acetylcholine and after removing them out. As observed in Figure 35 and Figure 36, 2 devices show an increase in the average network activity with the presence of Acetylcholine, while the other devices 2 show a loss in activity. Moreover the ones that

show a decrease of the activity keep the MFR roughly at the same level, while the ones that show an increase in activity in the first stage have two different behaviors (one becomes more than the double and one reaches the half) in the second stage.

Comparing the results with Cx-Cx devices we also don't see a clear distinction. In Figure 35, it is shown that the activity of the two compartments averaged, while in Figure 36 we show again only the activity of compartment B, the one where injected with Acetylcholine.

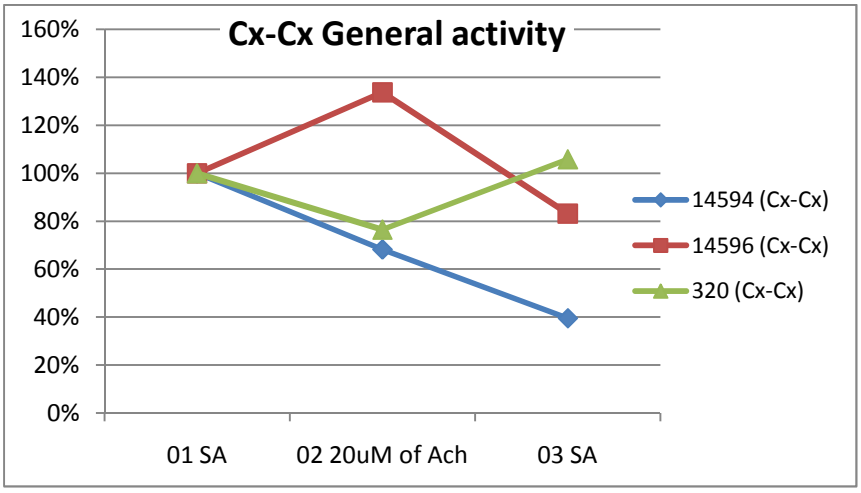


Figure 35: MFR of Cx-Cx devices before-during-after the application of Acetylcholine

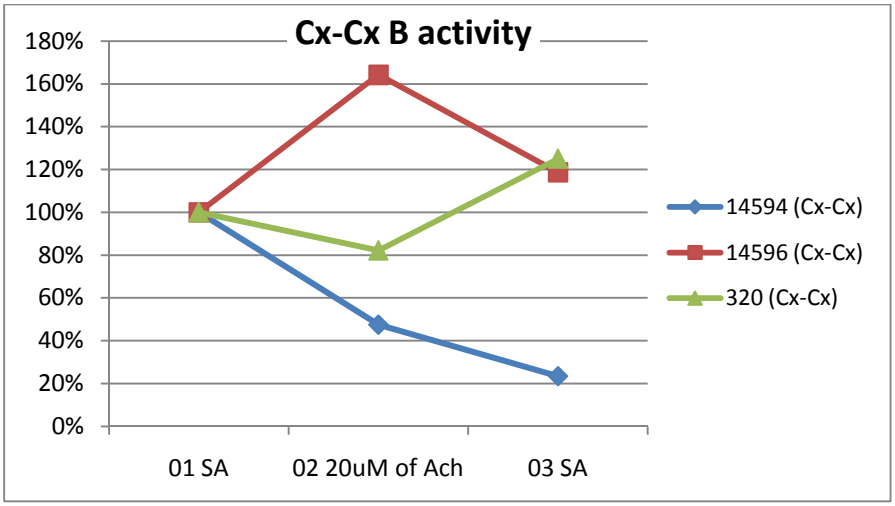


Figure 36: MFR of only compartment B of Cx-Th devices before-during-after the application of Acetylcholine

Also in this case it is not clear what the effect of the chemical compound on the cells is.

4.1.3 SYNAPTIC BLOCKER EXPERIMENTS

The compound that we called Synaptic Blocker (see paragraph 3.4.3) was used in one compartment at a time to observe the behavior of single cells without network communication. As documented in (9), the effect of synaptic blocker on cortical cells was observed to be a block of the synaptic communication, while keeping the pure activity of the individual pacemaker cells. The effect of synaptic blocker on

thalamic cells was not available in the literature and our experiments in this section were designed to understand the influence of this compound on the sub-cortical / thalamic cells.

4.1.3.1 INFLUENCE OF SYNAPTIC BLOCKER ON SUBCORTICAL/THALAMIC CELLS

The compound that we called Synaptic Blocker (see paragraph 3.4.3) was tested first in sub-cortical / thalamic cells to observe its efficacy. Since we showed that bursts are originated in cortical networks during our TTX experiments, the goal of the experiments was to observe the effect of the blockage of the synaptic communication between sub-cortical / thalamic cells in order to better observe the pure thalamic spiking activity.

In an experiment with dual-compartment device containing cortical cells in compartment A and sub-cortical / thalamic cells in compartment B, synaptic blockers were injected into compartment B and the change in network activity before and during the injection of synaptic blocker was analyzed as in Figure 38 and 39.

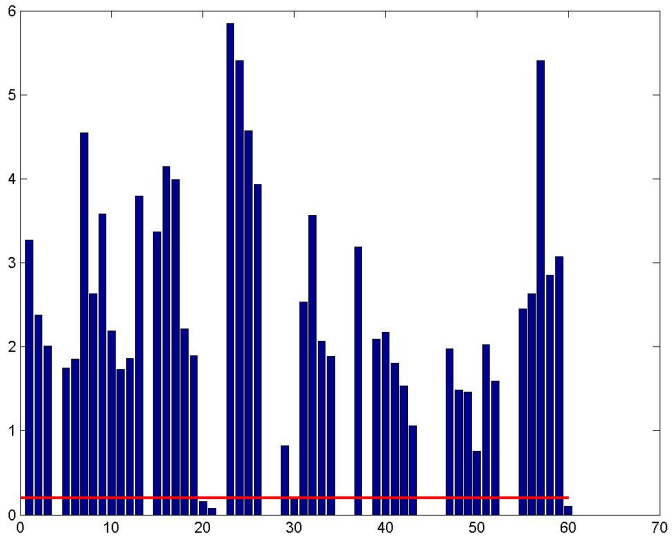


Figure 37: graph of the AFR of the device before Synaptic Blocker (Device #: 46_14596_15: SA before SynBlo)

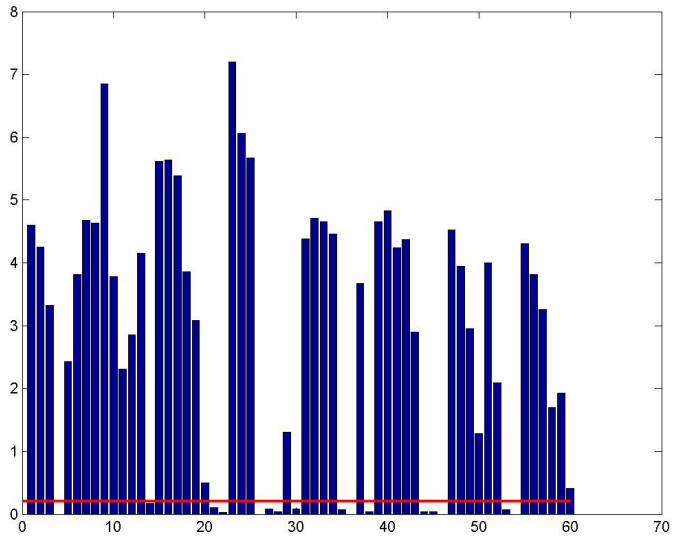


Figure 38: graph of the AFR of the device during Synaptic Blocker (Device #: 46_14596_15: SA during SynBlo)

The average AFR for compartment B with sub-cortical/thalamic cells in the first case (before the injection of synaptic blocker in compartment B) was 1.59 spikes/second, while in the second case (with synaptic blocker in compartment B) it was 2.58 spikes/second. Thus, the effect of synaptic blocker on sub-cortical/thalamic cells was not observed visually.

4.1.3.2 APPLICATION TO CORTICAL CELLS

The application of Synaptic Blocker to cortical cells has been studied to be antagonists of GABA-R, NMDA-R and AMPA-R channels on cell membrane. Applying synaptic blocker with cortical cells in both compartment A and B, the average firing rate (AFR) of the network activity in the compartment with synaptic blocker has considerably changed, as shown in Figure 39 and Figure 40.

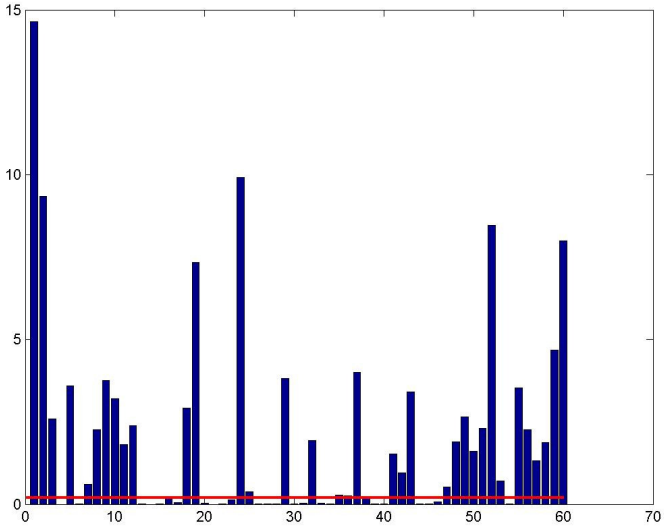


Figure 39: Average firing rate (AFR) of spontaneous activity in both the compartments in absence of synaptic blocker (device #46_I1377_DIV24)

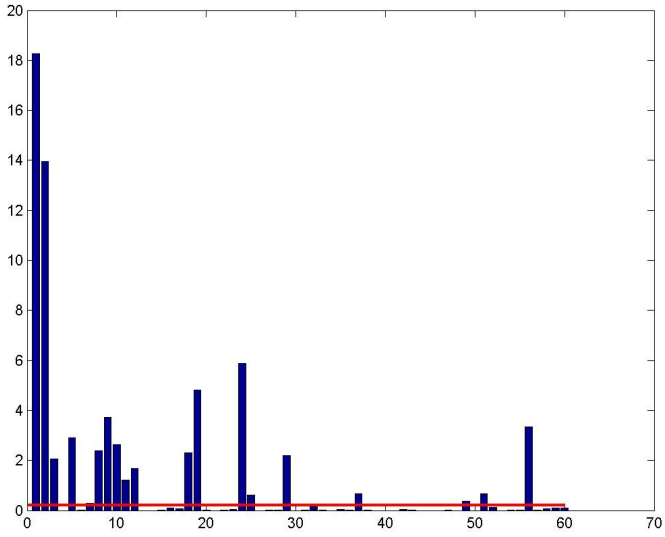


Figure 40: Average firing rate (AFR) of spontaneous activity in both the compartments with synaptic blocker in compartment B (device #46_I1377_DIV24)

As in Figure 41, the network activity in compartment B considerably decreased, while the activity in compartment A is retained. This further confirms the fluidic isolation between the compartments.

4.2 ELECTRICAL EXPERIMENTS

Electrical stimulation is a well known procedure able to manipulate neuronal activity. Many experiments have been done to set up the parameters for effective electrical stimulation (3). It has also been shown that, depending on the strength of the signal and on other parameters, electrical stimulation is able to evoke responses in neurons (7).

With the objective to observe the spread / influence of electrical stimulation in the array of electrodes when providing an electrode with electrical biphasic signal to use all the full potentiality of the dual compartmental devices, several experiments with both unipolar and bipolar electrical stimulations were performed.

In Figure 41, electrode 85 (represented by 'X') in compartment B was stimulated with a unipolar test stimulus of amplitude 760mV. As shown, the evoked responses can be observed across both the compartments mainly due to the current spreading across the ionic culture medium.

The problem was highlighted when a common PSTH response, as the one in Figure 41, was given from the electrodes of both compartment was discovered, even if a physical barrier existed between the two compartments (as our PDMS).

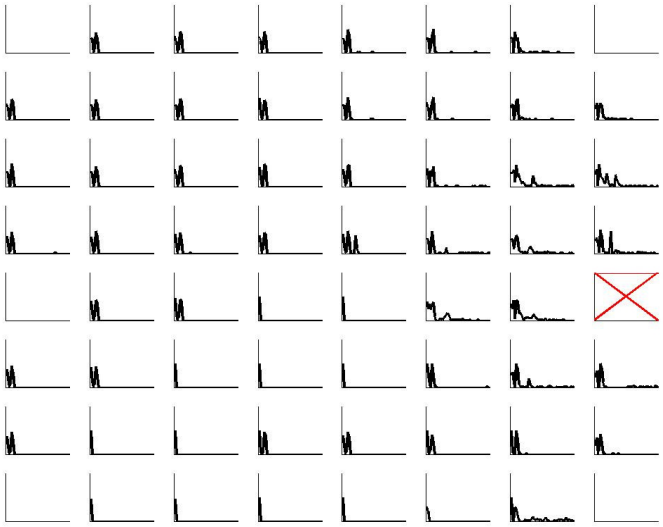


Figure 41: PSTH analysis of Unipolar electrical stimulation on a device on DIV 25 (Device #: 46_14589_25: Unipolar stimulation of electrode 85 (e85) (neurolid2))

Hence, considerable efforts were made in understanding the propagation of current in the two compartments in order to better develop a focused stimulation of cells in a compartment. Also, the best configuration to stimulate cells locally was studied based on our analysis of the stimulation artifacts spreading within the compartments.

4.2.1 PROPAGATION EXPERIMENTS

A problem, which has troubled electro-physiologists since early stimulation experiments, is the introduction of stimulation artifacts that obscure any neural activity near the stimulation site for tens or hundreds of milliseconds (65). The stored electrical charge, which ultimately generates the artifact, introduces problems for long-term stimulation protocols, as it could cause ion migration or in general saturation of recording system.

Whenever an electrical stimulation is provided those artifacts affect the recordings. Research groups have proposed different solutions for the elimination of the artifacts problem. In (66) an ad-hoc personalized circuit has been built to eliminate the residual charge of the electrodes using specific hardware, while in (61) an algorithm has been developed to eliminate artifacts post recording during analysis phase. Most of the research groups that have worked with electrical stimulations of neural cells on MEAs use a blanking time immediately after the stimulation is given.

Observing the amplitude of the artifacts recorded in the culture, a difference in the amplitude of the artifacts was noticed while using different stimulation methods. The same artifacts can be used as indicators of the current spreading while stimulating, to reach a better electrical configuration.

Originally the idea was to remove all the artifacts without the use of a big blanking time that would obscure the immediate response of the cells. Then, it was suggested that it is not important the complete elimination of the artifacts because not all the current that travels in the medium is able to elicit a response. Nevertheless a stimulation focused in a smaller area would improve all the experiments.

