

# Developing long-term *in vitro* microculture for neuropathology modelling

---

Ola Huse Ramstad

June 2016

Master thesis

Department of Neuroscience, Faculty of Medicine

Norwegian University of Science and Technology

Supervisors: Prof. Øyvind Halaas

Dr. Ioanna Sandvig

Dr. Axel Sandvig



## Abstract

**Background:** The regenerative capacity of the adult mammalian central nervous system is limited, while neuronal responses to injury, including axonal and synaptic plasticity are highly complex and evolve in a spatiotemporal manner. In animal models, the study of regeneration is costly ethically and materially. On the other hand, the study of regeneration *in vitro* is challenging due to poor long-term survival and lack of physiological complexity. Microfluidic platforms allow for highly controlled *in vitro* environments, and partial recapitulation of elemental characteristics of *in vivo* architecture. The development of such a platform is detailed herein.

**Aim:** To develop protocols enabling survival, establishment of modular neural networks and analysis of long-term neuronal co-culture on a microfluidic platform.

**Results:** A series of optimised protocols pertaining to cell loading, fluorescence staining, feeding, and contamination prevention were developed. In particular, cell loading and On-Chip fluorescence required substantial effort. Successful survival of neural culture up to 32 days was achieved.

**Discussion:** The key factors for longevity were found to pertain to initial seeding density, with ionic balance and contamination playing lesser roles than initially suspected. Furthermore, the issues of staining and cell loading protocols are examined, and improvements are detailed. Additionally, future uses and recommendations for long-term culture are given, and improvements to the microenvironment are laid out. In particular, the benefits of microelectrode integration are elaborated.

**Conclusion:** These protocols allow for long-term survival of neurons in culture, and enable study of neuropathologies and regeneration with minimal material cost compared to animal experimentation and conventional culture.



## Acknowledgements:

First and foremost, many many thanks go to my supervisors:

Prof. Øyvind Halaas for teaching me all I know of nanomedicine and pushing this project forward from start to finish.

Dr. Ioanna Sandvig for always being there to answer any question and worry I had, and encouraging me to achieve more with every part of my work.

Dr. Axel Sandvig for lending your immense knowledge on all things neuroscientific.

Special thanks go to Rosanne van de Wijdeven for helping me with damn near everything. Without you this project could never have happened.

The rest of the lab group, Ingrid Lovise Augestad, Vibeke Devold Valderhaug, Anna-Karin Jonasson, for their invaluable support and friendship.

Giovanna Perinetti Casoni, Patricia Moura Rosa, Bjørnar Sporsheim, and Kjartan Wøllo Egeberg for their help with training, protocols and suggestions.

# Contents

<b>1. INTRODUCTION.....</b>	<b>9</b>
1.1. LONG TERM <i>IN VITRO</i> CULTURE .....	9
1.1.1. <i>Issues of neuro specific long-term survival</i> .....	9
1.1.2. <i>Ionic balance</i> .....	10
1.1.3. <i>Cell density</i> .....	10
1.1.4. <i>Contamination</i> .....	11
1.1.5. <i>Glial and neuronal co-cultures</i> .....	11
1.1.6. <i>Mimicking in vivo architecture</i> .....	12
1.2. MICROFLUIDIC CULTURE.....	13
1.2.1. <i>Topography and compartmentalization</i> .....	14
1.2.2. <i>Micro level fluids</i> .....	15
1.3. POTENTIAL OF MICROFLUIDICS IN NEUROSCIENCE .....	16
1.3.1. <i>Basic network dynamics</i> .....	16
1.3.2. <i>Neuropathology modelling</i> .....	17
1.3.3. <i>Aim and objectives</i> .....	18
<b>2. MATERIALS .....</b>	<b>19</b>
2.1.1. <i>Chip manufacture and handling</i> .....	19
2.2. CHIP DESIGN .....	19
2.2.1. <i>The compartmentalized Migration Chips</i> .....	19
2.2.2. <i>The Neuro-Chips</i> .....	20
2.2.3. <i>Cell types</i> .....	23
<b>3. RESULTS.....</b>	<b>24</b>
3.1. IMAGING AND STAINING.....	24
3.2. LIVE STAINING.....	24
3.2.1. <i>PKH26</i> .....	24
3.2.2. <i>Calcein AM</i> .....	25
3.3. IMMUNOCYTOCHEMISTRY .....	28
3.3.1. <i>Hoechst on Chip staining</i> .....	28
3.3.2. <i>Staining on reversibly bonded chips</i> .....	30
3.4. REVERSIBLE BONDING.....	30
3.5. LOADING METHODS.....	31
3.6. MIGRATION-CHIP .....	31
3.6.1. <i>Pressure application</i> .....	31
3.6.2. <i>Pre-loaded pipette tips</i> .....	31
3.6.3. <i>Stationary loading pipettes</i> .....	33
3.7. NEURO-CHIPS.....	34
3.7.1. <i>Droplet loading</i> .....	34
3.7.2. <i>Loading summary</i> .....	34
3.8. COATING ASSAY.....	35
3.9. CHIP FEEDING, EVAPORATION AND CONTAMINATION CONTROL .....	35
3.9.1. <i>Long-term survival</i> .....	35
3.9.2. <i>Flooding protocol</i> .....	37
3.9.3. <i>Well flush feeding</i> .....	37
3.10. FLOW TESTING NEURO-CHIPS.....	38
3.10.1. <i>Full suction of side chamber</i> .....	38
3.10.2. <i>Test of flushing channels</i> .....	39
3.10.3. <i>Integrity of PDL bonding</i> .....	39

<b>4.</b>	<b>DISCUSSION .....</b>	<b>41</b>
4.1.1.	<i>Practical factors for long-term survival.....</i>	41
4.1.2.	<i>Improvements and tests of the loading methods .....</i>	41
4.1.3.	<i>Evaporation and contamination .....</i>	42
4.2.	IMAGING AND STAINING.....	42
4.2.1.	<i>Issues of long-term live staining.....</i>	42
4.2.2.	<i>On-Chip immunocytochemistry.....</i>	42
4.3.	CREATING A LAB-ON-A-CHIP FOR NEUROINFLAMMATION MODELLING.....	43
4.3.1.	<i>Potential issues of design and protocol.....</i>	43
4.3.2.	<i>Integration of biosensors for live monitoring and future recommendations.....</i>	43
4.3.3.	<i>Investigating the shift from acute to chronic neuroinflammation.....</i>	43
<b>5.</b>	<b>CONCLUSION .....</b>	<b>45</b>
<b>6.</b>	<b>BIBLIOGRAPHY.....</b>	<b>47</b>
<b>7.</b>	<b>SUPPLEMENTARY METHODS:.....</b>	<b>51</b>
7.1.1.	<i>Chip manufacture: .....</i>	51
7.1.2.	<i>Chip preparation: .....</i>	51
7.1.3.	<i>Media used: .....</i>	52
7.1.4.	<i>Cell expansion: .....</i>	53
7.2.	LOADING PROTOCOLS: .....	54
7.2.1.	<i>Pressure application:.....</i>	54
7.2.2.	<i>Standard pipette loading: .....</i>	54
7.2.3.	<i>Reversible bonding:.....</i>	55
7.2.4.	<i>Coating assay:.....</i>	55
7.3.	STAINING PROTOCOLS:.....	56
7.3.1.	<i>Calcein optimisation assay:.....</i>	56
7.3.2.	<i>Calcein On-Chip staining: .....</i>	56
7.3.3.	<i>PKH26:.....</i>	56
7.3.4.	<i>Hoechst On-Chip staining:.....</i>	57
7.3.5.	<i>Immunocytochemistry for reversible bonding chip:.....</i>	57

## Table of Figures

FIGURE 1: NEURONAL MICROFLUIDIC PLATFORM, PIONEERED BY TAYLOR ET AL. (2005). .....	13
FIGURE 2: NETWORK TOPOGRAPHIES COMMON IN IN VITRO CULTURE. ....	14
FIGURE 3: LAMINAR VERSUS TURBULENT FLOW. ....	15
FIGURE 4: THE MIGRATION-CHIP DESIGN: .....	21
FIGURE 5: NEURO-CHIP DESIGN:.....	22
FIGURE 6: ABSORPTION OF PKH26 INTO THE CELL SOMA. ....	25
FIGURE 7: CALCEIN CONCENTRATION ASSAY. ....	26
FIGURE 8: COMPARISON OF CALCEIN ON-CHIP STAINING IN WELL AND SIDE CHAMBER.....	27
FIGURE 9: HOECHST ON-CHIP STAINING.....	29
FIGURE 10: SETUP USED FOR MULTIPLE CONCURRENT SEEDINGS WITH PRE-LOADED PIPETTES.....	32
FIGURE 11: DEVELOPMENT OF LONG-TERM NCS CULTURE IN SIDE CHAMBER. ....	36
FIGURE 12: INTEGRITY OF CHANNELS ON A REVERSIBLY BONDED NEURO-CHIP. ....	39
FIGURE 13: INLET FLOW ON NEURO-CHIP WITH FULL SUCTION.....	40



# 1. Introduction

Damage to the adult mammalian central nervous system (CNS) can be devastating no matter if its cause is pathological or traumatic. Functional impairment is frequently a consequence of CNS damage, as the delicate circuits underlying behaviour unravel in the face of trauma or pathology (Siddique and Thakor 2014). Neuronal regeneration is thus a necessity to those injured if they are to regain any function lost.

Although neurons are not entirely lacking in intrinsic regenerative capabilities, the post-injury CNS microenvironment actively opposes their ability to heal. Primarily, the reestablishment of axonal connectivity is inhibited, as barriers to growth form at the injury site and establish a hostile microenvironment. These barriers are known as glial scars (Cregg et al. 2014). Furthermore, the upregulation of a host of growth inhibitory factors post-injury further inhibits axonal outgrowth and reestablishment of synaptic contacts with distal targets (Sandvig et al. 2004).

To study this regeneration, many turn to animals to mimic human injury, illness, and potential treatment. But such *in vivo* study is a burden ethically and may not translate accurately to human physiology. The animal sacrifice required, and the immense complexities of the models, provide strong incentive to reduce this animal use to a minimum. Unfortunately, the recreation of elaborate physiological processes *in vitro* is not a simple matter. Regeneration is a long-term affair, but the *in vitro* environment, due to its very simplicity is not accommodating to long-term assays involving live neuronal culture.

## 1.1. Long term *in vitro* culture

### 1.1.1. Issues of neuro specific long-term survival

To culture neurons, a neuroscientist today can draw upon a multitude of choices, the origin of tissue, size and density of the population, pattern of growth and a myriad of media. Combined, these factors can drastically vary both the nature and the lifespan of the culture (Potter and DeMarse 2001; Yang et al. 2010; Jeong Won Park et al. 2006). Furthermore, the increased understanding of neuronal development and steady improvement of protocol now allows neurons in culture to live for months (Potter and DeMarse 2001). Yet, the majority of current cultures are brief and 2D. Due to ease, conventional neuronal culture takes place in a dissociated monolayer rather than a more realistic 3D architecture. While this removes the *in vivo* character of the culture, it significantly reduces the complexity of experimentation and analysis, providing easy access to the cells for manipulation and high-resolution microscopy. This brief simplified architecture need not be the norm, but long-term survival in culture is challenging. The most significant factor of neuronal longevity is the lack of

proliferation and self-renewal. Once seeded, the population size of a neuronal culture will decline over time. Long-term neuronal survival is therefore a question of stability and prevention.

The stability of long-term culture rests on three main factors:

- Ionic balance (microenvironment control)
- Lack of contamination
- Cell density

### **1.1.2. Ionic balance**

Neurons are fragile cells, with demands for energy, environmental stability and protection exceeding nearly all other cells in the body. Moreover, their uniqueness, i.e. signalling, makes them even more susceptible to external perturbations. Their signalling, maintained by delicate ionic balances, can easily lead to their demise if misbalanced (Viviani et al. 2014). Too high an ionic concentration and the neuron succumbs to its own excitement (excitotoxicity), too low and it finds the same fate through idleness. Thus, the delicate nature of their membrane resistance gives rise to both their key function and key weakness. Moreover, as *in vivo*, this frailty holds true *in vitro* (Potter and DeMarse 2001).

The ionic balance is crucial to cell culture, but neurons face a two-fold issue as not only osmotic pressure, but also signalling demands ionic balance. Without either, neurons quickly face death. Balanced salt solutions are a central part in all cell culture media, but as the media gets depleted/evaporates over time this balance becomes skewed towards salinity. Given enough evaporation the nourishing media turns hyperosmotic, fatally tipping the scales against neuronal signalling and hyperosmolarity. Prevention of evaporation is thus a simple path to longevity in culture.

### **1.1.3. Cell density**

Impaired signalling does not only stem from lack of ions, but also lack of connections within a network. The result is the same however, as neurons starved for input are likely to die. This dependency on electrical stimulation shows with the longevity of the neuronal culture increasing on a near linear rate with the robustness of the network signalling. A common method to increase the longevity of the neuronal culture is thus to increase the density and number of neurons seeded (Yang et al. 2010). This increase in population size not only increases the length of time a viable culture is available for experiments, but also the longevity of each individual neuron (Shein-Idelson, Ben-Jacob, and Hanein 2011). Cell density also brings with it increases in trophic support between neurons, with nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) significantly reducing the death of neurons

early in culture (Delivopoulos and Murray 2011; Mika et al. 2015). The preservation of such factors is reflected in the feeding protocols, where only half the media is replaced per feeding (Yang et al. 2010). The build-up of trophic support is therefore preserved despite renewal of the culture media. Such feeding protocols also reduce the shock of changing osmotic levels inherent in complete media replacements. The increase in population size is the currently most reliable method to increase the longevity of the culture through both trophic and stimulation support. However, with a population increase, and more complex network, analysis becomes more problematic.

#### **1.1.4. Contamination**

Contamination followed by infection has long been an issue in experimental cell biology, and is in no way a problem unique to neurons. Infections by bacteria and mycoplasma, as well as mould and fungus, are hard to combat in any culture, and once active can quickly decimate a culture environment (Shimizu, Abt, and Meucci 2011; Potter and DeMarse 2001). As such, the best defence against contamination is prevention through sterility. Protocol improvement is the best way to ensure sterility, as the environments which cells favour, undoubtedly favour the contaminants as well. Infections can stem from any environment the culture contacts, be it incubator, safety hood or microscope. Isolating the culture and media from any direct contact with open air, fluids and surfaces closes most pathways to infection. In addition to vigilance, some reprieve is found in adding antibiotics and antimycoplasmic agents to the culture media used. Nevertheless, due to the repeated feedings and manipulations required for long-term cultures, contamination is always a potential risk.

#### **1.1.5. Glial and neuronal co-cultures**

The lack of self-renewal in neurons necessitates the addition of other cell types if the population is to maintain its cell density long term. *In vivo*, this is undertaken by glial cells, and in certain sub-regions by neural stem cells. Glial cells, specifically macroglia, are capable of self-renewal throughout the lifespan of the brain (Anderson, Ao, and Sofroniew 2014; Kriegstein and Götz 2003). Astrocytes, the most prominent of the macroglia, provide trophic, structural, metabolic and signalling support to surrounding neurons. This support is vital to prevent ionic misbalance, signal impairment and waste build-up in the neurons local environment. The most effective way to replicate this in culture is through co-culture, with neurons cultured on top of a glial feeder layer (Geissler and Faissner 2012; Götz et al. 2015; J. Park et al. 2012). As the glial culture is able to maintain its population as well as provide support for neurons, the health of the culture increases drastically. Prior to the advent of modern media and

culture practises, such co-culture was the most common manner in which to produce viable neurons *in vitro*.

Similar to *in vivo*, astrocytes also form a proficient supporting cell type *in vitro* (Wanner et al. 2008). The astrocytes in culture proliferate and mature to form a simplified form of the supporting network seen *in vivo*, with the cells acting as a buffer for ionic misbalances and metabolic waste build up. Secondly, the astrocytic effect on signalling in the network is seen through improved stability and rapid maturity of the network.

