LUND
UNIVERSITY

Let Us Put Our Brains in the Spotlight – Literally!

Elsa Karjalainen
Bachelor's Degree Project in Molecular Biology, 30 credits
2023, BIOK01
Department of Biology, Lund University

Acknowledgments

I could not have undertaken this journey without my assistant supervisor Carl-Johan. You have led me through the complex world of neurobiology and were always there to answer my questions, big or small. You guided me through 3D printing, computer coding, keeping cells alive, and the scary academic world. Without you, this thesis would not have been possible. I am also thankful to Viktor. Thank you for playing countless rounds of Yatzy with me during our lunch breaks. You got my mind off stressful moments regarding this project. Lastly, I would like to extend my deepest gratitude to Eric. You helped me check my grammar and syntax in this work. Foremost, you have been my biggest supporter. Thank you all.

Abstract

Neurons are the working force in each vertebrate's nervous system. These small cells have the important task of transferring information in the form of electrical charges called action potentials. Neurons make it possible for us to sense touch, process our environment, and store memory. To conduct studies on the brain outside of the body, one can culture neural cells in the lab. However, to accurately replicate our brain through cultured neurons is easier said than done. The formation of arbitrary networks must be accounted for when trying to emulate our inner environment. Arbitrary networks are not representative of the network activities found in vertebrate brains.

In my work, I have been trying to solve these random network formations by seeding human neural cells in microfluidic devices, a labyrinth for cells. The principle of culturing neural cells in a microfluidic device with a chevron pattern made up of a V-shape is to lead cellular growth in a desired direction. When the cellular growth is under control, it may be possible to activaley create the types of network more representative for our brain. For instance, I have tried to form feedforward networks in microfluidic devices. A feedforward network is a sleek organised structure where information is transferred from one layer of cells, through one or several hidden layers of cells, and eventually reaches an output layer of cells. My results point to the cell's viability is almost as good as when they grow freely. The activity is similar and the cells' growth is alike. The cells are also able to follow the chevron pattern in the desired direction and with a little tweaking of the design we expect to be able to better steer their growth and enable the formation of more representative networks.

Another aspect of my thesis was to evaluate the possibility of utlising optogenetics to these cultured neural cells. Optogenetics is a useful method to study and manipulate behaviour of animals and even singular cells. By transfecting the cells with the protein opsin, which is sensitive to light, it is possible to expose the cell to a visual stimulus wheruopon the cells open membrane channels and an action potential is elicited. Being able to control the amount of action potentials generated, we could potentially control how cells store memory. The information in form of memory is stored in the connections between neurons. One way to strengthen the connection is to generate multiple action potentials, which is possible with optogenetics. In my work, I found that it is possible to build an experimental setup and expose cells to visual stimulus as I simultaneously record the cellular activity. However, I did not add the opsin protein to the cells, meaning they were not actually sensitive to light. For future experiments, the opsin transfection should be performed to sensitise the cells to light.

To conclude, my work covers some basic ground for further future studies. With my chevron patterned microfluidicdevices I was able to steer the cellular growth. The cultured cells therefore exhibit potential to form feedforward networks with strong connection between the cells. When applying optogenetics to these cultured cells in coming experiments, the possibility to study memory formation in these networks will be possible. To manually strengthen the connections between the neurons in the created networks by using light, the prospect for memory research on mammalian brains outside of the body will open many doors.

1. Introduction

1.1. The Mammalian Brain and Its Complex Network of Neurons

Each vertebrate has a central nervous system that is the core of one of life's greatest mysteries – the brain. The brain consists of numerous parts, such as the thalamus, hippocampus, and cerebral cortex which, together with other parts, are grouped as the forebrain, midbrain, and hindbrain (Luo, 2020, p. 6-7). There are a great number of nerve cells spread throughout the organ as a complex network. It has been estimated that the human brain consists of around 86 billion neurons (Azevedo et al., 2009; Herculano-Houzel, 2009). Neurons are fundamental to the function of the brain and carry out the greatly important task of transferring information. Information transferring is the propagation of electrical signals known as action potentials through the neuron's axon, a cell projection able to conduct electrical signals, which is vital in the communication between neurons. The driving force behind the transferring of information is the difference in voltage outside and inside of the cell. When channels on the neuron respond to a specialised chemicals that can induce signal transmitting, called neurotransmitters, they open or close and enable or disable ions to pass through the channels. When the difference in voltage reaches a certain threshold, the cell depolarises, and an electrical impulse is sent through the axon of one neuron to the synapse of another.

Neurons are also important when it comes to storing and recovering memories. The forming of a memory is a dynamic process that is believed to occur over several hours, if not days. Although the process is not fully known, researchers have a good idea of how neurons play an important part of the process (Bisaz et al., 2014; Squire, 1986). What is known, is that the modifying change in synaptic strength plays an important role in memory. Long-term potentiation (LTP) is the increased synaptic strength between two neurons and long-term depression (LTD) is the weaking of synaptic strength between two neurons. The shift between LTP and LTD contribute to synaptic plasticity, neural circuits ability to form and dissolve connections, which is fundamental in memory formation (Bi & Poo, 1998; Bliss & Collingridge, 1993).

1.2. The Interplay Between Hebbian Plasticity, Feedforward Networks, and the Free Energy Principle

Donald Hebb introduced what he considered to be the mechanism for memory formation and learning in humans in his work *The Organization of Behaviour*. In his book, Hebb's postulates: 'When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased' (1949, p. 62). His theory of how neurons store and encode information is thereafter known as Hebbian plasticity, Hebb's theory, or Hebbian learning. What Hebb's postulate entails is that when a neuron is firing so that another neuron is repeatedly activated, the synaptic connection is strengthened and the network's ability to recognise and memorise patterns is facilitated.

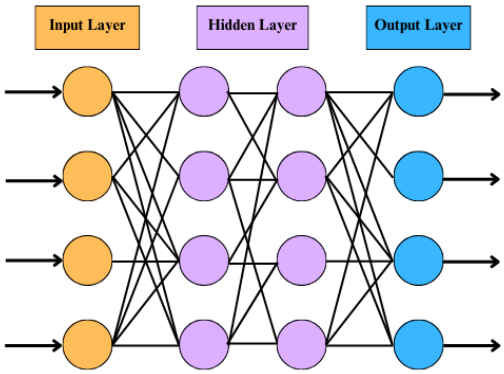


Figure 1. Schematic overview of a feedforward network and how information is propagated through several layers.

Hebbian plasticity is one of the driving forces in a feedforward neural network (FNN). In an FNN, information from an input layer is passed on through one or several hidden layers to eventually reach the output layer. The information travels sequentially, layer-by-layer, rather than simultaneously (Aguiar et al., 2019) and to several

neurons (Fig. 1). As stated by Hebb, the more one cell activates another, the stronger the synaptic connectivity becomes (Hebb, 1949). Therefore, FNNs have the potential to become great and effective at processing and forming memories according to Hebbian learning.

Another way of optimising the performance of FNNs is to account for the free energy principle. Friston et al. (2006) suggest that neural networks choose the path with the least surprises. This means that FNNs try to emulate their perception of the world based on their predictions. By making the correct prediction, the actual sensory stimulus will not surprise the system as significantly as it could, resulting in a decrease in free energy. The neural system is constantly updated so the predictions of the stimulus will be of better accuracy. Neurons play an influential role in minimising the free energy within a system and the better the synaptic connection the better accuracy (Friston, 2010). Therefore, the free energy principle together with Hebbian learning are powerful tools for improvements in FNNs.

1.3. Microfluidic Analysis and Its Applications

In order to employ microfluidic analysis, one can carry out experiments in little devices made from various materials that enable manipulation of small volumes less than one microlitre. For instance, with small volumes in small structures it is possible to create a laminar flow without disturbance which aids biomedical methods such as cell sorting (Gossett et al., 2010). Microfluidics has become a fast-growing practice used in biology and biochemistry by being cheap to fabricate and having a diverse field of use (Stone et al., 2004). Moreover, microfluidic analysis is used in other various areas including cell motility research (Hochstetter et al., 2015), astrobiology (Beebe et al., 2002; Theberge et al., 2010), and is on the rise in cancer research (Hajji et al., 2020).

One of the microfluidic device construction materials that has been used the most is Polydimethylsiloxane (PDMS) (Duffy et al., 1998). The silicone elastomer is very cheap and accessible, which is only one of the benefits of PDMS. According to McDonald et al. (2000), PDMS also reduces the formation of damaging by-products, decreases the usage of reactants, and enables a more replicable *in vivo* model.

A myriad of body systems can be replicated through microfluidic analysis. Some successful microfluidic designs include the microenvironment of a lung cancer patient (Xu et al., 2016), multi-layered tissue (Lee et al., 2009), and the network connection between neurons in the brain (Biffi et al., 2012; Morin et al., 2006; Tessadori et al., 2012).

1.4. Optogenetics and Its Use in Learning and Memory Research

Optogenetics has been in use since 2005, when scientists first successfully integrated the protein Channelrhodopsin-2 (ChR2) into the genome of mammalian neurons (Boyden et al., 2005). The microbial opsin ChR2 is a light-gated cation channel, that opens cell channels when stimulated by light, and enables ions to pass through generating action potentials. With this groundbreaking control technique, it is possible to manipulate cells by exposing them to light. As Deisseroth puts it, optogenetics fulfils the need to 'control defined events in defined cell types at defined times in intact systems' (Deisseroth, 2011). The possibilities of optogenetics are endless and can be used to induce behaviours such as feeding (Musso et al., 2019), mating (Tanaka et al., 2017), and even learning (Lak et al., 2020) in free-roaming animals.

With the possibility of inducing learning behaviours in mammals using optogenetics comes the utility of the technique in memory research. Opening the cell's channels and allowing ions to pass through and generate action potentials, it is possible to induce LTP and LTD which is important for memory formation. One example of this can be found in a paper Ramirez and his research team published on how they were able to elicit a false memory in mice through altering gene expression with ChR2 (Ramirez et al., 2013). After transferring ChR2 into mice, the research group exposed the animals to light, to optically activate neurons,

and simultaneously delivered a shock to their feet. Eventually, the mice started to associate the stimulus with the punishment and ultimately began to expect it. This led to the mice reacting as if they were about to get shocked in response to light, even if there was no shock. The mice learned how to predict what was coming next through the repetition of a stimulus. This concept can be applied to other types of cells as well. By presenting the transfected cells containing ChR2 with a light stimulus, they will eventually start to remember the pattern and possibly predict what is coming next.