A biphasic squared wave at 1.5 V peak to peak was used to stimulate at different electrodes positions a device with only neural basal medium (no cells). The stimulation lasted always 260 µs per peak, so 520 µs in total. During the stimulation the recordings were stopped in order to prevent saturation of the electrodes attached to the recording system. The time elapsed since the moment the recording stops until the stimulation is called pre-blanking time, while the time between the stimulation and the beginning of the recording is called (post-stimulus blanking time). If not specifically specified, the post-stimulus blanking time used was 100 µs and the electrode 15 was used as internal grounded electrode. When the neural basal medium was present in the dual compartmental PDMS, 2 external grounded electrodes (neurolid2) were connected, each one connected to one of the reservoirs of each compartment in the device. In the last experiments a configuration was tried with 4 external grounded electrodes (neurolid4) connected with the fluid through the 4 reservoirs.

The stimulation phase of the experiments was recorded for 300 seconds with stimulation provided during 250 seconds.

To calculate the amplitudes of the artifacts in the different positions of the matrix, the average of the artifacts amplitudes in 3 different moments of the recording was taken: the first stimulation, the one generated immediately after 150 second and the last one of the recording (250 seconds after the first one).

Extracted a noise threshold in the experiment (retrieving it in the part of the recording without stimulation), every amplitude below that threshold was considered as noise, so was considered of amplitude “0”. The letter “G” means that the particular electrode was logically connected to ground, while the letter “X” was the stimulated electrode. All the values shown are in µV.

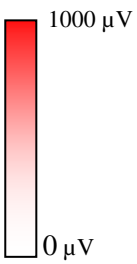
4.2.1.1 STIMULATION WITHOUT FLUID

Due to the uncertainty regarding the origin of the artifacts, a first check was done to assure that the system in use was free of any electrical issue (i.e. electrical coupling). The goal was to show that the artifacts are generated because of current propagation into the medium, instead of any other hardware problem in the recording system.

At first, stimulation was done in an empty MEA device (without PDMS and fluid). The results of this stimulation are shown in Table 3.

Table 3: Amplitudes of the artifacts in a dry MEA; stimulation in electrode 85 [values in µV]

	1	2	3	4	5	6	7	8
1			0	0	0	0	0	0	
2		0	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0	296
4		0	0	0	0	0	395	704	833
5	G		0	0	0	0	715	833	X
6		0	0	0	0	0	0	301	490
7		0	0	0	587	0	0	0	0
8			0	0	322	0	0	0	



The same experiment was then repeated with a PDMS device put on the MEA (results not shown, as they were roughly the same).

In both cases the noise level was between -120µV and + 120µV.

In a dry MEA where the electrodes are posed in a glass material supposedly no conductive, the visible artifacts in the experiment with the dry MEA are just near the stimulated electrode.

It is observable that the stimulation affects the nearest electrodes. We then can conclude that:

1. There are no electrical issues in the recording system so that all the artifacts we see in the recording comes from the electrical stimulation
2. The artifacts detected depend on the position of the stimulation on the Matrix

4.2.1.2 STIMULATIONS WITH MEDIUM AND UNIPOLAR STIMULI

We experienced the artifacts to change amplitude and shape, depending on the type and amplitude of the stimulation. Based on this, we were able to investigate what is the optimal configuration for stimulation of the devices with the neural basal medium that provides a strong electrical conductivity.

Injecting the fluid in a dual compartmental MEA device and connecting it to a ground such as the internal reference electrode or external grounded electrodes, the noise level decreases evidently, reaching a threshold of -8µV and + 8µV.

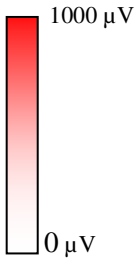
Experiments have been done attempting to recreate the “ground surface” theoretical configuration mentioned in (54). In order to understand the role of the grounds around the stimulation in dual compartmental devices, we changed the positions of the grounds logically connected to the electrodes in the device.

MEAs are normally thought to be used with a Ring that contains the whole neural basal medium (Ring MEA). They normally provide a big reference electrode the fluid is connected to. In the configuration used in our experiments, the dual compartmental PDMS device covers the big reference electrode, in a way that the medium cannot be physically connected to the ground. Due to this, *neurolids* were used, that are able to create a connection between the fluid and the ground of the system.

In order to have a reference, an experiment was done with the basic configuration where the stimulation is provided by the electrode 85, while the grounds were provided by the 2 external electrodes present in the neurolid2 and the big reference electrode (electrode 15). The averaged results are shown in Table 4.

Table 4: Averaged amplitudes of the artifacts in dual compartmental MEA with unipolar stimulation in 85 and ground in e15 and neurolid2 [values in µV]

	1	2	3	4	5	6	7	8
1		683	680	680	683	689	680	
2	679	686	477	657	699	697	770	671
3	638	656	669	684	682	694	798	705
4	655	615	632	667	693	705	40	755
5	G	633	578	457	480	590	741	X
6	551	554	479	525	454	507	555	474
7	476	461	551	531	587	496	505	515
8		411	374	417	468	464	484	



The results in Table 4 suggest that there is no difference in the current spreading between the 2 compartments. In Figure 42 the history of the artifact amplitudes is shown: per each electrode, the maximum amplitude is taken every 5 seconds, in a way that every artifact is taken only once per time.

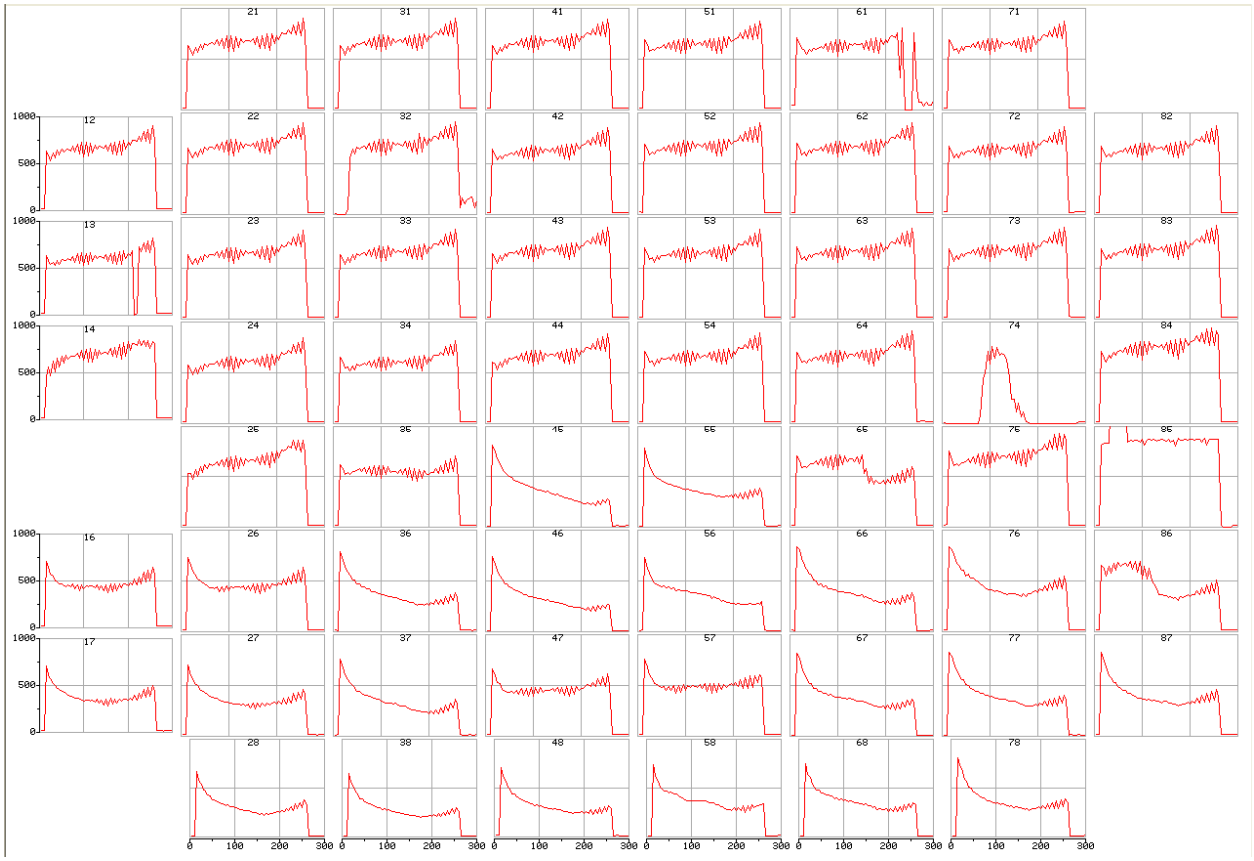


Figure 42: Artifacts history during stimulation of electrode 85 and ground in electrode 15 and neurolid2; electrode 74 is noisy [Values: x-axis 0-300 seconds, y-axis 1000 µV]

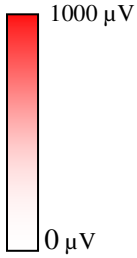
A sort of division is visible in the behaviors between the upper and the bottom parts of the device. The superior part is mostly increasing the amplitude when the stimulation is given for 5 minutes, while in the bottom part opposite to that is decreasing.

Taking inspiration from (54) and with the idea of recreating a grounded surface, one and subsequently two lines of electrodes next to the stimulation done in electrode 85 were logically connected to ground. The grounded electrodes in the first case are number 15, 74, 75, 76, 84 and 86 in presence of neurolid2. In the second case electrodes 63, 64, 65, 66, 67, 73, 77, 83, 87 were added as grounds. The averaged results are shown in Table 5.

Table 5: Averaged amplitude of the artifacts in dual-compartmental MEA with stimulation in e85 with neurolid2 and ground in e15 [values in µV]; A_ grounds logically put in 74,75,76,84,86; B_ grounds logically put in 63-67,73-77,83-84,86-87.

	1	2	3	4	5	6	7	8
1		850	843	844	842	862	859	
2	847	854	849	826	847	865	853	855
3	818	835	843	841	863	861	866	862
4	823	820	810	834	870	859	G	G
5	G	676	662	487	553	748	G	X
6	637	583	532	482	462	537	G	G
7	577	549	537	670	736	519	567	604
8		536	540	510	450	513	550	

	1	2	3	4	5	6	7	8
1		629	632	634	649	655	647	
2	622	629	632	624	641	659	600	560
3	614	618	624	635	650	G	G	G
4	603	610	607	624	659	G	G	G
5	G	468	446	399	416	G	G	X
6	427	426	397	359	571	G	G	G
7	401	399	394	497	538	G	G	G
8		389	402	395	543	414	403	

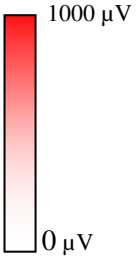


Further, a trial was made by putting the whole stimulated compartment to ground and even a column in the other side of the channels in order to better isolate the stimulation to one compartment. Results are shown in Table 6.

Table 6:Averaged amplitude of the artifacts in dual compartmental MEA with stimulation in 85, neurolid2 and ground in e15 [values in µV]; A_ grounds logically in the whole compartment B; B_ grounds logically in the whole B and in column 4

	1	2	3	4	5	6	7	8
1		401	394	393	G	G	G	
2	403	420	404	381	G	G	G	G
3	377	402	399	397	G	G	G	G
4	391	389	401	363	G	G	G	G
5	G	295	282	412	G	G	G	X
6	254	251	356	359	G	G	G	G
7	254	316	327	341	G	G	G	G
8		303	294	342	G	G	G	

	1	2	3	4	5	6	7	8
1		308	311	G	G	G	G	
2	295	315	321	G	G	G	G	G
3	375	289	307	G	G	G	G	G
4	310	281	279	G	G	G	G	G
5	G	276	267	G	G	G	G	X
6	250	240	300	G	G	G	G	G
7	229	271	269	G	G	G	G	G
8		246	230	G	G	G	G	



A comparison between all these configurations is necessary. A direct comparison was made by the differences between the averaged amplitudes of the artifacts taking all the experiments done with the same stimulation and by changing only the number of grounded lines.

A difference between the values of two tables per time was done. Starting from the first case, only the comparison with the best case is done.

The first comparison is made between Table 4 and Table 5A.

Table 7: Difference in the artifacts amplitude between Table 5A and Table 4 [values in µV]

	1	2	3	4	5	6	7	8
1		-167	-163	-164	-159	-173	-179	
2	-167	-168	-372	-170	-148	-168	-82	-184
3	-180	-179	-173	-157	-181	-166	-68	-158
4	-168	-205	-178	-167	-176	-154	G	G
5	G	-44	-84	-30	-74	-158	G	X
6	-86	-29	-53	43	-8	-30	G	G
7	-101	-88	14	-139	-149	-23	-62	-90
8		-126	-166	-93	18	-49	-66	

Negative values mean that the experiment in Table 5A is pejorative than the one in Table 4. As it is easy to see, there is no improvement putting only one line of grounds to the electrodes near the stimulated one.

Then the difference between Table 5B and Table 5A is shown in Table 8, while Table 9 represents the differences between case Table 5A and Table 4.

Table 8: Difference in the artifacts amplitude between Table 5B and Table 5A [values in µV]

	1	2	3	4	5	6	7	8
1		222	211	211	193	206	212	
2	224	225	217	203	206	206	253	294
3	204	217	219	206	213	G	G	G
4	221	210	203	210	211	G	G	G
5	G	208	216	88	137	G	G	X
6	210	157	135	123	-109	G	G	G
7	176	150	144	172	198	G	G	G
8	0	147	139	116	-93	99	147	0

Table 9: Difference in the artifacts amplitude between Table 5B and Table 4 [values in µV]

	1	2	3	4	5	6	7	8
1		55	48	47	34	34	33	
2	57	56	-155	33	58	38	171	111
3	24	38	46	49	31	G	G	G
4	53	5	25	43	34	G	G	G
5	G	165	131	58	63	G	G	X
6	124	128	82	166	-117	G	G	G
7	75	62	158	33	48	G	G	G
8		21	-28	23	-75	50	81	

From these three analyses it is possible to understand the order of improvement (from the worst to the best): Table 5A, Table 4, Table 5B. That is averagely true even though the values in Table 9 are small. That means that it is better to have two lines of grounds, while only one is not enough. Strangely, one line of grounds is pejorative than a configuration without any electrode connected logically to ground.

Continuing, in Table 10 and Table 11 comparisons are made between the values of Table 6A-Table 5B and Table 6B -Table 6A, respectively.

