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Controlling axon growth in microtunnel and microsieve devices

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Abstract

For studying brain diseases, a deeper understanding of axon signal exchange in neuronal networks is crucial. To study this, a researcher needs to culture predictable and consistent networks. Neuronal networks from autopsies and well-based devices cannot be consistently recreated. Chip-based devices can provide a solution. However, there remains a desire from researchers to exert more control over the architecture and functionality of the individual neurons and connections in a network. This report will propose two solutions that expand capabilities of microphysiological systems, i.e. a Nervous system-on-Chip (NoC) platform technology, to directionally guide axon growth paths and detail the experiments. These solutions are based on so called *microtunnel* and mainly *microsieve* devices using mechanical guiding cues contrary to chemical ones. The experiments show how mechanical barrier cues can be created on microsieves by 20 µl perfluorodecyl-1H,1H,2H,2H-trichlorosilane silanisation, 1000 mJ cm^{-2} UV-light exposure and 34 minute slow cooling. The findings provide guidance for others wishing to work with mechanical cues.

Contents

1 Introduction

To study brain diseases and neurological behaviour, it is usually necessary to observe the behaviour of and the interaction between individual brain cells called neurons. Neurons are the most rudimentary elements of the nerve system that can form networks and transmit signals. The main function of an individual neuron is to transmit a signal from one neuron to another. Studying the behaviour of groups of neurons and individual neurons forms the basis of modern-day neurological research.

Neurological experiments can be carried out using different testing environments. The three most common ones include well-based approaches, chip-based approaches, and autopsies. In a well based approach (like well-plates), pluripotent stem cells (hPSCs) are differentiated into neurons without confining the physical layout of the environment. The lack of structure allows the neurons to arrange themselves freely according to internal processes. Neurons behave and structure themselves like in the human body (in vivo), but do not replicate environmental effects caused by other tissue found in vivo. Due to the freedom of organisation, it is practically impossible to create the same neuronal network twice.

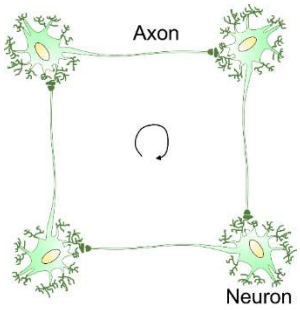


Figure 2.1: In an ideal case, a predetermined neuronal network like the one shown can reliably be copied to have the same network connections. This cannot be done with current methods.

Autopsies are directly taken out of an organism. What is retrieved is perfectly accurate with in vivo. However, studying behaviour of individual elements (like neurons) inside the autopsy is difficult, as the network is far too complex to study individual neuronal behaviour.

Chip-based research offers many possibilities when the self-assembly of neuron structures is not the research subject and therefore the network needs to stay constant. The freedom neurons have in making connections is limited by the physical layout of the microfluidic chip. This enables researchers to recreate the same network more consistently. This is of interest for researching individual neuron interactions through axon signalling, where action potentials are measured using calcium imaging. However, the ability to control axon growth to keep the neuronal network consistent is still undesirably limited. More specifically, many chips lack the capability for having predetermined one-directional axon growth limiting the choice of networks.

The goal of this project is to expand axon steering capabilities on the *microsieve*, a thin film made from an optical adhesive with regularly spaced holes in which hPSCs can accurately be placed. To gain a better understanding of the problem, solutions were also conceived for the *microtunnel* device as an exercise. This is a piece of polydimethylsiloxane (PDMS) with reservoirs in which pluripotent stem cells (hPSCs) can develop and with tunnels roughly as wide as axons. The microtunnel and microsieve concept are the work of G. Akçay, A. Bastiaens, R. Luttge and R. Sabahi-Kaviani. This project will expand on their development.

The report will start by analysing the goal (2 Goal of the project). A literature study was conducted to get a better understanding of axon growth behaviour and micro manufacturing techniques (3 Literature research) which was used to define requirements, preferences and constraints (4 Requirements, preferences, and constraints) upon which hypothetical designs are built
which is tested on manufacturability using experiments (6 Experiments) followed by a comparison between found and envisioned results (7 Results) from which conclusions are drawn (8 Conclusion). Most importantly, it concludes with a discussion useful for those who wish to continue development on this topic.

2 Goal of the project

The goal of this study is to **develop capabilities to research neuronal circuits by biomechanically controlling axon growth paths within microtunnel and microsieve devices**.

The solutions should aid brain researchers expand their knowledge about neuronal behaviour and the lack of predetermined signal unidirectionality holds back research. As such, the goal and many decisions are chosen to cater to the needs of brain researchers – the final user of these devices. To this end, interviews were conducted with CONNECT researchers to find out what they desire from potential new capabilities (Appendix A: Interview questions). Together with a literature study, their wishes were converted into requirements, preferences and constraints. The microtunnel and microsieve devices are the subjects for improvement, as their unidirectional growth capabilities are unsatisfactory. To limit the scope of this project, the solutions are purely conceived based on a biomechanical chip design, side-lining neurochemical approaches.

Solutions and concepts relating to the microtunnel device were investigated first. This provided some much needed experience for conceiving solutions for the microsieve device. The microsieve solutions were prioritised and the conclusion will only briefly touch on the microtunnel device.

3 Literature research

To be able to manipulate axons, it is necessary to know how axons behave and what has already been achieved toward the goal. This will mainly serve as the thought behind the hypothesis principles (5.1 Principles from observations) used to conceive the design. The RPCs (4 Requirements, preferences, and constraints) are also drafted using this knowledge.

3.1 Neurophysiology in vivo

Structure

Axons are long, thin "(between 0.2 μm and 20 μm in diameter)" [1] protrusions emanating from a neuron (nerve cell) that allows neurons to transmit and receive signals. On one side of the axon is the rest of the neuron (cell body) with a nucleus, mitochondria, and dendrites (points at which other neurons can attach). Axons can transmit and receive signals to and from other cells using synapses which are located on the other side of the axon. Multiple synapses are connected to axon-by-axon terminals, where the axon branches into several smaller terminals. There are many types of neurons and with it different types of axons. The difference can be the degree of myelination; how thick and how densely packed myelin sheaths are. There are also similar cells that do not conduct signals but are anatomically similar to neurons that provide structural support and regulate neurotransmitter concentrations called glia. Axons can range "from 0.1 mm to 1 m" in length [1] [2] [3].

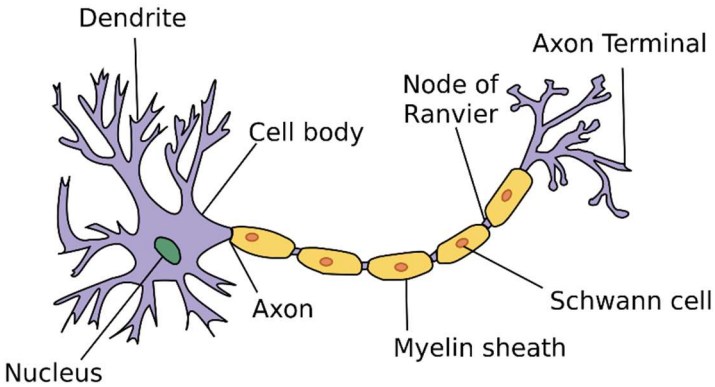


Figure 3.1: A neuron (Courtesy: "Anatomy and Physiology" by the US National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program)

Functioning

The axons transmit signals by means of electric conduction induced by ions. The base electric potential of a neuron is roughly -65 mV [3]. In- and outside the cell, there is a different concentration of potassium (K^+), sodium (Na^+) and calcium (Ca^{2+}) ions. The calcium ion concentrations can also be used for calcium imaging. When the axon is not transmitting a signal, the concentration of potassium ions is higher inside the axon and the concentration of sodium and calcium ions is higher outside the axon. When a threshold potential inside the neuron is reached by an incoming signal, "the cell membrane will *depolarize*, lowering the voltage within the membrane" [3]. This happens by opening the sodium ion channels inside the membrane, which allows diffusion of these ions to happen between the axon and its surroundings, causing the concentration of these ions to even out. With this, the electric potential becomes roughly 40 mV. This process reverses immediately afterwards by only letting the potassium channels stay open, which increases the potassium concentration and lowers the electric potential back to -65 mV. This charge travels through the axon in a pulse and either happens completely or not at all, which is why words like *trigger* and *fire* are often used in literature. The potential difference of the signal is between 70 and 110 mV [1]. These signals are called action potentials.

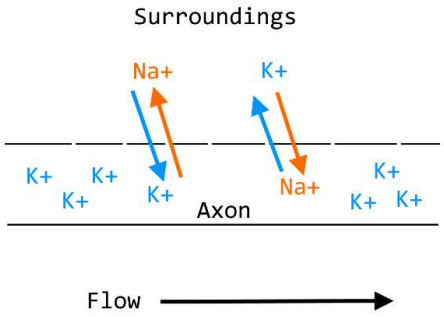


Figure 3.2: Action potential generation

Initial development
The initial phases of the growth of an axon are the exact same as that of a dendrite. The differentiation process happens gradually, meaning that the two types become more different over time in this initial stage. From a certain point onwards, the two types acquire different types of proteins. This destabilisation reconfigures the extracellular matrix (ECM) by creating a tubular opening, allowing the axon to escape. Afterwards, other nearby neuron extensions become dendrites by some chemical process. If the axon is damaged or is removed, however, another neuron extension can quickly fill the gap and become a new axon [1] [4].

Growth cone
The axon also has a "growth cone, highly motile distal tip of the axon" [4]. The environment the cone will move in is the extracellular matrix. The cone senses the environment, transmits signals back to the neuron and moves throughout the matrix. The movement is caused by polymerisation and depolymerisation within the axon [4].

Out of the cone extend filopodia and lamellipodia. The filopodia are rod shaped, flexible structures that rapidly grow and shrink anywhere there is space to grow inside the ECM. Chemical signals also play a role. The flexibility is derived from its thin membranes. The filopodia and lamellipodia make up the peripheral domain [1] [4].

The central domain or core contains tubulin, microtubules and mitochondria. The growth and retraction of filopodia is powered by myosin molecules [1]. The central domain becomes thicker when growth is paused and elongates "during fast advance mode" [4].

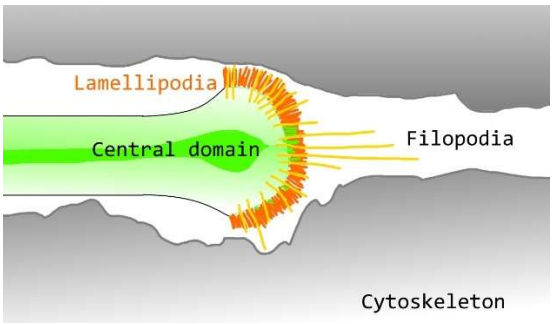


Figure 3.3: A rough sketch of the anatomy of a growth cone.

Growth
Growth happens in what can be summarised by a three-step process. Firstly, the filopodia fill up some space inside the ECM by having the myosin polymerise actin. Secondly, the increase in polymerisation compared to depolymerisation induces forward motion. Lastly, microtubules flow from the central domain to the peripheral domain which allows for new actin structures (filo- and lamellipodia) to grow atop the new central domain [1] [4].

This entire process is guided by chemicals inside the ECM that the filopodia react to. The membrane of a filopodium is a “signal-inducing receptor” [1] which allows certain ligands to bind to it. Crudely speaking, these ligands alter or destroy parts of the extracellular matrix which makes the filopodium move differently [1] [4] [5].

