Lab-on-a-Chip Platforms for Understanding Neuronal Cellular Interactions

by

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STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government’s Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Yiing Chiing Yap

September 2015
Neurons are highly polarized cells that connect and communicate with each other via electrical impulses and chemical synapses, usually between dendrites and axons. The brain alone consists of approximately 100 billion neurons that are present at birth and do not divide, and thus cannot be replaced. Damage or loss of neurons has been linked to many diseases of the central nervous system (CNS) including Alzheimer’s Disease (AD), Parkinson Disease (PD), Amyotrophic Lateral Sclerosis (ALS) and Traumatic Brain Injury (TBI). In 2005, the World Health Organization (WHO) reported that neurological disorders constitute 6.3% of the global burden of disease and 12% of total global death, making neurological disorders one of the most significant causes of mortality. Studies show that axonal pathology and degeneration can cause significant functional impairment and can precede, and sometimes cause, neuronal death in several neurological disorders, creating a compelling need to understand the mechanisms of axon degeneration. Microfluidic devices that allow manipulation of fluids in channels with typical dimensions of tens to hundreds of micrometers have therefore emerged as a powerful platform for such studies due to their ability to isolate and direct the growth of axons.

Chapter 1 offers an introductory overview of the nervous system and also briefly discusses the limitations of traditional primary cell culture methods and the compartmentalized campanot chamber. This is followed by a comprehensive overview on the development of microfluidic devices for neuroscience application. In Chapter 2, a new microfluidic platform to apply very mild (0.5%) and mild (5%) stretch injury to individual cortical axons was developed, allowing characterisation of the neuronal response to axonal stretch injury. This was realised by covering a pneumatic channel (90 µm wide, 17 µm high) with a thin, flexible poly (dimethylsiloxane) (PDMS)
membrane so that pressurising the pneumatic channel allowed a controlled deflection of the membrane. A compartmentalized microfluidic culturing chamber was positioned to guide axons across the pneumatic channel, allowing the axons across the pneumatic channel to be discretely stretched by pressurising the pneumatic channel. Results show that stretch injury in the range of 0.5% to 5% to a short (90 µm long) localized region of a cortical axon was able to trigger a degenerative response in both axons and soma. In Chapter 3, the neuronal response to axonal stretch injury was further investigated with a particular focus on repetitive injury. Closer investigations revealed growth cone collapse and significant abnormal cytoskeletal rearrangements in a time frame characteristic for TBI. Interestingly, a second very mild stretch within 24 h significantly exacerbated this response.

One of the limitations of the devices described above was the use of a two-step photolithography process to fabricate the compartmentalized microfluidic culturing device for isolation of axons from somas. Therefore, in Chapter 4, a novel, low cost method to fabricate small microchannels (in the range of 25-300 µm) on rigid poly(methylmethacrylate) (PMMA) microchips by using a low cost direct CO$_2$ laser micromachining system ($2500) was developed to simplify the two-step photolithographic fabrication process of the culturing chambers. A concept from near field scanning optical microscopy (NSOM) was applied to narrow the size of the laser beam using a stainless steel disk containing a small aperture – a pinhole. The pinhole significantly reduced the diameter of the laser spot, decreasing the ablated feature width from around 300 µm to around 60 µm when using the 50 µm diameter pinhole. With a 35 µm pinhole, the width could be reduced to around 25 µm. The separation efficiency for the electrophoretic separation of fluorescent dyes more than doubled when using the narrower channels made using the 50 µm pinhole, probably due to
more efficient heat dissipation. The laser engraver system was then used to pattern PDMS microfluidic device for culturing neurons. However, results demonstrated that primary neurons were unhealthy when cultured in laser engraved PDMS culturing chambers. Therefore, further modifications during the laser engraving process are required before these devices can be practically applied for studies using neural cells.

Finally in Chapter 5, to further enhance the understanding of the complex signaling cascades that occur in vivo signalling, a novel microfluidic compartmentalized co-culture platform was developed using a one step photolithography process. This multi-chamber device introduces a maze-like structure to restrict axonal outgrowth whilst facilitating the growth of glia, hence physically isolating the neuronal populations. The maze structure consists of multiple offset walls (50 µm wide, 22 µm high, 250 µm long) spaced at regular intervals (50 µm) with 150 µm gaps. Initial investigations using this device demonstrate its effectiveness in blocking axonal growth while being permissive to glial growth in a fluidically isolated microenvironment, confirming its potential for the investigation of the role of glial signaling in neuronal communication, for example the investigation of cellular interactions underlying the brains response to trauma and other neurological disorders.