Table 10: Difference in the artifacts amplitude between Table 6A-Table 5B [values in µV]

	1	2	3	4	5	6	7	8
1		227	238	241	G	G	G	
2	219	210	228	242	G	G	G	G
3	236	216	224	239	G	G	G	G
4	212	221	206	261	G	G	G	G
5	G	173	164	-14	G	G	G	X
6	173	175	41	0	G	G	G	G
7	147	82	67	156	G	G	G	G
8		86	108	53	G	G	G	

Table 11: Difference in the artifacts amplitude between Table 6B-Table 6A [values in µV]

	1	2	3	4	5	6	7	8
1		94	83	G	G	G	G	
2	108	105	83	G	G	G	G	G
3	3	114	92	G	G	G	G	G
4	81	108	122	G	G	G	G	G
5	G	19	15	G	G	G	G	X
6	4	11	55	G	G	G	G	G
7	26	45	58	G	G	G	G	G
8		57	63	G	G	G	G	

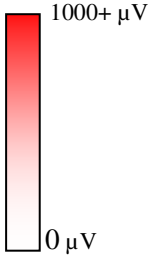
Finally, an order in the improvements is possible (from the worst to the best configuration): Table 5A, Table 4, Table 5B, Table 6A, Table 6B. The best case is the one shown in Table 6B, with the whole compartment B and column 4 grounded.

Going further in the investigation of the role of the grounds, a stimulation of the device was done putting off all the internal and external grounds. The result was that the artifacts were much bigger than the previous experiments and even the noise level increased reaching the amplitude of 150 µV (normally it is around 16 µV).

To further show the importance of the internal and external grounds, a test was done without the 2 external grounds in the dual compartmental device, but with the whole compartment B connected logically to ground so that the big grounded area could provide the same function as the external ground. Results are shown in Table 12.

Table 12: Averaged amplitude of the artifacts in dual compartmental MEA with stimulation in 85, without neurolid2 and ground in e15 and in the whole compartment B [values in µV]

	1	2	3	4	5	6	7	8
1		655	654	658	G	G	G	
2	648	649	917	663	G	G	G	G
3	1252	653	650	657	G	G	G	G
4	681	403	678	1265	G	G	G	G
5	G	657	645	572	G	G	G	X
6	639	639	641	686	G	G	G	G
7	634	640	638	670	G	G	G	G
8		643	647	393	G	G	G	



The case shown in Table 12 is pejorative than the one proposed in Table 6A. Some of the electrodes in Table 12 show amplitudes even higher than 1000 µV. The effect of this stimulation on electrode 33 is shown in Figure 43. It is possible to see that the charge in the electrode is strong that saturates.

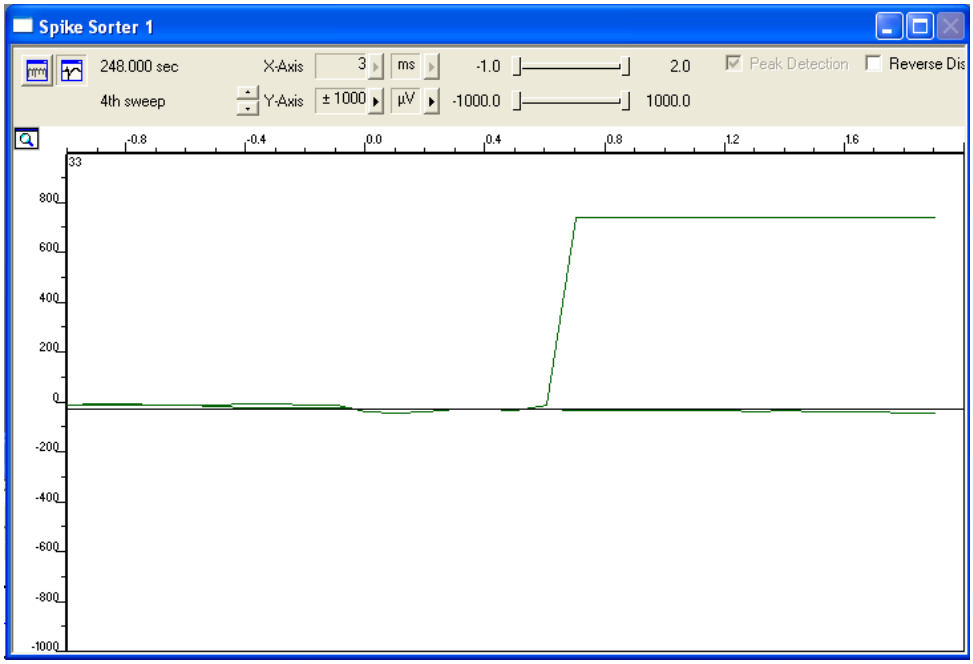


Figure 43: Saturation effect on electrode 33 during stimulation in electrode 85 without external ground; [values: x-axis in ms, y-values in µV]

Therefore the external grounds improve the configuration.

With the goal of obtaining electrical isolation between the two compartments, grounds were connected logically to the columns 4 and 5 of the MEA device, in a way that the current passing through the micro-channels can discharge its power into these grounds.The results of this experiment are shown in Table 13.

Table 13: Averaged amplitude of the artifacts in dual compartmental MEA with stimulation in 85, putting column 4 and 5 to ground [values in µV]

	1	2	3	4	5	6	7	8
1		586	604	g	g	626	602	
2	550	555	546	g	g	627	581	564
3	523	531	568	g	g	629	587	608
4	604	557	532	g	g	595	324	618
5	g	503	481	g	g	498	607	x
6	462	451	411	g	g	424	480	484
7	427	406	414	g	g	421	96	104
8		70	405	g	g	418	409	

1000 µV

0 µV

Unfortunately this experiment proves that the little surface of the electrodes in column 4 and 5 are not enough to discharge completely the current spreading in the liquid. This means that it is not possible to obtain electrical isolation with this configuration.

Electrical isolation should be reached then in other ways.

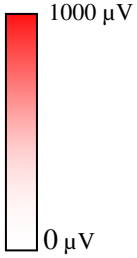
4.2.1.3 BIPOLAR STIMULI

In the following experiments we tried a bipolar stimulation in order to see if the stimulation is more focused compared to a unipolar one as it is documented in literature (54). So, we applied the bipolar configuration to our system and analyzed the results.

The first recording was made using a bipolar stimulation between two electrodes far from the micro-channels (from electrode 84 to electrode 85), connecting the ground with electrode 15 (covered by the PDMS) and putting two external grounds in contact with the liquid in the 2 reservoirs in the bottom part of the PDMS. This is the default configuration our experiments started from. The results of 5 minutes of stimulation without cells are shown in Table 14.

Table 14: Averaged amplitude of the artifacts in dual compartmental MEA with bipolar stimulation from 85 to 84 and 2 external grounds at the bottom of the device [values in µV]; letters "a" and "b" are now the stimulated electrodes.

	1	2	3	4	5	6	7	8
1		315	316	316	359	382	357	
2	279	425	308	324	357	355	351	500
3	172	231	294	315	346	382	614	584
4	137	126	149	311	342	430	829	B
5	G	267	262	283	298	479	570	A
6	218	289	284	269	308	330	317	484
7	193	239	283	328	367	326	286	349
8		281	237	271	433	342	264	



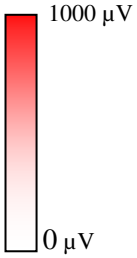
The stimulation is more concentrated near the stimulated area (i.e. electrode 74 compared to 14).

In Table 15A and in Table 15B the measurement made with unipolar configuration stimulating the same electrodes and a comparison between the unipolar and bipolar are reported.

Table 15:

A_ Averaged amplitude of the artifacts in dual compartmental device with unipolar stimulation in 85 and 2 external grounds at the bottom of the device [values in µV];

B_ Difference between unipolar and bipolar stimulation [values in µV];



	1	2	3	4	5	6	7	8
1		683	680	680	683	689	680	
2	679	686	477	657	699	697	770	671
3	638	656	669	684	682	694	798	705
4	655	615	632	667	693	705	40	755
5	G	633	578	457	480	590	741	X
6	551	554	479	525	454	507	555	474
7	476	461	551	531	587	496	505	515
8		411	374	417	468	464	484	

	1	2	3	4	5	6	7	8
1		368	364	364	324	307	323	
2	400	261	169	333	342	342	419	171
3	466	425	375	369	336	312	184	121
4	518	489	483	356	351	275	-789	B
5	G	366	316	174	182	111	171	A
6	333	265	195	256	146	177	238	-10
7	283	222	268	203	220	170	219	166
8		130	137	146	35	122	220	

As easily observable, the bipolar stimulation decreases for an average of 50% the amplitudes of the artifacts, providing a better stimulation only near the stimulated area. In fact, the negative values in the neighborhood of electrodes 84 and 85 show that in those electrodes the stimulation increases.

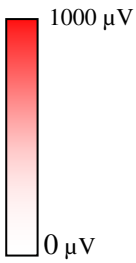
Found that the bipolar stimulation has a better focus, the new configuration was tested changing the position of the grounded electrodes in the device to reach a configuration without artifacts.

As done with the unipolar stimulation, the line next to the stimulation was connected to ground and subsequently also the whole compartment B, to ideally reach the configuration of a grounded surface. The results of this experiment are shown in Table 16.

Table 16: Averaged amplitude of the artifacts in dual compartmental MEA with bipolar stimulation from 84 to 85 and 2 external grounds at the bottom of the device [values in µV]; A_ ground is connected to the line next to the stimulation; B_ ground is connected to the whole compartment B

	1	2	3	4	5	6	7	8
1		290	311	308	295	255	305	
2	273	275	305	293	312	292	301	457
3	273	282	270	312	305	325	G	G
4	259	271	269	299	302	787	G	B
5	G	246	219	311	290	240	G	A
6	189	255	258	277	264	197	G	G
7	152	213	229	276	262	273	117	119
8		291	192	258	223	326	146	

	1	2	3	4	5	6	7	8
1		630	622	638	G	G	G	
2	605	633	610	647	G	G	G	G
3	560	573	624	637	G	G	G	G
4	505	532	557	619	G	G	G	B
5	G	370	346	21	G	G	G	A
6	285	181	73	68	G	G	G	G
7	196	106	63	320	G	G	G	G
8		179	63	79	G	G	G	



A comparison was then done to evaluate which one was the best configuration.

Table 17: Difference in the artifacts amplitude between bipolar experiments with grounded electrodes. A_ difference between Table 14 and Table 16A; B_ difference between Table 16A and Table 16B [values in µV]

	1	2	3	4	5	6	7	8
1		179	177	177	208	257	177	
2	186	190	172	180	207	193	159	281
3	184	180	188	176	195	175	G	G
4	188	184	186	179	193	-46	G	B
5	G	121	102	14	61	167	G	A
6	78	194	18	21	20	110	G	G
7	52	36	20	113	99	69	137	180
8		14	21	32	29	48	99	

	1	2	3	4	5	6	7	8
1		-340	-311	-330	G	G	G	
2	-333	-357	-305	-355	G	G	G	G
3	-287	-292	-354	-325	G	G	G	G
4	-247	-261	-289	-320	G	G	G	B
5	G	-123	-127	289	G	G	G	A
6	-96	75	185	209	G	G	G	G
7	-44	108	166	-44	G	G	G	G
8		112	128	178	G	G	G	

Table 17A shows that a first line of grounded electrodes decreases the amplitude of the artifacts. Curiously, in Table 17B the values of the artifacts amplitudes in the upper part are negatives, while the ones in the bottom part are positive. Considering that the experiments with the unipolar stimulation generally showed that putting many lines of grounded electrodes around the stimulation improve the focusing, a contradiction was obtained between the two experiments. In fact, putting many grounded electrodes around the stimulation does not always improve the focus.

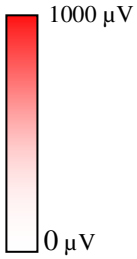
Given the results observed, hypothesizes were started to be thought about the fact that there should be a difference between the upper part and the bottom part of the device that makes the current flowing better in the bottom part.

Trials changing the position of the stimulated electrodes were done. In Table 18A results are reported regarding an experiment where stimulation was delivered in the upper part of the device, while in Table 18B the opposite part was provided with stimulation.

Table 18: Averaged amplitude of the artifacts in a dual compartmental MEA with bipolar stimulation. A_ stimulation between electrodes 61 and 51; B_ stimulation between 68 and 58 [values in µV];

	1	2	3	4	5	6	7	8
1		34	31,7	93,3	B	A	218	
2	27,7	29	33	83,3	286	223	147	183
3	25,3	25	29,3	69,3	58,3	180	225	255
4	25,7	25	24,3	32,3	160	41,3	39,3	34
5	G	27	25	22,3	21,7	21,7	24	35
6	49,7	297	22,3	22,3	19,7	18,7	21,3	224
7	20	22	22,3	32,7	27,3	20,3	17,3	17
8		264	19	20	54,7	19,7	18,3	

	1	2	3	4	5	6	7	8
1		880	876	876	874	873	878	
2	878	881	876	876	875	878	873	955
3	876	879	878	880	882	880	435	955
4	865	876	879	878	878	882	879	878
5	G	857	852	501	874	914	848	873
6	836	913	572	478	806	891	928	230
7	593	589	522	853	800	889	896	917
8		560	473	501	B	A	891	



It is evident that there is a strong difference between the two cases where the stimulation was delivered from different positions, keeping exactly the same signal features.

4.2.1.4 FROM 2 TO 4 EXTERNAL GROUNDED ELECTRODES

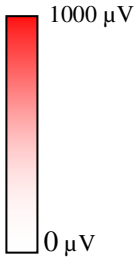
Given that the devices are perfectly symmetric horizontally and vertically, the only change that persists in any experiment with dual compartmental PDMS between the bottom part and the upper part of the MEA are the external grounded electrodes.

The stimulation was moved to a center position in the device to better study what is the behavior of the artifacts around the stimulation.

In order to have a reference, a test was done measuring the artifacts with the original configuration, so with external electrodes at the bottom of the device. The resulting averaged amplitudes of the artifacts are shown in Table 19.

Table 19: amplitude of the artifacts with bipolar stimulation in 65 and 55 with 2 external electrodes in the bottom part [values in µV]; noisy electrode in 56 and 75

	1	2	3	4	5	6	7	8
1		264	243	235	234	248	232	
2	303	296	247	218	158	238	229	238
3	328	313	287	242	229	237	234	236
4	191	295	311	234	239	233	232	235
5	G	248	253	70,3	B	A	645	228
6	559	229	127	67	680	289	186	372
7	170	150	118	137	131	398	196	91
8		221	97,3	69,3	248	381	380	



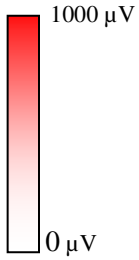
Trials were then done changing the positions of the two external grounded electrodes.