To summate, astrocytic feeder layers provide a buffer for the ionic balance within a culture, continuously increase its cell density, and improve the intercellular signalling of the neuronal network. In combination, all these factors vastly increase the longevity of neurons in culture, in some cases taking it from mere weeks to several months.

#### **1.1.6. Mimicking *in vivo* architecture**

Co-culture not only allows for long-term culture, but also enables the reproduction of complex *in vivo* networks. Given sufficient complexity of the cellular composition, the culture may begin to approximate the *in vivo* 3D environment as an organotypic culture (Daviaud et al. 2013; Geuna et al. 2015). Such organotypic culture is similar to conventional brain slice culture, where entire cortical structures are explanted and cultured as slices *in vitro*. The main distinction lies in the bottom up approach of organotypic culture, replicating the cortical structure through recapturing the cortical development *in vitro*. Successful organotypic culture allows for a greatly improved approximation of *in vivo* networks and far more accurate experimental data than conventional *in vitro* culture.

A balance must be found however, between the opportunities of *in vivo* modelling and the level of confounding factors arising from it. While glial co-culture greatly increases the longevity of neuronal culture, it similarly adds to the complexity of analysis (Liu et al. 2015; Geissler and Faissner 2012). Cell types respond differently to stimuli, which in turn increase the interaction between them when in culture. The simple addition of a single cell type greatly adds to the complexity of the extracellular milieu, as interlocking signalling factors act in unseen concert. Additionally, the creation of organotypic culture often utilizes 3D environments, which subvert most types of conventional analytic methods demanding transparency to provide accurate focus (Rocha et al. 2015; Kunze et al. 2011; Verhulsel et al. 2014). The balance therefore rests on creating a culture environment allowing long-term culture without compromising the potential for accurate analysis. One way to produce such a highly controlled environment while still allowing for advanced co-culturing is through microfluidic devices.

## 1.2. Microfluidic culture

Reducing the total size of both the culture and the culture environment allows for accurate compartmentalization and manipulation of the culture environment while retaining the resolution required for analysis (Taylor and Jeon 2011). Conventional plate culture typically relies on single compartmental culture consisting of  $10^4$ - $10^6$  cells. With such a uniform culture environment, any diffusible reagent or factor affects the culture systemically. This uniform treatment betrays the localized nature of the neural *in vivo* environment, especially poignant with regard to the gradients of trophic factors throughout the extracellular matrix (Mai et al. 2009). In order to replicate the variability of the *in vivo* environment while retaining the spatial and temporal control of the cells granted *in vitro*, more advanced culture platforms are needed than simple dish systems. Microfluidic platforms consist of culture environments reduced to only a few millimetres in size consisting of chambers and channels with sizes of only micrometres or less with equally small volumes of fluid (Sackmann, Fulton, and Beebe 2014). At such scales, the microenvironments offer control of single cells and even sub-cellular components, through the topography and fluid manipulation of the system (Bugnicourt et al. 2014; Millet et al. 2010). Combined with micro electromechanical sensors (MEMS) complex, live analysis can be made of the culture throughout the platform (Krause et al. 2006). When combined, in such a manner with MEMS, the microfluidic system is termed a Lab-on-a-Chip (LoC).

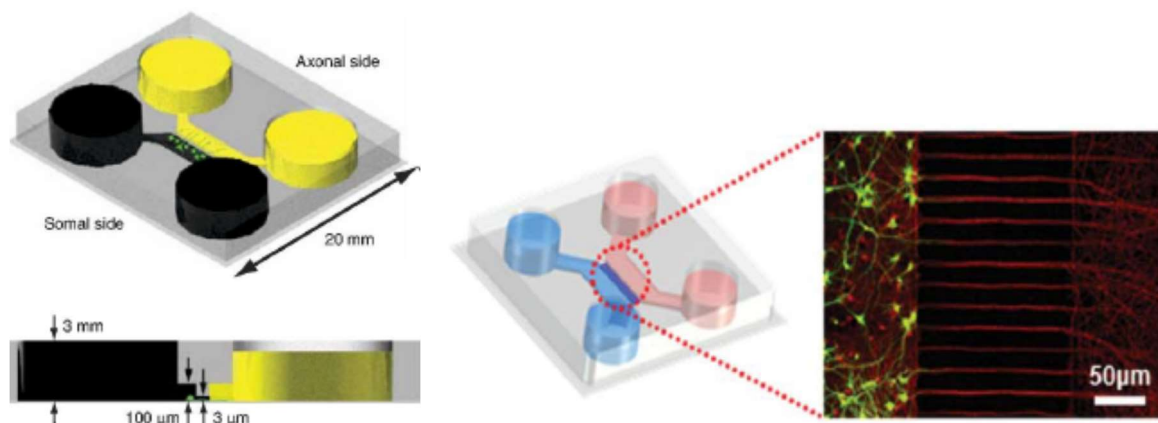


Figure 1: Neuronal microfluidic platform, pioneered by Taylor et al. (2005).

Note the large circular wells used for fluid manipulation, and the central culture area with connecting axon growth channels. The height of the axon growth channels,  $3\ \mu\text{m}$ , seen in the left bottom corner, is miniscule to exclude any soma from entering. Highlighted in the right image is axonal extension (red) through the axon growth channels (Taylor et al. 2005; Jae Woo Park et al. 2013).

The production of these microenvironments is most often performed using soft-lithography. Soft lithography utilizes the accuracy of photolithography to etch a microscale pattern onto a master, which is then be used to cast multiple replications of the pattern negative in a soft elastomer polymer. The preferred polymer for use in biology is polydimethylsiloxane (PDMS) which offers biocompatibility and gas permeability, with low cost and the transparency needed for high-resolution microscopy (Jae Woo Park et al. 2013). As the pattern is formed at the interface between the polymer and the substrate, most commonly glass, the structures formed are largely 2D. However, since the height of individual structures can be varied it is possible to produce chambers for multi-layered co-culture along with channels too narrow to permit cell migration (See figure 1).

### 1.2.1. Topography and compartmentalization

Not only cellular isolation, but also the behaviour of cells can be altered through the design of topography and structural elements (Delivopoulos and Murray 2011). Akin to the ECM *in vivo*, the topography effects migration, cellular adhesion, signalling and differentiation of neurons, something that must be considered during experimental design and comparisons (Bugnicourt et al. 2014; Tan et al. 2015; Moe et al. 2012). Topography permits production of networks with resolution down to single neurons and neurites. A major segment of this is structural compartmentalization.

Compartmentalization is the confinement of cells and cellular segments to distinct areas of the structure, for example, two cell types confined to separate growth chambers, with axon specific growth channels connecting them. The initial designs for neural microfluidic environments by Taylor et al. (2005) set this standard for such compartmentalization. The separation of neurons and neurites remains one of the main applications of compartmentalized microfluidics, due to the possibility of

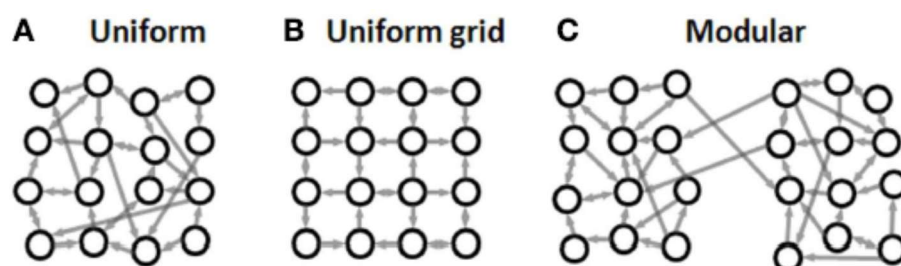


Figure 2: Network topographies common in in vitro culture.

Modular topographies (C) consist of discrete compartmentalized networks (A or B) sparsely connected together. Key to this modularity is the higher ratio of connectivity within the Uniform networks than that between the networks. This allows a hierarchy to be established. (Shein-Idelson, Ben-Jacob, and Hanein 2011).

differential analysis of each segment of the neuron (Taylor and Jeon 2011). Studies in regeneration, network dynamics and intercellular signalling relies heavily on localized manipulation of axons (J. Park et al. 2012; Taylor et al. 2005;

Fricke et al. 2011; Marconi et al. 2012). Compartmentalization also enables culture of multiple subtypes of cells by isolated except through axonal channels. Similar to co-culture, network interactions and connections formed by such cultures can mirror inter or intranuclei networks *in vivo* (See figure 2). Multiple cell types can also be loaded to the same chamber to examine the interactions of multiple co-cultures. In addition to topography, the interaction of cellular compartments can be controlled by creating one directional fluid flow or axonal growth to produce flow of processing akin to cortical hierarchies (Pan et al. 2011; Isomura et al. 2015). Similarly, the exposure of each compartment to differing cytokines is enabled through fluid control and good structural design.

### 1.2.2. Micro level fluids

With microliter volumes, fluid behaviour changes drastically compared to the macro level due to the changing strength of the forces controlling it. At the macro level, gravity is often the leading actor, but as the mass of the fluid decreases so too does the grip of gravity. What arises in its stead is capillary forces, surface tension, and viscosity (Sackmann, Fulton, and Beebe 2014). Combined with a low Reynolds number, a measure of viscosity versus inertial forces, laminar flow becomes the leading fluid dynamic in the microfluidic environment. Laminar flow, as opposed to turbulent flow (See figure 3), is highly ordered and predictable. Understanding the dynamics of laminar flow and the forces reigning on the micro level is essential to the control of microfluidic environments.

#### Laminar versus turbulent flow

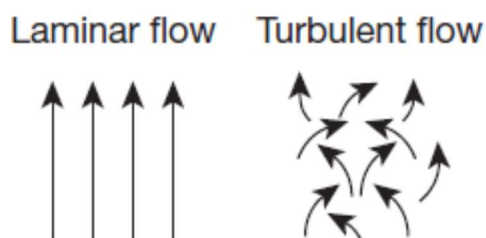


Figure 3: Laminar versus turbulent flow.

*Laminar flow is an unusual property of microvolume liquids, with highly ordered directional flow and nearly no turbulence. This is contrasted with chaotic turbulent flow, which is common to macrovolume liquids* (Sackmann, Fulton, and Beebe 2014).

Capillary forces are prominent due to the narrow constrictions of the channels, causing fluid movement along the length of the channel regardless of external pressure or gravity (Millet et al. 2010). This counter intuitive action stems from the interaction between the molecular polarity and surface tension of the fluid, with the polarity of the material of the channels. The low volume and high surface area of the fluid and channels makes this a significant force on the micro level. This capillary force makes passive control of fluids possible on the LoC, forgoing pump and valve systems needed for

active fluid control. Also enhanced is the force of diffusion, the movement of fluids and particulates from areas of high density to low. Finally, through maintaining uneven fluid volumes on differing areas of the LoC, pressure creates steady directional flow used for addition and removal of reagents to chambers or channels.

Microfluidic platforms are wholly or partially closed systems with the cells enclosed and potential sources of air contamination limited to the fluid inlets (Renault et al. 2015; Potter and DeMarse 2001). This creates far smaller air interfaces than those of conventional dish culture. Contamination is thus a more manageable through control of the fluid interfaces, though it still poses a significant risk. A second hazard is the effect of rapid fluid flow on cells. Shear stress arises when cells adhered to the substrate are caught at the boundary between the fluid and the substrate. Like to standing in a flowing river, if the flow is rapid enough the shear stress will overcome the adhesion and sweep away the cell. While this is unlikely to stem from capillary forces, large changes in diffusion may provide sufficient flow to pose a hazard to LoC cultures. This poses a risk when attempting large changes in media or reagents on the LoC.

In combination, compartmentalization and capillary channels produce the factors necessary for long-term *in vitro* culture by providing high-density culture and fluid control capable of reducing osmolality. Contamination and evaporation is reduced, but still poses a risk to longevity. Vitality, the low cell numbers needed for high-density culture creates a culture platform where complex processes can be modelled without greatly hampering analysis.

### 1.3.Potential of microfluidics in neuroscience

#### 1.3.1. Basic network dynamics

Neural development is a long-term process, taking place across years *in vivo*. Similarly, the development of stable and active neural networks in culture is a long-term process, with signalling patterns requiring weeks before stabilizing (van Pelt et al. 2004; Marconi et al. 2012; Cadotte et al. 2008). In turn, the patterning and topography surrounding the cells influences the stability and maturity of the network. Uniform patterning, though neatly ordered with equally spaced neurons, does not provide the best model for long-term culture (Shein-Idelson, Ben-Jacob, and Hanein 2011). Such spacing compromises two main features of the culture, the cell density and the stability of the network. The reduction in density reduces the number of interconnections between neurons, thus requiring a higher number of neurons to sustain spontaneous activity. In randomly patterned networks, density increases and the number of neurons needed to form spontaneous firing is far lower than that of regularly spaced networks. However, patterning can still be used to modify network dynamics by acting on the axonal connections rather than the cell placement itself. The directions of the axonal connections are influenced by the tapering and patterning of channels allowing unidirectional firing to occur (Honegger et al. 2013; Pan et al. 2011). The connection between several uniform networks can thus be utilized to produce more complex signalling activity than that of single networks.



This modular architecture can be further harnessed to produce hierarchical networks akin to those found *in vivo*. Reproducing this relationship of nuclei should be possible given sufficient interconnections between the compartments and high self-supporting networks within the chambers. For example, the motor pathways of the basal ganglia are mostly linear and consist of nuclei of singular cell types. The hierarchical nature of the cortical layer should also be reproducible within such a microenvironment.

### **1.3.2. Neuropathology modelling**

With stable and interacting networks, the effect of neuropathologies both acute and degenerative is possible to examine (Wanner et al. 2008; Gourov and Curran 2014; Gullo et al. 2014). Neurodegenerative disease is invariably a long affair, developing slowly across timespans of months or years. Similarly, the regeneration done by neurons is a process taking weeks or months to complete if full regeneration is possible at all (Geuna et al. 2015; Cregg et al. 2014; Vishwakarma et al. 2014). Due to the compartmentalized structure of microfluidic culture, localized injury can be induced on sub-populations or specific cell components within the culture (Siddique and Thakor 2014). Inflammatory factors, toxicological factors and nutrient imbalances can be selectively applied to replicate disease processes found *in vivo*, and with long-term culture the degeneration or potential recovery of the culture is open for analysis. A common model of such long-term *in vitro* degeneration and regeneration is axotomy and subsequent regeneration of the axonal pathways. Li et al. (2012) induced axotomy through exposure of the axonal compartment to acrylamide, a neurotoxin. The axonal degeneration was compared to the neurotoxin's effect on the somal chamber with axon exposure showing itself to reduce the subsequent degeneration. Following the degeneration, Li et al. rescued the axonal projections with monosialoganglioside pharmacological treatment and glial co-culture. Therefore, compartmentalization allowed for comparison of neurotoxicity on distinct cellular segments, as well as seeing the effect of co-culture interactions, all in the same microenvironment.

### 1.3.3. Aim and objectives

In the following study, the development of protocols for neuronal seeding, long-term survival, staining and microscopy on a microfluidic platform are detailed. The overall purpose is the production of protocols for operating compartmentalized microsystem devices for structured neuronal co-cultures and provide feedback to microfluidic design for future projects, with a long-term vision of creating replicable neuronal co-cultures of approximate neuropathological models. Protocol development initially focused on adaptation of an established microfluidic platform to suit neuronal culture, as methods for seeding, feeding and fluorescence imaging would be equivalent. Following this, a new platform for long-term neuronal culture was designed with continuing protocol development. The issues encountered and solutions attempted are detailed according to specific protocol goals, e.g. high-density cell loading, and where relevant to the platform design.

Specifically the objectives were:

- Establish protocols for neuronal staining
- Investigate the potential for long-term *in vitro* survival
- Provide feedback and recommendations for microsystem re-design
- Investigate the effect of astrocytic co-culture on survival of cortical neurons
- Utilize microsystem for neuroinflammatory co-culture

## 2. Materials

### **2.1.1. Chip manufacture and handling**

The microfluidic platforms (referred to as chips henceforth) were made by PhD student Rosanne v.d. Wijdeven. The Chips consisted of micropatterned PDMS with a glass surface functioning as the substrate (See Supplementary methods for full protocol). In brief, the PDMS is produced by pouring a 10:1 mixture of elastomer PDMS to curing agent onto the patterned master mould. In the mould, the polymer mixture is degassed to remove any air from the mix before the pattern is solidified through heating. Finally, the PDMS was bonded to the glass coverslip using a Plasma Cleaner and heating at a hot plate. The chips were produced in NTNU Nanolab and transferred to the laboratory in parafilm-sealed petri dishes.