1.5. Aim of the Thesis

My aim with this thesis was to investigate the possibilities of teaching neural cells while they grow and communicate in a feedforward network. While growing in PDMS with a pattern made up of chevrons, a V-shaped structure, the cells will grow differently far depending on which way they enter. The chevron pattern will also obstruct formation of arbitrary neural networks which exhibit electrical activity not normally found in the brain. Hopefully, the cells will individually reach each other, rather than forming network clusters, and create connections resembling that of a feedforward network. I also aim to evaluate the experimental procedure to eventually apply optogenetics on ChR2-trasnfected cells while they grow in microfluidic devices. This will be executed by building and assessing an experimental setup to expose cells to a light stimulus.

I hypothesise that we will be able to see the cells following the chevron patterns and that they will grow further in one direction. Moreover, I hypothesise that the optogenetic setup will enable us to acquire data and analyse the cellular response compared to the visual stimulus. I further hope that the setup will lay ground for future research when it is possible to transfect the cells with Chr2 and culture them in the microfluidic devices.

2. Methods and Materials

2.1.Cell Culturing

2.1.1. Seeding the Co-Culture of Neurons and Astrocytes on Microelectrode Arrays

We followed the steps according to the seeding protocol written by Cellular Dynamics (see Appendix A) for the PEI- and laminin-coating of the microelectrode arrays (MEA), preparation of the neural medium and thawing and seeding of the cells. However, we used larger falcon tubes (50 mL) than stated in the protocol to account for the large volume of cells. Ten of the MEAs had cells growing freely in them, and five of the MEAs had cells growing in PDMS. The residual cells that were not seeded on the MEAs were stored in flasks which we kept in an incubator together with the MEAs containing neurons.

We changed the neural medium daily for the first two days and later every other day in combination with recording the cells.

2.1.2. Seeding the Neural Stem Cells on Glass Slides

For the procedure of seeding neural stem cells on glass slides, we followed the protocols which can be found in Appendix B, C and D. Half of the seeded cells contained green fluorescent protein (GFP) which made them fluoresce under blue light. The cells were seeded on glass coverslips with PDMS on them, and were kept in an eight-well plate in the incubator. Note that in the protocol for medium preparation in Appendix B, the basic medium consists of all the ingredients listed and the complete medium contains all ingredients in the basic medium with the addition of the things listed under complete medium.

We changed the neural medium daily for the first two days and later every other day.

2.2.Fabrication of Microfluidic Devices

The microfluidic devices used in the experiments were produced by pouring liquid PDMS on a master plate made out of silicon with a laser-imprinted pattern. PDMS has a 1:10:100 ratio of co-polymer, curing agent and elastomer. The curing time for PDMS is approximately 48 hours but can be hastened by placing it in an oven at 80 °C for around an hour. We then took the fully cured PDMS and aligned it with another PDMS with pillars. Liquid PDMS was poured in-between the two PDMS stamps to create a pattern with chevrons and holes. After the final PDMS print is cured, we put it on the MEAs where it is attached to the glass surface. For the microfluidic devices we put on glass slides, we only used the chevron pattern without holes.

The patterned PDMS we used for our cells to grow can be seen in Figure 2. The chevron pattern allows the cells to grow in one direction (figure 2B) whilst they are stopped in the other (figure 2C).

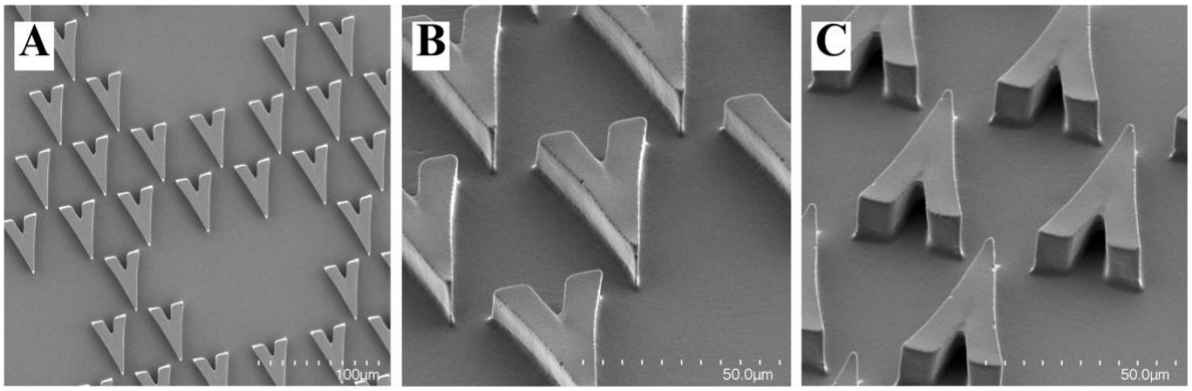


Figure 2. Scanning electron microscopic image of the PDMS with chevron pattern. A: Overview of several chevrons and how they align. B: Close-up and front view of the chevrons. C: Close-up and back view of the chevrons.

2.3. Optogenetic Setup

2.3.1. Equipment for Optogenetics

To be able to record the cells whilst we expose them to the video stimulus, we placed one dissection microscope on top of a phase contrast microscope. The dissection microscope had a hole in the bottom where we placed the amplifier connected to the USB-ME64-system. During the recordings, the MEA was placed in the amplifier. Furthermore, to display the video stimulus, a projector was mounted on top of the trinocular head (Fig. 3) of the phase contrast microscope. On top of the trinocular head of the dissection microscope, we mounted a camera connected to the computer. This made it possible to record a video of the cells when they received the video stimulus. To accurately capture the light stimulus from the video, we mounted a photodiode near the projector which was connected to the USB-ME64-system. This made it possible to compare the stimulus to the rest of the recording of cell activity.

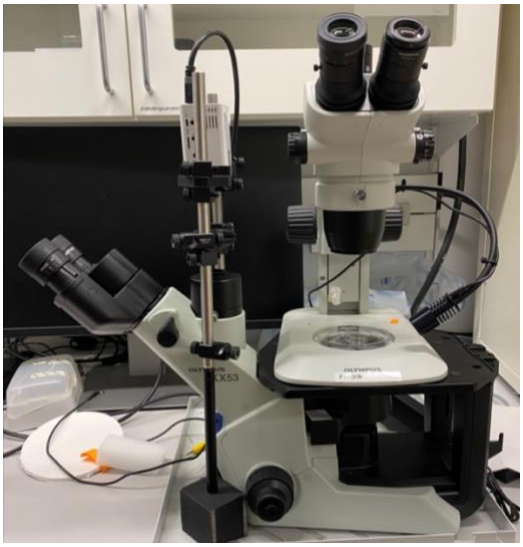


Figure 3. Experimental setup for optogenetic analysis. Two microscopes are mounted on one another with a projector staged above the trinocular head of the bottom microscope.

2.3.2. Video Stimulus

We played a video from a projector mounted on a microscope. The video consisted of blue flashes (HEX code: #0000FF) on a black background. A blue colour was chosen due to ChR2 being sensitive to that wavelength. The blue flashes lasted for 0.2 seconds, followed by a pause showing just a black background for 1 second, after which the video process was repeated for ten minutes. Meanwhile, a camera recorded the light stimuli from above so that the stimulus video and the MEA were pictured simultaneously.

2.4. Extracellular Recording of Action Potentials from Microelectrode Arrays

We recorded the neurons on the MEAs every second day through the USB-ME64-system. This data acquisition system can collect data from 64 electrodes and process and amplify the information before sending it to a computer. On the computer software MC_Rack, the recorded information is processed and analysed. The general procedure when recording MEAs was as follows: First, one MEA at a time was collected from the incubator where they rested in-between recordings. Then, we placed the MEA in the amplifier (Fig. 4). A five-minute waiting time was implemented to ensure that the neurons were not experiencing any sort of environmental chock. After the five-minute waiting time, the recording started and terminated after 10 minutes. Finally, we removed the MEA from the amplifier, changed the medium, and took photos of the neurons.

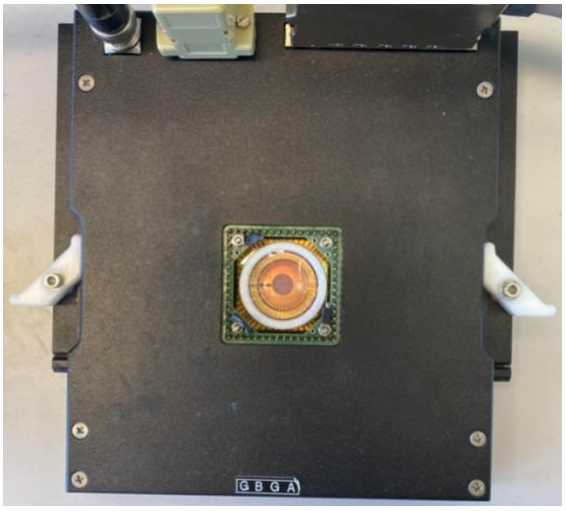


Figure 4. MEA containing cells in an amplifier connected to the USB-ME64-system.

2.5. Changing of Neural Medium

After recording, we changed the neural medium of each MEA. We started by weighing the MEA with cells and compared it to their initial weight right after we seeded them. We then calculated the weight difference and added the same amount in purified, or Milli-Q, water. We extracted and discarded 500 µL of the medium and added 500 µL fresh medium. After changing the medium, we placed the MEAs back in the incubator.

2.6. Fixing and Staining Cells for Imaging

Some of the cells already contained GFP, meaning we could observe them in a fluorescence microscope with a blue light. For the cells that did not contain a fluorophore, we fixed them with paraformaldehyde and later stained them with a 4',6-diamidino-2-phenylindole (DAPI) stain. The DAPI stain visualises every cell's DNA and can be seen under violet light, where the cells excite a blue colour.

3. Results

3.1. Fabrication of Microfluidic Devices

We casted the microfluidic devices from a silicon plate with a laser-engraved chevron pattern. For the microfluidic devices we placed on the MEAs, we aligned the chevron patterned PDMS with a PDMS stamp with pillars to then pour liquid PDMS in-between to create holes in the final device. The microfluidic devices we placed on glass slides only contained the pattern without holes. To make the PDMS with holes fit on the MEA we cut out a small piece, about 2.5 mm^2, so it covered the electrode grid.