The first experiment refers to a configuration where the two external grounds were put in the 2 reservoirs of compartment A, while in B there was no ground. The opposite experiment was made putting the two grounds in contact with the fluid in compartment B, while in A there was no ground. The results of these experiments are shown in Table 20A and B, respectively.

Table 20: amplitude of the artifacts in dual compartmental MEA with bipolar stimulation from 65 to 55 moving the external grounds in compartment A or B; A_ grounds only in A; B_ grounds only in B [values in µV];

	1	2	3	4	5	6	7	8
1		162	167	167	216	201	202	
2	173	160	169	209	234	211	196	491
3	198	181	176	173	210	210	478	233
4	280	241	235	169	201	236	176	231
5	G	256	248	155	B	A	401	175
6	300	556	171	133	722	338	618	377
7	157	174	158	228	301	458	307	1097
8		157	145	131	667	541	408	

	1	2	3	4	5	6	7	8
1		362	322	308	146	107	118	
2	408	403	329	293	232	127	138	190
3	467	443	246	322	136	137	388	352
4	311	442	453	323	239	353	372	345
5	G	439	459	305	B	A	328	313
6	510	612	356	310	256	198	707	953
7	430	381	373	391	308	374	147	255
8		350	339	334	627	414	278	



The differences can be easily observed in the amplitudes of the artifacts while moving the external grounds. In case of only grounds in compartment A shown in Table 20A, the artifacts are weaker in the same compartment A (i.e. 87 compared to 17), while in case of only grounds in compartment B shown in Table 20B, the artifacts are less in compartment B (i.e. 71 compared to 21). The artifacts are weaker in the compartment where we applied the external grounds.

Sometimes very high amplitudes have been observed in both the compartments going against theory. Due to the intrinsic random nature of these devices, this particular behavior has to be studied separately. The history of the artifacts of electrode 87 during the 5 minutes stimulation of the cases shown above - Table 20A and B - is shown in Figure 44.

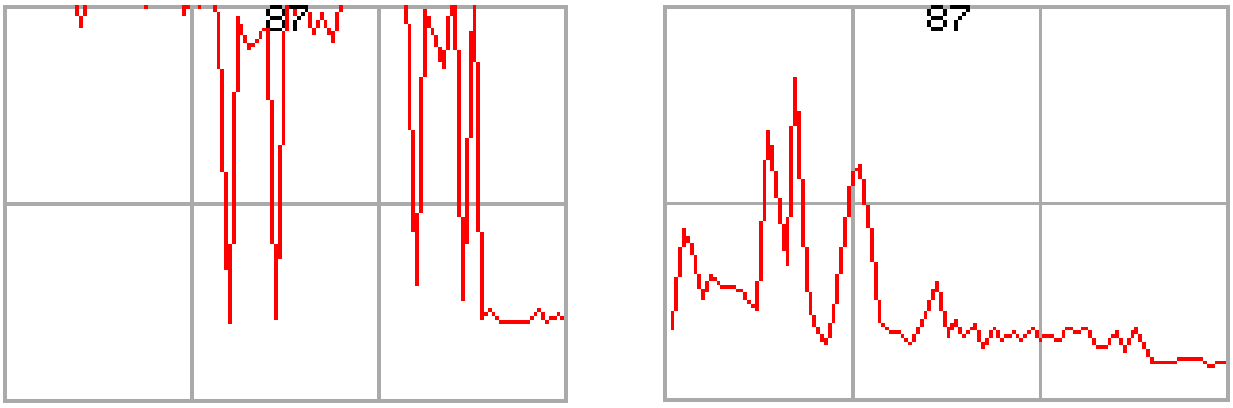


Figure 44: History of the amplitude of the artifacts in electrode 87 for the two experiments shown in Table 20 [scale: x-axis 0-300s, y-axis 0-1000 µV]

The particular electrode 87 doesn't have a linear behavior during the stimulation; that makes it unreliable. It was discovered that sometimes some of the electrodes have a noisy behavior, that was also documented in the several articles published using this technology i.e. (3). Before our recordings the presence of noisy electrodes in the device was always checked, using a threshold based on the majority of the other

electrodes and on previous observations. Because of that, the level of normal noise without stimulation was measured. During this recording no noisy electrode was detected. Results are shown in Table 21.

Table 21: amplitude of the noise without stimulation with neural medium connected to 2 external references [values in µV]

	1	2	3	4	5	6	7	8
1		11	12	13	12,5	12	12,5	
2	16	13	11	16	16,5	12	14	15
3	14,5	15	7	12,5	12,5	12,5	14,5	16,5
4	15	15	13,5	12	12	14,5	15,5	15,5
5	g	15	14,5	12,5	12	14,5	15,5	9,5
6	16	15	11,5	11,5	13	11	15	15
7	15	13	11	14	15,5	11,5	12	13,5
8		11	12	12,5	8	12	12	

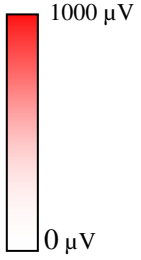
For example in presence of fluid connected to external grounds, the threshold that was empirically established was in the range of amplitudes of 20 µV, according to the measurements reported in Table 21.

Given the experiments done changing the position of the external electrodes in the four reservoirs, it was found that the only way to concentrate the stimulation in a smaller area is to provide grounds. The best way to reach the goal, so to provide the least amplitude of the artifacts in the not-stimulated compartment was to connect 4 external grounded electrodes to the neural medium through the 4 reservoirs. Therefore, a new configuration was needed.

An experiment was tried with the same test stimulation but with 4 external electrodes physically connected to the fluidic environment in the four reservoirs. Results are shown in Table 22.

Table 22: amplitude of the artifacts in dual compartmental MEA with bipolar stimulation from 65 to 55 with 4 external grounded electrodes [values in µV]; noisy electrodes are 75,76,87,86;

	1	2	3	4	5	6	7	8
1		80	62,3	69,3	147	150	154	
2	123	116	68	97,7	144	156	157	242
3	195	166	105	66,7	152	158	242	195
4	145	192	208	68,3	157	196	191	196
5	g	180	188	61	b	a	712	206
6	421	516	94,3	57	504	328	745	518
7	140	114	91	81	72,7	367	304	476
8		90	73,7	47	332	388	357	



Given that spontaneous neuronal activity is in the range of 100 µV, with this configuration the artifacts amplitudes were comparable with that spontaneous activity.

An immediate comparison between the values in Table 22-Table 20A, Table 22-Table 20B and Table 22-Table 19 is shown in Table 23, Table 24 and Table 25.

Table 23: comparison between the values in Table 22 and Table 20A

	1	2	3	4	5	6	7	8
1		-82	-105	-97	-69	-51	-48	
2	-50	-44	-101	-111	-90	-54	-39	-249
3	-3	-15	-71	-107	-58	-52	-236	-37
4	-135	-49	-27	-101	-44	-40	15	-35
5	g	-76	-61	-94	b	a	311	31
6	122	-40	-77	-76	-218	-10	127	141
7	-17	-60	-67	-147	-229	-91	-2	-621
8		-67	-71	-84	-335	-153	-51	

Table 24: comparison between the values in Table 22 and Table 20B

	1	2	3	4	5	6	7	8
1		-282	-260	-238	1	43	36	
2	-284	-287	-261	-195	-88	29	19	52
3	-271	-278	-141	-255	17	21	-146	-156
4	-166	-250	-245	-255	-82	-157	-181	-149
5	g	-259	-272	-244	b	a	384	-107
6	-89	-96	-262	-253	248	130	39	-435
7	-290	-266	-282	-310	-235	-7	157	221
8		-260	-266	-287	-294	-26	79	

Table 25: comparison between the values in Table 22 and Table 19

	1	2	3	4	5	6	7	8
1		-184	-181	-166	-87	-98	-77	
2	-179	-180	-179	-121	-14	-82	-72	4
3	-133	-148	-182	-175	-77	-79	8	-41
4	-46	-103	-104	-166	-82	-37	-41	-39
5	g	-68	-65	-9	b	a	67	-22
6	-138	287	-33	-10	-176	39	559	146
7	-30	-36	-27	-56	-58	-31	108	385
8		-131	-24	-23	84	7	-24	

In all the experiments it is shown that the one that has the best configuration and the least stimulation artifacts for the compartment A (not-stimulated) is the experiment with the 4 external electrodes (Table 22). Therefore we decided to create the *neurolid4* (explained in paragraph 3.2.2), which provides 4 grounded electrodes through the 4 reservoirs.

To make a direct comparison some experiments have been done between the old configuration (unipolar stimulation) and the new one (bipolar stimulation). Those experiments are explained in detail in paragraph 4.2.3.1 .

4.2.1.5 POST STIMULATION BLANKING TIME

In order for the artifacts to completely disappear, some more experiments have been tried testing the effect of the post stimulation blanking time, that is the time between the recording is stopped because the stimulation is provided, and the moment in which the recording starts. By default the post stimulation blanking time is set to 100 µs. It is normally implemented in stimulating systems as a safe measure to keep the recording system in healthy state.

The system used can rise the post-stimulus blanking time up to 4000 µs or more. Table 26 shows the result of the experiment done with a post-stimulus blanking time of 1000 µs that evidently decreases the amplitude of the artifacts.

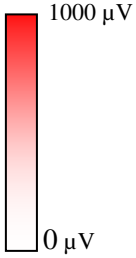
Table 26: amplitude of the artifacts with bipolar stimulation in 65 and 55 with 4 external grounded electrodes and a post-stimulus blanking time of 1000µs [values in µV];

	1	2	3	4	5	6	7	8
1		81	52,5	44,5	29	52,5	27	
2	119	115	60	26	24	30	28	29,5
3	148	142	100	46	28,5	26	30,5	29
4	358	94	124	46	29,5	30,5	17,5	26,5
5	g	75	111	19	b	a	562	61,5
6	466	121	49	19,5	354	31,5	41,5	56,5
7	91	68	52	18	50	32	33	40,5
8		232	35	16,5	14	47,5	32	

Increasing the post-stimulus blanking time up to 4000 µs, amplitudes of the artifacts under the noise threshold of the device were recorded. Results are shown in Table 27.

Table 27: amplitude of the artifacts with bipolar stimulation in 65 and 55 with 4 external electrodes and a post-stimulus blanking time of 4000µs [values in µV];

	1	2	3	4	5	6	7	8
1		15	12,5	14,5	15	13,5	16	
2	17	15	13	17	18	16	14,5	16
3	16	16	3	13,5	16,5	14,5	16	17
4	16,5	16	15	13,5	17	15	18	b
5	g	16	15	15	13,5	15,5	174	a
6	17	17	14,5	13,5	14,5	12,5	16	17
7	15,5	13	13,5	15	16	13,5	13,5	15
8		13	13,5	15	3,5	14	14,5	



Making a difference in the values of the amplitudes between this latter configuration (Table 27) and the normal level of noise (Table 21), the two tables are now comparable.

Table 28: comparison between Table 27 and Table 21

	1	2	3	4	5	6	7	8	
1			4	1	2	3	2	4	
2		1	2	2	1	2	4	1	1
3		2	1	1	1	4	2	2	1
4		2	1	2	2	5	1	3	b
5	g		1	1	3	2	1	158	a
6		1	2	3	2	2	2	1	2
7		1	1	3	1	1	2	2	2
8			2	2	3	2	2	3	

The only relevant difference between the two experiments is only in the electrode 75. In the other electrodes there is a maximum of 5 µV of difference in the values of the amplitudes. By the value of the two tables, the artifacts are under the level of the noise, so they can be considered to be disappeared during the stimulation. In Figure 45 a snapshot of the recording during the stimulation in the latter configuration is shown.

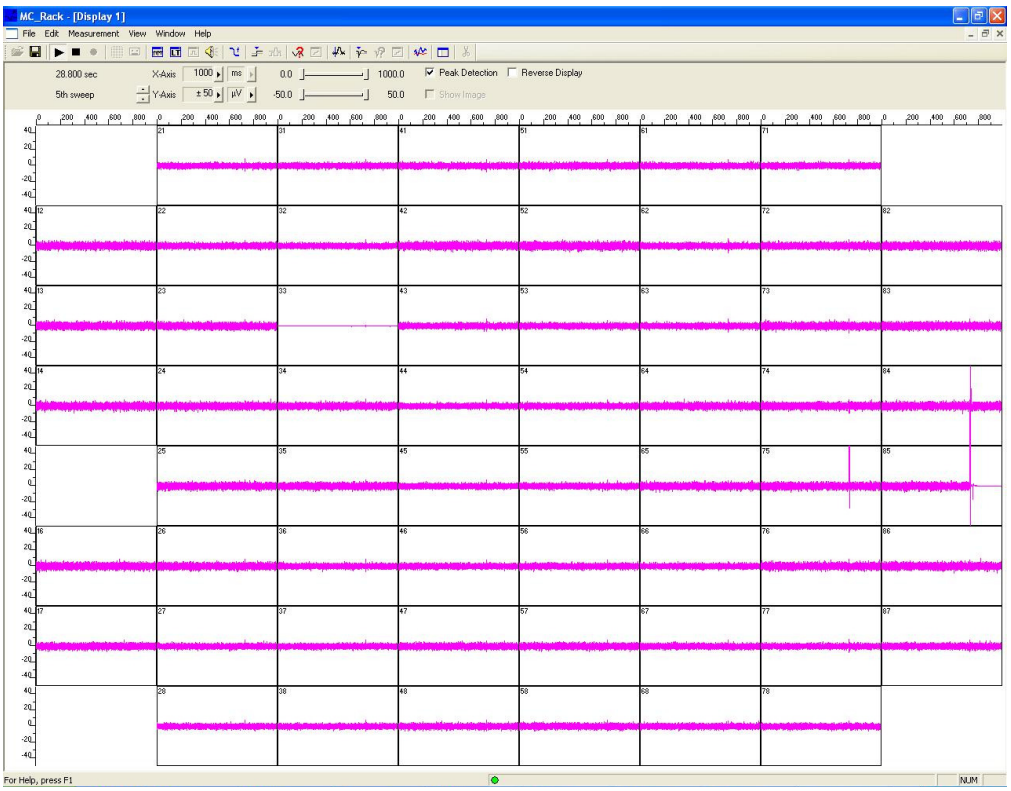


Figure 45: snapshot of the recording during stimulation in 84 to 85 [scale: x-axis 100ms,y-axis 50µV]

The artifact effect is due to the electrical stimulation given. The removal of this noisy effect provides clear recordings.

After found the best configuration to stimulate the neural cells, it was applied to the cultures.

4.2.2 EFFECTS OF THE TEST STIMULATION ON DIFFERENT CELLS

A study it is possible on the application of the documented electrical test stimulation to both cortical and sub-cortical / thalamic cells. To analyze the effects of an electrical stimulation on both cells, the PSTH graph was used. The PSTH algorithm explained in paragraph 3.5.2, is able to analyze the evoked response of the neuronal cells to an electrical stimulation at a low frequency.