### **2.2. Chip design**

Microfluidic platforms for cell culture are colloquially termed Chips. All Chips used in the following experiments were the first attempts of the laboratory group to establish neuronal microfluidic culture. The initial designs used were originally made for immunological migration assays, however the discrete chambers and connecting channels made the design adaptable for neuronal culture.

#### **2.2.1. The compartmentalized Migration Chips**

The Migration-Chip was designed by the Halaas group, in particular Nimi Gopalakrishnan, PhD.

The following features are common for all such microfluidic culture systems. Openings in the PDMS, termed wells, are used for all manipulations of the culture, from cell loading to feeding and adding reagents. The wells are connected to channels which create access to the culture chambers where the cells are housed. One well and channel is often designated the inlet, or entry channel, while an opposing channel is designated the outlet. Through the drainage of the outlet, flow from the inlet to the outlet occurs, thus allowing flow through the chambers. These chambers insulate against environmental fluctuations seen with open culture systems and allow for clear and consistent imaging compared to an open well.

Specific to the Migration-Chip is a single channel system connecting all growth chambers on the chip (See figure 4). Though initially intended for cell migration, it was hoped that this system of channels would be sufficient for axonal connections. Additionally, a drainage sink was present to provide consistent flow and gradient formation, though this was not used.

Cells were loaded from the top left feeding channel in the side chambers, and the left loading channel in the main chamber. All cells were loaded in the left side chamber and main chamber in this manner.

### **2.2.2. The Neuro-Chips**

The next generation of Chip was the first design specifically tailored to neuronal culture (See figure 5). It therefore had several improvements over the Migration generation. Flush channels were added to all chambers to allow for rapid exchange of reagents in a chamber and provide additional suction from the seeding well. Secondly, the channels connecting the culture chambers were specifically designed for axon outgrowth and imaging analysis. The axon growth channels also allow for future implementation of MEMS, with far wider channels than previously. To improve loading, pillars were added to the outlet and flush channels to discourage cell flow into these channels.

During all experiments with the Neuro Chips, cells were added to the main chamber, with side chambers being confined to provide flow.

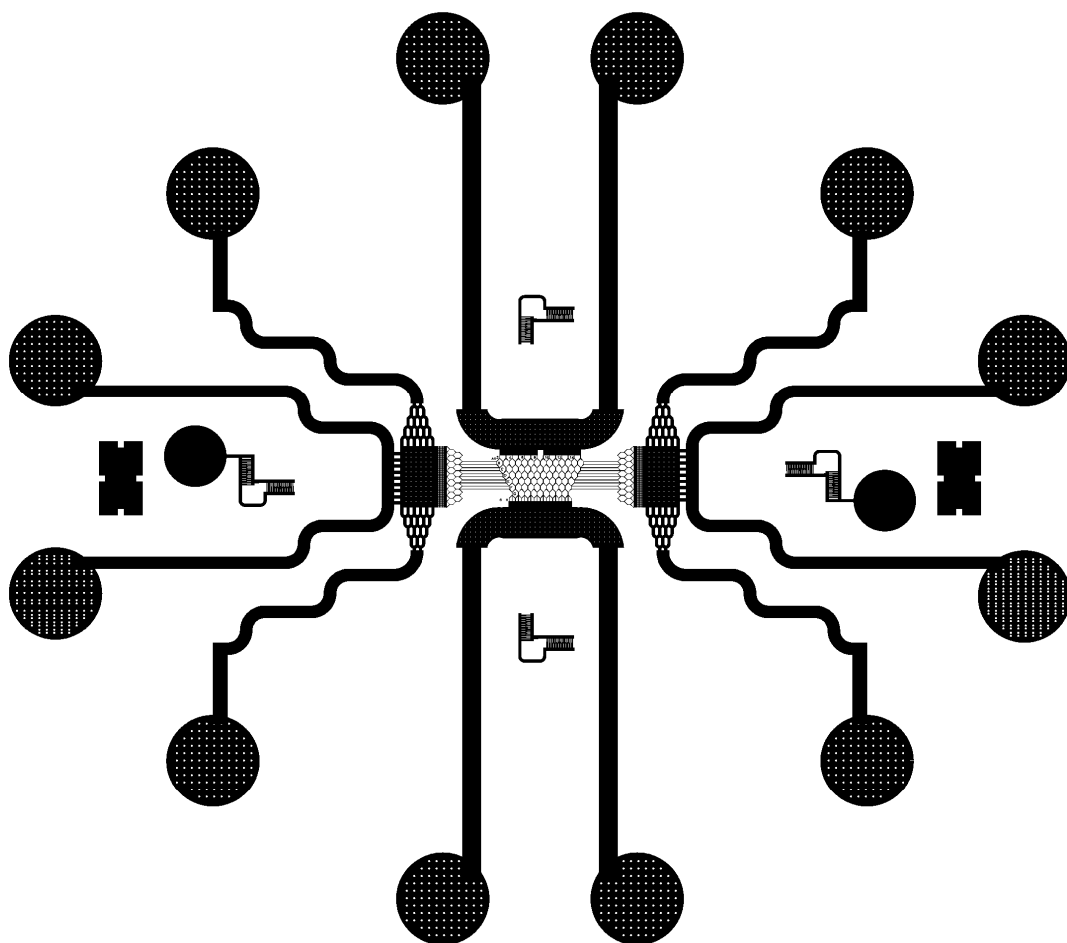


Figure 4: The Migration-Chip design:

The design consists of four core chambers.

The bottommost chamber is termed the Main Chamber, capable of supporting the greatest number of cells. It is connected, via migration channels, to two smaller growth chambers, termed the Left and Right Side Chambers. Narrow filter barriers prevent any cell migration into and out of the Side Chambers, but allows for fluid flow.

Directly opposing the Main Chamber is the Sink, which can be operated to engage flow to and from the migration channels

The Main Chamber is accessed through a direct inlet and outlet channel connected to respective inlet and outlet wells.

The Side Chamber access consists of four channels. The inlet and outlet channels are placed as the leftmost and rightmost channels and wells. Additionally, two Flush channels connect to each side chamber, placed as the topmost and bottommost channels. These enable rapid exchange of media in the side chambers.

The Side Chambers measure  $1000\ \mu\text{m}$  in length and  $600\ \mu\text{m}$  in width, not counting the connecting channels.

The Main chamber measures  $2600\ \mu\text{m}$  in longest and  $1200\ \mu\text{m}$  at the shortest. Its width is  $500\ \mu\text{m}$ .

All wells have a diameter of  $2000\ \mu\text{m}$ .

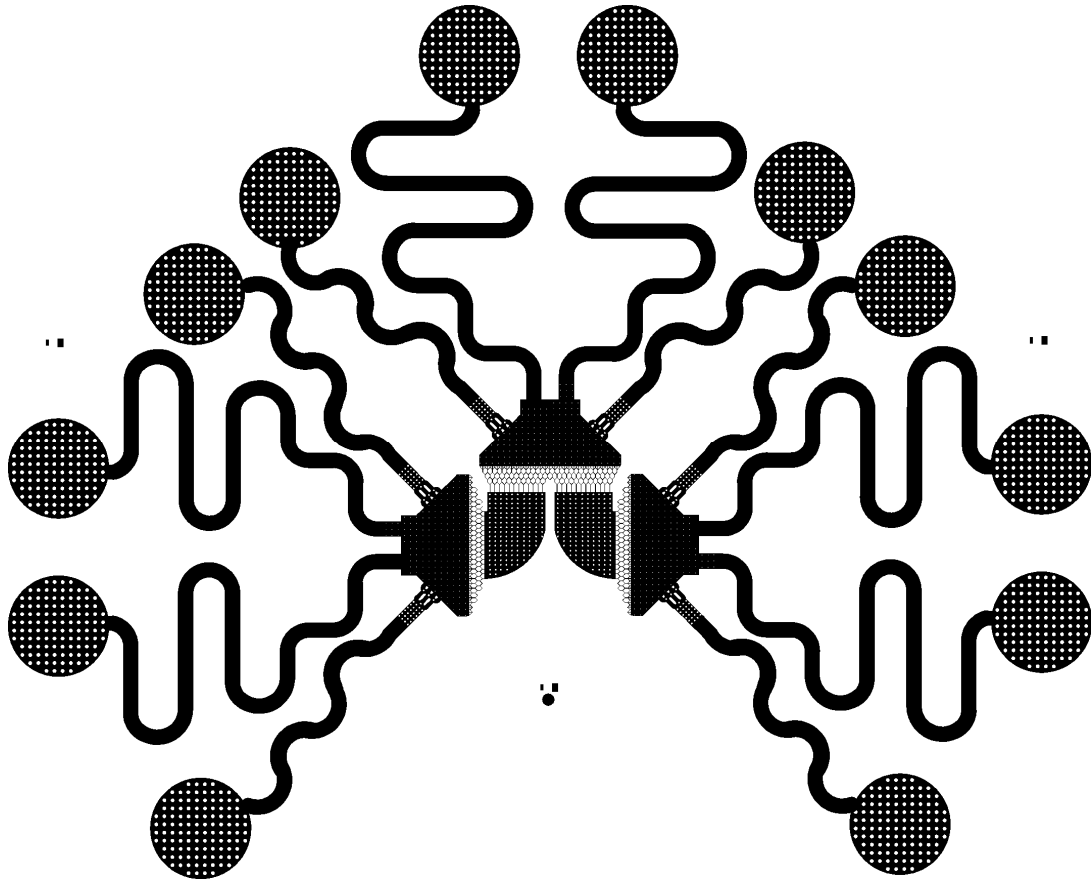


Figure 5: Neuro-Chip design:

*In the core of the design, three cell culture chambers are connected together by two discrete axon growth channels. All three chambers are accessed by an inlet and outlet channel, with two additional flush channels.*

*The inlet and outlet channels connect to the chambers' narrow middle, while the Flush channels, indicated by the splitting just prior to connecting to the chambers, attach nearer the axonal growth channels.*

*The Main Chamber is the topmost chamber of the design, which receives axonal channels from both Side Chambers.*

*The chambers measure  $2800\ \mu\text{m}$  at the widest and  $1200\ \mu\text{m}$  at the narrowest. The length of the chambers is  $1300\ \mu\text{m}$  and height  $28\ \mu\text{m}$ .*

*The axon growth channels measure  $3\ \mu\text{m}$  in height, with  $1000\ \mu\text{m}$  at the shortest connecting point between the chambers and  $2500\ \mu\text{m}$  at the longest.*

*As with the Migration-Chip, the wells' diameter is  $2000\ \mu\text{m}$ .*

### 2.2.3. Cell types

Neurons are non-proliferative, meaning they must be bought from a supplier or sourced directly from animals if they are to be used in culture. Such sourcing is highly expensive, or time consuming and requiring animal sacrifice. Secondly, they are fragile when handled *in vitro*, making them poor candidates for protocol development where rough treatment and loss of cells is to be expected. To circumvent this proliferating astrocytes and neural stem cells were used instead.

**Cortical rat astrocytes** (ThermoFisher): Astrocytes proliferate slowly, but steadily during culture making them suited for repeat experiments and long-term culture. Additionally, they are self-limiting, meaning that once they reach confluence they exit the cell cycle to prevent overgrowth. This combined with their supportive capacities allows them to easily form feeder layers. Strong adherence and ECM production is a key feature of astrocytes, allowing them to adhere directly to any culture vessel in the absence of pre-coated adherence factors. This feature also allows other cells to adhere to the astrocytes in turn. Combined, these abilities make astrocytes resilient cell well suited for *in vitro* culture.

**Neural stem cells:** Two types of neural stem cells (NSCs) were used, Rat Cortical NSCs (ThermoFisher) and Human (H9) NSCs (ThermoFisher). NSCs are multipotent and capable of differentiating into any cells of glial or neuronal lineage. Secondly, NSCs self-organize into neural networks and glial co-culture without the need for extraneous growth factors or manipulation. As *in vivo*, differentiation is highly dependent on both the topography and the chemical composition of the extracellular environment. In conventional monolayer culture, the extent of topographical and chemical manipulation is largely confined to the coating of substrates with ECM factors to enhance adherence.

## 3. Results

See the Appendix Supplementary Methods for detailed protocols and material overview of all procedures listed in these results.

### 3.1. Imaging and staining

As the microfluidic platforms were intended for long-term culture a significant effort was devoted to developing lasting staining protocols suitable for a microfluidic environment. With long-term live stains, time lapse could be utilized to examine axon growth and cell growth within the chambers. Secondly, immunocytochemistry would allow for cell specific and cell segment staining. Unfortunately, staining proved to be a recurring issue throughout the project.

Largely this was due to the minute volumes within the chips rapidly diluting most stains added. High molar concentrations for live stains and reversible bonding of the PDMS to provide direct access to the cells tried to overcome this. Similarly, several live stains were attempted at differing molar concentrations to both stain dilution and bleaching. The dilution factor of the small volumes would also counter immunocytochemistry as the dilution of the antibodies, both primary and secondary, would be pushed into the nanomolar concentration.

### 3.2. Live staining

Throughout the project, multiple staining protocols were tested for live staining both for cells already cultured on the Chip (On-Chip) and prior to seeding them (Pre-Chip). Initially, the focus was on Pre-Chip staining to allow for different stains to be used on different cell types, such as astrocytes and neurons. However, it was realized that the membrane specific live stains would eventually be taken up into the soma, and therefore unable to stain cellular extremities. A shift was therefore made towards staining On-Chip and immediate post-stain imaging.

#### 3.2.1. PKH26

PKH26 is a membrane stain with a very long half-life of over 100 days. It initially showed great promise during culture prior to chip seeding with astrocytes. However, beyond the first 3 days post staining the fluorescence gradually became absorbed into the soma. Within a week, the stain was largely confined to the area immediately surrounding the nuclei, with the cell membrane becoming very difficult to discern from the fluorescence alone (See figure 6). The stain would therefore be unable to act as a marker for any axonal growth long-term.



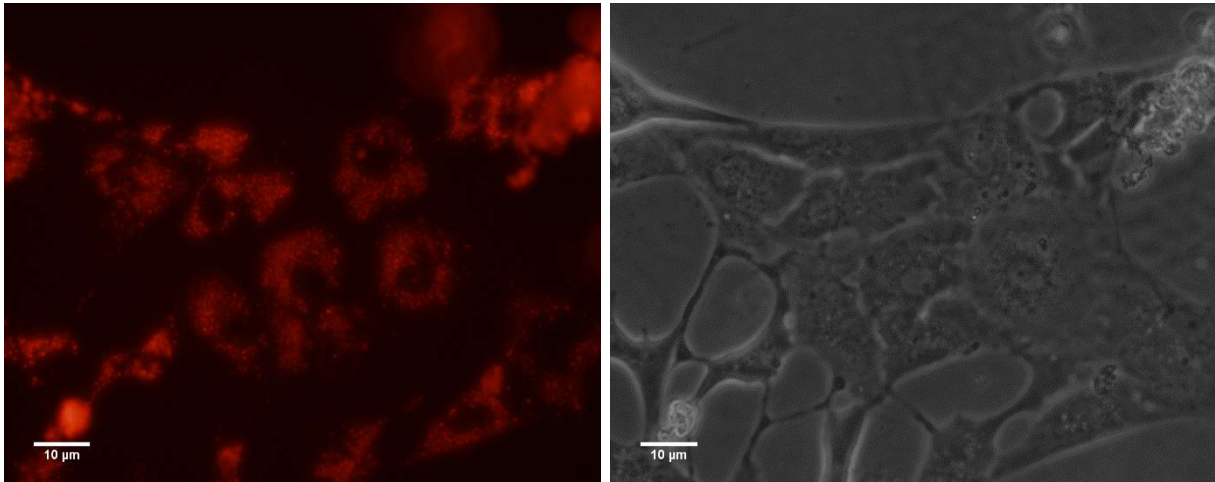


Figure 6: Absorption of PKH26 into the cell soma.