We often experienced that the PDMS was not completely cured when we tried to remove the aligned patterns. This problem was partly solved by curing the PDMS additionally in the oven at 80 °C for about an hour. By placing the PDMS in the oven to cure, we also obtained a more stable structure of the pattern. The risk of deforming the chevron pattern when pulling it off the mould was decreased after curing in the oven. Thermal fixing was also applied when adhering the PDMS to the glass. The thermal bonding made the attachment more secure to the glass of the MEAs and slides.

3.2. Microscope Images of GFP-Cells Growing in Microfluidic Devices

We studied the cell samples in an inverted fluorescent microscope. By using a specially built petri dish with a glass bottom, we could observe the cells growing under the PDMS. We edited the figures in Fiji Image J, to acquire both phase contrast and fluorescent images. At the point of the cell imaging, the cells had been growing in the microfluidic devices for four weeks. The cells had developed projections outside and inside of the PDMS (Fig. 5). The projections mostly grew along the chevron pattern, although they sometimes grew inside of the Vs (Fig. 6). The PDMS seemed to have detached in some places, which is visible where the pattern is out of focus in the images and where cells grow freely (Fig. 5). Some of the V-shapes are also deformed and have detached from the PDMS.

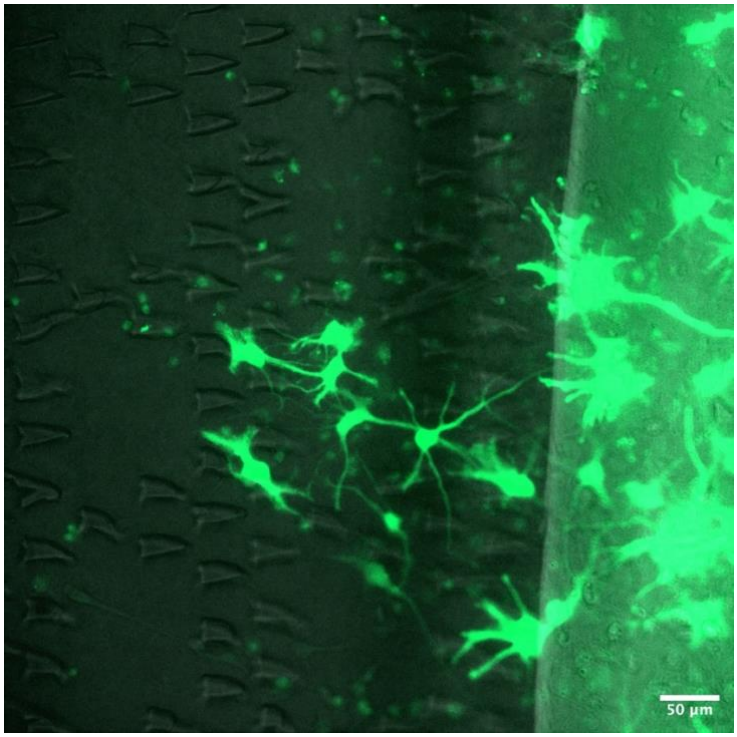


Figure 5. Fluorescence microscopy image of neurons growing inside and outside of the PDMS. The projections from the neurons grow down the pattern, and in some places through the pattern. A slight detachment of the PDMS can be seen at the border where the pattern is out of focus.

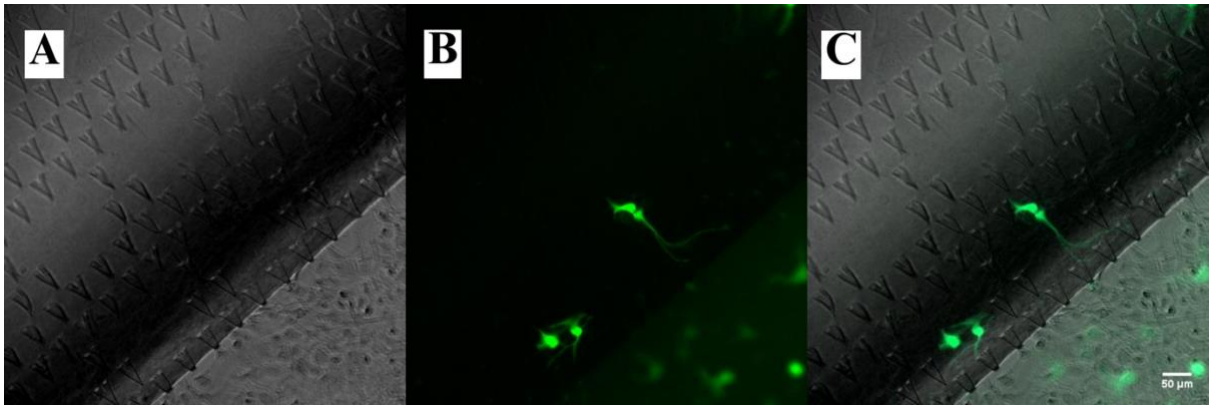


Figure 6. Montage of neurons growing into the PDMS's chevron pattern. A: Phase contrast image of the PDMS and its structure. B: Fluorescence image of GFP-cells showing cell bodies and projections. C: Combined image of the phase contrast and fluorescence image to visualise how the neurons grow in reference to the pattern.

When the Vs are attached to the glass and the cells grow in the undesired way, they are stopped by the pattern (Fig. 7). The cells tend to grow diagonally between the Vs in the undesirable way but are eventually stopped by the flat part of the V. However, they are able to travel quite far down the patterns, which means they are not stopped immediately.

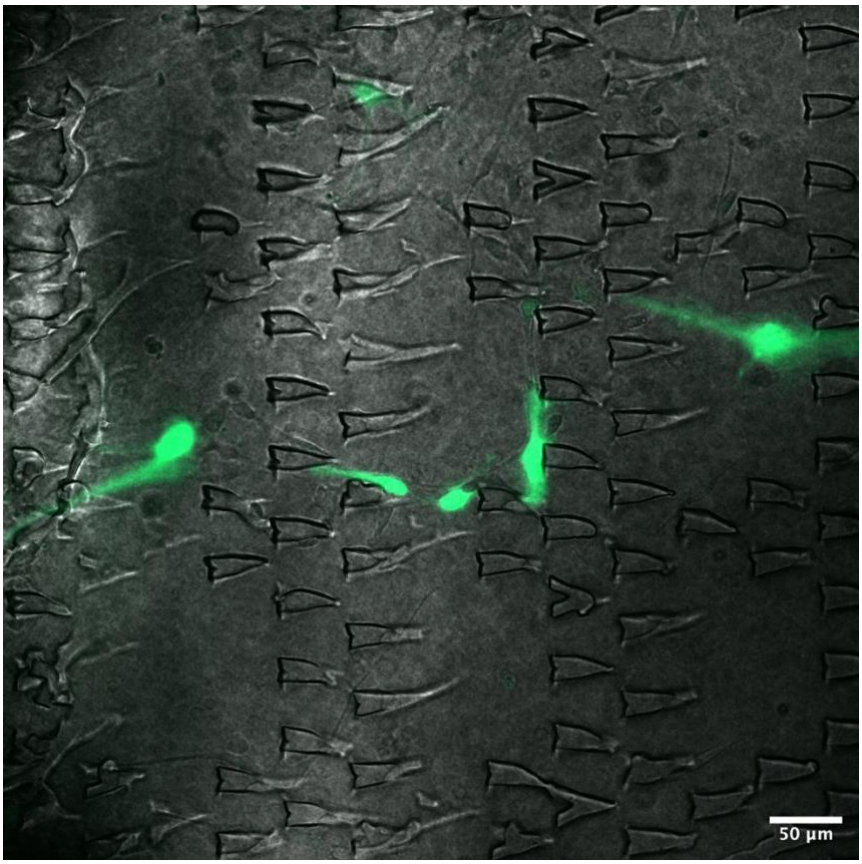


Figure 7. Combined phase contrast and fluorescence image of neurons growing inside of the PDMS's chevrons. The projections from the cell bodies are growing alongside of the chevron design. One neuron has been blocked by the flat part of a chevron and grows laterally in relation to the pattern.

Since not every cell contained GFP, we decided to stain the DNA of the neurons. After the cells were fixated and stained with DAPI, each nucleus was visible. Even the cells that did not

contain any GFP could be seen under the microscope (Fig. 8). The cells seem to have gotten further in the desired direction (Fig. 8A) than the undesired direction (Fig. 8B).

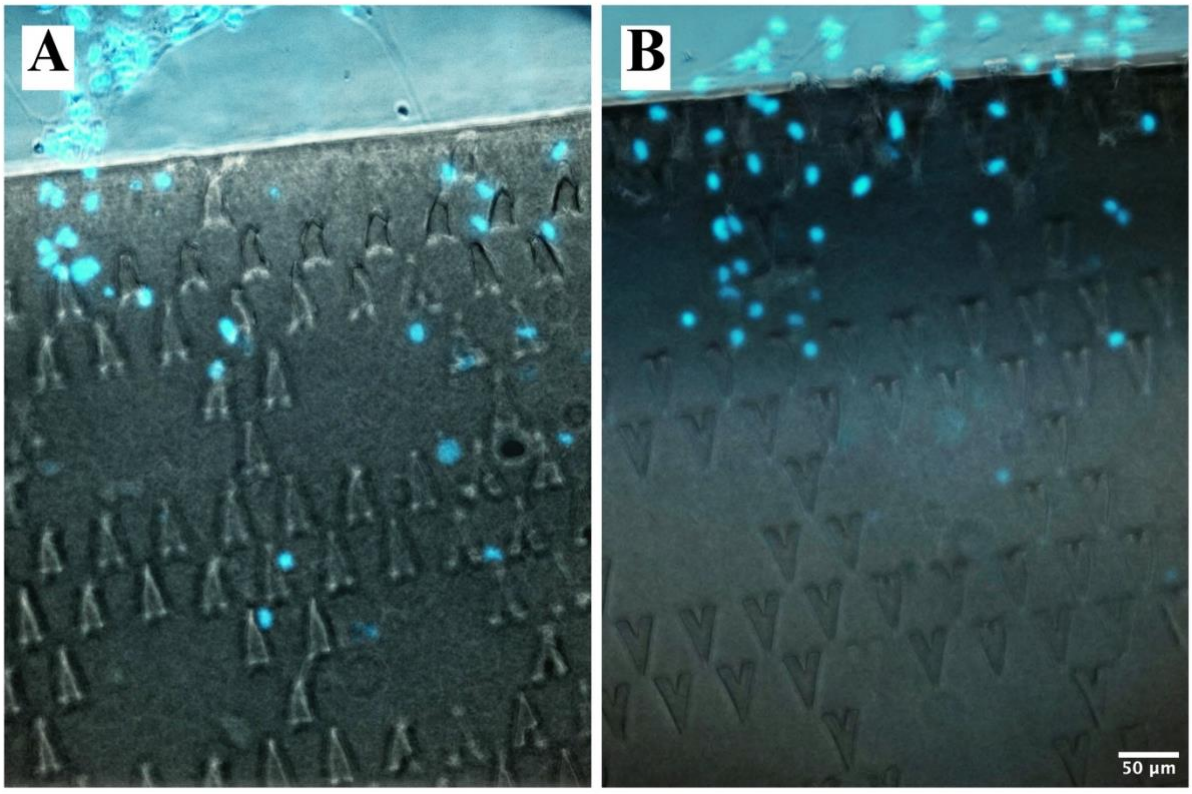


Figure 8. Merged phase contrast and fluorescence microscopy image of cells that grows in the PDMS pattern and has been DAPI stained. A: Cells growing in the desired direction. B: Cells growing in the undesired direction.