4.2.2.1 RESPONSE FROM CORTICAL CELLS

The effect on cortical cells has been consistently documented in several articles in literature i.e. (3). A typical response of these cells is shown in figure Figure 46, where a unipolar stimulation at the center of the device is given.

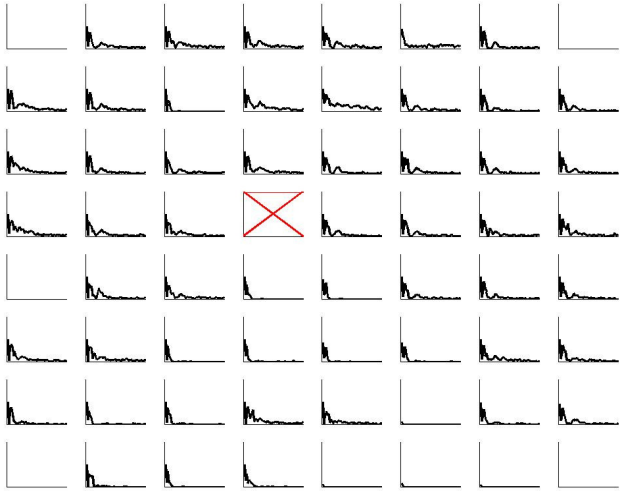


Figure 46: PSTH analysis of Unipolar electrical stimulation on a device on DIV 24 (Device #: 41_12549_24: Unipolar stimulation of electrode 44 (e44) (neurolid2))

As shown in Figure 46, electrode 44 (represented by 'X' in the figure) in compartment A was stimulated with a unipolar test stimulus of amplitude 760mV. Evidently, a response from the majority of the electrodes is received. Most of the experiments done with electrical stimulations are based on this response. The stimulation artifacts spread across both the compartments mainly due to the current spreading across the ionic culture medium.

4.2.2.2 SUB-CORTICAL / THALAMIC CELLS' RESPONSE

A trial with the same electrical test stimulation on sub-cortical / thalamic cells was done. In literature there are no articles that apply a similar stimulation on dissociated cultures of this kind of cells.Some experiments were therefore conducted with the aim at checking whether the test stimulus used for the cortical cells was affecting the sub-cortical / thalamic cells.

One of the active electrodes in compartment B containing sub-cortical / thalamic cells was electrically stimulated. The PSTH graph in Figure 47 shows the stimulation of the most active electrode in the compartment B of the culture.

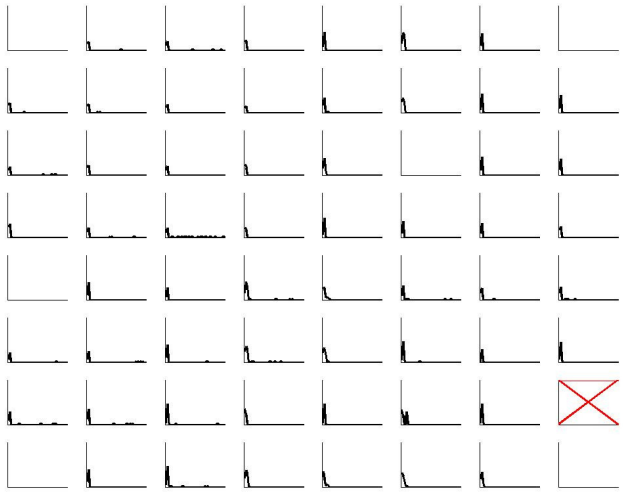


Figure 47: PSTH analysis of Unipolar electrical stimulation on a Cx-Th device on DIV 23 (Device #: 42_315_23: Unipolar stimulation of electrode 87 (e87) (neurolid2))

Any evoked response is elicited. Unfortunately after this stimulation the electrodes in compartment B showed a decrease of the activity passing from 0.86 to 0.34 spikes per second, where the stimulated electrode 87 reduced its activity from 11 spikes/sec to less than 4 spikes/sec. The two charts reported in Figure 48 and Figure 49 show the average of the activity before and after the stimulation.

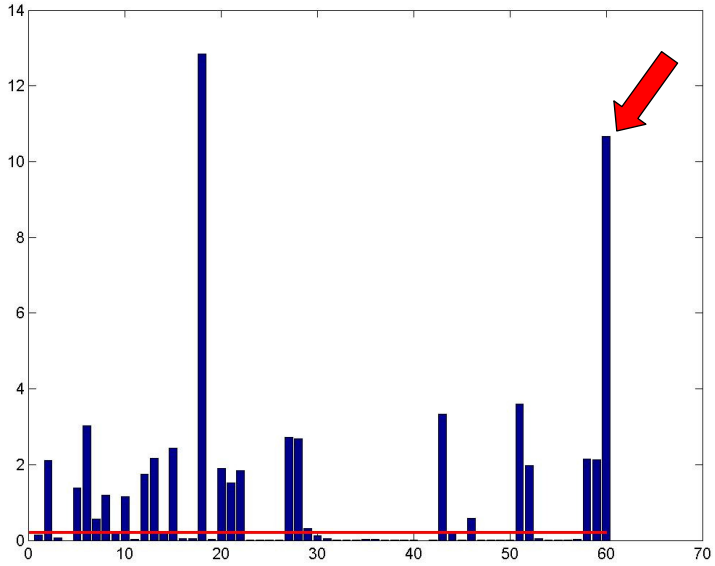


Figure 48: graph of the AFR of the device before stimulation (Device #: 42_315_23: SA before stimulation)

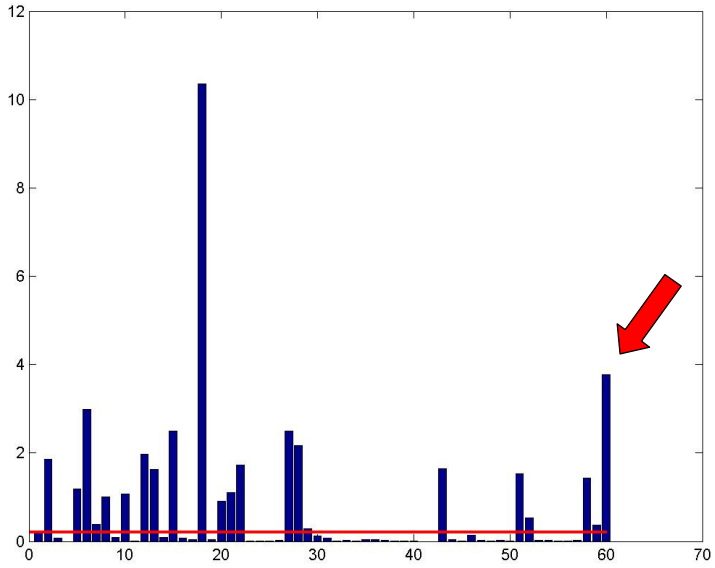


Figure 49: graph of the AFR of the device after stimulation (Device #: 42_315_23: SA after stimulation); we notice a strong decrease in the AFR in the stimulated electrode 87

The same experiment was done to prove again the effect of the same electrical stimulation, in this case with a bipolar configuration. The PSTH graph is shown in Figure 50.

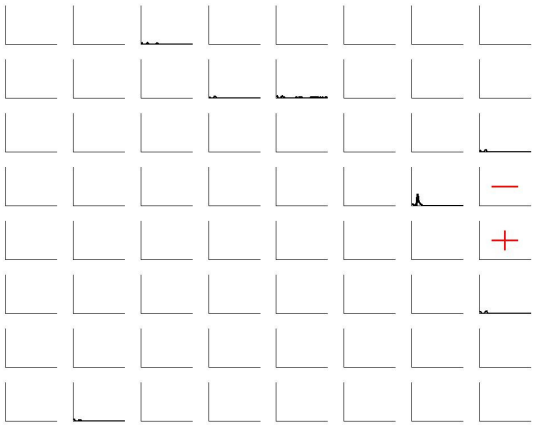


Figure 50: PSTH analysis of Bipolar electrical stimulation on a Cx-Th device on DIV 24 (Device #: 45_12554_24: Bipolar stimulation from electrode 85 to 84 (e87To84) (neurolid4))

Once again the stimulation doesn't elicit any response. More important than the PSTH is the graph of the average activity before and after the stimulation shown in Figure 51 and Figure 52. The activity of the compartment B completely disappears. It means the stimulation provided is not the appropriate one.

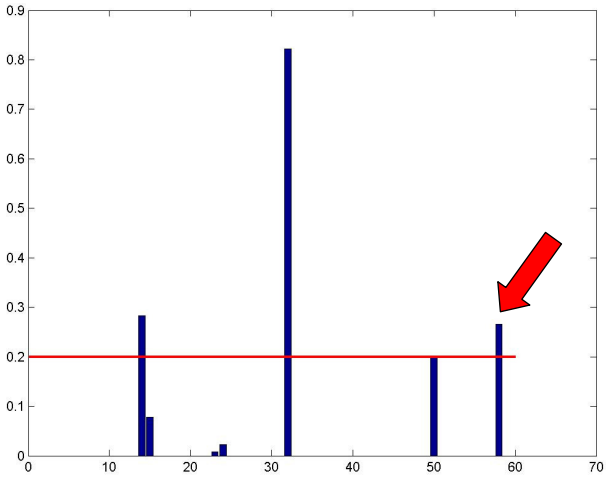


Figure 51: graph of the AFR of the device before stimulation (Device #: 45_12554_24: SA before stimulation)

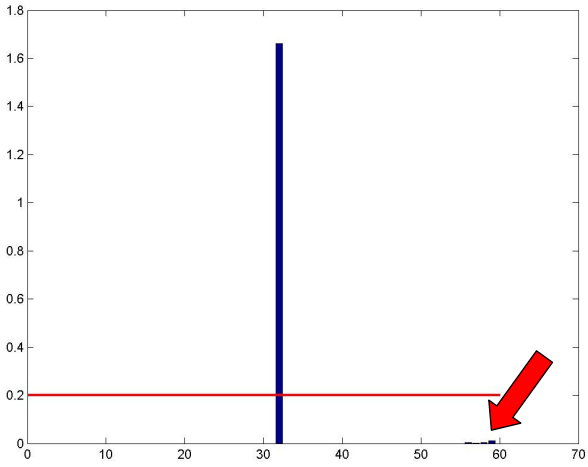


Figure 52: graph of the AFR of the device after stimulation (Device #: 45_12554_24: SA after stimulation)

By these two experiments it can be concluded that the test stimuli applied to cortical cells obtaining a response, cannot be applied to sub-cortical / thalamic cells.

In cortico-thalamic devices an electrical stimulation focused only on cortical cells can be very useful to excite only the cortical compartment and not to damage the sub-cortical / thalamic cells in the other compartment.

4.2.3 UNIPOLAR VS BIPOLAR STIMULATION

4.2.3.1 DIRECT COMPARISON

A typical unipolar test stimulation given at one electrode within the MEA array is able to evoke a response in cortical cells. However, a unipolar stimulation evokes responses in both the compartments of a device, even if a physical barrier is interposed between the two. As observed in Figure 53, the stimulation artifacts spread across both the compartments mainly due to the current spreading across the ionic culture medium.

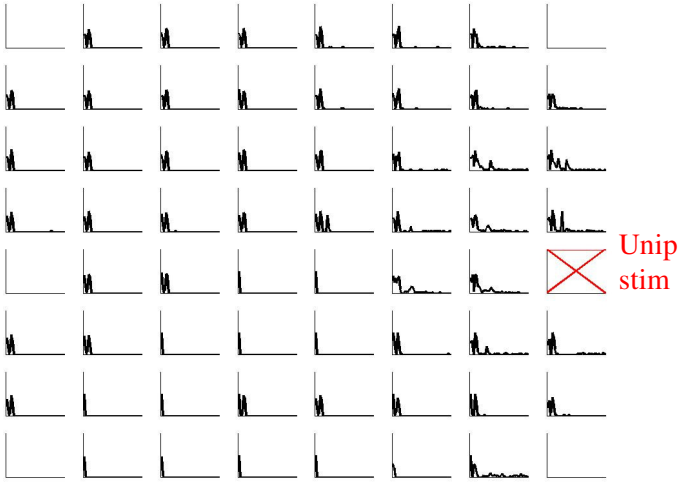


Figure 53: PSTH analysis of Unipolar electrical stimulation on a device on DIV 25 (Device #: 46_14589_25: Unipolar stimulation of electrode 85 (e85) (neurolid2))

In Figure 53 a direct stimulation of the cells of the two compartments is observed. A direct stimulation happens when the electrical current is propagated by the ionic medium where the cells are maintained and evokes direct electrically-evoked action potentials (dAPs) in the neurites and in the neural bodies. They are responses that do not depend on glutamatergic synapses. Opposite to dAPs, responses that do depend on glutamatergic synapses are synaptically-evoked action potentials (sAPs) can be evoked when a stimulated neuron sends its action potential through its neurites and make the following neuron fires.

As documented in (3) and (9), dAPs have been observed to have the following features:

- They are up to the immediate 20 ms after the stimulation
- Occurrence of more than 80% (very reliable)
- They have a low jitter (between 160 µs and 250 µs)
- Consistency in wave-form

While it's easy to watch dAPs, sAPs have been observed to have:

- Occurrences between 5 and 20 ms
- Temporal precision that varies around 2 ms
- Reliability up to 30 %, while common values are 10%
- A jitter larger than 1 ms

Further than dAPs and sAPs, a so called delayed response at about 50-100ms after the stimulus has been documented in (7).

Keeping the goal of having a much more focused stimulation in a way that only the cells in one of the two compartments are directly stimulated by the electrical stimulation provided, a passage from the original unipolar stimulation to a bipolar stimulation of the same amplitude and frequency was done, that in literature has been showed to have a more focused effect (54).

Thus, several experiments have been providing unipolar stimulation followed by a bipolar stimulation to show the difference in the observation of network response. In Figure 54A and Figure 54B the PSTH response of unipolar and bipolar stimulation of a cortical-cortical culture in both the compartments is shown.

The next two PSTH graphs are related to a cortico-cortico device at DIV 25 with 23 active electrodes at the 1st recording.