Though PKH26 provided good membrane staining the first three days post-staining, beyond this time point the stain was internalized. As seen in the left image, the fluorescence is largely centred around the nuclei, with little of the membrane seen in the right image being highlighted.

### 3.2.2. Calcein AM

As the Pre-Chip staining showed difficulty with both longevity and neurite staining, On-Chip cytoplasmic staining was tried instead to provide neurite staining with brief stains. Calcein, and its counterpart Ethidium homodimer-1, degrades rapidly compared to PKH26, and is intended for viability assays with immediate imaging after staining. Despite the brevity, it was thought to be adequate for imaging without disrupting the longevity of the culture. A long-term Migration-Chip culture was therefore selected for testing. The chip was especially promising due to several neurons showing axonal extensions into the central channels.

In order to first determine an optimal concentration for the calcein stain a test was performed with multiple concentrations as seen in figure 7 below. Though the low (1:5000) concentration provided clear membrane staining without overexposure, a high (1:2000) concentration was still chosen to counteract the expected dilution.

Despite the high concentration, the stain was largely absent on the chip, with little to no fluorescence seen. The effect of the dilution could be seen with the reduction in fluorescence between the seeding well and the culture chamber (See figure 8). The stain was also found to bleach rapidly, even within seconds, making it ill-suited for time lapses.

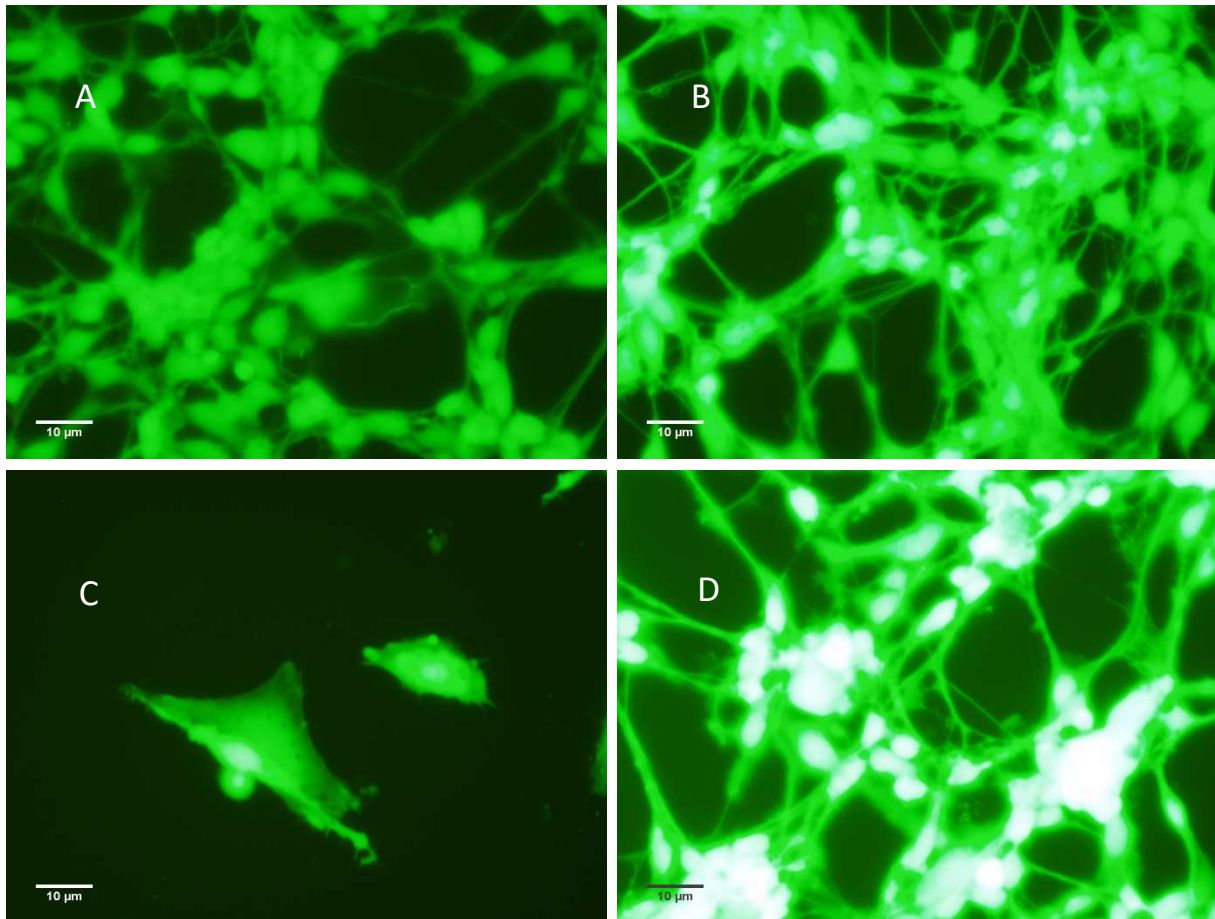


Figure 7: Calcein concentration assay.

The following concentrations were used:

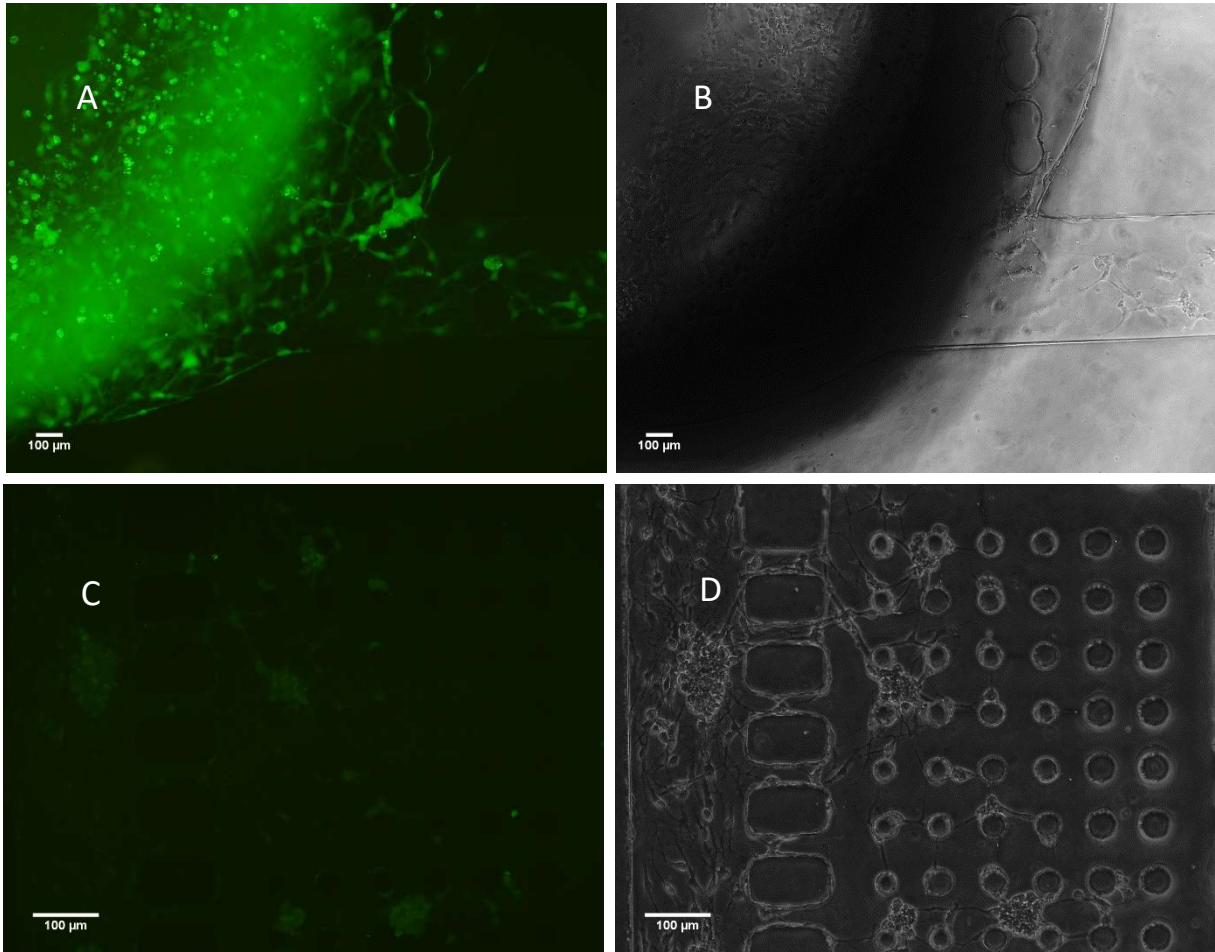
1:5000 (A)

1:2500 (B)

1:2000 (C)

1:1400 (D)

Exposure was set at 600 ms for all images to allow for comparison. While the exposure is suitable for the lowest concentration (A), some overexposure is present already in the 1:2500 concentration (B). In addition to the excessive fluorescence, the 1:1400 concentration (D) also exhibits substantial background fluorescence. The lack of background in the 1:2000 concentration made it a more promising candidate than 1:1400.



*Figure 8: Comparison of Calcein On-Chip staining in Well and Side Chamber.*

*While the inlet well (A & B) showed strong fluorescence akin to that of open culture, the cells in the connected Side Chamber (C & D) showed little to no fluorescence at the same exposure (600 ms).*

### 3.3. Immunocytochemistry

As the live stains largely proved unsuccessful and concentrated near the soma, indirect immunocytochemistry was attempted instead. Furthermore, the live stains used are all unable to differentiate between cell types, which became an issue once the switch to neural stem cells was performed. The dilution factor of the small volumes provided difficulty with immunocytochemistry as the dilution of the antibodies, both primary and secondary, would be pushed into the nanomolar concentration before reaching the cells. Hoechst however, was attempted On-Chip due to the single step staining and the very high affinity and strong fluorescence of the stain.

#### 3.3.1. Hoechst on Chip staining

As Calcein staining had shown to provide little viable staining despite high concentration it was decided that an attempt with differing time points would be made to establish sufficient time for stain penetration into all chambers. To that end the nuclei stain Hoechst was applied at set concentration (1:5000) with four differing time points (5 min 15 min, 30 min, and 45 min) using standard protocol for open culture ICC staining. As the Hoechst stain acts as a DNA binder and thus terminal stain, the protocol was applied to four chips at the end of long-term (>3 weeks) culture (See figure 9). The most effective stain timing was found to be 45 minutes, as brief stains showed almost no fluorescence at normal exposure. In conventional open culture, the timing for Hoechst staining is very brief, not exceeding 5-10 minutes due to the strength of the stain. The dilution of the stain On-Chip was therefore apparent in the length of time required for successful staining.

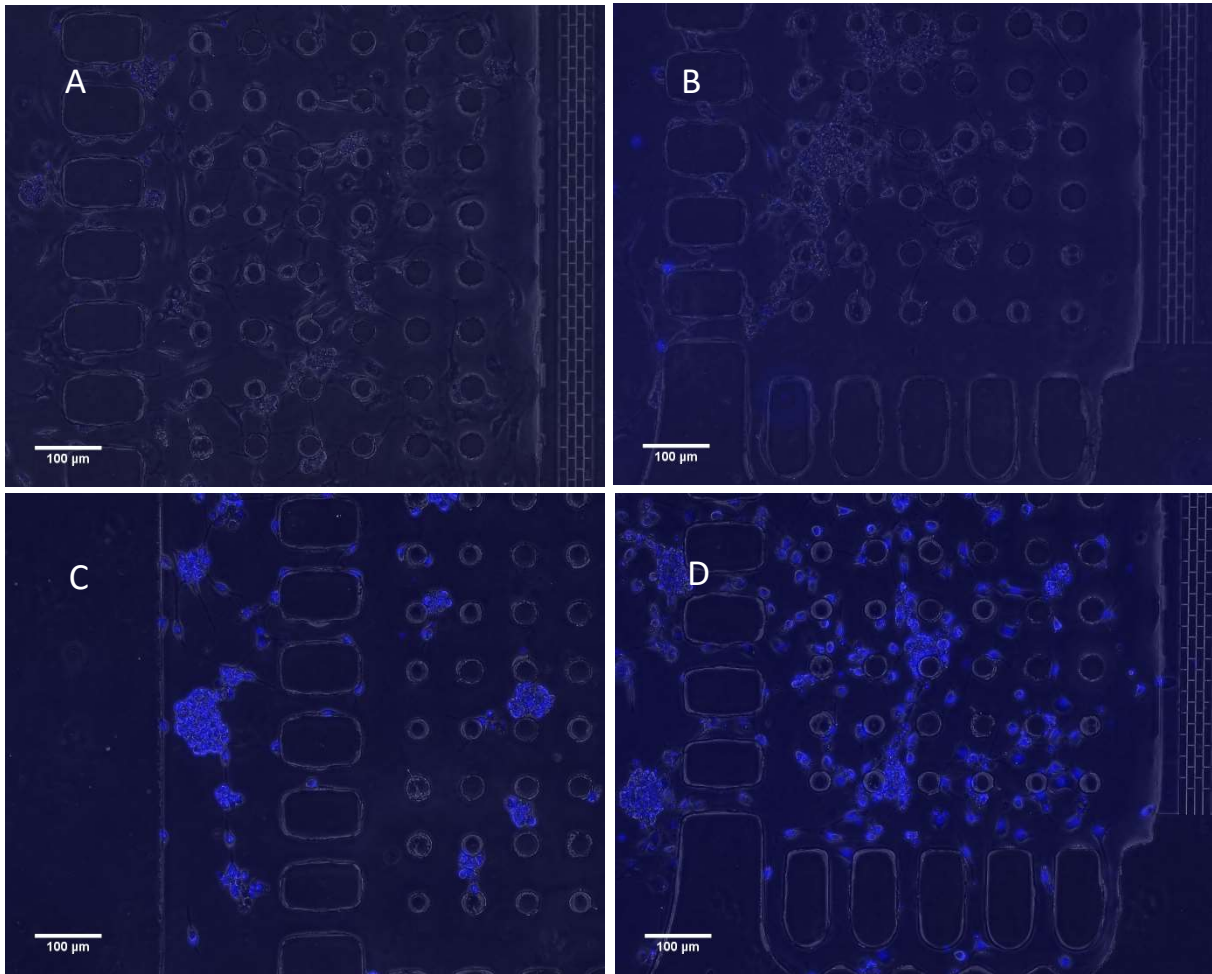


Figure 9: Hoechst On-Chip staining.

All stains were performed on Long-Term culture in Migration Chips using the following time points:

A: 5 minutes.

B: 15 minutes.

C: 30 minutes.

D: 45 minutes.

Note the lack of fluorescence at time points 5 and 15 minutes, compared to 45 minutes. Some background fluorescence is apparent however as an unfortunate effect of the longest stain timings. A set exposure of 20 ms was used for all images.

### **3.3.2. Staining on reversibly bonded chips**

Though the Hoechst was successful given long incubation, it was still hypothesised that reproducible immunocytochemistry would be difficult due to inadequate flushing of the whole chip and dilution of the factors used. To bypass the entire issue of the microfluidic volumes, reversible bonding of the PDMS was instead explored. By removing the PDMS from the glass, normal immunocytochemistry would become an option, without worry about dilution.

### **3.4. Reversible bonding**

Due to the difficulty with accurate immunocytochemistry within the structures of the PDMS and the limitations of live staining, strategies for reversible bonding of PDMS to the glass coverslips were investigated. If the PDMS could be easily removed following completion of the culture then immunocytochemistry could be performed directly on the coverslips akin to normal staining in a multi-well plate. The strength of the normal bonding between the PDMS and glass made removal of the PDMS post bonding impossible without greatly disturbing the cell culture. However, by providing an intermediate layer of coating between the glass and PDMS such removal could be performed easily without disrupting the neuronal culture. The molecular structure of PDL provides an ideal platform for such reversible bonding due to easy bondage to the glass and PDMS as well as its function as an adherence factor for the neuronal culture.