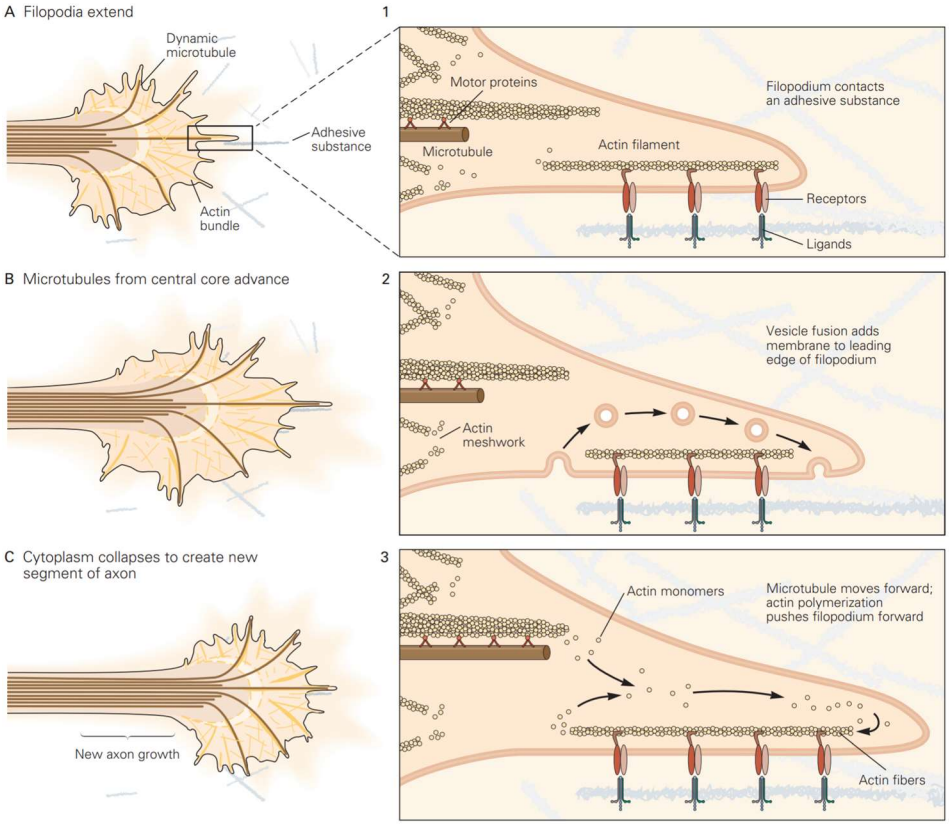


Figure 3.4: Three steps from the growth of a cone (Courtesy: [1])

Steering

There are molecules that guide axons to synapses. These mostly consist of ligands and receptors, where the ligands are on the outside of certain glia in the extracellular matrix on a path towards the synapse. The receptors are on the filopodia. They can support or hinder growth and cause a change in direction by having an increased concentration on one side. There are several ways the growth of an axon can be guided:

- Due to the spatial openings in the extracellular matrix;
- By attraction/repulsion from cell surface molecules;
- By attraction/repulsion from molecules emitted by cells (ligands);
- Using the openings created by another axon to grow along it;
- Extracellular molecules that induce branching (either at the dendrites of another cell or elsewhere).

Inside the human body, most steering is done using chemical cues. To limit the scope of this project, only physical approaches are investigated [1] [4].

3.2 Neurophysiology in vitro

The following section will establish how other researchers have achieved some form of controlled axon growth in Nervous system-on-Chip devices. Their ideas, successes and suggestions form a platform of collective experience and knowledge needed to design solutions. It is the second necessary ingredient beside knowledge on axonal behaviour (3.1 Neurophysiology in vivo) that serves as an inspiration and prediction tool for designing. The comparison to contemporary solutions also serves to keep the RPCs realistic (4 Requirements, preferences, and constraints).

3.2.1 Microtunnels and reservoirs device

A microtunnel device consists of a layer of patterned polydimethylsiloxane (PDMS) on a substrate. The PDMS layer has small grooves or tunnels on the side by which it is attached to the substrate. Only a handful of axons can pass through a single tunnel. Cylindrical holes are punched through the PDMS layer that serve as reservoirs where hPSCs can differentiate into neurons. Development and research on this device has mainly been performed by A. Bastiaens, R. Sabahi-Kaviani and R. Luttge [6].

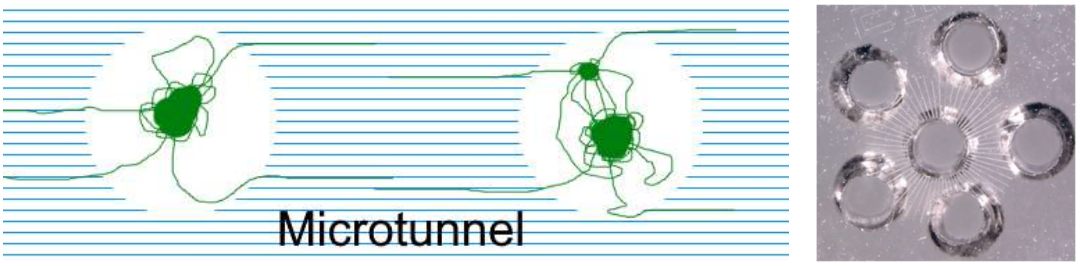


Figure 3.5: A schematic parallel microtunnel device with a neuronal culture (left) and a radial microtunnel device (right with courtesy: [6])

In Bastiaen's previous work, it was found that axons from SH-SY5Y cells (A brain sarcoma, similar to a tumour) can align with applied "nanogrooves" (small channels) in the brain-on-a-chip substrates for certain groove widths and depths. These cells also show this behaviour in a Matrigel (3.3 Material selection). They also found relations between the axon length, number of axon bearing cells, alignment of the axons and the distance of the grooves to the surface [7] [8].

Assembly

The microtunnel devices were made from PDMS using a combination of photo- and soft lithography. This method has been used in the following way illustrated in figure 3.6: Firstly, a layer of SU-8 negative photoresist is applied on a 100 mm Ø silicon wafer (step 1 and 2). After pre-baking at 95 'C for three minutes (step 3), a specifically patterned photomask is applied on top of the photoresist (step 4) and after exposing this to UV-light (step 5) cross-linking the polymer, the exposed parts will not be soluble to developer whereas the rest remains soluble after which it requires another round of baking at 150 'C for 5 minutes (step 6). The photomask can then be removed. The device is developed using mrDev-600 (step 7) This patterned silicon disc is then used as a *stamp* to create a mould in PDMS with the same features but inverted (a barrier becomes a tunnel). SU-8-on-silicon lithography is a well-developed process that yields very precise features. PDMS can also be peeled off easily. Hence, these materials are used. The stamp is then covered in a thick (between 1 and 4 mm) layer of PDMS using spin coating (step 8) to ensure a smooth, flat surface. After curing (step 9), the PDMS can then be peeled off (step 10). "Chips consisted of microtunnels in two different configurations, with either parallel, linear channels or channels in a radial design with a joint intersection at the center" [6]. The reservoirs (holes) are made using a punch, punching out the PDMS and creating the hole. The axons can grow into the tunnels. Plasma oxidation (step 11) "using oxygen plasma at 20 W for 30 s" can be used to join the PDMS and a glass sheet together [6] [7]. Glass has been chosen to allow for easier microscopy.

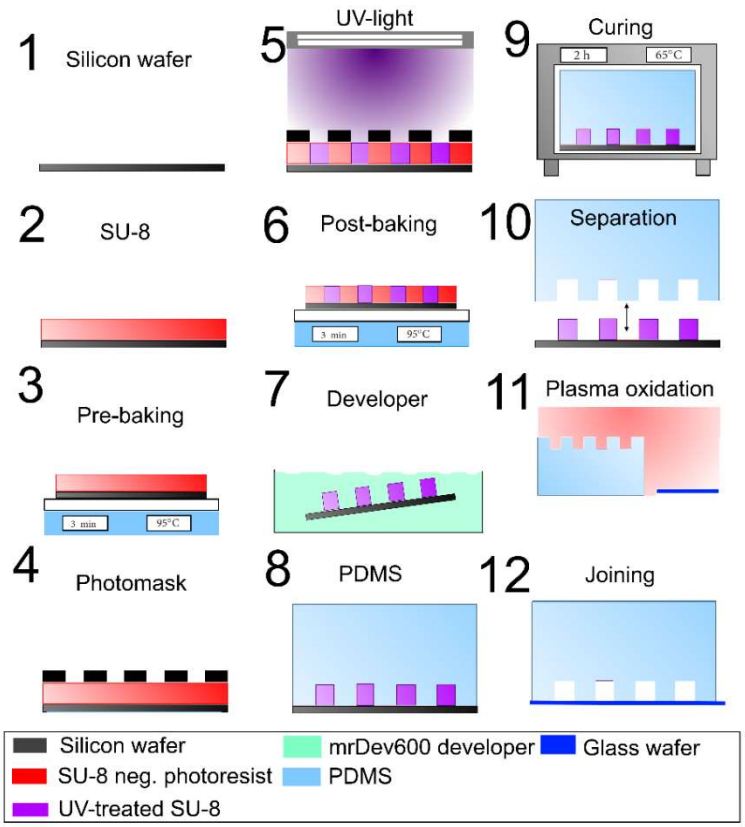


Figure 3.6: A process flow of making a microtunnel device.

3.2.2 Microsieve device

The microsieve is a thin film made from cured NOA81 (3.3 Material selection) with small, regularly spaced holes. Using a bioreactor as described by Moonen et al., hPSCs can be placed precisely into these holes using capillary pumping [9]. From there, they can develop into neurons and grow networks where axons traverse the flat NOA81 plane. Different microsieve devices exist with some being commercially available or having different purposes. The variant of interest for this study, is one designed by R. Sabahi-Kaviani [10]. The microsieve also contains channels that can be utilised for axon signal measurement by "pair[ing] single cells to electrodes". This capability was not used in this study.

The behaviour of neurons on this device is still poorly understood. Neurons are demonstrably able to form axonal connections and survive in the sieve holes. Neurons are also able to migrate out of the holes. The reason behind neuronal migration, survivability, and connection preference in the microsieve environment is not yet understood.

Figure 3.7: A schematic view of the microsieve from the top with three neurons (left) and a macroscopic picture of the sieve (right) (Courtesy of the right picture: [10])

After the differentiation of the hPSCs, the neurons can grow protrusions. According to van Boekel [11], a hPSC or a neuron in an early stage of differentiation can be modelled as a viscous drop. When modelling this viscous drop inside the pyramid-shaped sieve holes using computer aided design, it was discovered that there is a stress concentration at the seams as shown in Figure 3.8. It was then hypothesised that this could cause an increased likelihood of axons growing out on the seams using it as a biomechanical cue. Experiments done afterwards backed up this claim.