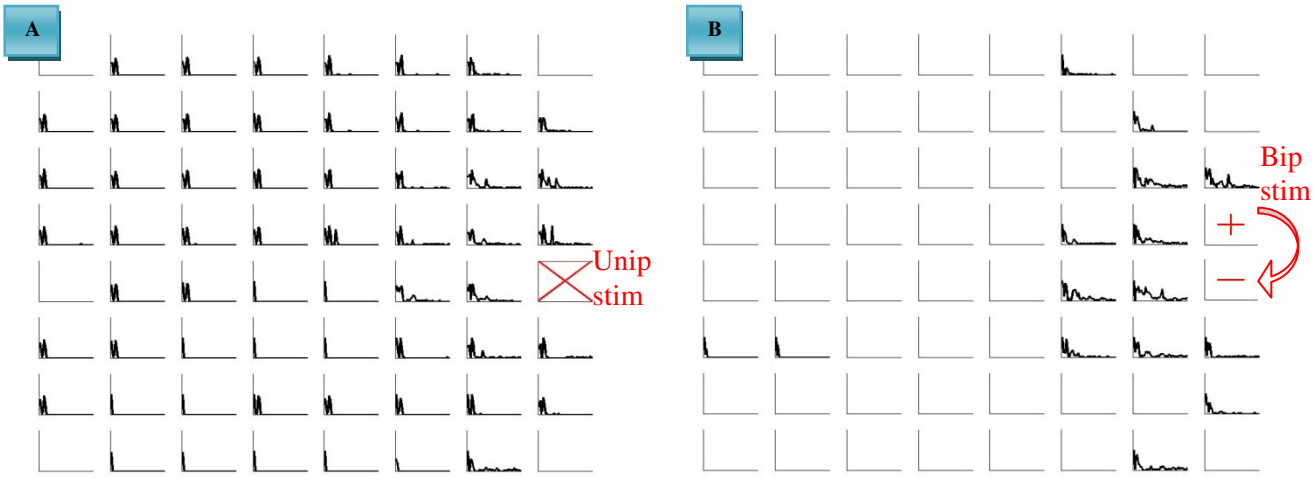


Figure 54: Observation of Unipolar and Bipolar electrical stimulation on a device on DIV 25 (Device #: 46_14589_25), [X-axis:bin1ms, window 50ms: Y-axis:2]; A: Unipolar stimulation of electrode (e85) with neurolid2; B: Bipolar stimulation between electrodes 84 (e84) and electrode 85 (e85) with neurolid4

The experiment shows that the bipolar stimulation is very focused around the stimulation site where the electrical field spreads within a very close region around the stimulating electrodes.

Given the results of the bipolar stimulation, an evident proof of its effect was needed. The TTX compound, described in paragraph 3.4.2, was used to further prove the localized effect of the bipolar stimulation.

4.2.3.2 TTX & ELECTRICAL STIMULATION

Since TTX blocks all sodium (Na+) channels on neurons, we wanted to utilize it to study the influence of stimulation on the adjacent compartment. Given the reliable chemical isolation between the two compartments (see paragraph 2.3.2), TTX mixed with neural medium was injected in one compartment and the same compartment was provided with electrical stimulation.

This experiment was made to check the effect on the compartment that was not chemically altered. If stimulation is provided in compartment B and the cells in compartment A respond, it means the electrical stimulation spreads by the medium through the small channels of the PDMS.

The two PSTH graphs [X-axis:bin1ms, window50ms: Y-axis:2] in Figure 55 are related to a Cortico-Cortico culture on device at DIV 22 with 55 active electrodes at the 1st recording.

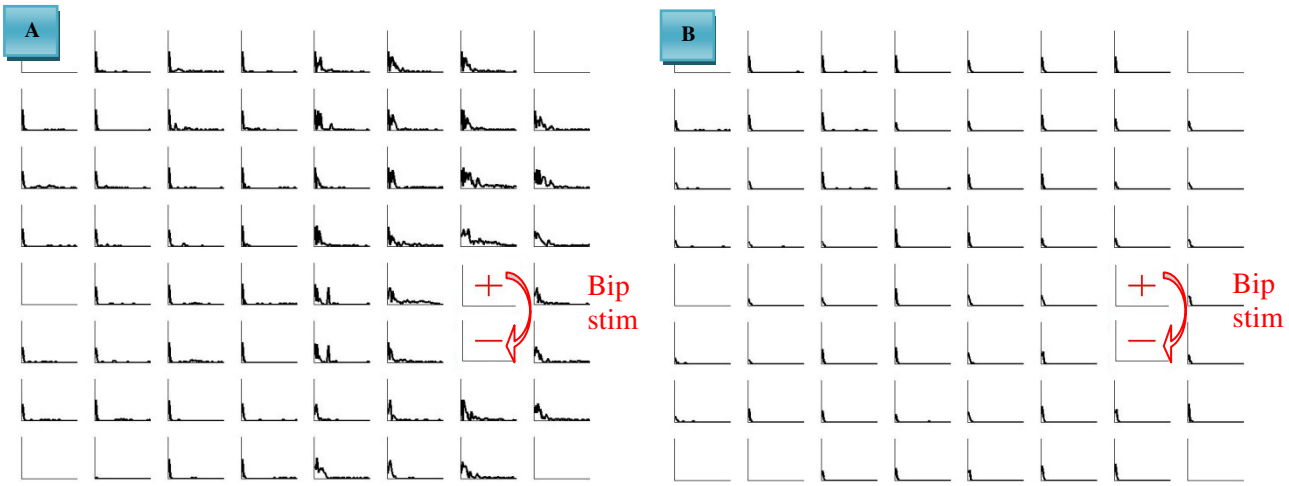


Figure 55: PSTH analysis of bipolar electrical stimulation on a device on DIV 22 (Device #: 46_14589_22). Bipolar stimulation was applied across the electrodes 75 (e75) and 76 (e76) with 4 External Grounded Electrodes; A: PSTH analysis of bipolar stimulation without TTX in any compartment; B: PSTH analysis of bipolar stimulation with TTX in compartment B

As can be seen in Figure 55A, response to stimulation was observed in compartment B which disappear on addition of TTX in the same compartment.

This further confirms that the bipolar stimulation affects only one of the compartments.

4.2.3.3 RESPONSIVENESS OF THE NON-ALTERED COMPARTMENT

To further confirm that both the compartments are electrically active and the observations in Figure 55A and Figure 55B are not due to complete electrical inactivity in compartment A, we performed experiments to show the electrical responsiveness of compartment A and compartment B separately.

As shown in Figure 56 and Figure 57A, stimulation of compartment A and compartment B were done under similar circumstances and the PSTH response shows the electrical activity in both the compartments. Figure 56 and Figure 57 show PSTH graphs [X-axis:bin1ms, window50ms: Y-axis:2] related to a cortico-cortico device at DIV 24 with 36 active electrodes at the 1st recording.

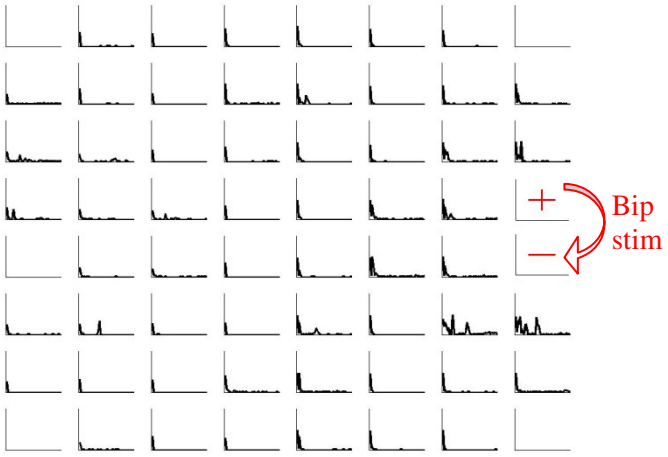


Figure 56: PSTH analysis of Bipolar electrical stimulation in compartment B (Device #: 46_I1377_24); Stimulation was applied across electrode 84 (e84) and electrode 85 (e85) with 4 external electrodes.

Figure 56 shows that compartment B is responsive to electrical stimulation. Similarly

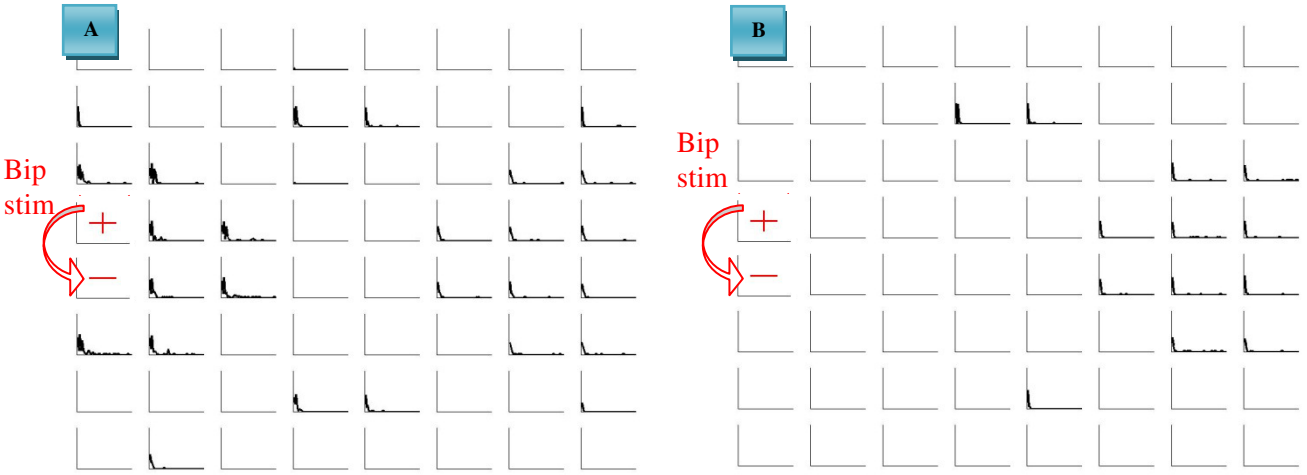


Figure 57: PSTH analysis of Bipolar electrical stimulation in compartment A (Device #: 46_I1377_24); Stimulation was applied across electrode 14 (e14) and 15 (e15) with 4 external electrodes; A: Stimulation without TTX in any compartment; B: stimulation with TTX in compartment A

As can be observed the cells were electrically active in both the compartments and addition of TTX to the compartment which is stimulated electrically confirms that the electrical stimulation is focused and doesn't spread to the adjacent compartment.

Further to reiterate that bipolar stimulation is focused within the region of origin when compared to the unipolar stimulation, the following four PSTH analyses are presented. The PSTH analysis as in Figure 58 and Figure 59 [X-axis:bin1ms, window50ms: Y-axis:2] are related to a cortico-cortico culture in device at DIV 24 with 40 and 41 active electrodes at the 1st recording. They show first a TTX experiment on the same device, then a unipolar stimulation after some days (3 days in this case, in order to avoid stressing the cells due to continuous recording) that represents the responsiveness of the whole device. On the same day after the unipolar stimulation, we repeated a bipolar stimulation that once again confirms the focused nature of stimulation.

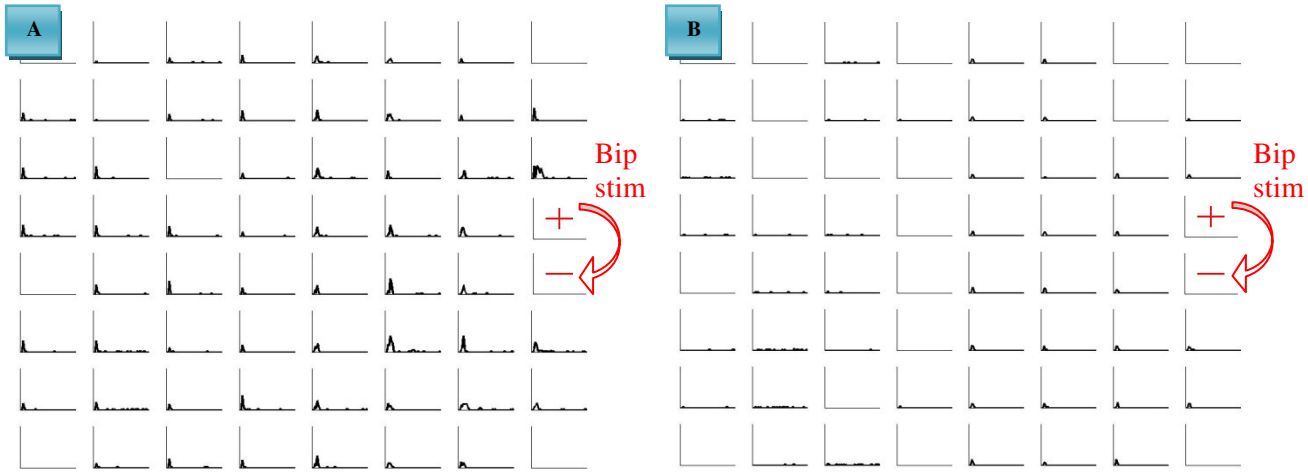


Figure 58: Influence of TTX on network activity. PSTH analysis of Bipolar electrical stimulation of electrode 84 (e84) and 85 (e85) in compartment B on DIV 22 (Device #: 46_12550_DIV22) with 4 external reference electrodes and after removing the artifacts from the recordings; Figure 6A: Without TTX in any of the compartment; Figure 6B with TTX in compartment B

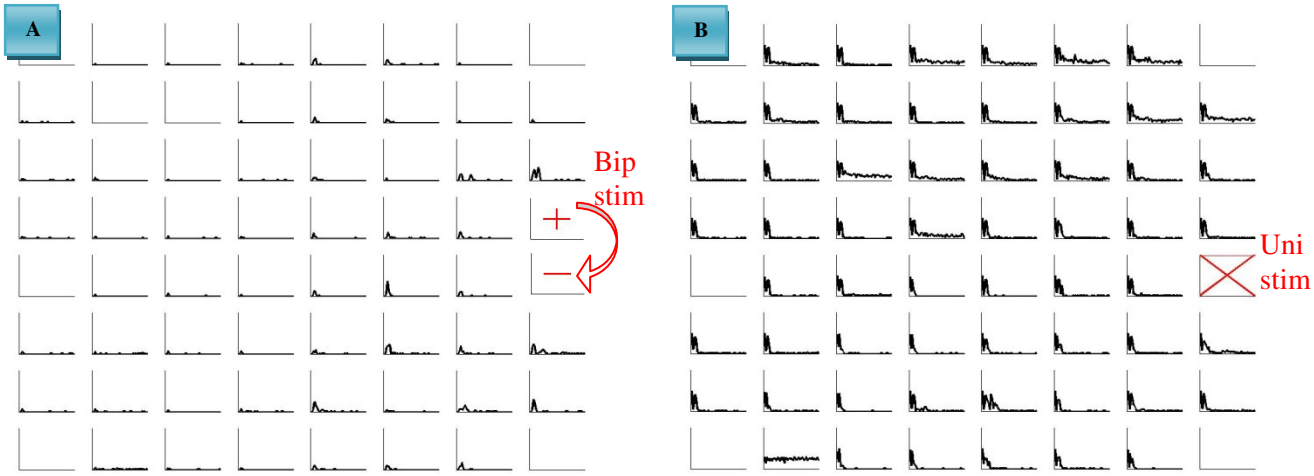


Figure 59: PSTH analysis to show confirm focused bipolar electrical stimulation (Device #: 46_12550_DIV25) with 4 external reference electrodes and after removing artifacts from the recordings; A: Bipolar stimulation of electrode 84 (e84) and electrode 85 (e85) in compartment B; B: Unipolar stimulation of electrode (e85) in compartment B

As shown from the analysis, after the TTX experiments with no response in compartment A in Figure 58, there is a complete response from the whole device in Figure 59B that shows the cells were responsive. Indeed, bipolar stimulation evokes only local response within a small region around its origin in the same compartment.