3.3. Optogenetics

3.3.1. Building the Optogenetic Setup

We successfully mounted the microscopes together to be able to record and observe the cells simultaneously. It was made possible through utilising two different microscopes mounted on to each other. However, we encountered a problem when placing the photodiode, so it accurately captured the light stimulus during the recording. Therefore, we 3D-printed a photodiode-holder to secure its position. At first, we placed the photodiode above the amplifier to capture the light as it went through the MEA with the cells. This setup poses problems partly due to the light being covered by the MEA, but also due to the distance from the light source to the photodiode. This problem was solved by placing the photodiode below the projector that showed the light stimulus to the cells. By doing this, we could accurately capture the light pulses.

3.3.2. Collection and Analysation of Data

The data was collected in the same way as for the neural cells growing on MEAs. By filtering out any noise or disturbances, and processing the raw data in MatLab, we can select distinct responses, average all channels' activity, or single out individual channels to evaluate them. With this method of analysing data, we can examine how the neurons respond to the light stimulus and detect any trends in the cell culture. We were not able to see any response of the cells since we did not transfect them. Therefore, there were no ChR2-protein to regulate the opening of channels in response to a light stimulus. However, we were able to accurately collect data from the photodiode so that we can compare that data to the rest of the electrodes (Fig. 9).

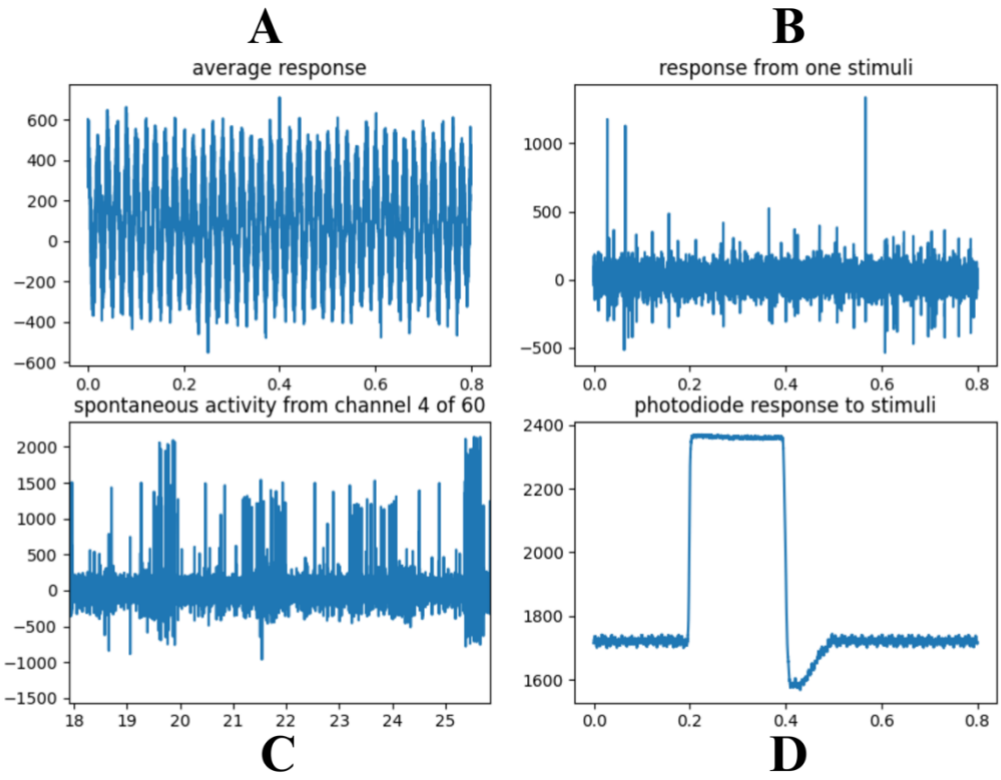


Figure 9. Diagrams representing the optogenetic experiments. The x-axis represents time in seconds, and the y-axis represents the measured activity in voltage (μV). A: The average activity collected from all channels on a MEA for 0.8 seconds in response to a light stimulus. B: Average activity collected from all channels in response to a single light stimulus. C: Spontaneous activity from channel 4 collected between 18 and 26 seconds after the recording started. D: What the photodiode captured during a light stimulus.

3.4. Cells Growing in Microfluidic Devices on Microelectrode Arrays

Cells growing in PDMS (Fig. 10A) showed clustering inside and outside of the structure. At the time of recording and imaging, the cells had been *in vitro* for 65 days. There are no, or very few, projections stretching through the pattern. However, there seem to be clusters in the holes, outside, and on top of the PDMS. The clusters inside of the holes are not as big as the ones outside or on top (Fig. 10A). Although it looks as if there are few cells in contact or nearby electrodes, the recorded activity resembles that of cells growing freely in a MEA. There is more electrical activity near the clusters (Fig. 10B) and electrodes close to each other and to these clusters seem to pick up slight synchronous activity.

Cell clusters are much bigger in the MEA with freely growing cells. At the point of recording and imaging, the cells had been *in vitro* for 63 days. The cells also have long projections connecting to other clusters (Fig. 11). The recorded activity from a MEA with cells growing in PDMS had similar properties to the activity from a MEA with freely growing cells (Fig. 12). They both showed distinctive bursts throughout the recording where more than one electrode picked up action potentials spread across the MEA.

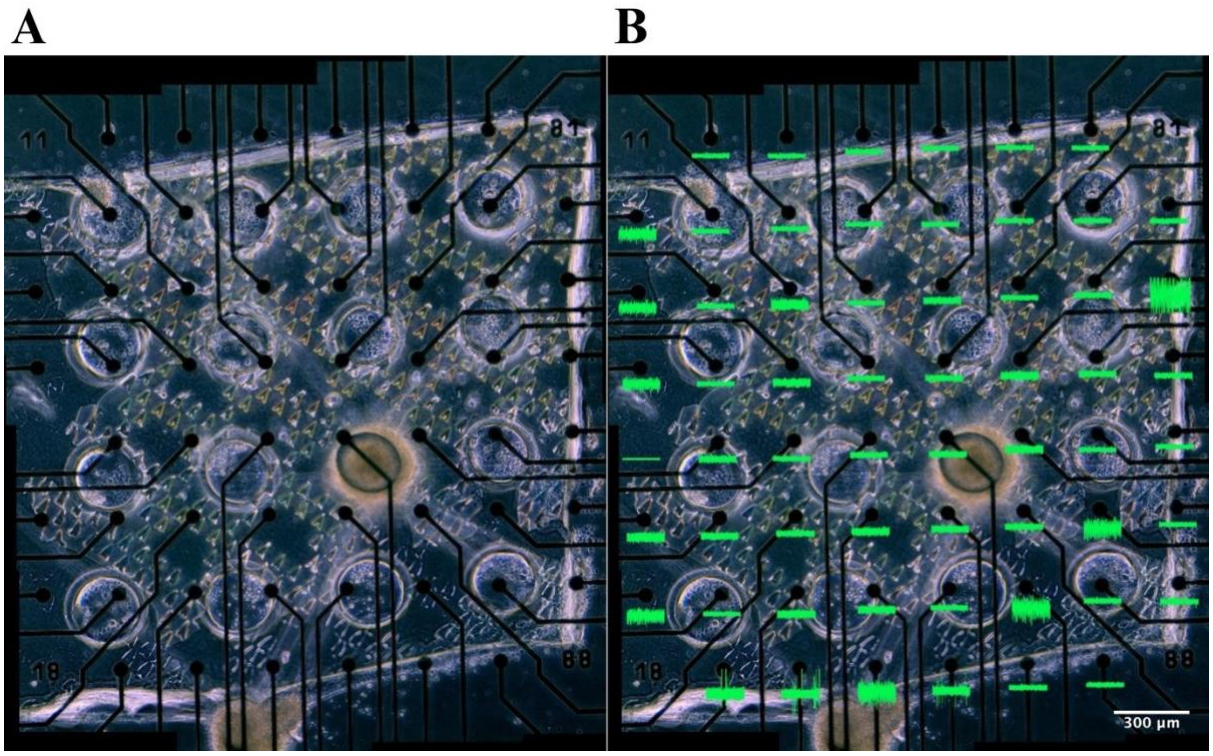


Figure 10. A: Image of PDMS placed on a MEA. Cell clusters can be seen outside, inside, and of top of the PDMS. The cells had been 65 days in vitro. B: Recorded activity of the cells in the MEA, 65 days in vitro, during the time frame 180 to 181 seconds. The green bars represent what each electrode captured during this time frame in the recording.