In summary, two new culture platforms were developed to advance the understanding of neuronal degeneration and neuron-glia signalling in TBI and other neurological disorders. A fast, inexpensive direct machining method for fabrication of microfluidic devices was also developed. However, further modifications are required before practical implementation for the fabrication of PDMS devices for application in neuroscience research can occur.
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<td>Computer Aided Design</td>
</tr>
<tr>
<td>CNC</td>
<td>Computer Numerical Control</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CTE</td>
<td>Chronic Traumatic Encephalopathy</td>
</tr>
<tr>
<td>DAI</td>
<td>Diffuse Axonal Injury</td>
</tr>
<tr>
<td>DIV</td>
<td>Days <em>in vitro</em></td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
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<tr>
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<td>EOF</td>
<td>Electroosmotic Flow</td>
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<tr>
<td>FCS</td>
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<td>FI</td>
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<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
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<tr>
<td>HAZ</td>
<td>Heat Affected Zone</td>
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<tr>
<td>HPC</td>
<td>Hydroxypropyl Cellulose</td>
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<tr>
<td>LED</td>
<td>Light-emitting Diode</td>
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<tr>
<td>MAP2</td>
<td>Microtubule Associated Protein 2</td>
</tr>
<tr>
<td>mTBI</td>
<td>Mild Traumatic Brain Injury</td>
</tr>
<tr>
<td>NFM</td>
<td>Neurofilament M</td>
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<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>NSOM</td>
<td>Near-Field Scanning Optical Microscope</td>
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<td>PBS</td>
<td>Phosphostre Bufffer Saline</td>
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1 INTRODUCTION

In 2005, the World Health Organization (WHO) reported that neurological disorders constitute 6.3% of the global burden of disease and 12% of total global death, making diseases such as Alzheimer’s Disease (AD) [1], Parkinson Disease (PD) [2], Amyotrophic Lateral Sclerosis (ALS) [3] and Traumatic Brain Injury (TBI) [4] some of the most significant causes of mortality. Axonal degeneration is an important pathological event that may underly many of these diseases [5] and the effects of axonal degeneration can be particularly devastating, due to the loss of connectivity of the neurons. Recent studies using animal models suggest protection from axon degeneration could be an important therapeutic strategy to improve clinical symptoms [6]. Thus, it is important to develop tools to elucidate the causes and effect of axonal injury and degeneration in neurological pathogenesis.

An important tool in neuroscience research is the use of primary cell culture using traditional culture dish methods. This approach allows the growth of neurons that polarise, create distinct dendrites and axons and interact with other neurons. However, there are distinct limitations with conventional culturing methods because the individual microenvironment of the neuronal soma and axons cannot be manipulated physically or biochemically. Microfluidic culture platforms (also called Lab-on-a-Chip platforms) have been developed for neuroscience research to overcome these limitations [7-11], delivering precise control over the microenvironment. They also have the ability to isolate and direct the growth of central nervous system (CNS) axons from cell bodies. The development of these microfluidic devices for neurological studies may provide informed insights into the pathogenesis of neurological diseases and in particular the mechanisms underlying axon degeneration.
1.1 Neurons and Glia

The nervous system, which consists of the brain, spinal cord, and peripheral ganglia, is undoubtedly the most complex part of the human body. The brain is an exquisitely complex structure that consists of approximately 100 billion neurons. Neurons are electrically excitable cells that function to transmit and process signals in both the peripheral nervous system (PNS) and central nervous system (CNS). A typical neuron possesses a cell body or soma, multiple dendrites, and a single axon. The soma contains the nucleus of the cell, and this is predominantly where protein synthesis occurs. Dendrites are the main site of the synaptic input to the neuron. The axon carries nerve signals away from the axon hillock, which emerges from the soma, to the distal synapses. Intercellular signaling molecules, extracellular matrix and growth factors all play critical roles in the development and maintenance of neuronal cells. Importantly, the unique structure of neurons means that the different neuronal compartments can be spatially separated within the body and therefore exposed to different factors, which can differentially affect their function in health and in disease. Mimicking this spatial separation experimentally will improve the understanding of the development of neurological disease and may aid in the development of appropriate therapeutics to reverse or delay the onset of clinical symptoms.