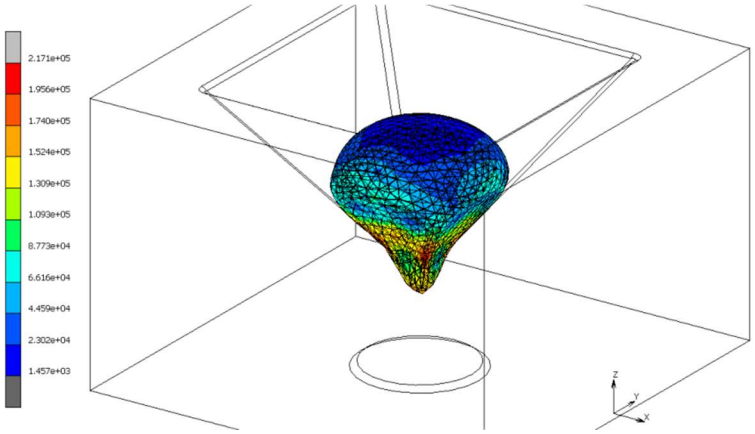


Figure 3.8: van Boekel's simulation of a viscous drop in a pyramid-shaped hole. It shows stress concentrations (red) near the seams. (Courtesy: [11])

Assembly

The microsieves are created using a double replica moulding process where a PDMS negative copy is created using a micro silicon electrode array (µSEA) master. The negative copy is then used to create the microsieve. The µSEA has been created by Schurink for prior research (step 1) [8]. The fabrication process is shown in Figure 3.9. After cleaning the µSEA using compressed air, non-cross-linked PDMS (its complete preparation can be seen at How are materials used, PDMS) is poured onto the µSEA (step 2) and cross-linked (step 3). The PDMS negative copy can then be peeled off (step 4). The negative copy can then be bonded to a glass plate for ease of handling by plasma oxidising the two parts at 3 Watts for 30 seconds. The negative copy can be reused a limited number of times. The "PDMS mo[u]ld needs to be hydrophilic to allow a wetting of NOA81 during spin-coating, a plasma oxidation step at 10 W for 30 s was performed" (step 5). NOA81 is then "poured in excess on the surface of the PDMS mo[u]ld shortly after plasma activation covering the full area of the mo[u]ld" (step 6). To ensure an evenly thin layer, the assembly is spin coated firstly at 500 rpm for 30 seconds, then at an angular velocity to determine the thickness (800 rpm was chosen in the experiments for a thickness of between 60 and 110 µm) for one minute and at an angular acceleration of 300 rpm s^{-1}. This is followed by a slow deceleration step. The excess NOA81 is irrelevant, as the angular velocity determines the thickness. The NOA81 was then partially cross-linked by UV-light exposure at 2100 mJ cm^{-2} and an irradiation of 15 mW cm^{-2} (step 7). The high intensity UV-light breaks bonds in the monomers that allow the broken monomers to create new bonds with other monomers, linking them together. The sieve needs to be peeled off shortly after (step 8). For optimal curing, it should again be put under UV-light with the same parameters (step 9). To keep the substrate flat during this process, it is recommended to put it between two glass sheets to keep it flat. Finally, a laser ablation step can be performed to open the holes using a 248 nm KrF laser with 5 ns pulse durations (step 10). This was not done in the experiments [10] [9].

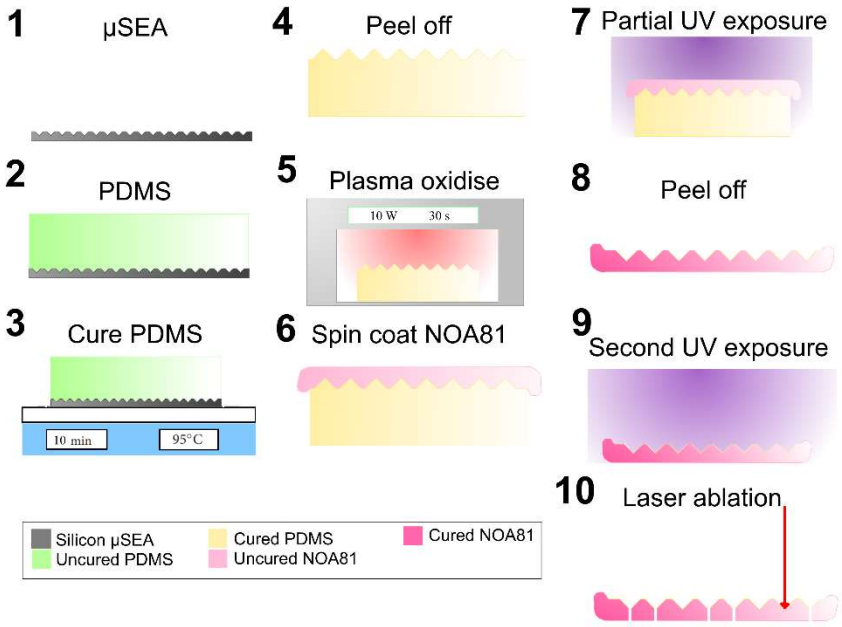


Figure 3.9: A process flow of making a microsieve device.

3.2.3 Funnel-chain method

Researchers from the Saint-Petersburg Academic University have achieved one-directional axon growth. They replaced a straight linear microchannel with a long chain of funnel shaped chambers that are at most 50 μm wide and narrow to 5 μm before they open again to 50 μm. Each funnel is 200 μm long. Another approach was to create wider *funnels* that are alternatingly skewed with respect to each other by twenty degrees as shown in Figure 3.10. Ideally, the probability that an axon grows into the narrowing of the funnel from the wider section is greater than that of a neuron outside the funnel. The straight design works better than the skewed design for achieving unidirectional growth. The probability of axons growing toward the intended direction is "4 ± 1.5 times higher than in [the] opposite direction" [12]. The method of manufacturing the brain-on-a-chips is almost identical to what has been done in Eindhoven (using a 5 μm high tunnels, SU8 as a negative photoresist applied by spin coating a silicon wafer, etc.). It also states that axons rarely grow past two segments in the opposite direction [12].

The paper has hardly any sources and its methods are relatively obscure. The paper also does not show any visual results. Furthermore, the researchers could not be contacted due to political arrangements. This set of circumstances raises doubts about validity.

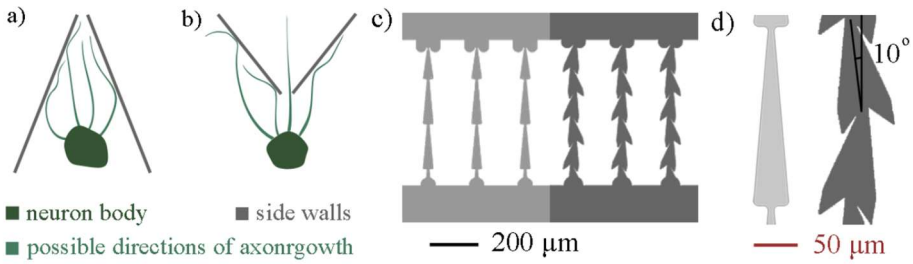


Figure 3.10: Funnel designs (Courtesy: [12])

3.2.4 Diodes

Researchers have made an array that "involves asymmetric micro-channels, imposing unidirectional axon connectivity with 97% selectivity". They noticed a property that can be exploited: when a feature the axon grows along makes a sharp turn, the axon will grow straight through. However, when the turn is slight, it will follow the feature. A funnel shaped geometry was also used. The channels are 3 µm high, 15 µm wide at the wide end and 1 to 3 µm at the narrow end. Furthermore, axons tend to keep the straight direction 50 to 100 µm after exiting the channel. Axons that are blocked travel along the wall and do not significantly enter the openings of the tunnels, instead they keep growing parallel. The device is made from PDMS using a similar process as Bastiaens but with an elastomer to curing agent ratio of 49:1 instead of 10:1 and BFR developer instead of mrDev-600 [13]. The 97% selectivity claim seems far-fetched and unrealistic for a novel technology. This raises scepticism, though the methods are clearly described and seem replicable.

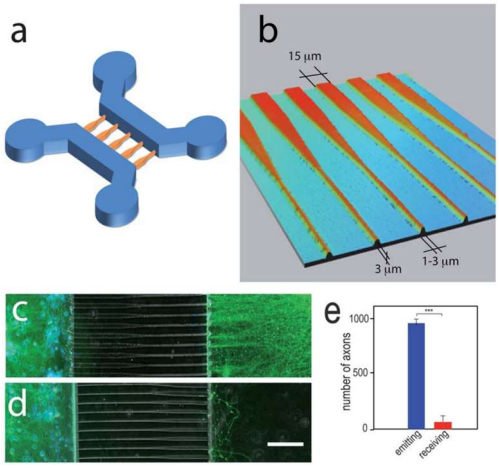


Figure 3.11: Diode geometries and results (Courtesy: [13])

3.3 Material selection

Many Nervous system-on-Chip devices would not be possible to create were it not for the materials and their specific properties. This section will explore the properties of the materials used in the microtunnel, microsieve and others that enable building the designs presented by the hypotheses.

Polydimethylsiloxane (PDMS)

PDMS "is an non-conducting silicone based elastomer that has been of widespread interest due to its flexibility and ease of micromo[u]lding for rapid prototyping of microdevices and systems" [14]. Its appeal for biomedical applications lends to its comparatively outstanding biocompatibility and its permeability for oxygen, carbon dioxide and nitrogen [15] at the thicknesses used for the microtunnel device. Sufficient gas flow for these gasses is essential for differentiating cells correctly. For making medical chip devices, the non-cross-linked elastomer is mixed with a cross-linking agent. A mass ratio of 10:1 has been chosen in all prior research on the microtunnels [7] [6]. The elastomer is readily available, whereas the curing agent is harder to come by [15]. Both liquids are transparent. It can have problems with matrix degeneration at high temperatures (thermal instability) and being unevenly distributed in a workpiece (inhomogeneity) which pose no problem for biomedical applications [14]. PDMS parts can be joined to silicon, silica, more PDMS and other materials by using plasma oxidation on both surfaces [16].

For both microtunnel and microsieve fabrication, the elastomer and cross-linking agent is stirred and put into a vacuum desiccator to remove bubbles for 20 minutes. The homogeneous mixture is then baked on a hot plate for 10 minutes at 95 'C to cross-link it. Removing the PDMS from the substrate is done by peeling where, if done carefully, it retains the features [6] [7] [10].

Hydrogels

Hydrogels may generally be considered as solid structures with a very low Young's modulus (under 10 kPa) that consist of roughly 99% (either mass or volume) water. Some biological structures may be considered hydrogels such as the extracellular matrix [3]. Hydrogels are therefore of particular interest for steering axons. Synthetic hydrogels also have properties that closely resemble brain tissue (largely *because* most brain tissue is a hydrogel). A type of hydrogel that is commonly used for brain research that was also used by Bastiaens et al. is Matrigel [6].

Matrigel from the Corning company is a hydrogel extracted from a mouse sarcoma; a tumour [17]. This is the extracellular matrix extracted from a mouse brain tumour. To preserve its condition, it is frozen in and then shipped to the customer, which means some original properties will be lost during the freezing. Moreover, since it is directly extracted from a mouse brain tumour, it models human brain conditions as much as the tumour is representative of the healthy human brain. This is a lofty assumption, but not as far-fetched for just the extracellular matrix.

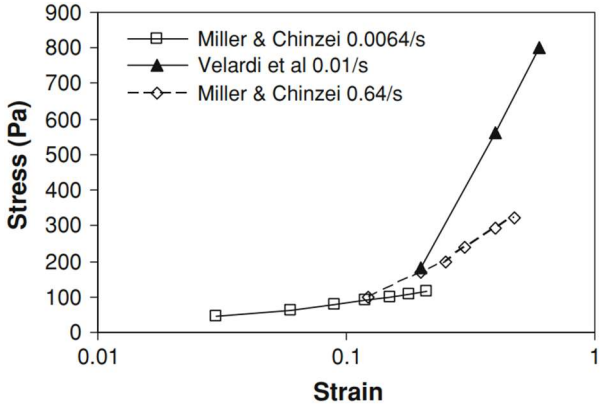


Figure 3.12: The stress-strain curve for low pressures (Courtesy: [18])

After development (which takes roughly ten minutes), the storage modulus (G') becomes 2000 Pa and the loss modulus (G'') becomes 200 Pa at 0.5% strain and an excitation frequency of 1 rad s^{-1} [19]. For real ECMs found in the human brain, "the shear moduli are of the order of a few kilopascals" [18]. Similarly, ECMs also "show shear thinning behaviour" [18]. As can be seen in Figure 3.12, the brain tissue also shows very similar stress-strain and excitation-relaxation behaviour compared to these gels.