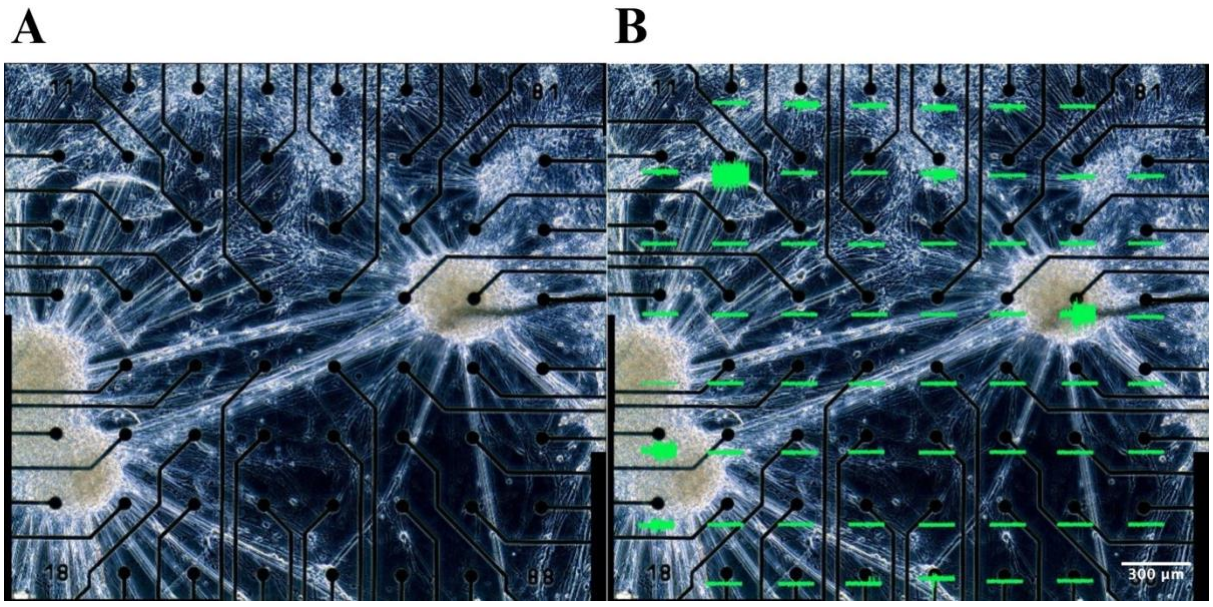


Figure 11. A: Image of cells placed on a MEA 63 days in vitro. Cell clusters can be seen and how their projections intertwine with each other. B: Recorded activity of the cells in the MEA, 63 days in vitro, during the time frame 271 to 272 seconds. The green bars represent what each electrode captured during this time frame in the recording.

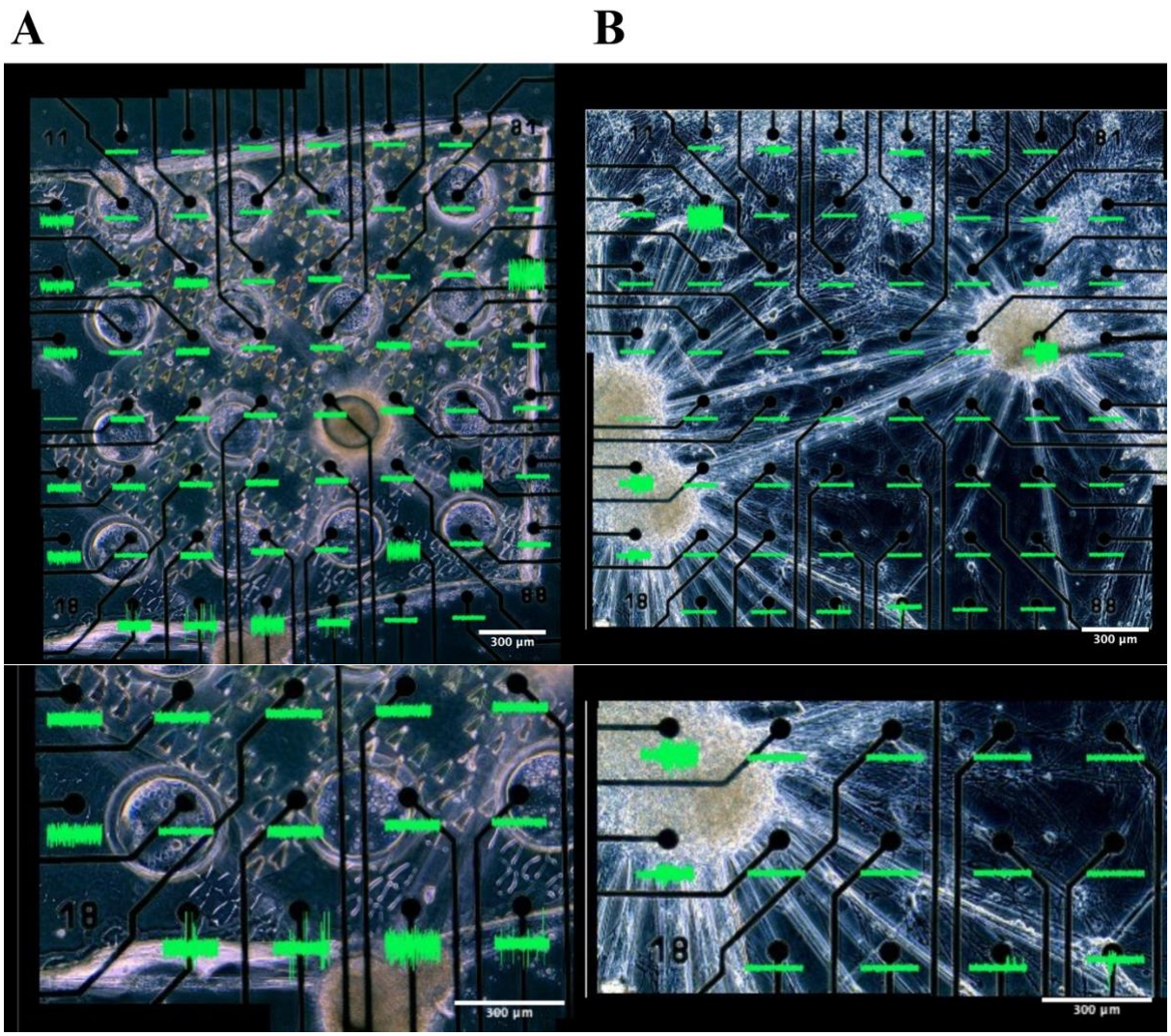


Figure 12. Comparison of the activity of cells that grow inside of PDMS (A) and cells that grow freely (B) in a MEA. A: Cells growing in PDMS on a MEA, the image at the bottom is to better visualise the activity of selected electrodes. B: Cells growing freely on a MEA, the image at the bottom is to better visualise the activity of selected electrodes.

4. Discussion

4.1. GFP-Cells Growing in Microfluidic Devices

4.1.1. Microfluidic Device Pattern Design

We were able to see the cells growing in the PDMS pattern. It is clear that they are able to get into the pattern and then follow the structure of the Vs (Fig. 5). However, due to the PDMS attachment not being completely perfect, the cells were able to grow through the design. This could be a potential problem if they cluster and do not try to reach further down the structure. Even if the PDMS pattern did not attach faultlessly, the design seems to serve its purpose. The V-shape enables the cells to grow in one direction due to the pointy tips. If they grow the other way, they are stopped by the flattened part of the V, and they cannot continue further (Fig. 13). For instance, this design idea where neurons are stopped in their growth is visualised in Fig. 7 where one cell is hindered by a V, and it cannot grow any further down the pattern.

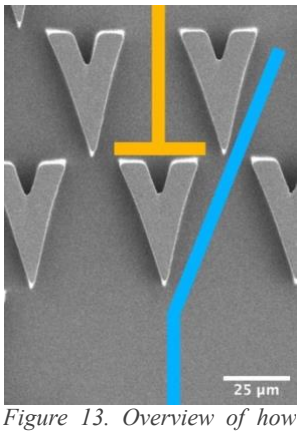


Figure 13. Overview of how neurons in theory should grow in the PDMS pattern. The blue line represents the preferred direction and the yellow line the unpreferred direction.

Empty spaces, where there are no Vs, can be seen in the PDMS. The idea is to combine this chevron pattern with a pattern filled with holes that fit seamlessly with each other. Through the holes, we will be able to seed the cells, so they are only present in the PDMS and not on the outside. Seeding the cells directly in the holes would also prevent the cells from forming clusters above the holes, where they do not enter down the pattern and make contact with the electrodes.

4.1.2. DAPI Staining of the Cells

Once we fixated and stained the cells with paraformaldehyde and DAPI, we could see each neuron's nucleus (Fig. 8). This stain made it possible to see every cell, not only those with GFP, and how far they had grown in the pattern. The cells seem to grow further on the desired side of the PDMS, looking at Fig. 8 it seems as if they have grown double the distance compared to the undesired side. Unfortunately, we were not able to see any cell projections after the DAPI stain due to it only staining DNA. This means that there is a possibility that the cells have grown even further than we can distinguish from the images.

4.2. Optogenetic Analysis

4.2.1. The Setup of the Experiment

The experimental setup seems to be working since we can acquire data from the cells, the photodiode, and simultaneously see the stimulus whilst we observe the cells. The photodiode accurately detects the light stimulus, with a duration of 0.2 seconds, which enables our ability to match the stimulus to the response in the data analysis. One shortcoming with the setup is that the projector constantly emits some sort of light. Even if we were to project a black image, there would be a vague pale light coming from the projector. Due to the build of the projector, this is hard to solve. One plausible way is to cover the lens of the projector when the black part of the pattern is shown. However, this cannot be done manually due to there is no way of ensuring that the pattern will be consistent throughout the recording. Moreover, the photodiode does not seem to significantly capture this vague light which eludes to it not being a big problem.

4.2.2. Expected Results from Future Experiment

The next step in the optogenetic experiment is to transfect the cells with ChR2. Once the cells have integrated ChR2 into their genome, they will be able to react to light stimulation. If we

were to show the pattern used to test the setup to the cells, we would expect them to respond either right when the stimulus starts, very shortly after it has started, or slightly after the stimulus has ended. Since we were not able to test transfected cells with our optogenetic method, it is hard to predict what we would have seen. Regardless of the results, we would use the same sort of data analytical methods to examine the response of the light stimulus. Through averaging the channels' response, we will be able to see how a prolonged light stimulus affects the reaction of the cells.