In addition to neurons, there are many different types of glia or non-neuronal cells that are also essential to CNS function, including astrocytes, microglia and oligodendrocytes [12] (Figure 1.1). Oligodendrocytes insulate axons, control axonal diameter and facilitate saltatory conduction with specialized membrane sheath called myelin [13]. Astrocytes, the most abundant glial cell type, are involved in homeostasis and nutritive functions [14]. Microglia are the immune cells within the brain because the blood brain barrier separates the brain from the blood borne immune cells [15].
Under normal conditions, glial cells perform a variety of functions including structurally supporting neurons, supplying vital nutrients and gases, insulating axons and facilitating action potentials, and destroying pathogens and removal of debris [16, 17]. The complex interactions between all cell types in the CNS allow fast and efficient transfer of electrical signals around the brain and to and from the brain stem and spinal cord. Therefore, understanding how neurons respond to each other and the role of glial cells is important for understanding normal brain function and the mechanisms of neurodegenerative disease and injury. However, elucidation of such mechanisms can be difficult using in vivo models and hence many investigators have turned to in vitro approaches.

1.1.1 Axon vulnerability in injury and disease

Neurons are among the largest cells in the human body and are highly polarized, often with more than 99.9% of the total cell volume corresponding to the axonal compartment [18]. Therefore, neurons are highly vulnerable to the effects of neurotoxins [19] and defects in protein transport [20], myelination [21], and oxygen delivery [22], through their axons. Recent studies proposed that axonal degeneration is a leading or contributing cause of several neurodegenerative diseases including ALS, AD and PD [23-27].

Axonal degeneration in neurological disorders and nerve injury may occur by a number of mechanisms. It can be triggered by insults that result in a blockage of axonal transport. For example, amyloid precursor protein (APP) is normally transported through axons by fast axonal transport, and is detected at low levels within the axon. However, in traumatic brain injury [28] and Alzheimer’s disease [26] axons that have been damaged accumulate APP, suggesting failure of axonal transport in these diseases. The axon degeneration that occur following TBI can be initiated
Figure 1.1 Cells of the CNS. Schematic representation of the different types of glial cells and their interaction with neurons. Oligodendrocytes are the myelinating cells of the CNS. They wrap myelin around axons to speed up neuronal transmission through nodes of ranvier. Astrocytes extend processes that close contact with blood vessel and synapse. They play important roles in maintaining local ion concentration and pH homeostasis in the extracellular space and transmitter homeostasis at the synapse. Microglia act as phagocytes and they are the primary immune cell type of the CNS. (Adapted from Allen and Barres [12])
directly by an external force resulting in acute stretching or breaking of the axon. The injury may also result in secondary damage or ‘diffuse axonal injury’ due to downstream pathological events [29]. In disease, axonal injury is unlikely to be caused by a direct force, but may be caused by the toxicity of the cellular environment. For example in ALS there is evidence that neurons are exposed to pathological levels of neurotransmitters such as glutamate (excitotoxicity) and reactive oxygen species resulting in oxidative damage [30]. These types of toxicity can lead to axonal damage through increased levels of calcium and downstream pathological processes. In some diseases such as multiple sclerosis, axon damage may occur through failure of non-neuronal cells such as oligodendrocytes to provide support for the axon [31]. However, in AD, axon damage may occur through the toxicity of beta amyloid plaques [32]. Furthermore, several chemical and toxic insults such as acrylamide, anticancer drugs such as vincristine, the antibiotic nitrofurantoin, and heavy metals also can cause axonal degeneration [33-37] and peripheral neuropathy. In many of these neurological diseases, it is still not clear where the site of primary pathology is and what is the role of the cell body is in the eventual death of the axon.