5 RESULTS AND DISCUSSION

5.1 APPLICATION OF CHEMICALS

5.1.1 INTER-COMPARTMENTAL CHEMICAL ISOLATION

An important study made in this project regards the chemical isolation between the two compartments in the PDMS device. Thanks to the 50 small micro-channels of 10µm height, 3µm width each, a small movement of substances between the two chambers is supposed.

A study in two directions has been done focusing on the presence of clearly visible substances on spectrophotometer graphs and on the change of activity in the cells when exposed to altering chemical compounds.

To detect the presence of substances, spectrophotometer analyses have been done. A sample empty PDMS device has been placed in a MEA and two different substances have been inserted in the two chambers. Those two compounds such as De-ionized (DI) water having no peak in the spectrophotometer graph, and other substances, such as neural basal medium or a food colorant having different peaks in the same graph, have been used to investigate the chemical isolation. As reported in paragraph 4.1.1, after leaving the two different substances one in every chamber for a certain time longer than the normal 5 minutes recording time, a spectrometer graph has been shown to demonstrate the non presence of neural basal medium or of food colorant in DI water. This has been showed in different experiments, changing the substance, extending the time up to 24 hours and with different devices. Moreover, an experiment with a leaking device proved that in case of damage of the PDMS the effect on DI water is visible. Hence, we could safely add a solution to one compartment and be sure that it wouldn't spread to the adjacent compartment during the course of the experiment.

Further, inhibition substances such as TTX or Synaptic Blocker have been applied in only one compartment in MEAs containing cells. No significant difference in the spontaneous activity of the cells in the non-altered compartment between the pure spontaneous activity and during the application of the chemical compounds has been observed. As a support for these experiments an article was also published (1).

These preliminary results allowed us the use of chemical compounds in the dual compartmental PDMS, with a certain confidence to alter the activity of only one compartment. The same chemical isolation was also used to support the other concepts within the project.

5.1.2 ACETYLCHOLINE

Due to the results showed in paragraph 4.1.2, some considerations have to be done regarding experiments with acetylcholine.

First, literature regarding the application of acetylcholine on neuronal cells is quite old and not updated. Moreover, none of the articles found refers to dissociated rats thalamic and cortical cells.

Based on our experiments, no clear conclusions can be drawn concerning the application of acetylcholine to cortical and sub-cortical / thalamic rat dissociated cells in culture. The results are inconclusive and it is very difficult to detect a common effect.

Therefore a deeper study on the area is recommended. Conclusions can be consolidated by elaborating a curve dose-response that was out of our goals.

5.1.3 SYNAPTIC BLOCKER EFFECT

Based on the results showed in paragraph 4.1.3, some interesting conclusions can be drawn.

In the experiments done, it is shown that the Synaptic Blocker compound doesn't have effect on sub-cortical / thalamic cells. No differences in the spiking activity of the same cells before and during the exposure have been noticed.

During the application of the Synaptic Blocker to cortical cells, a clear decrease of the spontaneous activity of the cells in the exposed compartment is shown. This fact was also used for the analysis of the inter-compartmental chemical isolation. However, some of the cells directly exposed to the chemical solution retained their typical spiking activity.

Analyzing the Average Firing Rate (AFR) in one of the devices exposed to Synaptic Blocker, interesting different changes in the activity have been noticed. A comparison between the AFR before and during the application of Synaptic Blocker is shown in Figure 60. It is possible to observe that in compartment B electrode 83 keeps its spiking activity even if exposed to the synaptic blocker (probably genuine spiking activity of single cells) and in compartment A an increase of activity for electrodes 12 and 13 is recorded, while at the opposite a strong decrease affects electrodes 35 and 42.

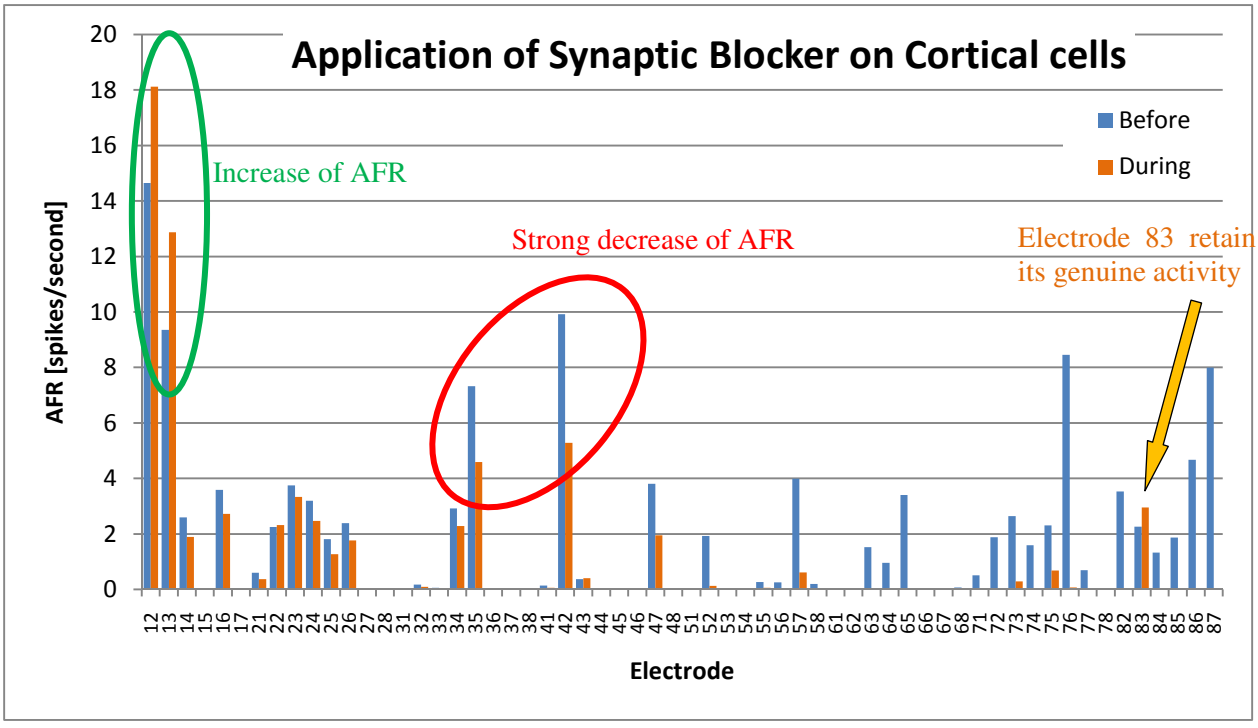


Figure 60: Comparison between the AFR before and during the application of Synaptic Blocker (device # 46_I1377_DIV24)

While the loss of activity in the electrodes nearby the micro-channels (column 4 and column 3) can be explained due either to a good connection with the compartment B or to a small leakage of the synaptic blocker compound, the increase of activity of electrodes 12 and 13 may probably be related to inhibitory synaptic connections with neurons in compartment B. In this case, when the communication is altered chemically, the cells are not anymore repressed.

Interesting studies in this direction can be further done on the influence of few active neurons on the network spiking and busting.

5.2 ELECTRICAL STIMULATION

5.2.1 PROPAGATION EXPERIMENTS

Electrical current propagation experiments have been useful to reach a better stimulation of the neurons in a MEA.

Several stages before reaching an optimal configuration have been reached.

Applying an electrical impulse to a empty dry MEA, it was proved that a stimulation provided by an electrode is recorded in the neighbor electrodes (Table 3 in paragraph 4.2.1) probably because of a small distance between them (200 μm). The same experiment proved that there is not any electrical issue regarding the recording device, as the electrodes physically far from the stimulation site are not affected by the stimulation.

Inspired by the work of S. Joucla in 2009 (54) we tried to recreate a grounded surface configuration connecting logically electrodes to ground nearby the stimulation site. A little peculiarity was observed while experimenting the increase of the number of lines of grounded electrodes nearby the stimulated one: a configuration with grounds on the first line of nearest electrodes to the stimulation has created a situation worse than no grounded electrodes. However, a second line of grounded electrodes got better results. The best case was observed to be the one with the whole compartment B and the first line beyond the channels (column 4 when stimulating compartment B) connected to ground. Putting grounds only near the channels (column 4 and 5) don't make a relevant difference in the amplitude of the artifacts between the two compartments.

We can conclude that the presence of grounded electrodes around the stimulation site averagely contributes in avoiding the spreading of current, demonstrated by the amplitude of the artifacts immediately after the stimulation. However, given that MEA arrays provide only 60 recording electrodes, 30 for every compartment, it's not advisable to put any of those electrodes connected to ground. Moreover, due to the strange results obtained while increasing the number of grounded lines nearby the stimulation site, we decided to use no grounded electrodes.

Once again inspired by the work of S. Joucla in 2009 (54), to improve the focus of the electrical stimulation a study on bipolar design was done.

Using a bipolar configuration compared to an unipolar one, a strong decrease of the amplitudes of the artifacts was immediately observed.

Moreover, doubts on the appropriate location of the external grounds have been raised, due to an observation of the concentration of higher artifact amplitudes in the upper part of the array. Those two external electrodes are useful in the dual-compartmented MEA, as disconnecting them from the device, the noise level and the artifacts are bigger, as reported in Table 12 in paragraph 4.2.1. The position of the external grounded electrodes was demonstrated to be relevant in the spread of the current. Stimulating the electrodes in the upper part of the device the artifacts were lower, while in every other experiment given with bipolar stimulation, higher amplitudes of the artifacts were highlighted in the region that was missing of external grounds.

Then, the old configuration with neurolid2 (two external grounds) was enhanced creating a neurolid4 with 4 external electrodes, that is able to reduce the noise level and that brought to a very low level of the artifacts amplitude.

Regarding the blanking time after stimulation, generally speaking it has been observed that the artifacts disappear easily with a longer blanking time post-stimulus. However, longer the post-time blanking, more data are lost. Considering that an early response is investigated and that the blanking time doesn't eliminate the spread of the current in the device, it was decided not to be used for further experiments. Theoretically even if artifacts are recorded everywhere in the device, not all them are able to stimulate the neurons. In fact only with the right amplitude and current the stimulation is effective as shown in (3). Other implemented methods are available to eliminate the noisy effect, i.e. the SALPA algorithm mentioned in (61).

All these experiments were made to reach an optimal configuration for electrical stimulation of the cells. Thus the new configuration was applied to the cells cultures.

5.2.2 UNIPOLAR-BIPOLAR STIMULATION

Previous studies showed that an unipolar stimulation applied to a cells culture on a dual compartmental PDMS, evoked a neural response in the two chambers. That was showed by a PSTH graph of an electrical stimulation in Cortical-Cortical device in a unipolar configuration. The stimulation artifacts are believed to spread across the compartments mainly due to the ionic culture medium.

A bipolar stimulation was observed to have a more focused effect when analyzing the amplitudes of the artifacts. Several experiments have been done providing unipolar stimulation followed by a bipolar stimulation or viceversa to observe the difference in the network response. The experiments show that the bipolar stimulation is very focused within a very close region around the stimulating electrodes. Together with bipolar stimulation, neurolids4 have been used.

Given the preliminary results obtained by the bipolar stimulation, a control of its localized effect was due. Since TTX compound blocks all sodium (Na+) channels on neurons, we wanted to utilize it to study the influence of stimulation on the adjacent compartment. Therefore TTX mixed with neural medium was injected in one compartment and the same compartment was stimulated with electrical stimulation. That was made to study the effect of a bipolar electrical stimulation on the compartment not chemically modified, to further prove that the stimulation provided didn't spread in a large area. Response to stimulation was observed to be evoked only in the same compartment which disappears on addition of TTX in the same compartment. This further confirms that the bipolar stimulation affects only one of the compartments, as explained in paragraph 4.2.3.2.

However, further to confirm that both the compartments were electrically active and responsive in the moment of the electrical stimulation so that the observations done are not due to complete electrical inactivity, experiments were performed to show the neuronal response of compartment A and compartment B in the same day. Compartment A and compartment B were stimulated under similar circumstances and the PSTH graphs show that there is an active response in both the compartments, as explained in paragraph 4.2.3.3.

Moreover, a unipolar stimulation was applied to the same culture to show the responsiveness of the whole device after some days (3 or days later to avoid stressing of the cells due to continuous recording). That was shown to have a response in the whole device.

Thus we conclude that the effect of a bipolar stimulation is very focused in a little area compared to the effect of unipolar stimulation.

6 CONCLUSIONS AND FUTURE WORK

My work was related to the study of the electrical and chemical stimulation of rat neuronal cells.

Since the chemical isolation between the compartments has been studied to be reliable, chemical neuromodulation of cells within a compartment is very promising. This kind of stimulation would affect cells within only one compartment and thus can be used reliably.

The influence of TTX and "Synaptic Blocker" on different populations of cells can be investigated using the technology demonstrated in this work. An investigation on the behavior of a population of cells in one compartment while the activity of the other communicating population is silenced would enhance those studies. Although within this project we have searched and tried a chemical stimulator that would excite populations of neuronal cells, further studies have still to be carried out.

We therefore used electrical stimulation. After the studies conducted on the propagation of the stimulation current, the system was improved with a bipolar configuration and creating the neurolid4. The new bipolar configuration was observed to be much more focused than the traditional unipolar stimulation.

In fact it was observed to affect only the area next to the stimulation, preventing not-desirable electrical application to other cells. As showed, a pattern of stimulation that has been well studied with cortical neurons cannot be applied to sub-cortical / thalamic ones without the risk of irreparably damaging the cells. Indeed, different types of cells require different stimulations. Studying the effect on a population of neurons while stimulating another population would be very useful to discover the intricate relations between families of neurons.

The final goal is in fact the harvesting and culturing of different families of neurons in compartment to recreate equivalent brain network pathways and to apply electrical stimulations to individual regions.