Another interesting reaction to examine would be if we changed the pattern. Showing the cells a pattern consisting of alternating stripes of blue and black (Fig. 14), we could investigate whether they can execute blind source separation. This phenomenon is the basis of the cocktail party effects, where one can focus on a conversation amongst several others occurring in the same space. In theory, we would expect the cells to react differently to the two patterns. One group of cells might respond stronger to the pattern in Fig. 14A and weaker to the pattern in Fig. 14B. The same principle was illustrated by Isomura et al. (2015). According to Isomura et al.'s study, they use two hidden sources in the form of sound. After a prolonged exposure, they noticed that groups of cells responded more to one type of sound whereas the activity got weaker to other sounds. The cells unintentionally form groups where they are sensitised to different patterns.

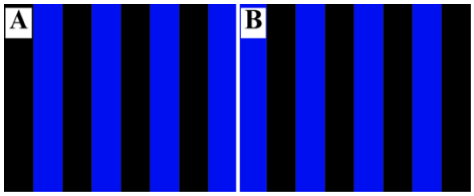


Figure 14. Example of alternating pattern of blue and black stripes. A: First frame. B: Second frame

An additional type of stimulus that could be exposed to the cells is a type of voltage-based sinusoidal wave. When alternating between a long-oscillating and short-oscillating pattern, we could emulate a sleep pattern. A longer oscillating sinusoidal wave would weaken the synaptic strength, emulating sleep, and a shorter one would once more strengthen the synapse, emulating being awake (Besing et al., 2022). The shift between strengthening and weakening the synaptic connections is the theory behind forming memories.

4.3. Cells Growing in Microfluidic Devices on Microelectrode Arrays

4.3.1. Comparison of Activity Between Cells Growing in Microfluidic Devices and Cells Growing Freely in a Microelectrode Array

When comparing the cells that grew in the PDMS pattern on a MEA (hereafter MEA1) to the cells that grew freely on a MEA (hereafter MEA2), there are some differences in the activity. MEA1 and MEA2 were seeded the same day and the recordings visualised in the results were obtained two days apart (MEA1: 63 days *in vitro*. MEA2: 65 days *in vitro*). They both show cellular growth, clustering, and branching. MEA1 had less clustering and branching which may be caused by the restriction of the PDMS (Fig. 10A). In contrast, MEA2 had big cell clusters and large projections reaching out from them (Fig. 11A). This points to the conclusion that the likelihood for cells growing in PDMS is almost as good as it is for cells growing freely in a MEA since there are cluster formations as well as some sort of spontaneous activity in both MEAs.

The upside of MEA1 not forming big and many clusters is that arbitrary networks are not as likely to form. By growing cells in the chevron pattern of PDMS, it is less likely to observe activity only found in artificial neural networks. These results entails that the cells growing in PDMS are more like the activity found in the brain than the cells growing freely on a MEA.

4.3.2. Cells Growing in Microfluidic Devices on Multielectrode Arrays – Problems and Solutions

The cells in MEA1 show an activity that resembles that of MEA2 (Fig. 12). According to the data collected from MEA1, the likelihood of cell survival in PDMS is almost as good as for the cells not growing in PDMS. However, there are sources of error that need to be corrected for future experiments. For example, how the cells tend to grow on top of the PDMS, not entering the pattern at all. This could be due to the piece of PDMS being too small. An idea to correct this is to create a piece of PDMS that has a larger unpatterned surface extending from the actual pattern. Another way of battling this is to seed the cells directly in the holes of the pattern. This would ensure that the cells would enter the pattern and grow inside of it, and not on top of it. An additional problem is that the cells grow under the chevron pattern, not following it as desired. Adhering the PDMS better to the glass surface of the MEA would combat this problem and hopefully have the effect of better cell growth. This would result in the cells having a harder time growing under the chevron pattern, as desired.

With a better attachment and an optimised PDMS pattern, this way of growing cells shows the potential of becoming a sized-down replicate of our brain. The ability to create feedforward networks where it is possible to simultaneously record the activity from the cells creates great grounds for further research.

4.3.3. Application of Optogenetics on Cells Growing in Microfluidic Devices on Multielectrode Arrays

The next step is to transfect cells with ChR2, seed them in the PDMS, and then apply optogenetics in form of exposing the transfected cells to a light stimulus. This is essentially a combination of the work I have been doing in this project, except for transfecting the cells. Since we were growing the cells in the PDMS without recording them and applied optogenetics to cells growing freely in a MEA without PDMS, we were not able to draw any conclusions on whether this will work altogether. The results from the different experiments are promising for the future.

4.4. Conclusion

A common pitfall in working with artificial neural networks is arbitrary networks forming. By seeding and growing neural cells in a microfluidic device made from PDMS, this problem is partly solved. Apart from being able to control the direction of cellular growth, we also somewhat control the formation of networks. With the chevron pattern, we favour a feedforward network alike ones we have in our brains. According to our data, neurons growing in microfluidic devices display activity similar to neurons growing without restriction. We were able to observe slight synchrony and bursts throughout the network in the cells growing in the microfluidic devices. For future experiments, the fabrication of the microfluidic devices needs some adjustments. For instance, creating a mechanism to seed the cells directly in the holes and to find a better way of attaching the PDMS to the glass.

The optogenetic experiment setup we built in this project serves its purpose of exposing cells to a visual stimulus consisting of a shifting blue and black pattern. We successfully acquired data from the cells as well as from the photodiode, facilitating the comparison between the light stimulus and the cellular response. We also managed to video record the cells as they were exposed to the stimulus. This setup is an important first step to later transfect the cells with ChR2, which will make the actual cells sensitive to the light stimulus.

As discussed, there are many a few shortcomings with these methods and some adjustments that need to be made to optimise the procedures. I believe these results are a great step toward replicating our brain outside of our body. At least in the means of trying optogenetics on transfected cells growing and communicating in a feedforward network.

5. References

Aguiar, M. A. D., Dias, A., & Field, M. (2019). Feedforward Networks: Adaptation, Feedback, and Synchrony. *Journal of Nonlinear Science*, *29*(3), 1129–1164. https://doi.org/10.1007/s00332-018-9513-7

Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., Filho, W. J., Lent, R., & Herculano-Houzel, S. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *Journal of Comparative Neurology*, *513*(5), 532–541. https://doi.org/10.1002/cne.21974

Beebe, D. J., Mensing, G. A., & Walker, G. M. (2002). Physics and Applications of Microfluidics in Biology. *Annual Review of Biomedical Engineering*, *4*(1), 261–286. https://doi.org/10.1146/annurev.bioeng.4.112601.125916

Besing, G.-L. K., St. John, E. K., Potesta, C. V., Gallagher, M. J., & Zhou, C. (2022). Artificial sleep-like up/down-states induce synaptic plasticity in cortical neurons from mouse brain slices. *Frontiers in Cellular Neuroscience*, *16*. https://www.frontiersin.org/articles/10.3389/fncel.2022.948327

Bi, G., & Poo, M. (1998). Synaptic Modifications in Cultured Hippocampal Neurons: Dependence on Spike Timing, Synaptic Strength, and Postsynaptic Cell Type. *Journal of Neuroscience*, *18*(24), 10464–10472. https://doi.org/10.1523/JNEUROSCI.18-24-10464.1998

Biffi, E., Menegon, A., Piraino, F., Pedrocchi, A., Fiore, G. B., & Rasponi, M. (2012). Validation of long-term primary neuronal cultures and network activity through the integration of reversibly bonded microbioreactors and MEA substrates. *Biotechnology and Bioengineering*, *109*(1), 166–175. https://doi.org/10.1002/bit.23310

Bisaz, R., Travaglia, A., & Alberini, C. M. (2014). The neurobiological bases of memory formation: From physiological conditions to psychopathology. *Psychopathology*, *47*(6), 347–356. https://doi.org/10.1159/000363702

Bliss, T. V. P., & Collingridge, G. L. (1993). A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature*, *361*(6407), Article 6407. https://doi.org/10.1038/361031a0

Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G., & Deisseroth, K. (2005). Millisecond-timescale, genetically targeted optical control of neural activity. *Nature Neuroscience*, *8*(9), Article 9. https://doi.org/10.1038/nn1525

Deisseroth, K. (2011). Optogenetics. *Nature Methods*, *8*(1), Article 1. https://doi.org/10.1038/nmeth.f.324

Duffy, D. C., McDonald, J. C., Schueller, O. J. A., & Whitesides, G. M. (1998). Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane). *Analytical Chemistry*, *70*(23), 4974–4984. https://doi.org/10.1021/ac980656z

Friston, K. (2010). The free-energy principle: A unified brain theory? *Nature Reviews Neuroscience*, *11*(2), Article 2. https://doi.org/10.1038/nrn2787

Friston, K., Kilner, J., & Harrison, L. (2006). A free energy principle for the brain. *Journal of Physiology-Paris*, *100*(1), 70–87. https://doi.org/10.1016/j.jphysparis.2006.10.001

Gossett, D. R., Weaver, W. M., Mach, A. J., Hur, S. C., Tse, H. T. K., Lee, W., Amini, H., & Di Carlo, D. (2010). Label-free cell separation and sorting in microfluidic systems. *Analytical and Bioanalytical Chemistry*, *397*(8), 3249–3267. https://doi.org/10.1007/s00216-010-3721-9

Hajji, I., Serra, M., Geremie, L., Ferrante, I., Renault, R., Viovy, J.-L., Descroix, S., & Ferraro, D. (2020). Droplet microfluidic platform for fast and continuous-flow RT-

qPCR analysis devoted to cancer diagnosis application. *Sensors and Actuators B: Chemical*, *303*, 127171. https://doi.org/10.1016/j.snb.2019.127171

Hebb, D. (1949). *The Organization of Behaviour*. Wiley.

Herculano-Houzel, S. (2009). The human brain in numbers: A linearly scaled-up primate brain. *Frontiers in Human Neuroscience*, *3*. https://www.frontiersin.org/articles/10.3389/neuro.09.031.2009

Hochstetter, A., Stellamanns, E., Deshpande, S., Uppaluri, S., Engstler, M., & Pfohl, T. (2015). Microfluidics-based single cell analysis reveals drug-dependent motility changes in trypanosomes. *Lab on a Chip*, *15*(8), 1961–1968. https://doi.org/10.1039/C5LC00124B

Isomura, T., Kotani, K., & Jimbo, Y. (2015). Cultured Cortical Neurons Can Perform Blind Source Separation According to the Free-Energy Principle. *PLoS Computational Biology*, *11*(12), e1004643. https://doi.org/10.1371/journal.pcbi.1004643

Lee, W., Debasitis, J. C., Lee, V. K., Lee, J.-H., Fischer, K., Edminster, K., Park, J.-K., & Yoo, S.-S. (2009). Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. *Biomaterials*, *30*(8), 1587–1595. https://doi.org/10.1016/j.biomaterials.2008.12.009

Luo, L. (2020). *Principles of Neurobiology* (2nd ed.). CRC Press.