With PDL coating on the glass, the PDMS could easily be peeled away without disrupting the culture. Subsequently the culture could be fixed and stained according to standard immunocytochemical protocol. However, this reversible bond proved far more fragile than expected, meaning handling and feeding had to be performed with far greater care to avoid accidentally peeling the PDMS. Similarly, an entirely new loading protocol had to be developed for this chip type.



### 3.5. Loading methods

Due to the distance between the Chip's wells and the culture chambers, cells need to be loaded into the chambers through manipulation of the fluid flow between the chamber and the well. Several methods were tested to find a way to ensure high density in the chamber, along with even dispersal and low damage to the cells. These methods varied depending on the chip design and cell type used. Laid out below is the development of the methods used towards the current protocol.

### 3.6. Migration-Chip

As mentioned previously, the Migration-Chip was designed as an immunological LoC, thus making it ill adapted for neuroscientific modelling. However, it did prove well suited for basic testing of protocols for microfluidic cell handling and simple assays of neuronal survival. In addition to testing handling protocols, it was useful for testing live and immunochemical staining. It therefore permitted significant protocol development prior to the production of a dedicated neuroscientific LoC.

#### 3.6.1. Pressure application

The first loading attempts made on the Migration-Chips consisted of forcibly seeding the cells into the chambers. Placing the pipette into the seeding well, pressure was applied to the pipette attempting to force the cells into the chamber. The tip was angled either straight into the well, or towards the channel. No drainage was performed in any of the outlet wells, making the fluid in the chip stationary. The loading was therefore fighting the fluid inertia from the rest of the chip, which led to poor seeding. In none of these loading attempts did the cells reach the chamber intact. Instead, the majority were confined to the well or initial segments of the loading channel. Several cells also burst during loading, seen as cell detritus throughout the loading channel. The high-pressure difference between the pipette and the loading channel as well as the lack of fluid movement within the chip led to the ineffectiveness of this approach. The pressure approach was therefore quickly abandoned once instruction on loading mechanisms was given by previous users of the Migration-Chip.

#### 3.6.2. Pre-loaded pipette tips

Following the failure of the pressure loading, a switch was made to stationary pipettes and capillary forces for loading. In brief, the approach consisted of loading the pipette with the cells then gently detaching the tip from the pipette. The loaded pipette would then be placed in the well and the outlets would be drained to provoke capillary forces towards the chamber (See figure 10). This approach had been highly successful with the immunological migration assays previously done on the Migration-Chip

and was therefore attempted with the astrocytes. Unfortunately, one significant problem that arose during this approach was cell clumping.

The initial attempt with this method proved unsuccessful as the cells clumped to such a degree as to be unable to enter the loading channel. The astrocytes strong adherence created significant clumping during seeding which was difficult to counteract with disassociation agents. This presented significant issues since the clumps formed frequently blocked the loading channels. After repeated failures with seeding astrocytes due to this problem, and failure of the pressure protocol, the astrocytes were replaced with neural stem cells. The neural stem cells presented two benefits compared to the astrocytes. Firstly, they have a far lower adherence and ECM production. Secondly, they possess a smaller cell size compared to the astrocytes, allowing them easier entry into the microstructures and enabling greater number of cells to be loaded simultaneously.

Having thus substituted the astrocytes for neural stem cells, this method showed far greater success. The lack of clumping and the smaller cell sizes allowed the capillary forces to pull the cells from the loading well into the chamber. The flow pull towards the outlets was strong enough to draw a large number of cells through the chamber and into the outlet channels and wells. Fortunately, due to the design of the chambers, competing flow forces would counter each other in regions of the chamber, thus allowing the cells to settle there. Also, the cells would frequently impact the columns in the chambers and adhere to them. Small clumps of cells would also become trapped between columns and act as a barrier for ensuing cells. This was both beneficial and unfortunate depending on the location of the blockage, as it could block the cells from exiting or entering the culture chamber.



*Figure 10: Setup used for multiple concurrent seedings with Pre-Loaded Pipettes.*

*When only one Chip was being seeded, the pipette tip would be held in hand to allow for manipulations of the flow and better angling of the tip towards the loading channel.*



To prevent the cells from overflowing the well, and to create good suction between the well and the chamber, the pipette tips were cut to size to fit the well. Creating a tight seal between the pipette tip and the well allowed the flow to run directly from the pipette into the chamber instead of upwards along the sides of the tip, and even spilling over to the surface of the chip. These features were not specific to the approach of stationary loading pipettes however.

The biggest issue of this approach was the time required due to the difficulty of positioning the pipette in the loading well. The time required placed the cells at risk due to keeping them at room temperature, an issue that was only slightly alleviated by keeping the cells at 4°C. The cells could not be kept on ice due to freezing because of the low resuspension volumes required for high density seeding. The wells of the irreversible chips, both Migration and Neuro, could contain approximately 4 µL due to the height of the PDMS. To cram as many cells into the wells as possible a highly concentrated suspension of cells was necessary. The high concentration meant resuspension of the cells was confined to merely 50 µL, which in turn reduced the survival of cells due to the lack of media per cell. Time was therefore vital during seeding. Initially, due to inexperience and poor protocol, the seeding time per chip could exceed 30 minutes, which proved detrimental to cell survival. Even with high seeding concentrations, the culture would still expire due to this delay. Reducing the timing was therefore vital to culture survival. This facilitated the development of the following loading protocol.

### **3.6.3. Stationary loading pipettes**

While the loading pipettes did prove successful, the time required for the seeding posed a risk. To reduce the seeding time, pipettes cut to fit the wells were placed *prior* to loading and angled towards the seeding channels. Cells were then loaded into the stationary pipette tips using Microloader tips. The Microloader was necessitated due to the inadequate reach of normal micropipette tips. Previous attempts with normal micropipette tips led to droplets of cells adhering to the midsection of the stationary loading pipettes.

This method produced consistent flow of cells from the seeding well, likely due to the gradual drift of cells into the seeding well from the loading pipette. The delay produced by this gradual loading, possibly also enhanced the effect of the capillary forces stemming from the drained well, as cells exhibited a rapid flow near the chambers, with cells adhering along the connector channels in main chamber. Up to two chambers on two chips could be seeded concurrently with this method at half the time required by the previous method.

Though as with previous seedings, cell clumping partially impeded the flow to the side chamber due to blockage of the inlets. The method led to good survival in all chambers, with the largest concentration of cells seen.

This loading method also significantly reduced the loading time, as several chambers and chips could easily be seeded concurrently, improving the timing of the previous stationary pipettes approach. The only intervention needed was to restart the cell flow following blockages or reduced capillary forces. Overall, this method proved the most successful of all tested, due to the steady flow from the well, ease of handling and short time needed for seeding.

### **3.7. Neuro-Chips**

With the new generation of chips, the loading protocols previously developed were adapted for use by merely altering the drainage order. The most vital seeding improvement over the Migration-Chip was the even dispersal of cells throughout the culture chambers. The new flushing channels which ensured this similarly reduced the number of cells drawn into the outlet which posed a significant issue with the Migration-Chip. The meandering of the channels did increase the potential for cells to become lodged prior to the chamber, an issue also seen with the Migration-Chip though not at this scale. One new protocol was needed however to allow seeding on the reversibly bonded chips.

#### **3.7.1. Droplet loading**

With the reversible bonding needed for staining cells, both stationary pipette approaches had to be abandoned. Unless extreme care was used during loading, the pipette tips would act as levers capable of peeling the PDMS from the glass. To further reduce this peeling risk the height of the PDMS was reduced by nearly half. Loading on these chips was performed by placing droplets on the seeding wells and draining the outlets as previously described. While successful, this did reduce the grasp of the flow on the cells in the seeding well and frequently led to lower cell numbers in the culture chamber. Spillage onto the surface of the chip was also far more frequent with this loading protocol. Unfortunately, this is thus far the only method found for seeding on the sensitive reversible bond chips.

#### **3.7.2. Loading summary**

Though large clumping of hundreds of cells, as seen with the astrocytes, could block loading channels, small clumps of less than a dozen cells acted beneficially flowing into the chambers and functioning as anchors for ensuing cells. The best cell loading was found with a steady flow of cells into the seeding well, at a high concentration, with little to no large clumping. The flow could be altered with minute changes to the volume of media in the outlets and flush wells, to create a steady flow and even

dispersal of cells into the chamber. Some further development is still required for the reversible chips however.

### 3.8. Coating assay

During the use of the Migration-Chips, CellStart acted as the main adherence coating for the neural stem cells. With the reversible Neuro-Chips, this coating proved incompatible with the PDL required. However, the reversible chips were found to have very poor survival soon after they were adopted, with no cultures surviving past one week. This low survival was suspected to stem from the lower density of the new cell loading method, or the PDL coating. To test this, a coating assay was devised to check survival of NCS on PDL versus, CellStart, and PDL with Laminin.

As suspected the survival of cells on pure PDL was poor, with no cells surviving past 5 days *in vitro* (DIV). CellStart, as expected, provided good survival till 7 DIV, akin to that seen on the Chip. Surprisingly, the survival of cells on PDL and Laminin proved at least as sufficient as CellStart. This was subsequently confirmed on the reversible Neuro-Chips with good survival on the cells with PDL and Laminin.

### 3.9. Chip feeding, evaporation and contamination control

#### 3.9.1. Long-term survival

With good coating and high density seeding, survival was achieved up to 25 and 32 DIV. Such long-term survival was seen on four Migration-Chips, where NCSs had been seeded to near confluence in the left side chamber. As with all Chips, the greatest loss of cells occurred within the first DIV as cells damaged from the loading procedure underwent apoptosis. Throughout the life span of the cultures, the greatest survival occurred in clusters of cells, which would be maintained near distinct topographical features such as columns or channel openings (See figure 11). Additionally, cells would clump together to maintain this high density, with isolated cells migrating towards high-density areas. As the cells formed these clusters, the adherence between the cells would maintain the cluster formation even after the cells had undergone apoptosis. Although the majority of apoptosis occurred during the first week DIV, a gradual decline of cells took place following this. Some cells would attempt to migrate into the channels of the Migration-Chips, but once isolated from the networks of the growth chambers, these cells would decline and become apoptotic within one to four days. Overall, cell density seemed to be the major factor determining survival.

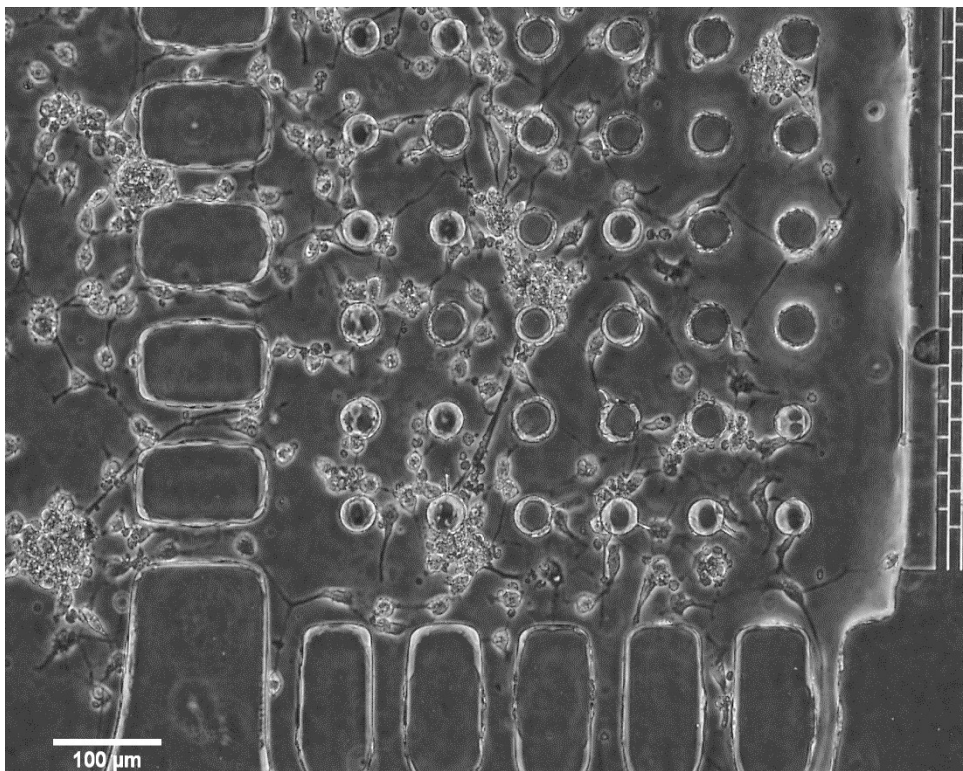
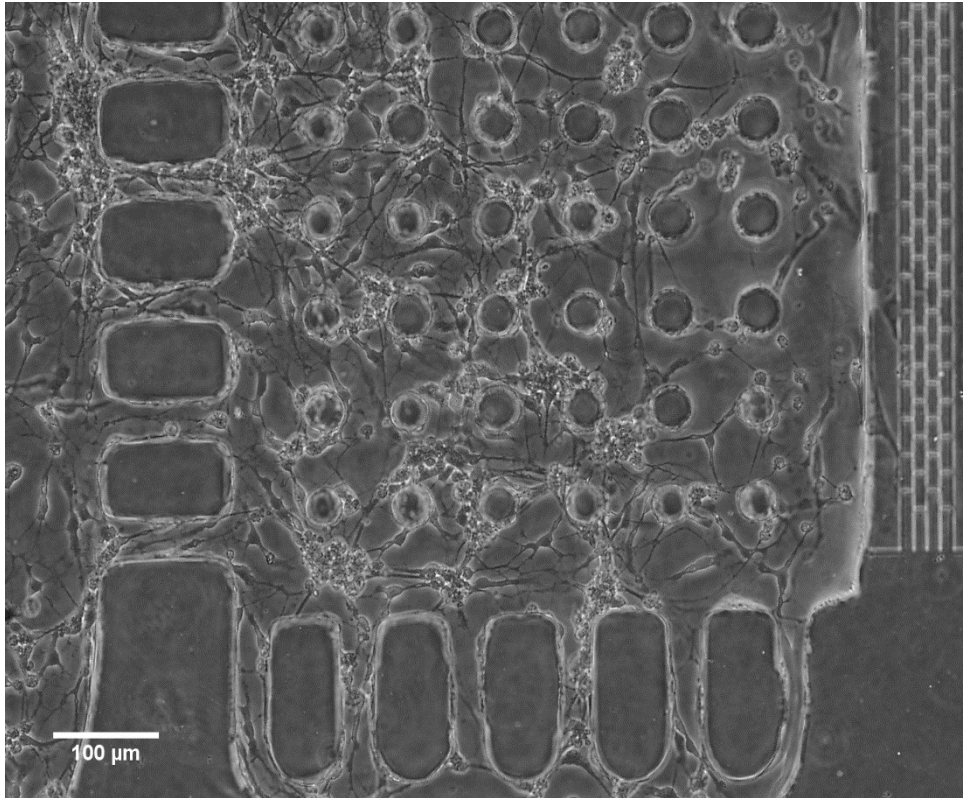


Figure 11: Development of Long-Term NCS culture in Side Chamber.

The top image shows a NCS culture at 7 DIV with high density seeding. Note the clustering of cells near the columns and openings into the chamber. The large number of cellular interconnections indicates the stem cells have undergone differentiation and begun network formation.

The bottom image shows the same culture at 23 DIV. Note the reduction in viable cells and the presence of dead cells still adhering to clusters. Such gradual decline of culture survival was seen in all Long-Term cultures. The bottom image has been brightened to better align with the top image.

Throughout the experiment, three Chips were lost to evaporation and one Chip showed potential contamination. Developing protocols to counteract these risks turned out to require balancing between the two, as measures to prevent one increased the risk of the other.

The majority of contamination risk stemmed from the handling and feeding protocols of the chips. As the chips were contained in parafilm-sealed petri dishes, the only regular air contact came during feeding. However, leakage of media from the petri dish posed an unforeseen possibility for contamination.