Recent evidence indicates that axon degeneration has a degree of independence from the cell body ([38] [39] [40]) and importantly degeneration of the axon can occur without cell death [41]. The evidence for the independence of axon degeneration comes from the discovery of a type of transgenic mouse called the slow Wallerian degeneration (Wld⁺) mouse [42]. Remarkably, the distal axons of these mice can survive for a long time after transection and can conduct action potentials for up to 3 weeks [43, 44]. It has also been shown that protection of apoptosis is not sufficient to protect against axon degeneration in disease. For example, proto-oncogene, Bcl-2 is a repressor of cell body apoptosis in the nervous system [45, 46]. Sagot et al. [47]
studied the effect of Bcl-2 overexpression in a genetic mouse model of progressive motor neuronopathy (pmn). Interestingly, the overexpression of Bcl-2 in this mutant mouse did not prevent axon degeneration. The mice still lost motor neurons and myelinated axons and died at around 6 weeks of age, which was the same as those without Bcl-2 overexpression. Results from Sagot et al. [47] proved that degeneration of the axon and neuronal cell death are distinct events. It remains unclear how these two processes are linked. It is clear that neuronal apoptosis results in loss of the axon. However, studies show that axon degeneration can precede, and sometimes cause, neuronal death in several disorders [25, 26, 48, 49] so the need to understand the mechanism of axon degeneration is compelling.

**1.1.1.1 Axonal degeneration**

Several types of axon degeneration have been described such as Wallerian degeneration, dying back degeneration and dying forward degeneration [38]. Wallerian degeneration, first described by Waller [50] is the mechanism of axon degeneration that occurs in the distal segment of an injured nerve following transection or crush injury. Normally, axonal degeneration will occur within 24 to 48 h following lesion [50]. Axon degeneration that begins at the distal regions of the long axon, followed by distal to proximal progression, is referred to as dying back degeneration [38]. Alternatively, pathology may initially occur in more proximal axon segments and spread to the distal axon, which is also known as dying forward degeneration [38]. The relationship between Wallerian degeneration and dying back or dying forward degeneration, and the involvement of common mechanisms is as yet unclear.

The observable pathological features of the degenerating axon may include swelling, beading and fragmentation [38]. It is important to note that the site of primary
pathology may not represent the site of initial insult. Thus, we need powerful platforms to elucidate pathogenesis of a range of neuronal diseases and disorders, focusing on the axonal response to a range of insults that may occur in different parts of the cell.

1.1.2 Neuronal cytoskeletal involvement in disease and injury

A key common process that is likely to be involved in all modes of axon degeneration is the disruption of the neuronal cytoskeleton [51]. The cytoskeleton consists of three key components; the microfilaments (actin), the intermediate filaments (such as neurofilaments) and microtubules (Figure 1.2). The cytoskeleton is important for a number of cellular functions such as maintenance of neuron shape, axon outgrowth and intracellular transport [51]. Thus, it is not surprising that in many neurodegenerative diseases, and following trauma, a number of cytoskeletal changes may be present including abnormal accumulations of cytoskeletal proteins within the axon, abnormal phosphorylation and mislocalization of neurofilament proteins within the cell [52]. Many of these cytoskeletal changes can be replicated in cell culture models [53-56] and may result in increased intracellular calcium levels [57] or axonal transport disruption [55, 56]. Although many neurodegenerative diseases are characterized by abnormalities in the organization of the cytoskeleton, the link between cytoskeletal changes and development of disease is poorly understood. It still remains unclear whether cytoskeletal changes have a causative role during axon degeneration or whether they represent only a pathological hallmark of neurodegenerative disorders and may thus be potentially important targets for therapeutic interventions.