Matrigel has become the gold standard in this field, with most publications regarding medical hydrogel using Matrigel exclusively. Given the market dominance, a global shortage of Matrigel has affected research worldwide, including this project. This has given rise to other companies developing hydrogels that mimic Matrigel as closely as possible. Other hydrogels used for the gelstop design that mimic Matrigel are expected to show the same results because the material properties are in the same ballpark as extracellular matrices.

Hydrogel-PDMS interaction

PDMS is a hydrophobic material with the water contact angle being greater than 100 degrees, whereas hydrogel is mostly water [20]. This becomes problematic for the gelstop solution (5.2 Gelstop). Depositing hydrogel in a reservoir would lead to a large drop of hydrogel accumulating in the centre, staying away from all tunnels and edges. To combat this, several techniques can be employed to increase hydrophilicity of the PDMS.

Plasma oxidation manages to improve hydrophilicity by bombarding a surface with high energy oxygen ions (O^{2-}) that engages in a redox reaction with the surface to absorb these oxygen ions. The surface is damaged by the high energy plasma, which also releases hydrogen atoms. The formed hydroxyl groups in the surface molecules increase hydrophilicity. The damaged surface is not problematic for biocompatibility. The surface slowly reacts with other molecules, decreasing the hydrophilicity over time. Another method "is to coat substrates with polyvinyl alcohol". This method does not result in a loss of hydrophilicity over time though less biocompatible and less researched [20].

Several pressures are at play when the gel encounters the narrow tunnels. To minimise the potential energy surface, a capillary pressure pushes the water into the tunnels. However, this behaviour is dependent on if the surface loses potential energy by being in contact with the PDMS with respect to the air. If the PDMS is too hydrophobic, the potential surface energy is higher, and no capillary pressure will form. Another force preventing water from further creeping into the tunnels is the energy needed to compress the collagen structure when water is removed by migrating into the tunnels. Experiments (6 Experiments) will investigate hydrogel-tunnel interactions for untreated and plasma oxidised PDMS.

Norland optical adhesive 81 (NOA81)

NOA81 (formally mercapto-ester triallyl isocyanurate) "is a single component liquid adhesive that cures in seconds to a tough, hard polymer when exposed to ultraviolet light" [21]. Developed as a material to bond fibre optic cables, it forms strong bonds to most materials after exposure to UV-light between 320 and 380 nm and is designed to cure under energies supplied by handheld UV lamps (2100 mJ cm^{-2}). Uncured, its dynamic viscosity is 300 mPa s which requires a spin coating step if it needs to become a thin layer [21].

Originally, "microsieves were made with a high pattern fidelity by silicon micromachining" [10]. NOA81 is used instead because it is a photo-cross-linking polymer which means it allows for rapid prototyping, is optically transparent, more affordable than machined silicon and makes changing the design a less time-consuming process. After curing, the adhesive bonds well to most materials, though plasma oxidised PDMS remains an important exception [10].

Because NOA81 is relatively unused, many of its properties remain unknown, as many microfabrication techniques have never been attempted with the adhesive. The experiments (6 Experiments) try to gain understanding by performing NOA81-on-NOA81 and SU8-on-NOA81 photolithography.

SU-8 negative photoresist

SU-8 is a commonly used permanent epoxy negative photoresist because it allows for very accurate and fine details that do not readily collapse when developing. Protrusions can also be made very tall and narrow compared to other photoresists. The liquid comes in several variants with differing viscosities that determine the final layer and feature thickness after spin coating. [16] [22].

The procedure for performing photolithography that was used in the experiments and recommended in the guideline is to spin coat it onto the substrate followed by soft baking it, exposure to UV-light with another baking step afterwards and finally developing it. The intense UV-light (which happens optimally at the soft baked temperature) breaks bonds inside the monomers which releases an acid. This acid catalyses the epoxy cross-linking process, which occurs only with sufficient heat in the post exposure baking. The non-cross-linked monomers are soluble in 1-methoxy-2-propyl acetate, whereas the cross-linked polymer is not. This washes away the uncured SU-7. If the features break loose from the substrate during development, it is advised to perform a slower cool down step after the post exposure bake as that reduces the stress build up in the polymer [22] [23].

SU-8 "exhibits hydrophobic behavio[u]r with water contact angle (WCA) of 90'C" [24]. This presents a problem for adhering it to cross-linked NOA81 as it contains hydroxyl groups that repulse hydrophobic liquids.

Its biocompatibility is contested in literature where some claim that "The biocompatible nature of polymerized SU-8 is evidenced by cell viability and minimal hemolytic activity" [25] whereas others state that "SU-8 might not be completely biocompatible, existing surface modification techniques, such as O2 plasma treatment or grafting of biocompatible polymers, might be sufficient to minimize biofouling" [26]. Biocompatibility in general is an ambiguous term, as some immortalised neuroblastoma can survive more challenging conditions than hPSC derivates.

NOA81 lithography

Since NOA81 only cross-links after UV-light exposure and is an adhesive, it can be utilised as a negative photoresist. Although it has been used to adhere two photolithographed pieces together, it has not been used as a photoresist. Compared to SU-8, it requires a significantly higher exposure intensity. This can create effects for the substrate; if it is patterned on a NOA81 substrate for example, it can further cross-link the substrate, which can have unintended consequences. Its main advantage, as previously mentioned, is that it adheres to most substrates very strongly. Considering SU-8 bonds poorly to precured NOA-81 (7 Results), this is a major advantage [27] [10].

Huang et al. have shown that a stamp of thoroughly cured SU-8 can pick up parts of NOA81 with reduced fluidity (slightly cross-linked) from a glass plate [28]. The bond this creates is 3.3 MPa strong. This hints at a possibility of doing the reverse; bonding liquid SU-8 to cured NOA81.

SU-8-on-NOA81 bonding

Performing photography onto pre-cured NOA81 seems never to have been done before. As seen in the results of the experiments (7 Results), SU-8 adheres very weakly to cross-linked NOA81. Generally, the bonding strength can be increased using an adhesion promotor.

The SU-8 manual recommends MCC Primer 80/20 as a general adhesion promoter. MCC Primer 80/20 is the brand name of a mixture from the same company as SU-8 consisting of 80% propylene glycol monomethyl ether acetate and 20% hexamethyldisilazane (HMDS) [23]. It improves the adhesive properties of the substrate to SU-8 for steel, glass, ceramics, polypropylene, and polyethylene [29]. The acetate “is a slow evaporating solvent with both ether and ester functional groups” [30] and is an ingredient also found in developer whereas the HMDS can create siloxane bonds between different hydrophobic molecules, though water-soluble, amine-containing alternatives exist that can deliver similar results such as polyallylamine [31].

Another way to increase adhesion is through 3-aminopropyl triethoxysilane (APTES) silanisation. The hydroxyl groups in the NOA81 substrate react with the alkoxy groups (hydrocarbons bonded to the rest of the molecule by one oxygen bridge) in the APTES, causing the hydroxyl groups to be replaced with siloxane bonds which quickly receive the hydrophobic part of the alkoxyl making the NOA81 surface more hydrophobic. “This allows homogeneous control over the wetting behavio[u]r, which is difficult to achieve with vapor-phase surface treatments” [27].

4 Requirements, preferences, and constraints

Since the final product is intended to be used by other researchers in the CONNECT group, the product needs to be suited to their demands. To get a detailed understanding of their needs, they were asked several questions in an interview (Appendix A: Interview questions). Using their responses (Appendix B: Responses from individual interviewees), a list of specifications can be compiled.

Beside wishes specified by the end users, more specific requirements need to be set that will determine the specifications of the design. These requirements narrow down the solutions.

General Requirements

1. The device must be able to directionally guide axons growing from pluripotent stem cells into a predetermined path on the chip

Given that interviewees deemed controlled axon growth useful for their research, this poses a requirement. This corresponds with the goal of the entire project, confirming the usefulness of the goal.

Some understand the concept of '*controlled axon growth'* as purely physical steering whereas others also included chemical options. A focus will be placed on physical steering rather than chemical steering in this research.

2. The device must allow for stem cells to differentiate into desired neuron types using the queues used by the CONNECT research group
3. Neurons must be able to stay alive for at least 21 days

One of the major problems mentioned by researchers was their inability to produce consistent control populations. 21 days is what can already be achieved by Bastiaen's et al. [6], so any lifespan less than 21 days would *worsen* the problem and is therefore not permissible.

4. Each neuronal circuit must be allowed to grow into a circuit with at least three nodes

A neuronal network that consists of only two nodes can only give insight into the signal exchange between two nodes and means the signal transmission is always dependent on both nodes and cannot be independently altered. It also means the neuron never has time when it is not busy transmitting or processing a signal. The minimal number of nodes to ensure independence is three nodes. To make observations easier, the number of nodes is to be kept at a minimum. Therefore, three nodes are ideal. Note that a node may consist of multiple neurons or potentially other cell types and may internally interact or form networks. These interactions need not be observable, though this would be preferred.

5. Each axon must be uniquely distinguishable using calcium imaging

This requirement is necessary to ensure meaningful results about axonal behaviour can be obtained. It requires the entire device to be sufficiently flat and non-fluorescent. It also stipulates that axons should not grow on top of or underneath each other.

Preferences

1. Mimic in vivo *conditions* as closely as possible
2. Recreate in vivo *structures* as closely as possible
3. Allow for the control groups to be as consistent as possible

These preferences were the most frequently mentioned in the enquiries, though it can come to mean different things. Researchers want to research interactions between different regions inside the body which have different environment properties. Controlling these properties is important. This would also allow for establishing control research groups, which is a problem many interviewees alluded to.

Conditions itself is an ambiguous term. Temperatures, pressures and the presence or absence of certain chemicals are some examples of variables that can be matched to in vivo and are relatively

easy to measure. However, hPSCs are sensitive to more than just three variables [3]. Specific directional stresses or pressure regions inside a liquid can also influence stem cell differentiation and must remain similar to in vivo and constant unless it can *controllably* be altered [4].

Another important aspect is the geometry of the surroundings. Axons (and probably also stem cells) react to differences in rigidity in their environments. This is what is meant by *structures*.

4. The directionality of the neuronal connections within the loop should be predictable and predetermined

To establish consistent control groups and allow some form of customisation, the physical layout of the device – the key parameter that can be controlled – should predetermine axonal behaviour.

Constraints

1. The manufacturing methods are limited to those already proficiently harnessed by the Microsystems department.
2. The axons should be steered solely using biomechanical cues, not biochemical cues.
3. The device must be able to be manufactured (but not cultured) in two weeks.

These constraints limit the scope of this project given the limited time and expertise available in this project.

5 Hypotheses and design

This chapter will propose two hypothetical solutions as design elements to incorporate unidirectional growth into a neuronal network. It will start by establishing design principles based on observations from literature about axonal growth (3.1 Neurophysiology in vivo) and the work of others (3.2 Neurophysiology in vitro). The manufacturability will be tested using experiments (6 Experiments).

5.1 Principles from observations

The fundamental basis of these hypotheses rests on the following observations established in the literary research:

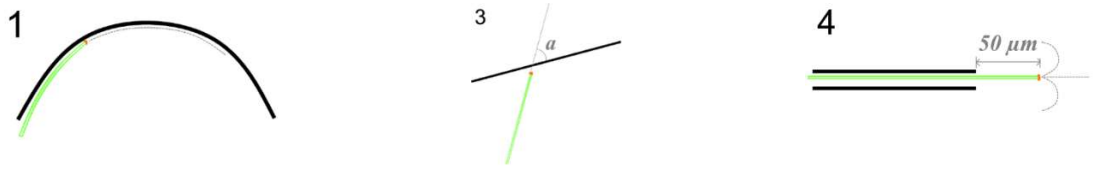


Figure 5.1: Illustrations for the principles where axons are depicted in green and barriers in black

1. Axons are more inclined to follow the edges of physical features in an environment, more specifically the separation between impenetrable and penetrable material. Even if this feature *makes a turn* (Figure 5.1.1).
2. Sharp turns and straight corners make it less likely for an axon to keep following the edge of a physical feature.
3. When approaching from a featureless environment and encountering a physical feature, an axon is more likely to start to follow the feature if the angle of approach (*a* in Figure 5.1.3) is smaller.
4. Axons tend to preserve their orientation after following physical features even when they are no longer present for roughly 50 µm (Figure 5.1.4).
5. If no other hard physical cues are present, axons retain their orientation more in medium compared to a randomly ordered hydrogel.
6. Axons will grow towards other neurons when within a certain range (this range depends on the type of axon and varies wildly).
7. Axons grow significantly faster in medium compared to a randomly ordered hydrogel.
8. When stem cells develop into neurons inside the sieve holes of a microsieve, axons will grow out of the hole either randomly or at the corner edges and largely maintain their orientation afterwards.
9. Axons can grow along other axons

Based on these observations, several designs can be made that could serve as building blocks for building more complex architectures that can mimic nerve systems inside the (human) brain and body of ever-increasing complexity.