### **3.9.2. Flooding protocol**

Chip feeding was initially performed through flooding protocol, i.e. placing the entire chip underneath media and filling the chip container to the brim. The reason for this was two-fold, first to prevent nutrient deficiency due to media evaporation, secondly to prevent PDMS drying and hydrophobic groups forming. During flooding protocol, a full media replacement was initially performed twice a week, but once a week was found to be sufficient given the slow evaporation rate. Though effective, this feeding protocol was eventually abandoned due to fears of contamination. To replace it a well specific feeding protocol was adopted.

### **3.9.3. Well flush feeding**

The seal of the chip containers posed a significant worry throughout the Migration-Chips. The spacing between the bottom and top plate of the allowed media to seep through the gap and pose a potential source of contamination. To remediate this, chips were initially covered in parafilm to seal the gap. However, the amount of media in the chip containers during flooding protocol placed enough pressure on the parafilm to allow seepage through the sealing. As this placed both the chip and the incubators housing them in risk of contamination, it was decided to place all chips containers in petri dishes in addition to the normal containment. Both the petri dish and the chip container were henceforth sealed in separate layers of parafilm.

In addition, the media volume normally present in the chip containers was greatly reduced. The media surrounding the chip was kept at a maximum of half the height of the chip, or approx. 1.5 mL of media. As the inlet wells were not covered by the media, flushing of fresh media was performed every 2 days. This flushing was performed by removing all media from the outlet wells and placing media over all cell connected inlet wells. The chips were allowed to sit for 2 minutes to engage the capillary forces before all wells were submerged in media.

Eventually, the media was removed from the containment dish entirely, and only the wells were left with media. This removed any chance of contamination due to media seepage and spillage, and therefore the need for the external petri dish. With small containers and good sealing, this method was eventually successful at reducing the risk of contamination. However, it did require vigilant checking of the media levels to avert evaporation.

Fortunately, evaporation proved to be a far smaller issue for the long-term cultures than expected, as the only Chips lost to it were affected within the first days of culture after deviation from standard protocol. Secondly, the risk of short-term evaporation was largely confined to the reversible Neuro Chips.

With the reversible bonding of PDMS, a larger well diameter was attempted to allow for high-density cell seeding despite the lowered PDMS height, and thus size, of the wells. This larger diameter proved fatal however, when combined with a large unsealed container to host the chips. Large petri dishes hosted several chips concurrently, while remaining unsealed by parafilm. The increase evaporation from this set up, proved enough to almost completely dry out three chips within the first DIV. Despite the successful seeding and high density of the cultures, all three chips showed no survival following the first DIV in the incubator. This complete drought was the only major effect of evaporation seen, but long-term osmolality changes may have produced minor effects. But through design, the flow within the Neuro Chips allowed for rapid and major exchanges of media which would likely counteract the worst of these changes.

### 3.10. Flow testing Neuro-chips

With the Migration-Chip, testing had been performed by previous researchers to determine the direction and integrity of flow between the chambers and channels. This was replicated on the Neuro-chip to determine the same and test the integrity of the reversible bond. All flow testing was done with commercial Food Colouring.

#### 3.10.1. Full suction of side chamber

The full suction protocol was attempted to establish how quickly new reagents could be made to enter the side chamber and if even reagent dispersal was possible (See figure 13). This was done by emptying both flushing channels and the main outlet. The result was surprising as the dye entering the chamber was akin to normal outlet suction, i.e. >10 minutes. This may be due to the flow channels mainly creating suction from the axon channel rather than the side chamber itself. Secondly, it may be due to the reagent diffusing partially based on diffusion through the media, rather than direct flow.



### 3.10.2. Test of flushing channels

A flush test was performed by emptying one flush channel, termed flush outlet, and adding dye to the opposing flush channel, termed flush inlet. The test of the flush channels was partially successful as the dye quickly (<20 seconds) entered the side chamber. However at this point the dye diverged from the expected flow path into the flushing outlet. Instead the flow continued into the connecting axon growth channel. Eventually the dye permeated the entire chamber, and entered the flushing outlet as well as the main inlet and outlet. This occurred after >10 minutes.

### 3.10.3. Integrity of PDL bonding

Due to the greatly reduced bonding strength of the PDMS to the PDL layer, the integrity of the channels and chamber was in question. To test this dye was added to both side chambers and the main chamber in a standard seeding configuration (See figure 12). While monitoring the spread of the dyes, no significant air bubbles were seen to form in any chambers or channels beyond that seen normally. Secondly, no leakage of dye beyond the pattern was seen indicating good integrity of all channels and chambers despite the reduced strength of the bonding.

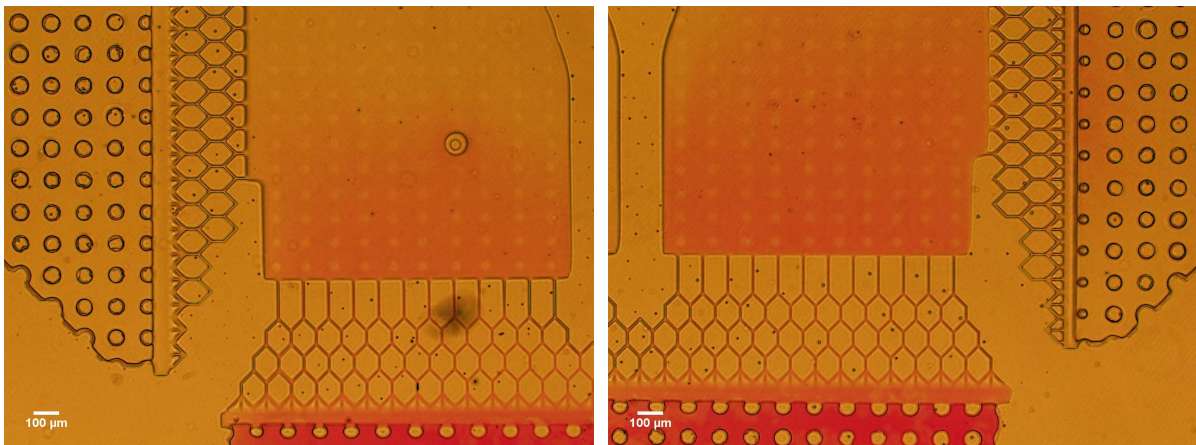


Figure 12: Integrity of channels on a reversibly bonded Neuro-Chip.

*Image shows dye flow from the main chamber towards the side chambers with no apparent leakage outside the minute axon growth channels. The increased flow towards the right side chamber is likely due to the proximity between the main chamber inlet and the right side chamber outlet. The small air bubble seen in the left growth channel likely stems from the flushing of the chip during production, as such bubbles are an occasional issue with irreversibly bonded chips.*

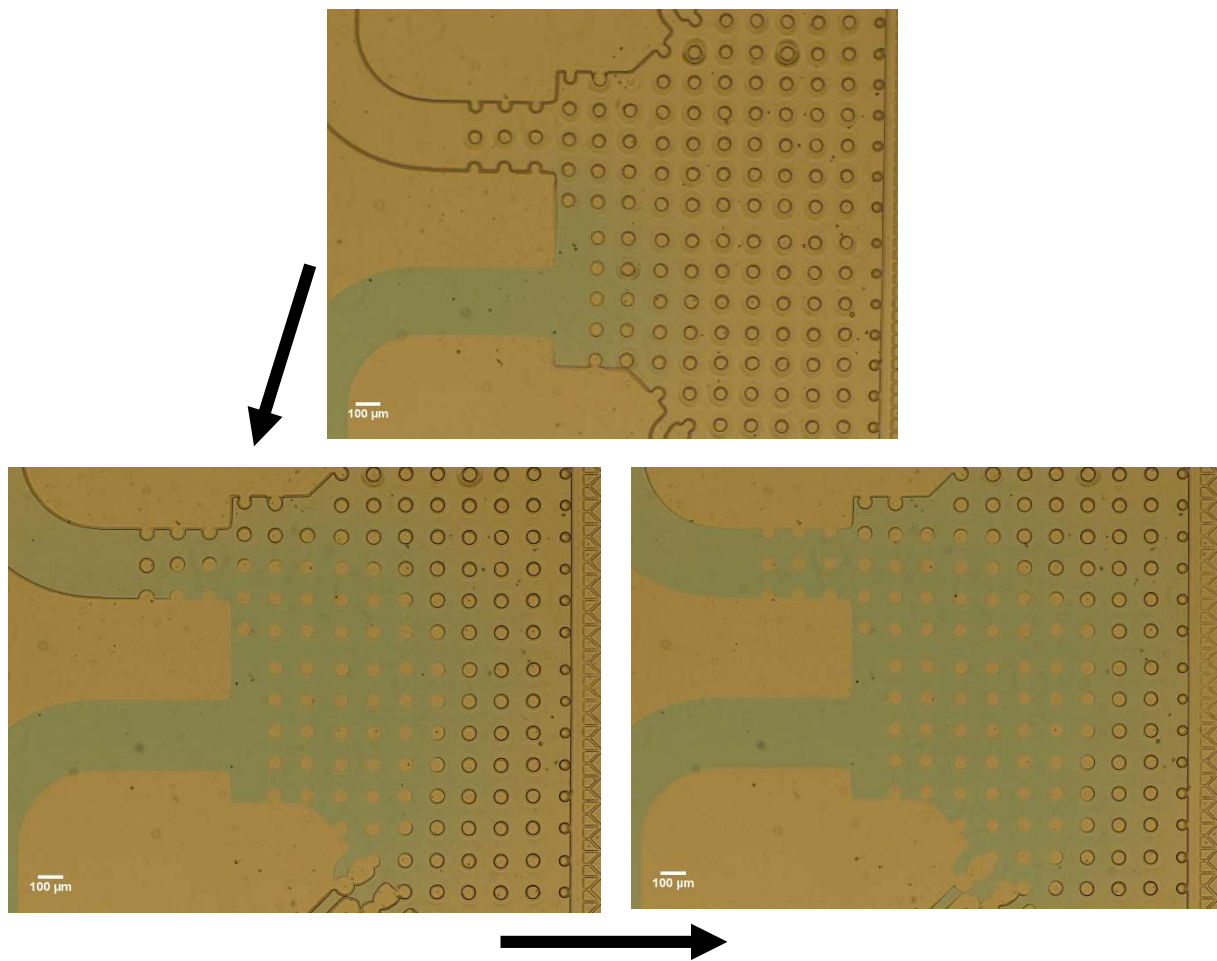


Figure 13: Inlet flow on Neuro-Chip with full suction.

The flow test, read top left to right, shows dye dispersal over a time period of 10 minutes following full drainage of outlet and both flush channels. Though substantially slower than cell loading, the dispersal of dye shows the even flow throughout the Neuro-Chip chambers. The effect of the outlet drainage is shown by the filling of the outlet channel prior to the saturation of the chamber itself. If not countered during cell loading, this effect can pull a great number of cells towards the outlet before they are able to reach the centre of the chamber.



## 4. Discussion

### 4.1.1. Practical factors for long-term survival

The goal of long-term *in vitro* culture was achieved, though the factors underlying the achievement were not aligned with expectations. Once a culture had survived beyond the first week DIV, its lifespan would extend into long-term. Though all cultures saw rapid decline of cells within the first DIV, a few were able to sustain a cell density robust enough for long-term survival. This first DIV appeared to be the crux of the cultures' long-term survival, and almost entirely dependent on the loading method.

### 4.1.2. Improvements and tests of the loading methods

The droplet method was adopted for reversible chips, due to the fear of peeling the PDMS with loading pipette tips. Unfortunately, it did not reach the effectiveness of the stationary loading pipettes as the cells were far more likely to settle in the well than previously. The gradual flow of the pipette tips was missing, and angling of the cells towards the channel was impossible with this method. However, it may be possible to adapt the stationary loading pipette for use on the reversible PDMS. If a holder is made for stationary placement of the pipette tip without placing any load on the PDMS it should be possible to retain the success of stationary loading on the reversible chips. The holder may be a ring placed directly onto the PDMS with a central opening for the tip, or a rigid scaffold to prevent lateral movement of the pipette above the well. The production of this is unfortunately beyond the scope of this project.

An easier test to preform would be the implementation of current loading protocol to astrocytes. The switch to neural stem cells was largely because of their ease of handling and rapid proliferation, which allowed for far more rapid protocol testing. With the protocols currently available it is tempting to return to the more troublesome astrocytes to produce clear co-cultures with feeder layers. With the gradual seeding of cells facilitated by stationary pipette loading, it is possible to seed astrocytes without the difficulty of constant clumping. The gradual flow and the small diameter of the Microloader tips should counteract most of the detrimental clumping. Additionally, the use of astrocytes on the reversible chips would bypass the issue of coating on those chips, as the feeder layer itself would be adherent for subsequent cells.

This implementation of astrocytes is one of the more exciting prospects for future projects, as a feeder layer would not only enhance adherence for cells seeded afterwards, but their strong adherence would likely counter any sheer stress of secondary loadings. If a feeder layer would enhance both short and long-term survival of NCSs and or neurons seeded is intriguing and testing of the co-culture would be relatively easy given successful seeding of the feeder layer.

### **4.1.3. Evaporation and contamination**

Overall, evaporation and contamination proved to be a far smaller counter to longevity than expected. Of the 26 Chips seeded throughout this project, three were lost to evaporation and only one to potential contamination. The risk and fear of both did guide development of several protocols, but ultimately survival of the cultures hinged on other issues. While evaporation may have reduced the life span of the cultures with build-up of salinity, the crux of survival oft appeared to be within the three days of culture, to brief for major build of salinity. The sole exception to this was seen with the three chips lost due to evaporation of the entirety of the media.

## **4.2. Imaging and staining**

### **4.2.1. Issues of long-term live staining**

Longevity of the live stains used was a significant issue throughout the project. The long-term survival of the cells required stains that would not interfere with the cells longevity or normal function. However, stains would either fade rapidly, require a concentration too high to produce staining without substantial background, or face absorption by the cell. While PKH26 appeared to be a good candidate, the uptake of the stain into the soma eventually made it unsuitable for accurate staining of the neuronal extension. This does not mean it was useless, as a long-term somal stain could still be useful for cell counting and migration assays. The unresolved issue would thus be to find a stain capable of highlighting neurite extension without impacting the longevity or behaviour of the cells. Currently the best solution for this would appear to be intermittent use of short-term stains such as CellTrace, which did highlight the entirety of the cytoplasm, but not for longer than 72 hours. The frequency of potential use and associated risk would have to be assessed before any data could be derived from this imaging. A search for better stains, cytoplasmic or otherwise, would likely be better.

### **4.2.2. On-Chip immunocytochemistry**

Unlike live staining, immunocytochemistry is able to stain nearly any cell type or segment of the cell. But the multiple steps required for staining coupled with the issue of dilution made immunocytochemistry difficult on the chips. The reversible chip did offer a partial solution, though the absence of the Chip topography as a reference in the images is unfortunate. Direct immunofluorescence may counter some of the dilution issue by reducing the number of steps required for On-Chip staining. Direct immunofluorescence uses primary antibodies with directly conjugated fluorophores, thus removing the need for blocking and secondary antibodies. The dilution issue would still necessitate significant testing of optimal concentration and stain timing, but it should hopefully be possible to overcome.

## 4.3. Creating a Lab-on-a-Chip for neuroinflammation modelling

### 4.3.1. Potential issues of design and protocol

This project is thus far the only of its kind not only at NTNU, but also in Norway, therefore external guidance for the project is difficult to find. The rarity of such projects nationally necessitated the development of protocols from scratch. The need for high density loading protocols and long-term staining was anticipated, but the substantial challenge and time required for their development was unexpected. On-Chip co-culture and in-vitro modelling of neuroinflammation was therefore not performed as planned at the outset of the project. The effect of coating on long-term survival and differentiation is still unknown, as is the effect of topography on the cells. Moreover, the only long-term survival seen was on the Migration Chips, where channels were not designed for axonal outgrowth. Axonal connectivity between chambers should therefore be examined, both with regard to substrate coating and chemotactic factors. Beyond potential current experiments, integrating more MEMS into the chip would be an advantageous next step in the development of the chips.