During development of the central and peripheral nervous system, the tips of neurites form highly mobile structures, known as neuronal growth cones (Figure 1.3). These structures regulate neurite growth and navigate by responding to a large variety of
guidance cues during development [58]. The can be divided into two distinct regions. The peripheral, or P-domain, consists of a thin region with actin filament from which the motile and sensory lamellipodia and filopodia emerge. Normally, actin filaments are densely bundled into filopodia, or into a meshwork that extend throughout lamellipodia [59]. The central, or C-domain contains the advancing microtubules that are bundled in axons and splayed within lamellipodia. The distal tips of microtubules always reach the leading edges of the lamellipodia and sometimes enter filopodia [60]. After injury, the formation of a growth cone at the tip of a transected axon is a crucial step during subsequent axonal regeneration. The tips of lesioned axonal stumps located in the PNS transform into growth cones of stereotypic spiky morphology capable of sustained growth [61]. On the contrary, damage to adult CNS axons results in the formation of axonal retraction bulbs at the tip of their proximal stumps. These structures seem to be remarkably stable as they can be found in brain injury sites often years after damage has occurred and are regarded as the visible static marker for failed regeneration growth responses of damaged axons [62, 63]. Immunohistochemical analysis of post mortem brain sections of TBI victims very often reveals diffuse axonal injury (DAI) characterized by end bulbs, also called retraction balls, retraction bulbs or dystrophic axons (a generic term of misshapen axons carrying larger spheroids and smaller varicosities) in the corpus collosum, thalamus, brainstem, cerebellum or cerebral hemispheres [39]. The mechanisms underlying the pathological hallmarks of TBI remain to be fully elucidated.
Figure 1.2 Diagrammatic representation of the neuronal cytoskeleton. The cytoskeleton consists of three main components, namely microfilaments, neurofilaments and microtubules. Microtubules are composed of tubulin molecules. Neurofilament are comprised of subunits with a filamentous structure. Microfilaments are composed of helically intertwined strands of polymerized actin molecules. (adapted from [64]).
Figure 1.3 The neuronal growth cone. The peripheral (P) domain predominates with actin filaments, forming longitudinally arranged bundles in filopodia, but few microtubules. Lamellipodia are broad, fan shaped structures which consist of crosslinked F-actin and microtubules. The central (C) domain is abundant with microtubules with some microtubules extending to the base of filopodia at the leading edge. (adapted from [58]).
1.2 *In Vitro* Models of Neuronal Degeneration

*In vitro* primary cell culture methods, typically using glass coverslips placed in petri dishes or a multiwell plate, are commonly used for studying various properties of neuronal cells and have been used to achieve remarkable insights into axonal, dendritic and synaptic development. However, this standard conventional cell culture method cannot be used to study the spatial compartmentalization of neuronal signals due to the random arrangement of neurons seeded in homogeneous media (Figure 1.4). On the other hand, neurons are highly sensitive to their surrounding microenvironment such as substrate properties [65] and temperature [66]. This makes it extremely challenging to study neuronal development and degeneration *in vitro*.

1.2.1 *In vitro* compartmentalized Campenot chambers

Limitations of the standard primary cell culture approach to study neuronal networks has lead to the development of new culture devices to control the network architecture *in vitro*. An *in vitro* culture device allowing compartmentalized culture of neurons with localized fluidic environmental control was first introduced by Campenot in 1977 [67, 68]. This chamber uses a Teflon divider sealed to the substrate via a silicone grease layer (Figure 1.5). Neuronal cell bodies are plated in one compartment while the axons can grow through the twenty scratches made in silicon grease using twenty insect pins. Typically, nerve growth factor (NGF) is used to promote the axonal growth. Some researchers have used the Campenot chamber to isolate PNS or retinal ganglion neurons axons from cell bodies, which has helped improve our understanding of the axon-glia interaction and axonal biology of PNS and CNS. For example, they have been used to study the transport of neurotrophic factors [70-71] and microtubule network [72, 73] along axons. In addition, Guertin and colleagues [69] utilized this Campenot compartmentalized model to study the wallerian degeneration following
Figure 1.4 Cortical neurons grown with conventional *in vitro* cell culture methods. Culturing neurons on a coverslip results in a chaotic neuronal growth in a steady microenvironment and does not allow separate treatment of specific neuronal compartments. (MAP2, green, dendrites; NFM, red, axons). (Image credit Dr K. Southam)
Figure 1.5 Diagram of Campenot chamber. The device consists of a petri dish (35 mm) divided into three chambers using a Teflon divider that is sealed to a collagen coated coverslip with silicon grease. Twenty insect pins were used to make 20 parallel scratches about 200 µm apart on the collagen-coated coverslip. Neurons were plated into a narrow central chamber (1x 5 mm) (a) and, axons were guided towards the left (b) and right (c) side chambers through the 200 µm wide collagen regions between the scratches. The growth of neurites into the left or right chamber was typically promoted by the use of nerve growth factor (NGF). (adapted from[81]).
microanatomy of axon to Schwann cells signaling. Besides, Ishibashi and coworkers have shown that astrocytes promote myelination in response to an electrical impulse [74]. Biochemical analysis [75, 76] of isolated axons in Campenot chambers using polymerase chain reaction (PCR), Western blot and thin layer chromatography have been successfully conducted. Additionally, regenerative responses of sympathetic axons have been successfully studied with Campenot chambers [77].