5.2 Gelstop

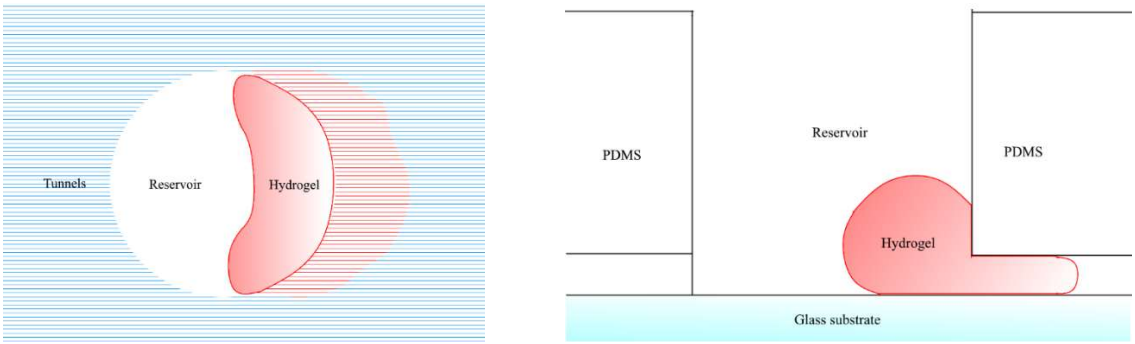


Figure 5.2: A top view (left) and a side view (right) of the *gelstop*. It is a drop of uncured hydrogel deposited onto one side of the reservoir which then creeps slightly into the tunnels which is cured afterwards

Purpose
Hydrogel on one side of a reservoir (henceforth called a *gelstop*) should prevent axons from the reservoir the hydrogel is put into from growing into the tunnels covered by the gel, whilst letting axons entering the reservoir from the tunnels through. It should function as a one-way valve.

Hypothesis
The cultured neurons will spawn axons. The axons will not be subject to any mechanical cues initially. According to observation 7, the axons will likely grow straight towards the edge of the reservoir, the tunnels or the hydrogel.

- When it encounters the edge of the reservoir, it is likely to keep following the edge of the reservoir according to observation 1. The axon is unlikely to enter a free tunnel as it approaches the feature perpendicularly and the tunnel opening is smaller than the orientation preservation range according to observation 2 and 3. The axon can penetrate the hydrogel, whereafter it will start to grow more randomly and/or keep following the edge according to observation 1 and 4. The axon is also unlikely to enter blocked tunnels due to similar reasons as before, though the added randomness makes it even less likely.
- When it encounters an unblocked tunnel, either randomly or due to it being the perceived source of attractive chemicals, it will grow into the tunnel and keep following the edge of the tunnel according to observation 1 and 3.
- When it encounters the hydrogel from the medium in the reservoir, it will start to grow slower more randomly according to observation 5 and 6. The growth speed difference could be large enough, that incoming axons grow faster into the reservoir than outgoing axons. Furthermore, since no fluid flows through the hydrogel, attractive chemicals from the neuron on the other side cannot reach the reservoir, making it also less likely the axon will grow into the blocked tunnel.

An axon approaching the gel from the tunnel side, however, is more likely to continue through the tunnel according to observation 1 and 4. When incoming axons exit the hydrogel on the reservoir side, the axon is very likely to connect to the neuron(s) in the reservoir, according to observation 5. In total, the *gelstop* makes it much more likely for axons to grow from the tunnels to the reservoir compared to the other way around (illustrated in Figure 5.3). Thereby facilitating predetermined one-way axon connections.

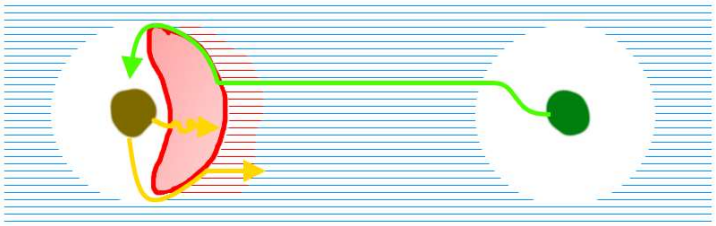


Figure 5.3: A top view of the hypothetical working when the axon grows into a gelled reservoir (green) and out of a neuron in a gelled reservoir (yellow). The green axon is reaching the yellow neuron faster than the yellow axon will reach the green neuron

5.3 Barrier funnel

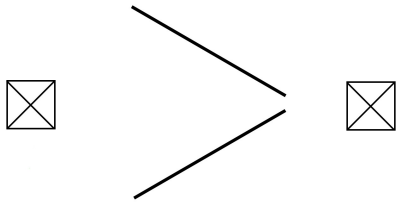


Figure 5.4: The two impassable barriers on the microsieve

Purpose
The two narrowing elevated barriers (henceforth called a funnel or a V-piece) should – similar to the gel stop on microtunnel devices – prevent axons from growing through from the narrow end to the wide end, whilst enabling growth through the funnel. It should function as a one-way valve.

Hypothesis
If hPSCs are trapped by the sieve-holes and grow into neurons from that position, axons will grow out and maintain their orientation according to observation 7. Since there are no other physical cues than the substrate it rests on and possibly chemical cues emanating from surrounding neurons, there are no cues steering the axon in any direction and as such grow in a random but maintained trajectory according to observation 4 and 6.

In a situation where only two adjacent holes are filled with neurons with this funnel placed in between, some neurons are expected to encounter this feature.

- When the axon approaches the feature from the wide side, it is expected to follow the physical cue towards the narrowing according to observation 3. It can then be expected that the axon will follow into the cavity because the axon only needs to follow the current orientation to grow through the hole according to observation 3 and 3.
- When the axon approaches from the narrow side, it is expected that the axon will also grow along the physical cues provided by the funnel due to its sharp angle of approach according to observation 3. The axon is then expected to grow away from the other neuron according to observation 4 and 6.

Besides, the probability of an axon randomly growing through the hole from the narrow side compared to from the wide side (heavily based on the assumption of random outgrowth as with observation 8) is very small. Hence, axons should only grow from the neuron on the wide side to one on the narrow side and not the other way around. Possible growth trajectories are visualised in figure 5.4.

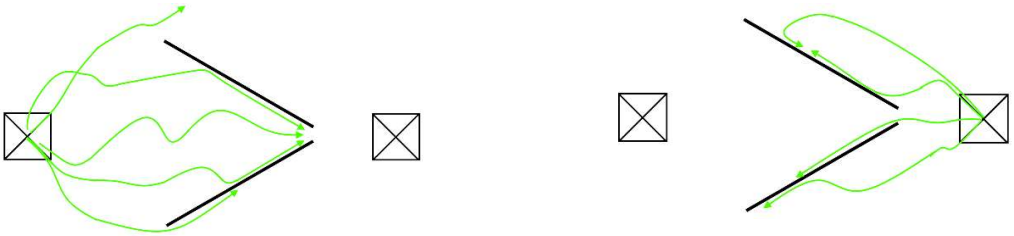


Figure 5.5: The two scenarios for when the axons approach from the wide side (left) and from the narrow side (right). Even if the axon makes contact with the funnel from the outside, it is still likely to follow its intended purpose, as the contact angle is still small. Axons are shown in green.

6 Experiments

Hydrogel

The purpose of the hydrogel experiments is to assess attachment and to characterise gel behaviour in the reservoir and with the tunnels.

Several preliminary experiments were conducted in a kitchen setting using gelatine both as hydrogel and as device material to assess difficulties for the following experiment. The procedure and results can be found in appendix C. Its most important findings were that gelatine can reasonably be placed on one side of a reservoir without accurate pipetting techniques and that the gelatine quickly creeps into any cavities.

The hydrogel experiment used the following procedure: Firstly, a microtunnel device was created (as described in 3.2.1 Microtunnels and reservoirs device). This was a radial microtunnel device with one central and five surrounding reservoirs with a diameter of 2 mm. The device was 4 mm thick and the tunnels were 15 µm wide. The tall, narrow tunnels made for a worst-case scenario for accurate pipetting as shallow wide reservoirs are easiest. As a hydrogel, gelatine was used again as biocompatibility was not strictly necessary for this experiment and since gelatine is also a randomly ordered hydrogel, it makes for a reasonable substitute for an extracellular matrix. The collagen was mixed with water with a ratio of 2:3 in mass. The gelatine was pipetted into the reservoirs one µl at a time. The gelatine was cured for one hour in a refrigerator. The result was imaged using a microscope.

Untreated SU8-on-NOA81 photolithography

The microsieves have been made as described in the literature research (3.2.2 Microsieve device). The rest of the procedure differs per type of photolithography. SU8-on-NOA81 was tried first followed by NOA81-on-NOA81.

First, a (cleaned) glass sheet was plasma oxidised using a plasma asher at 70 W for one-and-a-half minutes. The Ordyl was then laminated onto the glass sheet at 115 ‘C and 3 bar for easier removal. On top of this, the microsieve is placed and attached to the Ordyl using some consumer grade tape (not over the sieved area). The flat side was attached to the Ordyl. Then, SU-8 was spin coated on top of the microsieve first at 500 rpm for ten seconds and then 1500 rpm for thirty seconds. This thins the SU-8 out. As the Microchem SU-8 guide stipulates [32], This should result in a 10-20 µm layer for the SU-8 2010 used. The UV-exposed structure can be seen in figure 9.1. The structure was then soft baked on a hotplate at 95 ‘C for three minutes (again in line with the guide). After aligning the photomask (which involves some complications discussed later), the photoresist was exposed to UV-light at 140 mJ cm^{-2}. The post exposure baking was done at 95 ‘C for four minutes as without a slow cooling process. The result was then developed in mrDev600 for three minutes. The final cleaning step involved isopropanol alcohol and compressed air.

As the first experiment proved not to have satisfactory adhesion, several variables were changed to increase adhesion. The second spin coating step was done at 2500 rpm instead of 1500 rpm. The soft baking step was prolonged by thirty seconds and at a temperature of 110 ‘C instead of 95 ‘C. The exposure intensity was drastically increased to 400 mJ cm^{-2}. The post exposure baking was also done at 110 ’C. All variables have been tested separately.

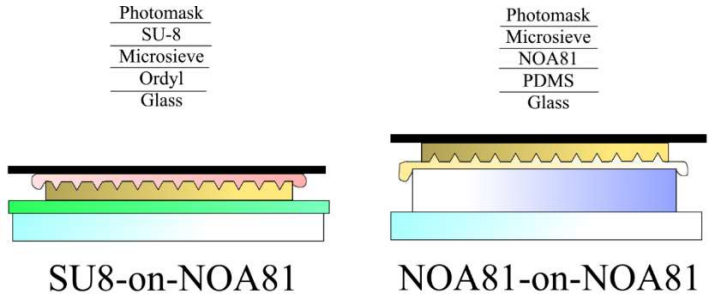


Figure 6.1: The final assemblies to be UV-exposed. The UV-light will shine from the top down. The structure for SU8-on-NOA81 photolithography is seen on the left and for NOA81-on-NOA81 photolithography on the right. Note that there is a larger distance between feature and photomask on the right, indicating a possible inaccuracy.