McDonald, J. C., Duffy, D. C., Anderson, J. R., Chiu, D. T., Wu, H., Schueller, O. J. A., & Whitesides, G. M. (2000). Fabrication of microfluidic systems in poly(dimethylsiloxane). *ELECTROPHORESIS*, *21*(1), 27–40. https://doi.org/10.1002/(SICI)1522-2683(20000101)21:1<27::AID-ELPS27>3.0.CO;2-C

Morin, F., Nishimura, N., Griscom, L., LePioufle, B., Fujita, H., Takamura, Y., & Tamiya, E. (2006). Constraining the connectivity of neuronal networks cultured on

microelectrode arrays with microfluidic techniques: A step towards neuron-based functional chips. *Biosensors and Bioelectronics*, *21*(7), 1093–1100. https://doi.org/10.1016/j.bios.2005.04.020

Ramirez, S., Liu, X., Lin, P.-A., Suh, J., Pignatelli, M., Redondo, R. L., Ryan, T. J., & Tonegawa, S. (2013). Creating a False Memory in the Hippocampus. *Science*, *341*(6144), 387–391. https://doi.org/10.1126/science.1239073

Squire, L. R. (1986). Mechanisms of Memory. *Science*, *232*(4758), 1612–1619. https://doi.org/10.1126/science.3086978

Stone, H. A., Stroock, A. D., & Ajdari, A. (2004). Engineering Flows in Small Devices: Microfluidics Toward a Lab-on-a-Chip. *Annual Review of Fluid Mechanics*, *36*(1), 381–411. https://doi.org/10.1146/annurev.fluid.36.050802.122124

Tessadori, J., Bisio, M., Martinoia, S., & Chiappalone, M. (2012). Modular Neuronal Assemblies Embodied in a Closed-Loop Environment: Toward Future Integration of Brains and Machines. *Frontiers in Neural Circuits*, *6*. https://www.frontiersin.org/articles/10.3389/fncir.2012.00099

Theberge, A. B., Courtois, F., Schaerli, Y., Fischlechner, M., Abell, C., Hollfelder, F., & Huck, W. T. S. (2010). Microdroplets in Microfluidics: An Evolving Platform for Discoveries in Chemistry and Biology. *Angewandte Chemie International Edition*, *49*(34), 5846–5868. https://doi.org/10.1002/anie.200906653

Xu, Z., Li, E., Guo, Z., Yu, R., Hao, H., Xu, Y., Sun, Z., Li, X., Lyu, J., & Wang, Q. (2016). Design and Construction of a Multi-Organ Microfluidic Chip Mimicking the in vivo Microenvironment of Lung Cancer Metastasis. *ACS Applied Materials & Interfaces*, *8*(39), 25840–25847. https://doi.org/10.1021/acsami.6b08746

Appendices

Elsa Karjalainen
Bachelor's Degree Project in Molecular Biology, 30 credits
2023, MOBK01
Department of Biology, Lund University

Appendix A

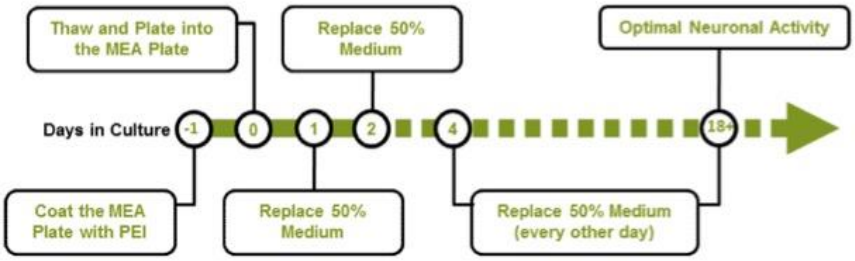
Seeding protocol, recipe for the neural medium, and how to maintain the neurons and astrocytes.

As Performed By Carl-Johan in Seeding 22/8-19

Updated: 22.08.2019

Co-culture of iCell GlutaNeurons with iCell Astrocytes on Multielectrode Arrays (MEAs)_Chronic Cultures

WorkFlow



Coating the MEAs with 0.07% PEI

1. Working in sterile biological safety cabinet, sterilize external part of the **Multichannel MEAs** by wiping with tissue moistened with 70% EtOH and place it on a sterile 10 cm plastic petri dish and label it appropriately.
2. Fill the MEA culture chamber of the chip with 96% EtOH. Incubate for 20 min at room temperature (RT).
3. Wash 4x with MilliQ water and let it dry.
4. Add 500 µl/well or 100 µl/chamber of ~0.07% PEI solution to each MEA / chamber.
5. Incubate at 37°C for 1 hour. Use membrane lid.
6. Aspirate the PEI solution and immediately rinse twice with sterile D-PBS. Do not allow the wells to dry.
7. Rinse once more with MilliQ water and air-dry the MEA overnight in a sterile biological safety cabinet.

   Note: It is critical to allow the MEA plate to air-dry overnight to achieve optimal cell attachment and maximal performance.

Coating the nanofibrous scaffolds with PLO and laminin (Optional)

1. Working in sterile biological safety cabinet, place the scaffolds supports in each well of a 24-welle plate.
2. Place the fiber scaffolds on the supports (middle part). Optional: place another support on top, and lock the fibers in place.
3. Add 500 µl of sterile Poly-L-Ornithine (PLO) solution 0.01%. Incubate for 1 h at room temperature (RT).
4. Aspirate the PLO solution and rinse twice with sterile D-PBS and the third time with MilliQ water.
5. Immediately after, add 300 µl of fresh **DOTTING MEDIUM** (10 µg/ml laminin). Incubate for 1 h at 37°C.

Preparing the Medium

1. Thaw the iCell Neural Supplement B, iCell Nervous System Supplement at RT on the day of medium preparation.

   Note: Do not thaw supplements in a 37°C water bath. Do not refreeze individual medium components.

2. Prepare 100 ml of **COMPLETE BRAINPHYS MEDIUM** (1 µg/ml laminin) by adding the following components

Component	Amount (ml)	Final Concentration
BrainPhys Neuronal Medium	95	Not Applicable
iCell Neural Supplement B	2	Not Applicable
iCell Nervous System Supplement	1	Not Applicable
Laminin, 1 mg/ml	0.1	1 µg/ml
N-2 Supplement, 100X	1	1X
Penicillin-streptomycin, 100X	1	1X

3. Filter through a 0.22 µm filter unit.

   Note: CDI recommends using room temperature **COMPLETE BRAINPHYS MEDIUM** to thaw iCell GlutaNeurons and iCell Astrocytes. Store the complete BrainPhys medium at 4°C for up to 2 weeks. Equilibrate the COMPLETE BRAINPHYS MEDIUM at RT before starting the co-cultures.

   Note: Thaw stock laminin solution at room temperature or at 4°C overnight. Do not thaw the stock laminin solution in a 37°C water bath. Do not vortex the stock laminin solution.

4. Prepare the **DOTTING MEDIUM** (10 µg/ml laminin) solution by diluting 250 µl of 1 mg/ml stock laminin solution in 25 ml of COMPLETE BRAINPHYS MEDIUM. Gently mix by inverting the tube.

Thawing

Maintain the iCell GlutaNeurons and iCell Astrocytes in liquid nitrogen/ -150°C until immediately before thawing to ensure maximal performance of the cells. Complete the following steps of the thawing procedure in a time-efficient manner to facilitate optimal iCell GlutaNeurons and iCell Astrocytes viability and performance.

Note: Recommended to start the co-culture by thawing one vial of iCell Astrocytes first. Thaw the vial of iCell GlutaNeurons after finishing with the thawing steps of the iCell Astrocytes.

5. Remove cryovial (with approximately 1M iCell Astrocytes) from -150°C freezer and place on dry ice. Do not keep on dry ice for over 10 minutes.
6. Immerse the cryovial in a 37°C water bath for exactly 3 minutes (avoid submerging the cap) holding the tube stationary (no swirling). Use of a floating microcentrifuge tube rack is recommended.

   Note: *Precise timing is critical to maximizing viable cell recovery.*

7. Immediately remove the cryovial from the water bath, spray with 70% ethanol and place in a biological safety cabinet.
8. Gently transfer the iCell Astrocytes cryovial contents to a sterile 50 ml centrifuge tube using a 1 ml pipettor.

   Note: Use of a 50 ml centrifuge tube facilitates suitable mixing to minimize osmotic shock and increase neuron viability.
9. Add 1 ml of **DOTTING MEDIUM** (10 µg/ml laminin) to the empty cryovial to recover any residual cells from the vial.

   Note: Avoid repeated pipetting of the thawed iCell Astrocytes cell suspension.
10. Transfer the 1 ml rinse from the cryovial **drop-wise (~1 drop/sec)** to the 50 ml centrifuge tube containing the iCell DopaNeurons cell suspension. Gently swirl the tube while adding the medium to mix the solution completely and minimize the osmotic shock on the thawed cells.

    Note: Drop-wise addition of the medium to the cell suspension is critical to minimize osmotic shock and ensure maximum viability and subsequent attachment of the cells to the plating substrate.
11. Further dilute with 8 ml of **DOTTING MEDIUM** (10 µg/ml laminin) to the 50 ml centrifuge tube **drop-wise (~1 - 2 drops/sec**). Gently swirl the centrifuge tube while adding the medium.

    Note: It is critical to add the 8 ml of **DOTTING MEDIUM** (10 µg/ml laminin) slowly to ensure maximum viability and attachment of the cells once plated.
12. Gently mix the contents of the 50 ml centrifuge tube by swirling 3 - 4 times.