Furthermore, studies regarding the treatment and neuromodulation of thalamic neuronal cells are outdated and further investigations on this subject are necessary to better understand the spontaneous behavior and the effects of chemical / electrical stimulations on this cells type. Though we have been trying to harvest sub-cortical / thalamic cells, a deeper observation of these cell types is advisable. Characterization of spontaneous activity of thalamic cells would create a good basis for the analysis. To proceed further, a study on the proper electrical stimulation of thalamic cells such as tetanic stimulation would enhance the studies related to DBS. Furthermore, creating a bridge between cortical cells and pure thalamic cells would give interesting results to the whole research.

On this track, the idea of a multi-compartmented device simultaneously with the electrical stimulation we have studied would be an interesting base for all the studies related to DBS. It would be possible to reproduce the main part of the neuronal loop involved in Parkinson's disease by connecting populations of neurons from the cortical, thalamus, striatum and the internal segment of the globus pallidus as described earlier.

Development of such multicompartment devices to accommodate several neuronal sub-populations in culture for long-term studies is underway at the DBS research group and it is believed to be a promising tool for developing a comprehensive in-vitro neuronal network model.

BIBLIOGRAPHY

1. *Dual compartment neurofluidic system for electrophysiological measurements in physically isolated neuronal cell cultures.* **Kanagasabapathi, TT, et al.** 2009, Conf Proc IEEE Eng Med Biol Soc., p. 1655-8.

2. *Towards in-vitro neuronal network models: In-vitro dual compartment neurofluidic system and functional connectivity in physically isolated neuronal cell culture.* **Kanagasabapathi, T. e Ciliberti, D., Decré M.** 2010. 7th Int. Meeting on Substrate-Integrated Microelectrodes.

3. *Effective parameters for stimulation of dissociated cultures using multi-electrode arrays.* **Wagenaar, D. A., Pine, J. e Potter, S. M.** 2004, Journal of Neuroscience Methods, Vol. 138, p. 27-37.

4. *Deep brain stimulation.* **Breit, S, Schulz, JB e Benabid, AL.** 1, 2004, Cell and tissue research, Vol. 318, p. 275-288.

5. *The neurochip: a new multielectrode device for stimulating and recording from cultured neurons.* **Maher, M.P., et al.** 1, 1999, Journal Of Neuroscience Methods, Vol. 87, p. 45-56.

6. *Towards an embodied in vitro electrophysiology: the NeuroBIT project.* **Martinoia, S., et al.** 2004, Neurocomputing, Vol. 58–60, p. 1065 – 1072.

7. *Activity modulation elicited by electrical stimulation in networks of dissociated cortical neurons.* **Massobrio, Paolo, et al.** 2007, Proceedings of the 29th Annual International Conference of the IEEE EMBS, p. 3008-3011.

8. *In-vitro compartmented neurofluidic system for studying neural networks.* **Kanagasabapathi, T., et al.** 2008. 6th Int. Meeting on Substrate-Integrated Microelectrodes. p. 317-318.

9. *Long-Term Activity-Dependent Plasticity of Action Potential Propagation Delay and Amplitude in Cortical Networks.* **Bakkum, D. J., Chao, Z. C. e Potter, S. M.** 5, 2008, PLoS ONE, Vol. 3.

10. *Rat cortical neurons in cell culture: culture methods, cell morphology, electrophysiology, and synapse formation.* **Dichter, MA.** 1978, Brain Res, Vol. 149, p. 279 –293.

11. *Multi-electrode analysis of coordinated, multisite, rhythmic bursting in cultured CNS monolayer networks.* **Droge MH, Gross GW, Hightower MH, Czisny LE.** Journal of Neuroscience, Vol. 6, p. 1583–1592.

12. *Making real neural nets: design criteria.* **Curtis AS, Breckenridge L, Connolly P, Dow JA, Wilkinson CD, Wilson R.** 1992, Medical and Biological Engineering and Computing, Vol. 30, p. CE33–CE36.

13. *Research on information processing by neural networks cultured on substrates.* **Wilkinson, CDW.** 1993, Journal of Applied Physics, Vol. 32, p. 6210–6212.

14. *In vitro 2-D networks of neurons characterized by processing the signals recorded with a planar microtransducer array.* **Bove, M, Grattarola, M e Verreschi, G.** 1997, IEEE Transactions on Biomedical Engineering, Vol. 44, p. 964–977.

15. *Odor, drug and toxin analysis with neuronal networks in vitro: extracellular array recording of network responses.* **Gross GW, Harsch A, Rhoades BK, Gopel W.** Biosensors and Bioelectronics, Vol. 12, p. 373–393.

16. *Learning in networks of cortical neurons.* **Shahaf G, Marom S.** 2001, Journal of Neuroscience, Vol. 21, p. 8782– 8788.

17. *Miniature Microelectrode Array To Monitor Bioelectric Activity.* **Thomas, C.A. e al., et.** 1, 1972, Experimental Cell Research, Vol. 74, p. 61-&.

18. *Recording Action-Potentials From Cultured Neurons With Extracellular.* **Pine, J.** 1, 1980, Journal Of Neuroscience Methods, Vol. 2, p. 19-31.

19. *Multi Channel Systems MCS GmbH, Microelectrode Array (MEA) User Manual.* 2005.

20. **Morin, F.O., Takamura, Y. e Tamiya, E.** *Investigating neuronal activity with planar microelectrode arrays: Achievements and new perspectives.* 2005. p. 131-143.

21. *Controlling bursting in cortical cultures with closed-loop multi-electrode stimulation.* **Wagenaar, Daniel A., et al.** 3, 2005, Journal of Neuroscience, Vol. 25, p. 680-688.

22. *Dynamics and plasticity in developing neuronal networks in vitro.* **Pelt, J. van, et al.** 2005, Prog. Brain Res., Vol. 147, p. 173-188.

23. *Selective electrical interfaces with the nervous system.* **Rutten, W.L.C.** 2002, Annual Review of Biomedical Engineering, Vol. 4, p. 407-452.

24. *Study of neuronal cell culture in microfluidic compartmented systems.* **Cinque, L.** 2007.

25. *Micropatterned substrates for the growth of functional neuronal networks of defined geometry.* **Vogt, A.K., et al.** 5, 2003, Biotechnology Progress, Vol. 19, p. 1562-1568.

26. *Constrained synaptic connectivity in functional mammalian neuronal networks grown on patterned surfaces.* **Wyart, C., et al.** 2, 2002, Journal Of Neuroscience Methods, Vol. 117, p. 123-131.

27. *Biocomplatible parylene neurocages.* **Tooker, A., et al.** 6, 2005, Engineering in Medicine and Biology Magazine, IEEE , Vol. 24, p. 30-33.

28. *Neuroelectronic interfacing with cultured multielectrode arrays toward a cultured probe.* **Rutten, W., et al.** 7, 2001, Proceedings of the IEEE , Vol. 89, p. 1013-1029.

29. *Simultaneous Induction of Pathway-Specific Potentiation and Depression in Networks of Cortical Neurons.* **Jimbo, Y., Tateno, T. e Robinson, H. P. C.** 1999, Biophysical Journal, Vol. 76, p. 670–678.

30. *A microelectrode array (MEA) integrated with clustering structures for investigating in vitro neurodynamics in confined interconnected sub-populations of neurons.* **Berdondini, L ., et al.** 1, 2006, Sensors and Actuators B: Chemical , Vol. 114, p. 530-541 .

31. *Microfluidic Multicompartment Device for Neuroscience Research.* **Taylor, Anne M., et al.** 5, 2003, Langmuir, Vol. 19, p. 1551-1556.

32. *Mechanisms controlling bursting activity induced by disinhibition in spinal cord networks.* **Arbon, P, et al.** 2002, European Journal of Neuroscience, Vol. 15, p. 671– 683.

33. *Suppression of axonal conduction by sinusoidal stimulation in rat hippocampus in vitro.* **Jensen, A.L. e Durand, D.M.** 2, 2007, Journal of neural engineering, Vol. 4, p. 1-16.

34. *Dissociated cortical networks show spontaneously correlated activity patterns during in vitro development.* **Chiappalone, M., et al.** 2006, Brain Research, Vol. 1093, p. 41-53.

35. *Spontaneous periodic synchronized bursting during formation of mature patterns of connections in cortical cultures.* **Kamioka H, Maeda E, Jimbo Y, Robinson HPC, Kawana A.** 1996, Neurosci Lett, Vol. 206, p. 109–112.

36. *Experimental analysis of neuronal dynamics in cultured cortical networks and transitions between different patterns of activity.* **Canepari M, Bove M, Maeda E, Cappello M, Kawana A.** Biological Cybernetics, Vol. 77, p. 153–162.

37. *Synchronization of neuronal activitypromotes survival of individual rat neocortical neurons in early development.* **Voigt T, Baier H, deLima AD.** 1997, European Journal of Neuroscience, Vol. 9, p. 990–999.

38. *Origins of activity patterns in selforganizing neuronal networks in vitro.* **Gross GW, Kowalski JM.** 1999, Journal of Intelligent Material Systems and Structures, Vol. 10, p. 558–564.

39. *An extremely rich repertoire of bursting patterns during the development of cortical cultures.* **Wagenaar, D. A., Pine, J. e Potter, S. M.** 2006, BMC Neuroscience, Vol. 7.

40. *Physiological effects of sustained blockade of excitatory synaptic transmission on spontaneously active developing neuronal networks—an inquiry into the reciprocal linkage between intrinsic biorhythms and neuroplasticity in early ontogeny.* **Corner, MA, et al.** 2002, Neuroscience & Biobehavioral Reviews , Vol. 26, p. 127–185.

41. *Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same.* **Turrigiano, GG.** Trends Neuroscience : s.n., 1999, Vol. 22, p. 221–227.

42. *Spontaneous firing as an epigenetic factor in brain development—physiological consequences of chronic tetrodotoxin and picrotoxin exposure on cultured rat neocortex neurons.* **Corner MA, Ramakers GJA.** 1992, Developmental Brain Research, Vol. 65, p. 57– 64.

43. *Investigating dynamic aspects of brain function in slice preparations—spatiotemporal stimulus patterns generated with an easyto-build multi-electrode array.* **Heck, D.** 1995, Journal of Neuroscience Methods, Vol. 58, p. 81– 87.

44. *NGF and the local control of nerve terminal growth.* **Campenot, R.B.** 6, 1994, Journal of Neurobiology, Vol. 25, p. 599-611.

45. *Enhanced Extracellular Recordings from Axons Patterned through PDMS Microtunnels in a Neuronal Culture.* **Wheeler, B.J.D.a.B.C.** Reutligen, Germany : s.n., 2008. MEA Meeting.

46. *Novel MEA platform with PDMS microtunnels enables the detection of action potential propagation from isolated axons in culture.* **Dworak, B.J. e Wheeler, B.C.** 2009, Lab on a Chip, Vol. 9, p. 404-410.

47. *Long-term characterization of firing dynamics of spontaneous bursts in cultured neural networks.* **van Pelt, J., et al.** 11, 2004, Ieee Transactions On Biomedical Engineering, Vol. 51, p. 2051-2062.

48. *Patterned cell culture inside microfluidic devices.* **Rhee, Seog Woo, et al.** 1, 2005, Lab on A Chip, Vol. 5, p. 102-107.

49. *Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies.* **Sia, S.K. e Whitesides, G.M.** 21, 2003, Electrophoresis, Vol. 24, p. 3563-3576.

50. *How to Culture, Record and Stimulate Neuronal Networks on Micro-electrode Arrays (MEAs).* **Hales, Chadwick M., Rolston, John D. e Potter, Steve M.** 2010, Journal of Visualized Experiments, Vol. 39. http://www.jove.com/index/Details.stp?ID=2056.

51. *Serum-free B27/Neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus.* **Brewer, G.J.** 1995, Journal of Neuroscience, Vol. 42, p. 674-683.

52. *Mechanisms Underlying the Early Establishment of Thalamocortical Connections in the Rat.* **Molnar, Z., Adams, R. e Blakemore, C.** 15, 1998, The Journal of Neuroscience, Vol. 18, p. 5723–5745.

53. *Spike manager: a new tool for spontaneous and evoked neuronal networks activity characterization.* **Vato, A., et al.** 2004, Computational Neuroscience: Trends in Research, Vol. 58-60, p. 1153-1161.

54. *Improved Focalization of Electrical Microstimulation Using Microelectrode Arrays: A Modeling Study.* **Joucla, S. e Yvert, B.** 3, 2009, PLoS ONE, Vol. 4, p. 1-13.

55. *Mechanism of excitation by acetylcholine in cerebral cortex.* **Krnjevic, K., Pumain, R. e Renaud, L.** 1, 1971, Journal of Physiology-London, Vol. 215, p. 447-465.

56. *Two types of muscarinic response to acetylcholine in mammalian cortical neurons.* **McCormick, D. A. e Prince, D. A.** 1985, Proceedings of the National Academy of Sciences of the United States of America, Vol. 82, p. 6344-6348.

57. *Acetylcholine induces burst firing in thalamic reticular neurones by activating a potassium conductance.* **McCormick, D.A. e Prince, D.A.** 6052, 1986, Nature, Vol. 319, p. 402-405.

58. *Acetylcholine-sensitivity of thalamic neurones: its relationship to synaptic transmission.* **McCance, I., Phillis, J. W. e Westerman, R. A.** 1968, Br. J. Pharmac. Chemwther., Vol. 32, p. 635-651.

59. *Tetrodotoxin poisoning.* **Hwang, DF e Noguchi, T.** 2007, Advances in Food and Nutrition Research, Vol. 52, p. 141–236.

60. *Intrinsic dynamics in neuronal networks.* **Latham, PE, et al.** 2000, J Neurophysiol, Vol. 83, p. 828–835.

61. *Real-time multi-channel stimulus artifact suppression by local curve fitting.* **Wagenaar, Daniel A. e Potter, Steve M.** 2002, Journal of Neuroscience Methods, Vol. 120, p. 113/120.

62. *A novel algorithm for precise identification of spikes in extracellularlyrecorded neuronal signals.* **Maccione, A., et al.** 2009, Journal of Neuroscience Methods, Vol. 177, p. 241-249.

63. *An approach to the quantitative analysis of electrophysiological data from single neurons.* **Gerstein, G. L. e Kiang, N. Y. S.** 1960, Biophysical Journal, Vol. 1, p. 15-28.

64. *Current methodologies for detection of tetrodotoxin.* **Noguchi, T e Mahmud, Y.** 1, 2001, Toxin Reviews, Vol. 20, p. 35-50.

65. *Electrode recovery potential.* **Mayer, S., et al.** 1992, Annal. Biomed. Eng., Vol. 20, p. 385–394.

66. *Stimulus-Artifact Elimination in a Multi-Electrode System.* **Brown, E. A., et al.** 1, 2008, IEEE TRANSACTIONS ON BIOMEDICAL CIRCUITS AND SYSTEMS, Vol. 2, p. 10-21.