### 4.3.2. Integration of biosensors for live monitoring and future recommendations

The most straightforward, and likely most beneficial, MEMS to integrate would be a microelectrode array (MEA). MEAs, as the name suggests, consist of numerous electrodes situated underneath the neuronal culture (Wheeler, Nam, and Wheeler 2011). These electrodes are capable of reading any electrical activity from the neuronal networks, as well as stimulate the neurons in return. Additionally, feeder layers, though situated between the neurons and the substrate, do not interfere with activity to and from the electrodes (Odawara et al. 2014). By simply replacing the current glass substrate of the PDMS chips with a MEA, live readouts of all network activity is possible throughout the life span of the culture. Furthermore, the MEA can be used to provide electrical activity from the first DIV, thereby enhancing survival of the culture by ensuring minimum stimulation for the neurons.

### 4.3.3. Investigating the shift from acute to chronic neuroinflammation

With a long-term culture established, live electrical readouts and fluorescence available, numerous neuropathologies mimicking mechanical lesions or neurodegenerative diseases are open for *in vitro* study. Key to several pathologies, and a component of the glial scar, is inflammation, in particular chronic inflammation. Acute neuroinflammation is a vital necessity for damage reduction in CNS lesions such as stroke, traumatic brain injury, and spinal cord injury (Lucas, Rothwell, and Gibson 2006). Nevertheless, if this inflammation persists, the acute protective effect warps to a detrimental chronic one. This shift has been difficult to examine *in vivo* due to the incredible intricacy of the signalling networks involved. But with a microfluidic environment, confounding signalling factors can

be reduced to a minimum, and networks and cell types can be exposed to inflammatory factors at will. Pro- and anti-inflammatories can therefore be tested in parallel on multiple long-term networks without incurring the costs and difficulties of *in vivo* testing. Thus, the effect of the factors, and any treatment and recovery, is accessible for testing. Such testing will be the next step for these microfluidic platforms.

For example, astrocytes form a significant component of the inflammation present in the glial scar (Ma et al. 2013; Wanner et al. 2008). Specifically, these astrocytes are termed reactive astrocytes due to their altered behaviour compared to normal function. Such reactive astrocytes secrete a substantial number of pro-inflammatory cytokines, which can be harnessed through conditioned media. Neuronal networks co-cultured with reactive astrocytes could be compared with a neuronal monoculture exposed to conditioned media, i.e. seeing the impact of astrocytic contact with regards to the inflammatory response. A comparison could also be made between the neuronal response to a reactive astrocyte conditioned media versus that of a defined pro-inflammatory media. Calcein, and its counterpart Ethidium homodimer-1 (EthD), would lend themselves well to such assays as cell survival could be assessed prior to and following any acute inflammatory response.

Furthermore, by utilizing the modular architecture of the chips, a neuronal culture in the main chamber of the chip could be assessed on its ability to extend axons towards a reactive and a non-reactive co-culture present in either side chamber. One can thus see how patterning of growth cues in the axonal channels, or the presence of anti-inflammatory factors is able to overcome a potential growth inhibition towards the reactive side chamber.

A multitude of inflammatory factors and growth cues are available for such testing, and the compartmentalized architecture will be exciting to employ for these assays.

## 5. Conclusion

As mentioned at the start of this dissertation, *in vivo* modelling of CNS damage and repair has certain caveats. Though it enables realistic physiological modelling, the costs in animal life and complexities of analysis pose a recurring issue. To overcome some of these issues, the realism of *in vivo* physiology must be brought to the *in vitro* culture. The realism of *in vivo* models and control of *in vitro* modelling form a delicate balance, and at the moment microfluidics sit above the pivot. Overcoming the brevity of normal culture, and controlling the complexities of physiology allows for the study of CNS pathology, regeneration, or development without the cost of animal life. However, the development of a neuronal microculture is not only beneficial for preserving animal life, but also enables testing of human cells. Rather than forcing human neuropathology onto animal models, the human cells themselves could be subjected to experimentation. By deriving cells from a patient, one could potentially bypass the physiological inaccuracies of animal modelling. The use of induced pluripotent cells opens the door to realistic, i.e. donor/patient-specific culture and, combined with the unparalleled spatiotemporal control of microfluidics, it enables testing of a multitude of CNS pathologies.



## 6. Bibliography

- Anderson, Mark A, Yan Ao, and Michael V Sofroniew. 2014. "Heterogeneity of Reactive Astrocytes." *Neuroscience Letters* 565 (April): 23–29. doi:10.1016/j.neulet.2013.12.030.
- Bugnicourt, Ghislain, Jacques Brocard, Alice Nicolas, and Catherine Villard. 2014. "Nanoscale Surface Topography Reshapes Neuronal Growth in Culture." *Langmuir* 30 (15): 4441–49. doi:10.1021/la5001683.
- Cadotte, Alex J, Thomas B DeMarse, Ping He, and Mingzhou Ding. 2008. "Causal Measures of Structure and Plasticity in Simulated and Living Neural Networks." *PLoS One* 3 (10): e3355. doi:10.1371/journal.pone.0003355.
- Cregg, Jared M, Marc A DePaul, Angela R Filous, Bradley T Lang, Amanda Tran, and Jerry Silver. 2014. "Functional Regeneration beyond the Glial Scar." *Experimental Neurology* 253 (March): 197–207. doi:10.1016/j.expneurol.2013.12.024.
- Daviaud, Nicolas, Elisa Garbayo, Paul C. Schiller, Miguel Perez-Pinzon, and Claudia N. Montero-Menei. 2013. "Organotypic Cultures as Tools for Optimizing Central Nervous System Cell Therapies." *Experimental Neurology* 248 (October): 429–40. doi:10.1016/j.expneurol.2013.07.012.
- Delivopoulos, Evangelos, and Alan F. Murray. 2011. "Controlled Adhesion and Growth of Long Term Glial and Neuronal Cultures on Parylene-C." *PLoS ONE* 6 (9). doi:10.1371/journal.pone.0025411.
- Fricke, Rita, Peter D. Zentis, Lionel T. Rajappa, Boris Hofmann, Marko Banzet, Andreas Offenhäusser, and Simone H. Meffert. 2011. "Axon Guidance of Rat Cortical Neurons by Microcontact Printed Gradients." *Biomaterials* 32 (8). Elsevier Ltd: 2070–76. doi:10.1016/j.biomaterials.2010.11.036.
- Geissler, Maren, and Andreas Faissner. 2012. "A New Indirect Co-Culture Set up of Mouse Hippocampal Neurons and Cortical Astrocytes on Microelectrode Arrays." *Journal of Neuroscience Methods* 204 (2): 262–72. doi:10.1016/j.jneumeth.2011.11.030.
- Geuna, S, S Raimondo, F Fregnan, K Haastert-Talini, and C Grothe. 2015. "In Vitro Models for Peripheral Nerve Regeneration." *The European Journal of Neuroscience*, August. doi:10.1111/ejn.13054.
- Gourov, Andrei V, and Bridget Curran. 2014. "An in Vitro Model to Study Brain Tissue Recovery." *Medical Hypotheses* 82 (6): 674–77. doi:10.1016/j.mehy.2014.03.001.
- Gullo, Francesca, Irene Manfredi, Marzia Lecchi, Giorgio Casari, Enzo Wanke, and Andrea Becchetti. 2014. "Multi-Electrode Array Study of Neuronal Cultures Expressing Nicotinic  $\beta 2$ -V287L Subunits, Linked to Autosomal Dominant Nocturnal Frontal Lobe Epilepsy. An in Vitro Model of Spontaneous Epilepsy." *Frontiers in Neural Circuits* 8 (January): 87. doi:10.3389/fncir.2014.00087.
- Götz, Magdalena, Svetlana Sirko, Johannes Beckers, and Martin Irmeler. 2015. "Reactive Astrocytes as Neural Stem or Progenitor Cells: In Vivo Lineage, In Vitro Potential, and Genome-Wide Expression Analysis." *Glia* 63 (8): 1452–68. doi:10.1002/glia.22850.
- Honegger, Thibault, Mark a Scott, Mehmet F Yanik, and Joel Voldman. 2013. "Electrokinetic Confinement of Axonal Growth for Dynamically Configurable Neural Networks." *Lab on a Chip* 13 (4): 589–98. doi:10.1039/c2lc41000a.
- Isomura, Takuya, Kenta Shimba, Yuzo Takayama, Akimasa Takeuchi, Kiyoshi Kotani, and Yasuhiko Jimbo. 2015. "Signal Transfer within a Cultured Asymmetric Cortical Neuron Circuit." *Journal of Neural Engineering* 12 (6). IOP Publishing: 066023. doi:10.1088/1741-2560/12/6/066023.

- Krause, Guido, Stefanie Lehmann, Mirko Lehmann, Ingo Freund, Erik Schreiber, and Werner Baumann. 2006. "Measurement of Electrical Activity of Long-Term Mammalian Neuronal Networks on Semiconductor Neurosensor Chips and Comparison with Conventional Microelectrode Arrays." *Biosensors & Bioelectronics* 21 (7): 1272–82. doi:10.1016/j.bios.2005.05.014.
- Kriegstein, Arnold R, and Magdalena Götz. 2003. "Radial Glia Diversity: A Matter of Cell Fate." *Glia* 43 (1): 37–43. doi:10.1002/glia.10250.
- Kunze, Anja, Michele Giugliano, Ana Valero, and Philippe Renaud. 2011. "Micropatterning Neural Cell Cultures in 3D with a Multi-Layered Scaffold." *Biomaterials* 32 (8): 2088–98. doi:10.1016/j.biomaterials.2010.11.047.
- Li, Li, Li Ren, Wenming Liu, Jian-Chun Wang, Yaolei Wang, Qin Tu, Juan Xu, et al. 2012. "Spatiotemporally Controlled and Multifactor Involved Assay of Neuronal Compartment Regeneration after Chemical Injury in an Integrated Microfluidics." *Analytical Chemistry* 84 (15). American Chemical Society: 6444–53. doi:10.1021/ac3013708.
- Liu, Rui, Zhe Wang, Lin Gou, and Hanpeng Xu. 2015. "A Cortical Astrocyte Subpopulation Inhibits Axon Growth in Vitro and in Vivo." *Molecular Medicine Reports* 12 (2): 2598–2606. doi:10.3892/mmr.2015.3702.
- Lucas, Sian-Marie, Nancy J Rothwell, and Rosemary M Gibson. 2006. "The Role of Inflammation in CNS Injury and Disease." *British Journal of Pharmacology* 147 Suppl : S232–40. doi:10.1038/sj.bjp.0706400.
- Ma, Di, Shijie Jin, Endong Li, Yukiko Doi, Bijiya Parajuli, Mariko Noda, Yoshifumi Sonobe, Tetsuya Mizuno, and Akio Suzumura. 2013. "The Neurotoxic Effect of Astrocytes Activated with Toll-like Receptor Ligands." *Journal of Neuroimmunology* 254: 10–18. doi:10.1016/j.jneuroim.2012.08.010.
- Mai, Junyu, Lee Fok, Hongfeng Gao, Xiang Zhang, and Mu-Ming Poo. 2009. "Axon Initiation and Growth Cone Turning on Bound Protein Gradients." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 29 (23): 7450–58. doi:10.1523/JNEUROSCI.1121-09.2009.
- Marconi, Emanuele, Thierry Nieu, Alessandro Maccione, Pierluigi Valente, Alessandro Simi, Mirko Messa, Silvia Dante, Pietro Baldelli, Luca Berdondini, and Fabio Benfenati. 2012. "Emergent Functional Properties of Neuronal Networks with Controlled Topology." *PloS One* 7 (4): e34648. doi:10.1371/journal.pone.0034648.
- Mika, Johann K., Karin Schwarz, Heinz D. Wanzenboeck, Petra Scholze, and Emmerich Bertagnolli. 2015. "Investigation of Neurotrophic Factor Concentrations with a Novel in Vitro Concept for Peripheral Nerve Regeneration." *Journal of Neuroscience Research* 93 (11): 1631–40. doi:10.1002/jnr.23598.
- Millet, Larry J, Matthew E Stewart, Ralph G Nuzzo, and Martha U Gillette. 2010. "Guiding Neuron Development with Planar Surface Gradients of Substrate Cues Deposited Using Microfluidic Devices." *Lab on a Chip* 10 (12): 1525–35. doi:10.1039/c001552k.
- Moe, Aung Aung Kywe, Mona Suryana, Guillaume Marcy, Sandy Keat Lim, Soneela Ankam, Jerome Zhi Wen Goh, Jing Jin, et al. 2012. "Microarray with Micro- and Nano-Topographies Enables Identification of the Optimal Topography for Directing the Differentiation of Primary Murine Neural Progenitor Cells." *Small* 8 (19): 3050–61. doi:10.1002/sml.201200490.
- Odawara, A, Y Saitoh, A H Alhebshi, M Gotoh, and I Suzuki. 2014. "Long-Term Electrophysiological



- Activity and Pharmacological Response of a Human Induced Pluripotent Stem Cell-Derived Neuron and Astrocyte Co-Culture." *Biochemical and Biophysical Research Communications* 443 (4): 1176–81. doi:10.1016/j.bbrc.2013.12.142.
- Pan, Liangbin, Sankaraleengam Alagapan, Eric Franca, Gregory J Brewer, and Bruce C Wheeler. 2011. "Propagation of Action Potential Activity in a Predefined Microtunnel Neural Network." *Journal of Neural Engineering* 8 (4): 046031. doi:10.1088/1741-2560/8/4/046031.
- Park, Jae Woo, Hyung Joon Kim, Myeong Woo Kang, and Noo Li Jeon. 2013. "Advances in Microfluidics-Based Experimental Methods for Neuroscience Research." *Lab on a Chip* 13 (4). The Royal Society of Chemistry: 509–21. doi:10.1039/c2lc41081h.
- Park, Jaewon, Hisami Koito, Jianrong Li, and Arum Han. 2012. "Multi-Compartment Neuron-Glia Co-Culture Platform for Localized CNS Axon-Glia Interaction Study." *Lab on a Chip* 12 (18): 3296–3304. doi:10.1039/c2lc40303j.
- Park, Jeong Won, Behrad Vahidi, Anne M Taylor, Seog Woo Rhee, and Noo Li Jeon. 2006. "Microfluidic Culture Platform for Neuroscience Research." *Nature Protocols* 1 (4). Nature Publishing Group: 2128–36.
- Potter, Steve M, and Thomas B DeMarse. 2001. "A New Approach to Neural Cell Culture for Long-Term Studies." *Journal of Neuroscience Methods* 110 (1-2): 17–24. doi:10.1016/S0165-0270(01)00412-5.
- Renault, Renaud, Nirit Sukenik, Stéphanie Descroix, Laurent Malaquin, Jean-Louis Viovy, Jean-Michel Peyrin, Samuel Bottani, Pascal Monceau, Elisha Moses, and Maéva Vignes. 2015. "Combining Microfluidics, Optogenetics and Calcium Imaging to Study Neuronal Communication in Vitro." *PLoS One* 10 (4): e0120680. doi:10.1371/journal.pone.0120680.
- Rocha, Daniela N, José P Ferraz-Nogueira, Cristina C Barrias, João B Relvas, and Ana P Pêgo. 2015. "Extracellular Environment Contribution to Astroglial Scar: Lessons Learned from a Tissue Engineered 3D Model of the Glial Scar." *Frontiers in Cellular Neuroscience* 9 (January): 377. doi:10.3389/fncel.2015.00377.
- Sackmann, Eric K, Anna L Fulton, and David J Beebe. 2014. "The Present and Future Role of Microfluidics in Biomedical Research." *Nature* 507 (7491). Nature Publishing Group: 181–89. doi:10.1038/nature13118.
- Sandvig, Axel, Martin Berry, Lee B Barrett, Arthur Butt, and Ann Logan. 2004. "Myelin-, Reactive Glia-, and Scar-Derived CNS Axon Growth Inhibitors: Expression, Receptor Signaling, and Correlation with Axon Regeneration." *Glia* 46 (3): 225–51. doi:10.1002/glia.10315.
- Shein-Idelson, Mark, Eshel Ben-Jacob, and Yael Hanein. 2011. "Engineered Neuronal Circuits: A New Platform for Studying the Role of Modular Topology." *Frontiers in Neuroengineering* 4 (September): 1–8. doi:10.3389/fneng.2011.00010.
- Shimizu, Saori, Anna Abt, and Olimpia Meucci. 2011. "Bilaminar Co-Culture of Primary Rat Cortical Neurons and Glia." *Journal of Visualized Experiments : JoVE*, no. 57 (January): 1–5. doi:10.3791/3257.
- Siddique, Rezina, and Nitish Thakor. 2014. "Investigation of Nerve Injury through Microfluidic Devices." *Journal of the Royal Society, Interface / the Royal Society* 11 (90): 20130676. doi:10.1098/rsif.2013.0676.
- Tan, Kenneth K B, Jason Y Tann, Sharvari R Sathe, Seok Hong, Dongliang Ma, Eyleen L K Goh, and Evelyn K F Yim. 2015. "Biomaterials Enhanced Differentiation of Neural Progenitor Cells into Neurons of the Mesencephalic Dopaminergic Subtype on Topographical Patterns." *Biomaterials*