Aligning the photomask without a mask aligner was not too difficult. The required accuracy is not very high, as the only requirement is that the radial barriers are in the microsieve area but not the exposed centre. Since both features are visible with the naked eye, precision alignment is unnecessary. It was done by using a consumer grade permanent marker on the glass plate to mark where the microsieve area is on the other side of the photoresist. By using a torch underneath the assembly, the mask taped to a glass plate can be aligned. For more precise features such as the ones described in the hypothesis, a mask aligner is advised.

One challenging aspect was to keep the layer between the mask and the photoresist as thin as possible (preferably directly on the photoresist). A thick layer can distort the UV-light after the mask, reducing accuracy or not let the light reach the bottom of the photoresist layer meaning it does not cross-link.

Another challenging aspect was keeping the photomask as level as possible. The thickness of the photomask is greater than the width of the slits of the would-be tunnels in the radial mask. This means that even small angles can block all UV-light from passing through the slits, thereby incorrectly patterning the tunnels. The mask has been tried to be levelled by taping it to the top glass plate and by simply supporting the mask onto two other objects of a similar height.

NOA81-on-NOA81 photolithography

After cleaning a glass substrate and a ~2 cm² by ~2cm² flat PDMS sheet, both were plasma oxidised for 30 seconds at 3 W to bond them together. This step is necessary as the NOA81 cannot be put directly atop glass as NOA81 sticks to it. The NOA81 was then spin coated on the PDMS at 500 rpm for 10 seconds followed by 800 rpm for one minute to create a thin, even layer. A microsieve was then put atop the structure with the cavities making contact with the NOA81. One difficulty that was experienced was locating the sieve after this step, so it is advised to mark this spot beforehand on the glass sheet to assist mask alignment. The structure as can be seen in figure 9.1 was then exposed to UV-light. Several experiments were performed at different intensities (2100, 4500 and 6000 mJ cm^{-2}). The patterned microsieve was then peeled off and rinsed with acetone followed by isopropanol alcohol and compressed air.

Treated SU8-on-NOA81 photolithography

Due to the unsatisfactory results of the first SU8-on-NOA81 experiments, experiments were conducted using adhesion promoters. These adhesion promoters are described in the literature research (SU-8-on-NOA81 photolithography). To create a faster method for creating flat homogeneous cured NOA81 layers, the NOA81 was spin coated directly onto glass using the same spin coating settings and cured at a dose of 2100 mJ cm^{-2} and an irradiation of mW cm^{-2}.

The MCC primer 80/20 was experimented with first. Since this mixture appeared not to be readily available, it was made during the experiment by mixing half a gramme of HMDS with two grammes of mrDev-600 developer acetate. After removing moisture using compressed air, the 2.5 grammes of mixture was put onto the NOA81 layer. This was left to interact for 20 seconds. Afterwards it was ‘spin dried’ for 30 seconds at 4000 rpm and then baked at 110 ‘C for 2 minutes. The same SU-8 lithographic steps were then taken as described before.

The second adhesion promoter that was experimented with was silanation. This was performed using a vacuum desiccator with 20 µl of perfluorodecyl-1H,1H,2H,2H-trichlorosilane (which functions very similar to the earlier described APTES). The samples were left in the desiccator for 13 hours.

Since many silanated samples were created, half of the samples were not put into developer immediately after the post exposure bake of 4 minutes, instead they were left to bake for 20 minutes and then the hot plate was turned off to cool down back to room temperature which lasted 34 minutes. This was also done with additional non-silanated samples. Different exposure doses were tried to retrieve which dose is optimal. The doses tested were 280, 420, 560, 700, 840, 1000 and 1500 mJ cm^{-2}.

7 Results

Hydrogel

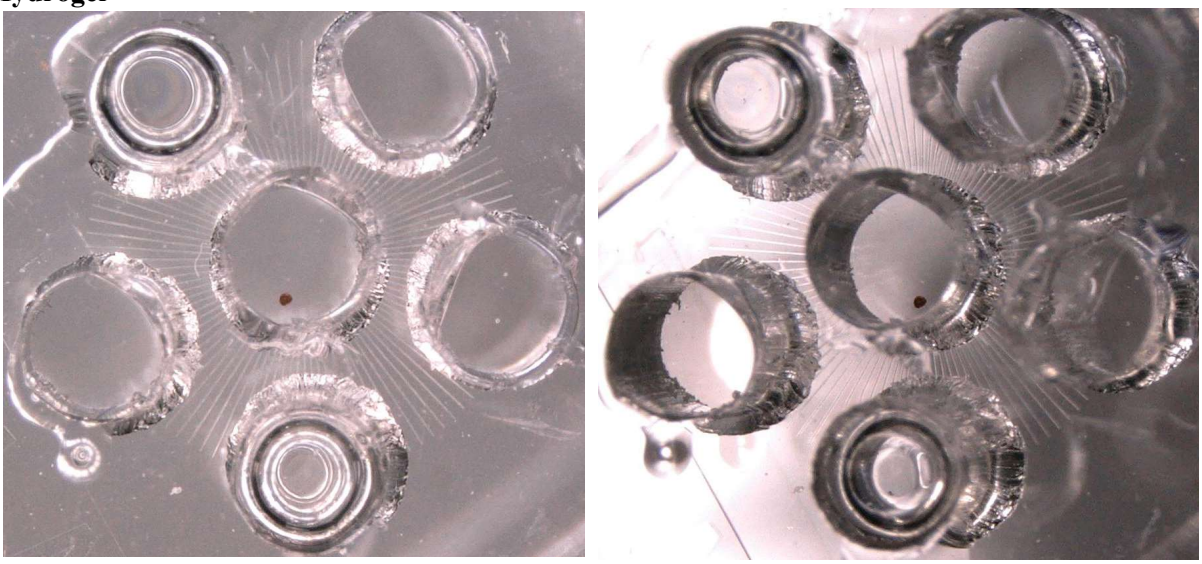


Figure 7.1: A top (left) and angled (right) view of the reservoirs in the gel experiments

As can be seen in the top left and bottom reservoirs, when too much hydrogel is used, the hydrogel spreads across the entire perimeter of the reservoir without any gel presence in the centre. This indicates that potential energy is lost when in contact with the PDMS with respect to both the glass substrate and open air. From the top view, the hydrogel seems accurately placed on one side in the other reservoirs, when looking at the angled view though, it becomes clear not all the gel has reached the bottom of the reservoir.

Less obvious in Figure 7.1 is that some tunnels are filled with hydrogel, whereas others are perfectly devoid of it. This indicates (as expected in the literature study) that no forces impede the hydrogel from creeping further into the tunnel once already in.

Untreated SU8-on-NOA81 photolithography

The SU8-on-NOA81 photolithography was unsuccessful. The first experiment yielded only some dispersed ridges with a shallow slope that appear to be somewhat concentric where the original opening and the radial tunnel device would be placed. Though, the circle seems to have shifted over time.

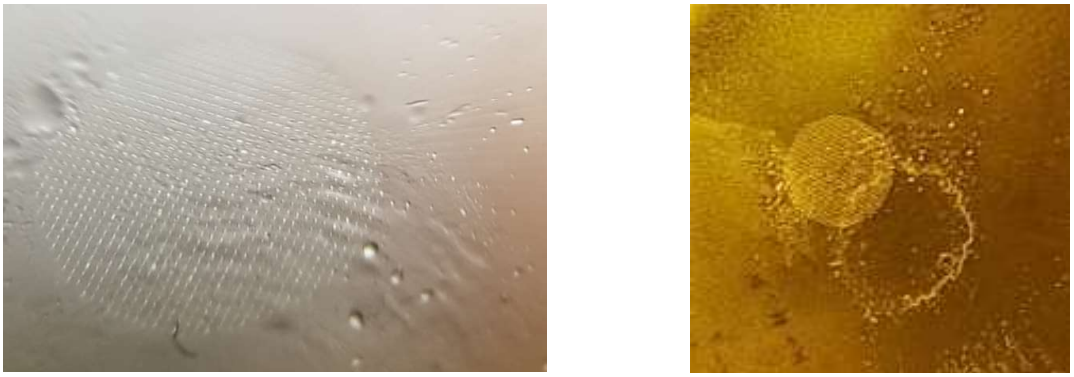


Figure 7.2: The result of SU8-on-NOA81 photolithography from under a microscope (left) and in a larger view (right). The waves seen in the microscopic picture are SU-8 ridges. These ridges appear to be concentric as observed in the larger view

It indicates one or several required aspects went wrong. It is possible that the final rinsing step involved too much or too pressured isopropanol alcohol that damaged the structures. In any case, the features did not appear cross-linked enough nor strong enough attached to the microsieve. The second experiment attempted to combat this by increasing the UV-light intensity, prolonging the baking steps, thinning the NOA81 more and reducing the final amount of isopropanol alcohol. None of these measures appear to have had strong enough an effect on changing the outcome. This means that for SU8-on-NOA81 photolithography to work, a pretreatment step needs to be taken in order to ensure appropriate bonding.

NOA81-on-NOA81 photolithography

The NOA81-on-NOA81 photolithography was more promising than untreated SU-7. It shows the intended radial barrier features which indicates that the mask alignment was sufficient. It shows radial barrier outgrowth though this seems to stop once approaching the microsieve itself, which likely indicates that the microelectrode structure present on the microsieve hinders the growth of these barriers. The barriers are also significantly wider and coarser than intended. The microsieve holes are 20 by 20 µm and each barrier is supposed to be 15 µm wide. As can be seen, the barriers are significantly wider than 20 µm. the central reservoir is also much larger done it should be. These are signs of over exposure where more NOA81 is cross-linked than desired due to too intense or too long UV-light exposure

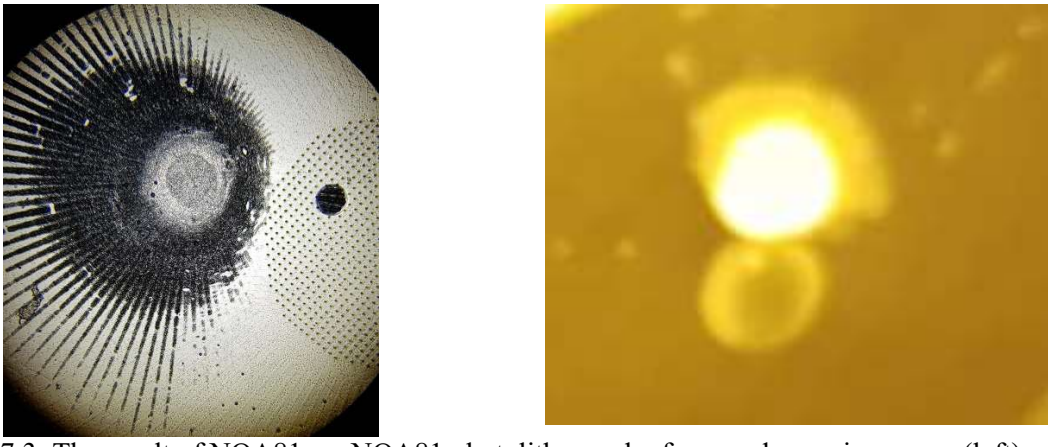


Figure 7.3: The result of NOA81-on-NOA81 photolithography from under a microscope (left) and in a larger view (right).