    Note: Gentle mixing is critical to ensure maximum viability. Avoid vigorous shaking or vortexing of the cell suspension.
13. Transfer the cell suspension to a 15 ml centrifuge tube and concentrate the cells by centrifuging at 300 x g for 5 minutes.
14. Carefully aspirate the supernatant, leaving 1 ml in the centrifuge tube, and resuspend the pellet.
15. Count the iCell Astrocytes using a hemocytometer or automatic cell counter to verify the viability and total number of cells listed in the Certificate of Analysis.
16. Reserve this cell suspension at RT.
17. Repeat steps 7 to 17, but using a cryovial of 6M iCell GlutaNeurons.
18. To pool the suspensions into a 1:1 ratio of cell types, use this formula:

    (Cell Conc A * Cell Vol A) / (Cell Conc B) = Volume of Cell B to transfer to Cell A.
19. After counting iCell GlutaNeurons concentration in 1 ml, mix the reserved iCell Astrocytes cell suspension with the iCell GlutaNeurons.
20. Count the cell mixture using a hemocytometer or automatic cell counter.

Plating iCell GlutaNeurons with iCell Astrocytes into the MEAs

21. Thoroughly mix the cell suspension by gently inverting the tube 2 - 3 times.
22. Dilute the cell suspension such that 10 µl contains the wanted seeding number of cells.
23. Take 10 µl of cell suspension and place it directly on top of the active area of the MEA.
24. Cover each individual MEA with a sterile lid with porous membrane.
25. Place a smaller (3.6 cm^2) petri dish with sterile water inside the bigger (10 cm^2) petri dish with the MEA. Cover the big petri dish.

    Note: This is important to keep the humidity levels inside the big petri dish during incubation.
26. Incubate in a cell culture incubator at 37°C, 5% CO2, 95% humidity for 1h.
27. Gently and slowly add 200 µl/well of *COMPLETE BRAINPHYS MEDIUM* containing 1 µg/ml laminin down the side of the well. Adding the medium too quickly will dislodge the adhered neurons.

    Note: Timing is critical in this step. The performance is compromised if the droplets are allowed to dry. CDI recommends adding a small volume of medium to all wells first rather than adding the total volume in each well at once.

    Note: Take out the MEAs from the incubation IN SEQUENCE. They may not dry in the incubator, but once they are subjected to the dry air in the lab they dry out very quickly!
28. Add an additional 800 µl/well of *COMPLETE BRAINPHYS MEDIUM* to the side of the well to reach a final volume of 1 ml.
29. Cover each MEA with the membrane lid and incubate at 37°C, 5% CO2, 95% humidity.

Maintaining iCell GlutaNeurons with iCell Astrocytes

30. Immediately before use, equilibrate the *COMPLETE BRAINPHYS MEDIUM* to RT for at least 30 min

    Note: Do not equilibrate the medium to 37°C.
31. Perform 50% medium change on Day 1 with RT *COMPLETE BRAINPHYS MEDIUM* (700µl/ MEA).
32. Perform 50% medium change on Day 3/4 with RT *COMPLETE BRAINPHYS MEDIUM* (700µl/ MEA).
33. Maintain co-cultures in culture by replacing 50% of spent medium every 2nd day.
34. Culture iCell GlutaNeurons with iCell Astrocytes in a cell culture incubator at 37°C, 5% CO_2.
35. **Note:** Neuronal activity can be detected on day 4. Optimal synchronous neuronal activity is observed at approximately day 14 post-plating.

Appendix B

Recipe for the neural medium used for human induced pluripotent stem cells.

Marina Castro Zalis

March 3rd, 2017

L-glutamine P/S HNPC Culture Medium

Ingredients	50 ml	100 ml	500 ml
BASIC MEDIUM			
DMEM/F12 1x (Fridge) *ThermoFisher no 21331-020*	47 ml	94 ml	470 ml
N2-supplement, 100x. (Freezer) *ThermoFisher no 17502-001*	0.5 ml	1 ml	5 ml
L-Glutamine P/S. (Freezer) (Stock 200mM, 10000 units/ml Penicillin, 10000 µg/ml streptomycin) *ThermoFisher no 10378016*	0.5 ml	1 ml	5 ml
Glucose 0,6% (Dry storage) (Stock 30%) + Mill. Q *Sigma no G70213*	1 ml	2 ml	10 ml
Heparin 2 µg/ml. (Dry Storage) (Stock 10mg/100ml (Fridge)) *Sigma no H3149* + DMEM	1 ml	2 ml	10 ml
Can keep media for approx 1 month	***STERILE FILTER***		
COMPLETE MEDIUM			
EGF, rec. human EGF (Freezer) (Final conc 20 ng/ml) (Stock conc 100 µg/ml) *Prospec, CYT-217*	10 µl	20 µl	100 µl
bFGF, rec. basic human FGF (Freezer) (Final conc 20 ng/ml) (Stock conc 100 µg/ml) *Prospec, CYT-218*	10 µl	20 µl	100 µl
LIF, recombinant human LIF (Freezer) (Final conc 10 ng/ml) (Stock 10 µg/ml) *Sigma, no L5283* *Prospec CYT-644*	50 µl	100 µl	500 µl
Can keep media for approx 1 week			

Appendix C

Thawing and expansion protocol for human induced pluripotent stem cells.

Marina Castro Zalis

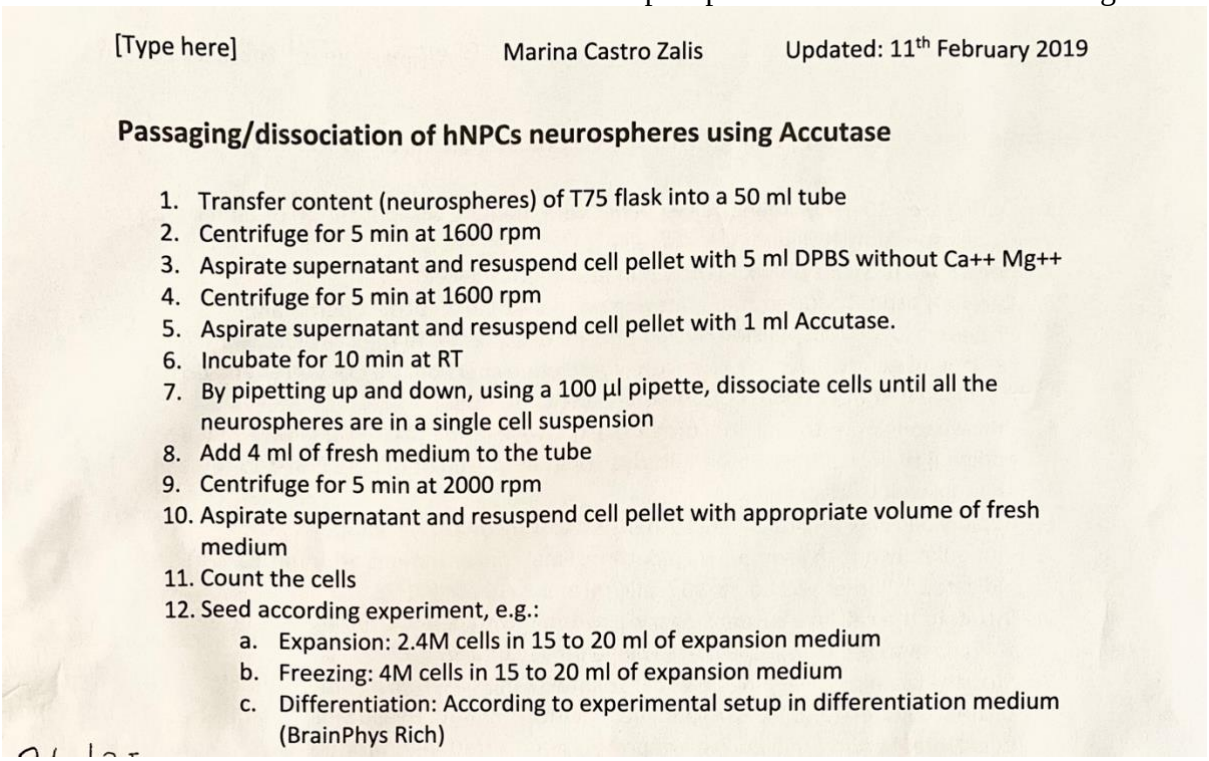
March 3rd, 2017

Thawing and expansion of hNPCs

1. Get frozen cells in the cryovials in dry ice
2. Prepare 50 ml tubes for every 2 cryovial of the same batch and add 7 to 10 ml of basic medium.
3. Quick thaw the cryovials in a 37°C water bath.
4. Add 1 ml of basic medium to the cells to dilute the freezing medium, as DMSO is toxic to the cells and immediately transfer the content to the rest of the basic medium in the 50 ml tube.
5. Centrifuge at 300 x g for 5 min.
6. Aspirate supernatant and resuspend the pellet in 2 ml expansion medium.
7. Transfer the cells into a T75 flask containing 18 ml of expansion medium.
8. Check cells under the microscope.
9. Incubate in a cell culture incubator at 37°C, 5% CO2, 95% humidity.
10. Two (2) days after, add 20 ml of fresh expansion medium to each T75 flask and then remove 20 ml of the suspension into a new T75 flask.
11. Incubate in a cell culture incubator at 37°C, 5% CO2, 95% humidity.
12. Feed cells (each flask) with 3 to 4 ml every 3rd to 4th day until the neurospheres are big enough and ready for dissociation.

Appendix D

Protocol for how to dissociate the human induced pluripotent stem cells before seeding them.



[Type here] Marina Castro Zalis Updated: 11th February 2019

Passaging/dissociation of hNPCs neurospheres using Accutase

1. Transfer content (neurospheres) of T75 flask into a 50 ml tube
2. Centrifuge for 5 min at 1600 rpm
3. Aspirate supernatant and resuspend cell pellet with 5 ml DPBS without Ca++ Mg++
4. Centrifuge for 5 min at 1600 rpm
5. Aspirate supernatant and resuspend cell pellet with 1 ml Accutase.
6. Incubate for 10 min at RT
7. By pipetting up and down, using a 100 µl pipette, dissociate cells until all the neurospheres are in a single cell suspension
8. Add 4 ml of fresh medium to the tube
9. Centrifuge for 5 min at 2000 rpm
10. Aspirate supernatant and resuspend cell pellet with appropriate volume of fresh medium
11. Count the cells
12. Seed according experiment, e.g.:
    a. Expansion: 2.4M cells in 15 to 20 ml of expansion medium
    b. Freezing: 4M cells in 15 to 20 ml of expansion medium
    c. Differentiation: According to experimental setup in differentiation medium (BrainPhys Rich)