43. Elsevier Ltd: 32–43. doi:10.1016/j.biomaterials.2014.11.036.
- Taylor, Anne M, Mathew Blurton-Jones, Seog Woo Rhee, David H Cribbs, Carl W Cotman, and Noo Li Jeon. 2005. "A Microfluidic Culture Platform for CNS Axonal Injury, Regeneration and Transport." *Nature Methods* 2 (8): 599–605. doi:10.1038/nmeth777.
- Taylor, Anne M, and Noo Li Jeon. 2011. "Microfluidic and Compartmentalized Platforms for Neurobiological Research." *Critical Reviews in Biomedical Engineering* 39 (3): 185–200.
- van Pelt, Jaap, Pieter S Wolters, Michael A Corner, Wim L C Rutten, and Ger J A Ramakers. 2004. "Long-Term Characterization of Firing Dynamics of Spontaneous Bursts in Cultured Neural Networks." *IEEE Transactions on Bio-Medical Engineering* 51 (11): 2051–62. doi:10.1109/TBME.2004.827936.
- Verhulsel, Marine, Maéva Vignes, Stéphanie Descroix, Laurent Malaquin, Danijela M. Vignjevic, and Jean Louis Viovy. 2014. "A Review of Microfabrication and Hydrogel Engineering for Micro-Organs on Chips." *Biomaterials* 35 (6). Elsevier Ltd: 1816–32. doi:10.1016/j.biomaterials.2013.11.021.
- Vishwakarma, Sandeep K, Avinash Bardia, Santosh K Tiwari, Syed A B Paspala, and Aleem A Khan. 2014. "Current Concept in Neural Regeneration Research: NSCs Isolation, Characterization and Transplantation in Various Neurodegenerative Diseases and Stroke: A Review." *Journal of Advanced Research* 5 (3): 277–94. doi:10.1016/j.jare.2013.04.005.
- Viviani, Barbara, Mariaserena Boraso, Natalia Marchetti, and Marina Marinovich. 2014. "Perspectives on Neuroinflammation and Excitotoxicity: A Neurotoxic Conspiracy?" *NeuroToxicology* 43. Elsevier B.V.: 10–20. doi:10.1016/j.neuro.2014.03.004.
- Wanner, Ina B., Andres Deik, Miguel Torres, Andrew Rosendahl, Joseph T. Neary, Vance P. Lemmon, and John L. Bixby. 2008. "A New in Vitro Model of the Glial Scar Inhibits Axon Growth." *Glia* 56 (15): 1691–1709. doi:10.1002/glia.20721.
- Wheeler, Bruce C., Yoonkey Nam, and Bruce C. Wheeler. 2011. "In Vitro Microelectrode Array Technology and Neural Recordings." *Critical Reviews<sup>TM</sup> in Biomedical Engineering* 39 (1): 45–61. doi:10.1615/CritRevBiomedEng.v39.i1.40.
- Yang, Hao, Rui Cong, Luo Na, Gong Ju, and Si Wei You. 2010. "Long-Term Primary Culture of Highly-Pure Rat Embryonic Hippocampal Neurons of Low-Density." *Neurochemical Research* 35 (9): 1333–42. doi:10.1007/s11064-010-0189-0.

## 7. Supplementary Methods:

### 7.1.1. Chip manufacture:

All Chip manufacture was performed by PhD student Rosanne v.d. Wijdeven at NTNU NanoLab.

1. Polydimethylsiloxane (PDMS) solution at a weight ratio of 10:1 (Base: Curing agent).
2. The PDMS solution was onto the patterned master mold.
3. It was then degassed in a desiccator for 15 min. An Airgun is used to remove any remaining bubbles if necessary.
4. PDMS is baked in an oven at a temperature of 70 °C for 1 hr to allow for curing. The cured PDMS layer is then removed from the master mold.
5. A sharpened needle (2 mm  $\varnothing$ ) is used to punch holes at the inlet and outlet connections for the loading and flush channels.
6. Any PDMS debris is removed from the surface with clear scotch tape
7. The PDMS features are placed in a glass container with acetone and a magnet, and left in a magnetic stirrer for 1 min. The acetone is then changed to ethanol (70%) and left for an additional 5 min.
8. The PDMS is then blown dry with compressed air and left to sit overnight.
9. The following morning, glass slides are rinsed with acetone and ethanol (70%) and dried under nitrogen.
10. The PDMS chip (with the patterned surface upwards) and a glass coverslip, are treated in a plasma cleaner for 1 min with 80% oxygen and a power of 80 W.
11. The PDMS chip (patterned surface down) then placed onto the glass coverslip directly after the plasma generator is finished.
12. The sealed chip is then placed onto a hotplate at 70°C for 30 sec.
13. The chip is subsequently loaded with sterile water by pipetting it into the punched inlets and outlet from the loading and flushing channels.
14. Finally, the structure is placed in a petri dish and wrapped in parafilm, then stored at a temperature of 4°C till transfer to the cell laboratory.

### 7.1.2. Chip preparation:

The chip preparation prior to seeding was as follows: Rinse with PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  which was applied to each well with a 1 mL pipette. The PBS was then aspirated from each well and CellStart was applied with a 10  $\mu\text{L}$  pipette to each well in order to coat the chip for NCS seeding. The CellStart was left on for 1 hour before the chip was rinsed with sterile  $\text{H}_2\text{O}$ . Following this the Chip was filled with media and left to equilibrate.

### **7.1.3. Media used:**

#### **Astrocyte media**

85% D-MEM (High Glucose; Gibco)

15% Fetal Bovine Serum (Gibco)

#### **NCS growth media**

Knockout DMEM/F-12 (1X; Gibco)

GlutaMax I Supplement (2 mM; Gibco)

StemPro Neural Supplement (2%; Gibco)

Anti-Anti (1:100; Gibco)

bFGF (20 ng/mL; Sino Biological)

EGF (20 ng/mL; Sino Biological)

#### **NCS differentiation media**

Knockout DMEM/F-12 (1X; Gibco)

GlutaMax I Supplement (2 mM; Gibco)

StemPro Neural Supplement (2%; Gibco)

AntiAnti (1:100; Gibco)

NCS growth media was utilized for cell expansion on culture flasks, while differentiation media was reserved for use on the Chips. Astrocyte media was used both for cell expansion and on Chips.

#### **7.1.4. Cell expansion:**

All cells were expanded on T12.5 or T25 flasks to >90% confluency prior to seeding the chips. The following protocol was used to passage the cells. All ratios are specific to T12.5 flasks.

1. 1 hour prior to passaging a new T12.5 flask is coated with CellStart (Gibco) and incubated at 37°C.
2. The Culture flask is removed from the incubator and media is aspirated and discarded.
3. The cells are rinsed once with 3mL pre-warmed D-PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> which is added to the side of the flask. The flask is gently rocked throughout this to cover the entire surface with D-PBS.
4. The D-PBS is aspirated off and 500 µL of StemPro Accutase (Gibco) is added to the flask.
5. The flask is incubated for 2 minutes at 37°C.
6. Following incubation the flask is tapped on the bottom to detach any cells still adhered.
7. 3.5mL of warm Growth media is added to quench the StemPro Accutase, and the cells are subsequently transferred to a 15mL centrifuge tube.
8. The cells are centrifuged at 300 x *g* for 2 minutes.
9. The supernatant is aspirated off and the cells re-suspended in 1mL of growth media.
10. 25µL of this is mixed 1:1 with Trypan blue and used for counting on a Hemocytometer to determine total cell number.
11. Approximately half the cells would be used to seed a new T12.5 flask to be used for further expansion, though this number depended on the number of Chips to be seeded.
12. The remaining cell suspension would be transferred to a 1.5mL Eppendorf tube and spun down. The cells would then be resuspended in 50µL of Growth media and used for seeding the Chips.

## 7.2. Loading protocols:

### 7.2.1. Pressure application:

Placing the pipette into the seeding well, pressure was applied to the pipette attempting to force the cells into the chamber. The tip was angled either straight into the well, or towards the channel. This was maintained for 2-5 min per loading. After this the cells were placed in the incubator to be examined the following day. The cells were added to the lower far right well, which feeds into the right cell chamber.

### 7.2.2. Standard pipette loading:

Prior to loading, all Chips were prepared as noted in the Chip preparation protocol, and cells were prepared according to cell expansion protocol.

First, to start the flow towards the chamber, the outlet is partially emptied and flow channels are completely emptied of media.

4 $\mu$ L of cells, the maximum volume of the seeding wells, is drawn into the pipette tip which is then placed in the seeding well and gently detached from the pipette by twisting the tip. Unless the tip had been cut to fit the well, it would be angled towards the loading channel to reduce the chance of the cells remaining in the loading well.

Throughout this procedure the flow and cell density in the chamber would be monitored on Phase-Contrast microscope.

The following adjustments would be made if cell flow from the well was low or ceased. If the low flow could be traced to one of the outlet channels, it would be drained to restart the flow. As the outlet wells gradually filled over time this was a common occurrence. Unseeded side chambers could also be drained to provide additional flow. Flow in the seeding well itself could also be poor which was alleviated by gently moving the pipette tip up and down in the well. Similarly, twisting the tip was found to influence flow of cells. Occasionally, severe clumping would occur in the entrance to the loading channel. This was partially countered by placing a 5- $\mu$ L pipette tip and re-suspending the cells towards the channel entrance. However as this was rough on the cells, akin to the pressure loading, it was rarely performed.

Cell flow would be maintained for a maximum of 30-40 minutes before media would be placed in all wells, the chip container sealed and the chips placed in the incubator.

Stationary loading followed this protocol with the exceptions noted in the Results.

Droplet loading utilized the same adjustments for low flow, except where a stationary pipette is noted.

### **7.2.3. Reversible bonding:**

The PDL (Sigma Aldrich) coating was performed using the following protocol. First the glass coverslips (22x22 mm or 24x32  $\emptyset$ ) were soaked in 70% ethanol for 1 hour then aspirated and left to dry vertically, ensuring the removal of all ethanol. PDL (5  $\mu\text{g}/\text{mL}$  in PBS) was then added for 5 min. After coating the coverslips were rinsed quickly 3 times with  $\text{dH}_2\text{O}$ .  $\text{dH}_2\text{O}$  was chosen due to the salt crystal formation of drying PBS potentially impairing the PDMS bonding. After washing the coverslips were left to dry for 2 hours, then placed in storage at  $-20^\circ\text{C}$  before being transferred to the cleanroom for PDMS bonding.

### **7.2.4. Coating assay:**

The coating assay was performed on an Ibidi 12 Well  $\mu$ -culture chip, where 6 wells were utilized.

Each coating was done on two wells to reduce misinterpretation of any cell death due to loading or poor feeding. The following coatings were used: CellStart (Gibco), PDL (Sigma-Aldrich), and PDL + Laminin (Gibco).

$4.5 \times 10^4$  Rat NCSs were seeded in each well with 250  $\mu\text{L}$  NCS growth media. After 2 days in culture the growth media was removed and replaced with NCS differentiation media. Half the differentiation media was replaced every 2 days. The cultures were maintained on differentiation media for 7 days and imaged daily on phase contrast microscope.

## 7.3. Staining protocols:

### Imaging Microscope:

All images were taken on an Olympus IX72 Microscope, with an Olympus DP72 Camera and Cell<sup>^</sup>B imaging software. Post-processing was performed in ImageJ to convert non-fluorescence images to greyscale.

#### 7.3.1. Calcein optimisation assay:

The following protocol was used to determine an optimal concentration for the On-Chip Calcein (Molecular Probes) stain with Rat NCSs: First, the following concentrations were made by diluting Calcein stock in DPBS.

2  $\mu$ L/mL Ratio 1:5000

4  $\mu$ L/mL Ratio 1:2500

5  $\mu$ L/mL Ratio 1:2000

7  $\mu$ L/mL Ratio 1:1400

Rat NCSs were maintained with differentiation media for 3 days on CellStart using 4 wells on a 24-Well. The media was aspirated off and the stains were all added as 500  $\mu$ L to the wells, then placed in the incubator at 37°C for 30 minutes. Following incubation the wells were washed once with 500  $\mu$ L warm DPBS. Imaging was then performed immediately. Exposure on the microscope was set to 600 ms for all images.

#### 7.3.2. Calcein On-Chip staining:

The Calcein stain of 5  $\mu$ L/mL (4mM) was applied On-Chip using the following protocol. First capillary movement was engaged by emptying non-seeded wells. After 5 minutes at room temperature the remaining wells were emptied and the Calcein was added to the seeded wells. After 5 minutes, Calcein was added to the remaining wells and incubated at 37°C for 30 min to settle the stain. Post incubation the stain was flushed from all wells with dPBS first, and secondly with media. After resting the cells for 15 minutes, imaging began immediately.

#### 7.3.3. PKH26:

The astrocytes were first stained by applying 1 mL of dilute C (Sigma-Aldrich) to the well as a wash. While the wash was being applied to the membrane, 4  $\mu$ L of PKH26 (Sigma-Aldrich) was diluted in 1 mL dilute C in an Eppendorf tube. The wash was then removed and the stain was added to the well



and the culture placed in the incubator for 5 minutes. Following incubation the stain was removed and 1 mL PBS with 10% FBS was applied as a wash. The wash was removed and Astrocyte growth media was added.

#### **7.3.4. Hoechst On-Chip staining:**

For all four Chips, Hoechst (diluted 1:10000 in PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ; Thermo-Fisher Scientific) was added to the seeding wells containing cells, i.e. the right side chamber inlet well on the Immuno-Chips. All outlet wells were emptied immediately prior to staining. The stains were maintained for 5, 15, 30, and 45 minutes. Post-staining the Chip channels were flushed by replacing the staining solution with cold PBS and repeatedly draining the outlet channels for 2 minutes before adding PBS to all wells. Imaging began immediately after washing.

#### **7.3.5. Immunocytochemistry for reversible bonding chip:**

All primary antibodies used were supplied by Abcam and secondary antibodies (Alexa Fluor) were supplied by Molecular Probes.

1. The cells are fixed On-Chip by replacing all media in the wells with 4% paraformaldehyde for 15 minutes at room temperature.
2. The PDMS is then peeled from the glass by grasping an unseeded well firmly with a pair of tweezers and lifting the PDMS off in one careful motion.
3. Once the PDMS has been removed the paraformaldehyde is removed and the coverslip is washed 3x 10 minutes in PBS.
4. The cells are then incubated in blocking solution consisting of 5% goat serum (Gibco) and 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 1 hour at room temperature.
5. The blocking solution is aspirated off and the primary antibodies are added in a solution of 1% goat serum and 0.1% Triton X100 in PBS. The primary antibodies are then incubated overnight at 4°C.
6. The following day the cells are washed 3x 15 minutes in PBS.
7. The secondary antibodies are added in 1% goat serum and 0.1% Triton X-100 in PBS for 3 hours at room temperature in the dark.
8. Following this the secondary antibodies are aspirated off and Hoechst 1:10000 is added for 5 minutes at room temperature.
9. The cells are then washed 3x 15 minutes in PBS before being mounted using Fluorsave. Imaging was done the following day.