Halving the exposure intensity seems to have reduced the size of the central overexposed region. Another observation that can be made is that only a narrow set of barriers was created. This likely has to do with the mask being two angled with respect to the collimated UV-light. Since the photomask is thicker than the weight of these tunnels, even a small angle can block UV-light from passing through these slits. This highlights the importance of keeping the mask levelled. The barriers are thinner, but still overexposed.

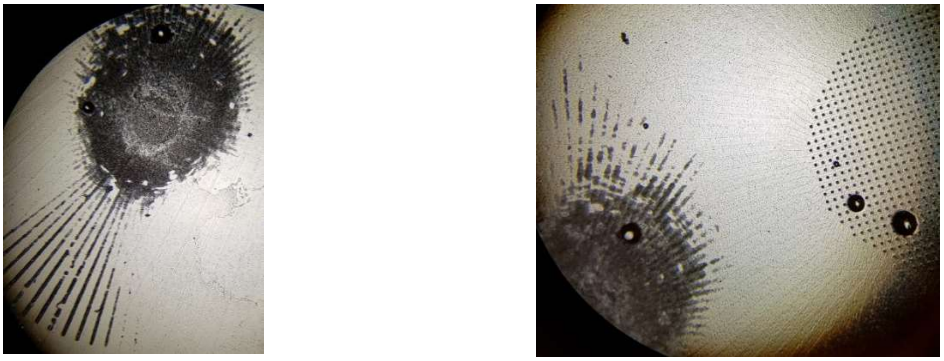


Figure 7.4: The result of NOA81-on-NOA81 photolithography with halved UV-light intensity from under a microscope (left and middle) and in a larger view (right).

Another attempt of half intensity photolithography with extra care for levelling the photomask showed finer barriers though once again only at a band of the total circle. The centre of the circle seems to have broken off somewhere during exposure and some cross-linking seems to have taken place after this point, or some barriers were more thoroughly cross-linked before the centre broke off. The barriers are thinner and closer to the intended 15 µm. It still does not show much consistency with regard to putting tunnels atop the microsieve.

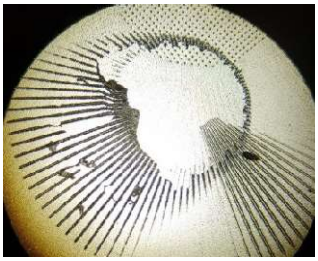


Figure 7.5: The result of NOA81-on-NOA81 photolithography with halved UV-light intensity from under a microscope (left) and in a larger view (right).

Treated SU8-on-NOA81 photolithography
Both treatment methods the managed to increase adhesion.

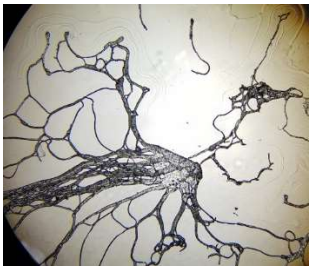
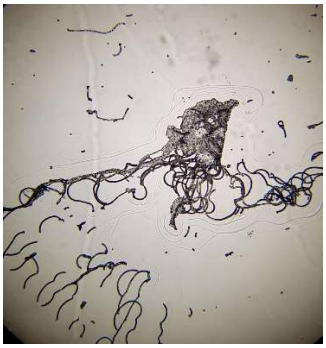


Figure 7.6: The result of SU8-on-NOA81 photolithography with acetate and HMDS treatment.

The acetate and HMDS treatment managed to increase adhesion to a degree where some SU-8 stuck to all areas where it had been intended to be placed. The bonding was still too weak for the features to retain their shape.



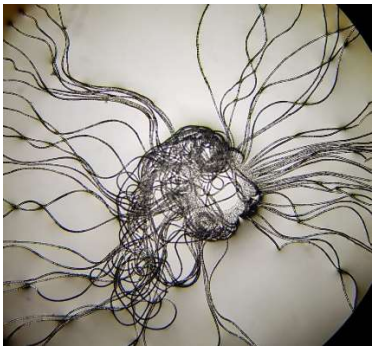


Figure 7.7: The result of SU8-on-NOA81 photolithography with a slow cooled PEB (left) and increased UV-light intensity (right).

A slow cooling step after a longer post exposure bake increased adhesion and additional UV-light intensity cross-linked the barriers enough that the barriers did not break upon developing. It still does not sufficiently adhere to the substrate, though it adheres notably better at the ends compared to near the centre.

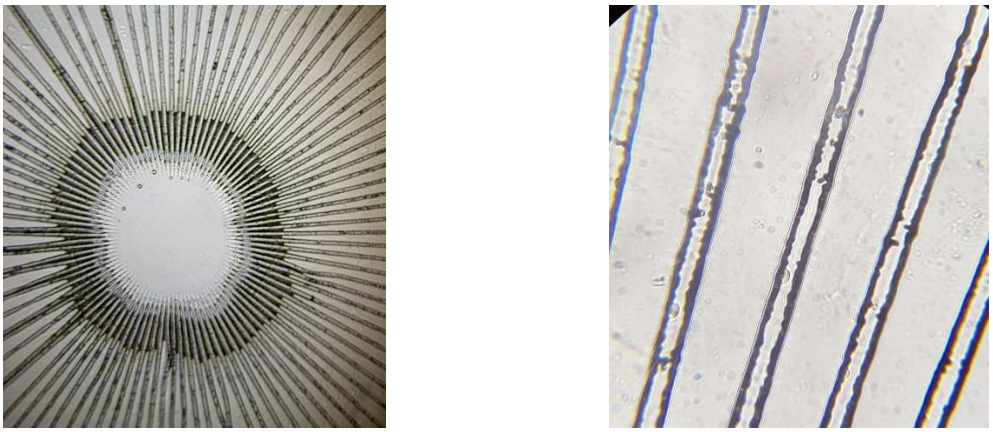


Figure 7.8: The result of SU8-on-NOA81 photolithography with a slow cooled PEB, silanation and an intensity of 840 mJ cm^{-2} or 1000 mJ cm^{-2} at the centre (left) and in detail (right).

Silanation, a slow cooling step and an intensity of either 840 mJ cm^{-2} or 1000 mJ cm^{-2}. This showed the most successful barriers. Below these intensities, the same effect as shown in Figure 7.6.2 occurs, whereas too much exposure leads to a larger centre comparable to Figure 7.3. It still shows slight imperfections which leaves room for improvement (for example by better cleaning). At these conditions, the barriers are still not perfectly conform the photomask, as some barriers still bundle together (as can be seen in Figure 7.7.1). It is a minor version of the entanglement effect where adhesion to the substrate is lacking. This seems to be a rare occurrence, however.

8 Conclusions

Hydrogels can be placed on one side of the reservoir and they penetrate the entire tunnel or not at all. Wide and shallow reservoirs make for more accurate gel placement and care must be taken that the bottom of the reservoir is reached if reservoirs are narrow. It is advised to check the capillary gel-tunnel behaviour of a specific hydrogel used before placing it into the desired device. Axon behaviour with these hydrogels remains untested.

SU8-on-NOA81 photolithography requires some pre-treatment step to ensure either both materials are hydrophobic or hydrophilic or can at least bond to each other. Silanation using, a slow post exposure bake cool down and a UV-light intensity of 1000 mJ cm^{-2} yielded the best results among the methods tested and is capable of creating barriers with the desired width. Axonal interaction with these barriers has not been tested.

NOA81-on-NOA81 photolithography is capable of producing barriers, though they cannot reliably reach the microsieve, are overexposed, and only a seemingly random band of the entire circle spawns barriers. An intensity of 2100 mJ cm^{-2} or lower is recommended.

The micro electrode structure on the microsieve hampers the ability of barriers to grow. During fabrication, care needs to be taken to align the mask as to not cause any unnecessary angle blocking light from passing through the slits.

9 Discussion

Results and literature

The results of the experiments may be more influenced by control variables not being kept constant enough than made out in the conclusions section. Considering the limited laboratory experience at hand and small sample groups, this is not surprising and is expected to generally cause inaccuracies. The conclusions that have been made from the observations are carefully made with this discrepancy in mind. The distance and angle between photomask and substrate in particular is the most concerning variable that was not kept very constant, that still influences that fineness of the barriers greatly. Another issue that greatly affects adhesiveness is the degree of cleanliness of the substrates, which was subjectively unsatisfactory.

A study or manual on how to perform SU8-on-NOA81 lithography or SU-8 lithography on unusual substances in general seemed to be non-existent. Instead, papers on single complete devices using SU-8 or NOA81 as an ingredient were abundant. This presents an opportunity for new researches to be conducted to either summarise all findings of others (literature oriented) or conduct experiments like these to obtain optimal methods for more substrates (experimentally oriented).

Project

The results that have been achieved are minimal, though useful. During the project, a conscious decision was made to forego culturing cells on an unfinished barriered microsieve and instead focus on more thoroughly developing SU8-on-NOA81 lithography. The conclusions of this paper are already being used to further develop axon steering capabilities, which can be seen as a success.

In the initial phase of the project, the central problem was not sufficiently analysed in the beginning phase. This mainly led to difficulties in adapting to change, as the procedure to reach the strictly defined goal also seemed fixed rather than flexible. It ultimately led to unnecessary work and trouble keeping order. The root cause of it were incorrect expectations. Considering minimal but useful results were obtained, it still leads to this project on a whole to be a success.

Appendix A: Interview questions

Interviews were held with different researchers from the CONNECT research group. Each of the interviewees were asked these same questions. The questions that were asked are:

1. How do you understand "*controlled axon growth*"?

Controlled axon growth is an ambiguous term. *Axons* are unambiguous, but *growth* and *control* can be interpreted differently depending on the person in question. *Control* can either perceived to be directional steering, chemical/protein steering or even refer to control theory. *Growth* is less ambiguous, but some may interpret it as the growth of the signal within the axon, not the axon itself. This question serves to clarify what the interviewee means with *controlled axon growth* irrespective of what I assume it to be.

2. Could controlling axon growth be useful for your research and if so, in what way?

This is a very open-ended question devised to discover what exactly the researcher wishes to use this device for. The product should be tailor-made for this purpose. Ultimately, product only meets the requirements if the answer to this question can be achieved.

3. What axon paths and neuron connections would you want to make if possible?

As the previous open-ended question may not always achieve the desired level of depth in the answer, this question is asked to obtain this level of depth as it forces interviewees to think about a practical use case. Furthermore, as predicted "accurately *simulate in vivo conditions*" is a very common demand. However, this can mean different things depending on what aspects where in the body the researcher wishes to replicate. After all, brain tissue does not behave the same as peripheral nerve attachment points.

4. What data would you want to collect in a chip where the axon growth path can be controlled?

The only way to know what happens any chip is by some form of data collection. This can be done in different ways, and it is important to know which methods are preferred. It also drastically changes the layout of the design. For example, if the only method of data collection is via electrodes, autofluorescence is not a problem anymore. However, if optical methods are preferred, then autofluorescence must be accounted for.

5. Do you experience any issues with reliably growing SH-SY5Y or cultivating hPSCs into axon-growing neurons with current chips?
6. Do you experience any issues with collecting data with current chips?
7. What pitfalls can you not overcome (if any) or would you like a less cumbersome method for when working with nervous system-on-Chip?

The last three questions are there to locate current problems. It is not necessarily the intention to improve upon each of these areas. Rather, it is intended to know which constraints are in place and which problems may not be exacerbated further. For example, if cells are not able to stay alive for long enough, this project does not aim to completely alleviate this problem by making them stay alive long enough, more so not to exacerbate the problem making the cells die even earlier. It would be nice to solve all problems, given the scope of this project that is likely to be unfeasible.

From the enquiries, the following requirements, preferences, and constraints are derived (important focus points are bold faced).