However, CNS neurons, such as cortical, hippocampal and spinal cord neurons that are involved in the pathology of many neurodegenerative diseases and injuries have not been successfully cultured in these Campenot chambers. This is because cerebral neurons are typically more difficult to culture and do not have the same dependency on NGF for axonal growth as dorsal root ganglion (DRG) and retinal ganglion neurons. Other drawbacks of Campenot chambers include their tendency to leak owing to an imperfect grease seal and difficulty in assembly. In addition, Campenot chambers also have limitations in their use with sophisticated microscopy including live cell imaging because of the fluorescence background from the grease barrier.

1.2.2 Lab-on-a-Chip devices (microfluidic devices)

During the last few years it has been recognized that microfluidic platforms or Lab-on-a-Chip devices, that allow the manipulation of fluids in channels with typical dimensions of tens to hundreds of micrometers, are another important tool for studying living cells [78]. Microfluidic platforms overcome many of the limitations of traditional cell culture methods by offering precise spatially and temporally controlled extracellular environments mimicking the in vivo environment of cells. Other significant advantages over conventional techniques, include the use of low volumes of expensive media, hormones and growth factors, the capacity for highly sequential or parallel processing and also the potential of low cost disposable devices. In
addition, microfluidic devices are compatible with high resolution microscopy and immunocytochemistry, and can therefore be used for long term culturing, with live time-lapse microscopy [79, 80]. Investigation of axonal biology in the CNS is hindered by a lack of an appropriate in vitro method to probe axons independently from cell bodies. Jeon’s group [7, 8] developed the first poly (dimethylsiloxane) (PDMS) microfluidic device that allowed growth of the CNS axons into a fluidically isolated environment (Figure 1.6). This was achieved through hydrostatic pressure, and incorporating a hydrodynamic barrier via embedded microgrooves (width 10 µm, height 3 µm and length 150-900 µm) that separated two larger compartments. These platforms were fabricated using a two-step photolithography process and they are now commercially available. Previous studies also show that when there is a volume difference between the somal side and axonal side (50 µl), the chemical microenvironment of axons will be isolated for over 20 h due to the high fluidic resistance of the microgrooves [8].

Therefore, this device enables the isolation of CNS axons without soma or dendrites, resulting in a fluidically isolated axonal environment without the need for neurotrophins. This means that both physical treatment and pharmacological interventions can be performed on axons or neuronal soma of a single neuron subtype. In addition, microfluidic devices with embedded microstructures as well as electrical, optical and protein or chemical cues can be utilized to control where neurons will be patterned and attached, differentiate and grow [82]. Growth properties such as length and growth and retraction rates of axons and dendrites, can also be accurately quantified, largely due to the straight growth processes within the microgrooves [83]. Compartmentalized microfluidic platforms have also been used to create neuronal
Figure 1.6 Commercially available microfluidic culture platform demonstrating fluidic isolation of the axons from cell bodies. (A) The commercially available culture chamber consists of a soma compartment and axonal compartment connected by microgrooves (arrow head) (3 µm high, 450 µm long, 10 µm wide). Four wells (arrow) are the reservoirs for media. Neurons are cultured in the soma compartment and after a few days the axons extend into the axon compartment through the microgrooves. (B) Cross sectional view of the platform showing dimensions of chambers. The fluidic isolation of the axon compartment is established when the volume of axon compartment (yellow) is lower than the soma compartment (black) due to hydrostatic pressure in the microgrooves. (C) Immunocytochemical image of neurons at 14 DIV shows that microgrooves prevent growth of dendrites into the axon chamber. Axons were immunostained for NFM (red) and dendrites for MAP2 (green). (A, B: adapted from [8], C: image credit Dr K. Southam)
connections between distinct populations. By adding a second population of neurons to the axonal compartment, synapses can form within the microgrooves and each neuronal population can be independently manipulated genetically or pharmacologically within its compartment [84]. From this original design several adaptations have been made by individual labs in order to improve the device for specific needs. Park et al. [9] have developed a microfluidic co-culture platform composed of a soma compartment and a large axon/glia compartment through embedded microgrooves (Figure 1.7). This platform also provides physical and fluidic isolation between the two compartments. Their novel circular design demonstrated excellent cell loading characteristics where cells are positioned right next to the inlet of axon-guiding channels. This is due to the open access soma compartment in the center for neuron loading and flow occurring radially toward the axon-guiding channel inlets. This design enabled formation of a denser axonal network in comparison to the square design from Taylor et al. [8]. Oligodendrocyte progenitors were successfully co-cultured with axons inside the axon/glia compartment and differentiated into mature oligodendrocytes [9]. These results indicate that this platform can be used as a powerful tool for future study of the interactions of, and signaling between, axons and glia.