Appendix B: Responses from individual interviewees

Name: Dr. Demircan Yalçin

Requirement:	- consistent control groups.
Preference:	- "recreate structure present in human body" as closely as possible ↳ flexible enough to do this
Constraint:	- autoflourescence - no work on stemcells

1	"manipulate neurons, to control the axons to grow in specific paths using chemical or physical cues."
2	Yes, we try to have a control and disease groups to ensure consistent growth and ~~see~~ compare accurately
3	i don't know, recreating the ~~tu~~ structure present in human body found in literature, it needs be flexible enough to do this,
4	Neural network ~~neuro~~ electrophysoligical and morphological data to see if it meets expectations
5	Lots! Generally, too much variability, either due to materials, coating ~~mat~~ materials or freezing procedure
6	Yes, very few problems with electrode variant, but optical one has autofloorecense
7	No not really, it is difficult to deal with stemcells because they are super vulnerable to change in behaviour when subjected to work.

Name: Drs. Rantaxtaro

Requirement:	- Less noise when measuring transcent electrical signals. - Just mimic the body well.
Preference:	- Very precise bonding allignment if we wish to fit electrodes. - cell consistency should not be a problem at all, if the cells are biocompatible
Constraint:	

1	"control the path of the axon "using" the specific device surface and direction geometry".
2	No, for collaborators this is useful, he makes electromechanical electrodes. Positioning of synapses huge problem or just the distance from the electrodes. 1 micron good
3	enteric – central nerve connection, he does not care. As long as it ~~is~~ mimics the human body.
4	Current. from oxidation and reduction. You cannot measure action potential. Electrochemical responce > electrophysiological.
5	He does not use them - he does not like using immortalised cells. If they are biocompatible, there should not be any issues
6	He has not, transent (over time) signal measurement was hard to do due to noise.
7	Bonding allignment must become very accurate. sensor → □ ═══ channel | allignment error.

Name: Dr. Gómez-Giró

Requirement:	- SHOW THAT growth is unidirectional - Must closely resemble brain
Preference:	- Nice if we can combine cell-types - Distinguish between neurons.
Constraint:	

1	way of driving growth of ~~w~~into physically steering axons.
2	Yes, we try to connect organoids, we try to do 3d-structures neuro. we model regions of the brain for parkinssons research "resemble how this is done in the brain" unidirectional happens the tunnels could be useful for her
3	~~indiv~~ Connection between organoids high to mid brain bundles of neurons mid to stratum
4	Show that growth is unidirectional distinguish between ~~int~~ neurons using immunofluorescence She can already see action potentials only used organoids
5	* Longest time one month. (no problems)
6	Bad access view with microscope
7	In the future, problems may arise with combining cell types

Name: Drs. Akçay

Requirement:	- Optical transparency
Preference:	- Improve replicability - Less autofluorescence
Constraint:	- Not have neuron soma grow through the tubes.

1	"How axons can grow through the channels" "mimic brain conditions by layered gels" "cell soma go through unwanted" "Axonal growth preference"
2	"Axonal growth means communication"
3	"Mimic healthy conditions" "Rahman is working on Alzheimer's"
4	Read-out-techniques: micro-electrode array, calcium-imaging, immunostaining
5	"We use SHSY5Y ~~to~~ because it is fast" "Handling hPSCs is difficult" from AKEL is difficult.
6	Of course, "replicability is difficult" which is important
7	Optical transparency important. PDMS is not als biocompatible as desired.

Appendix C: Kitchen gelatine experiment

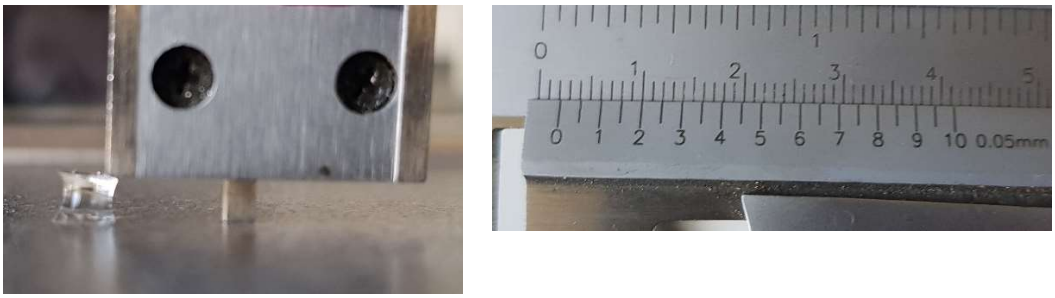
The second experiment was done to get an indication of whether it could be possible to achieve one-sided gel deposition without the use of barriers. In all other aspects, the experiment is very similar to the previous experiment

The premise of this 'experiment' is as follows: It does not simulate the microtunnels.

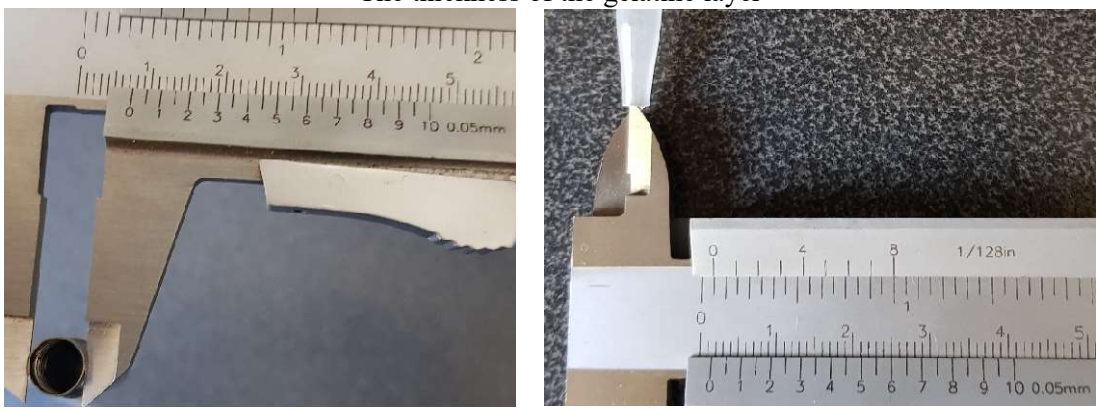
1. Create a base layer of uncoloured gelatine gel in an oven plate. The gelatine mimics the PDMS and the oven plate mimics the glass wafer substrate. More gelatine was used than in the previous iteration
2. Punch holes small from this base layer using a disassembled pen. This will mimic the biopsy reservoir holes.
3. Make new a coloured gel and deposited on the sides of the holes using various methods
    a. using a pipette at an angle
    b. using a pipette pressed against the side of one hole
    c. using a dentistry tool
    d. using a paper clip
    e. using a toothpick
4. After curing, see whether the coloured gel stays on one side of the hole

Process

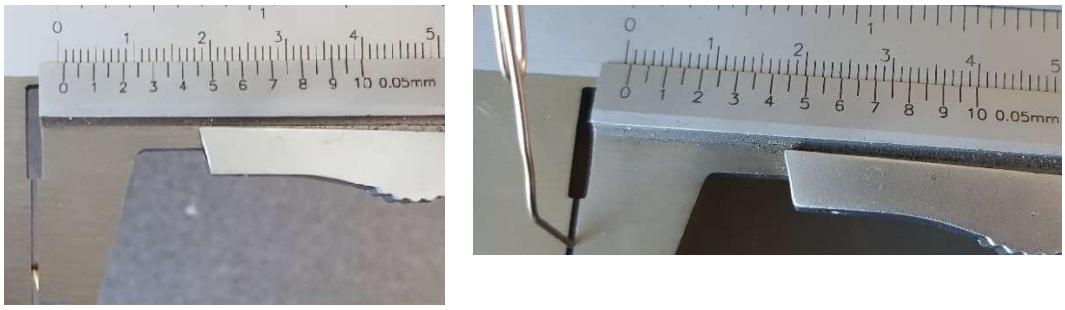
The gelatine layer was significantly thicker this time being 3.20 mm thick (compared to 0.75 mm). The gelatine felt slightly stiffer than last time (which in theory, should be more in line with PDMS). The hole diameter is 6.50 mm. The pipette inner diameter is 1.60 mm. The toothpick is 0.85 mm thick. The paper clip is 0.75 mm thick and the dentistry tool 0.50 mm.



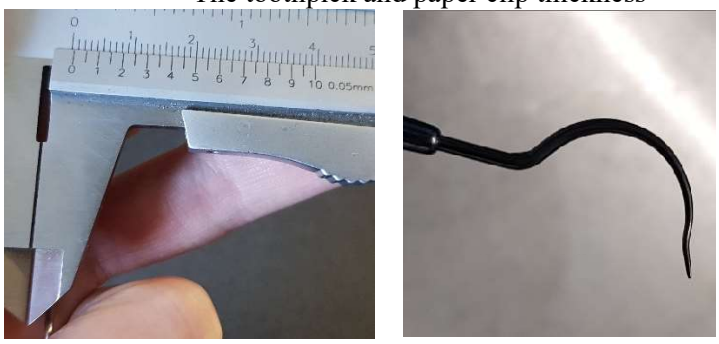
The thickness of the gelatine layer



The hole (left) and pipette (right) diameter



The toothpick and paper clip thickness



The tip of the dentistry tool and its thickness

Figure ?

Results

Once again, the holes could be made without damaging the rest of the structure. The only deposition method that does not work is using the toothpick for the given diameter. However, not all methods were equally successful in distributing the gel *cleanly*. Straight pipetting was successful in dropping a small amount of gel to one side. The gel concentrates a small distance away from the wall to the side though it also creeps towards the wall when contact is made and spreads outwards along the wall. This means a small narrowing occurs between the droplet centre and the wall. This narrowing has the size of the thickness of the pipette tube (which visually was roughly equal to the pipette diameter).

The liquid gel did not sufficiently stick to the toothpick probably because the angle is too sharp. Hence, it was of no use. The liquid did however stick to the dentistry tool and the paper clip. This made it possible to deposit the gel either by sliding it off the wall of the hole or by tapping it on the glass surface. Sliding it off the side made it stick largely to the side but was not very consistent as it depended on how much gel stuck to each bit of the length of the paper clip. Tapping it on the glass surface was even more inconsistent as it depended not only on where the gel stuck to but also to the way it was tapped onto the surface.

Solidifying gel was less consistent and controllable than still very liquid gel.

Pipetting at angle directly pushes the liquid to the seam between the glass and the gelatine. It then spreads around the seam not pushing much upward.

Overview of the results

The result of pipetting pressed against one side of the hole

The result of using a toothpick (left two) and using the dentistry tool (other)

The result of using the paper clip with hot (left four) and already solidifying (right four) gel

The result of using the pipette at an angle

Conclusion

Considering the diameter of the reservoirs in the real chip do not differ much from that of the real devices, these results are somewhat indicative of the real situation. Though, the diameter of the holes on this board are still 150% as large as on the chip, so some methods lose accuracy.

Pipetting at an angle may seem preferable because it pushes the liquid directly onto one side of the reservoir without still have a large presence in the middle of the hole. However in the real situation, this would mean a more liquid creeps into the microtunnels which does not necessarily help with the stated goal; disabling axons from the hole to grow into these tunnels but have external axons still be able to grow towards the hole. Therefore, it is advantageous to block incoming axons as little as possible whereas outgrowing axons must be stopped. This means that less liquid in the tunnels and more liquid on the edge of the reservoir is desired. Furthermore, professional pipettes are designed around straight pipetting, so it may be unsuited to pipetting at an angle.

Having the gel stick to a rod and then sliding or tapping it into the hole is less consistent when it can also be done using pipetting, though it remains an option nonetheless. Besides the inconsistent spread of the gel, tapping or sliding can also damage the reservoir or alter the internal make-up of the hydrogel. Considering that one of the common ideas to expanding the brain-on-a-chip toolbox is to engineer a specific gel structure, this idea is also not future-proof.

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