1.2.2.1 The fabrication of microfluidic device

The microfluidic devices can be fabricated in various polymers, glass or silicon using microfabrication techniques. Typically, PDMS, a thermally stable elastomer is used to produce microfluidic devices for neuronal studies [7, 8, 85] because PDMS is biocompatible and permeable to gas, which aids in cell aspiration [86]. It can also be simply sterilized by ethanol immersion. This is an important property, since all equipment used for cell culture has to be sterile. It is also ideal for biological
applications because it is optically transparent [87, 88] to visible and UV light wavelengths ranging from 230 nm to 700 nm. Most of the fluorescence markers and the UV light used for sterilization (~265 nm) are within this range. In addition, PDMS has a low curing temperature (<100 °C), which can be easily achieved in a conventional oven. Most importantly, PDMS can be used to fabricate complicated microfluidic devices with simple cast and cure molding processes [79, 87].

The most common method for fabricating PDMS based microfluidic devices for biological applications, uses photolithography techniques [79, 89-92] to create a template using the photosensitive epoxy SU-8 (as shown in Figure 1.8). This is an epoxy based negative photoresist that is ideal for micromachining and other microeletronic applications. Similar to other types of negative photoresist, SU-8 has good adhesion to various substrates such as silicon and glass and can be used to create high aspect ratio microfeatures. Additionally, it exhibits excellent thermal and chemical stability. SU-8 polymerizes when exposed to ultraviolet light and can be used to create relatively high structures, in the range of hundreds of microns, which are suitable for cell culture [79].

The first step in building a PDMS based microfluidic device involves drawing the design using computer software such as AutoCAD, a Computer Aided Design (CAD) software application for 2D and 3D design, and developing the drafts with Autodesk, Inc. This design is then printed on a photomask, placed over a substrate (for example glass or silicon) that is precoated with a thin layer of the photoresist (for example SU-8), and then placed under a UV exposure system. After exposure, the photoresist will be polymerized in areas that have been exposed to UV light through the mask, and the un-polymerized photoresist can be removed using the developer. This leaves a flat substrate with raised structures with a pattern defined by the transparency. This
1.2.2.2 Application of microfluidic culture platforms in neuroscience research

Microfluidic platforms have been used in a number of neuroscience applications such as axon growth guidance [93], pharmacological interactions [94], electrophysiology [95], axon myelination [9], drug induced axonal degeneration [96] and local protein synthesis in axon [97]. One important application of these devices is the investigation of the biochemical composition of axons. Taylor et al. [8] have successfully harvested axonal mRNA from the axonal compartment of 6 day in vitro neurons (developing axons). Confirming the presence of axonal mRNA species has been difficult in standard culture due to the presence of the somatodendritic compartment and in vivo it is complicated by the presence of glial cells. Taylor et al. [8] provided the first evidence that mRNA coding for the presynaptic vesicle protein, synaptophysin, but not postsynaptic (CamK2a), was present within developing axons of rat cortical and hippocampal neurons. The mRNA population present in mammalian cortical axons after 13 days in culture, in both the naïve state and in regenerating axons following axotomy, was also characterized using this compartmentalized microfluidic platform [98]. This study provided the first evidence that naïve, mature CNS axons contain
Figure 1.7 Schematic illustration of the microfluidic CNS neuron co-culture platform developed by Park and Colleagues [9]. This platform consists of a circular somal compartment surrounded by a large compartment connected by microgrooves. (A) 3D view of the circular design. (B) cross-sectional view showing the physical isolation of the axons and oligodendrocytes from neuronal soma and neurites through the microgrooves. (adapted from [9])
Figure 1.8 The fabrication process of the PDMS based microchips. The template is typically fabricated using photolithographically in SU-8, an epoxy based negative photoresist. This template is then used for casting PDMS, a process called soft lithography. The steps required for the photolithography and soft lithography processes include: (A) Create coating with desired height by spin coating SU-8 onto the silicon substrate; apply a pre-exposure bake to evaporate the solvents and cure the SU-8. (B) Expose SU-8 to UV radiation using a photomask. (C) Apply a post exposure bake to complete the crosslinking reaction. (D) Develop to remove unexposed SU-8, leaving the exposed areas. (E) Pour PDMS onto the SU-8 template, de-gas and cure 60 min at 80°C. (F) Release the cured PDMS mould (G) Punch the inlet and outlet holes (H) bond with glass substrate to close the microchannel. (adapted from [89])
>300 mRNA transcripts that can serve to support intracellular transport, the cytoskeleton, mitochondria maintenance, and synaptic function, within the axonal compartment.

In other experiments researchers have used microfluidic chambers to investigate the role of the axon or somatodendritic compartment in the toxicity of a variety of drugs and thus determine mechanisms of toxicity or protection. For example, Yang et al. [96] used the chambers to determine whether the toxicity of the chemotherapeutic drug paclitaxel is on the DRG cell body or axon. By using these chambers they show that paclitaxel causes degeneration of axons through local mechanisms and recombinant human erythropoietin can protect against this axonal toxicity even when it is applied to the neuronal cell body compartment. A few microfluidic devices that have the ability of spatial segregation of the axons have also been developed to study axonal injury in vitro because this type of axonal degeneration is closely related to the pathophysiology of TBI and many neurodegenerative diseases. For example, Jeon’s group who developed the neuron culture devices for isolating axons also utilized this device to generate an injury of axons by vacuum aspiration and monitored the effect of myelin inhibitors on regeneration of injured axons [83]. They demonstrated that both the Nogo-66 (100 nM) and MAG-Fc (250 nM) inhibited CNS axonal regeneration compare to the control. In addition, Kim and colleagues [111] adapted this system into their neuro-optical microfluidic platform that also consists of femtosecond laser to enable complete axotomy of live mammalian axons and a mini incubator for continuous long term monitoring of post injury events. Recently, Hellman and Colleagues [99] integrated less harmful pico and nanosecond pulsed laser microbeams to microfluidic based surface culture devices that allowed for precise and localized axonal damage to study the dynamics of axonal injury and regeneration. These
previous studies indicate that microfluidic technology has the potential to investigate the cellular and molecular mechanisms underlying neurodegenerative diseases and response to trauma.
1.3 **Hypothesis and Aims**

Neurobiology is a field that is developing very fast, hence the demand for new tools is growing and changing. In the conventional petri dish culture system, neurons are randomly seeded onto the homogenous substrate. Because cells respond to their local microenvironment (cells, substrate and biomolecules), neurobiologists have had difficulties in obtaining a clear dynamic cellular response through conventional culture system. Microfabrication technology allows for the fabrication of a nearly indefinite number of shapes and designs, providing neurobiologists with endless opportunities. Microfluidic devices offer unique functions and advantage over traditional *in vitro* systems through the ability to precisely control the dynamic cellular microenvironments, together with the low cost, high throughput production and rapid prototyping capacity. The use of microfluidics in neuroscience is relatively recent, with much of the research not yet dedicated to solving any hypothesis driven biological questions, but instead simply focused on proving the successful culturing of neurons. Advancement in this area is often hindered by a lack of understanding between the disciplines that provide technological advances and those having the experimental needs; understanding of both the microfabrication process and the neuronal culture process is needed. Close collaboration between the neurobiologist and fabricator is crucial for the development of innovative and practically useful microfluidic devices for studying neurons. The transdisciplinary approach utilized in the current project bridges neurobiology, microfabrication and microfluidics with the aim to provide a better understanding of neurological disorders and thus greater flexibility in design relative to needs.

The main purpose of this thesis was to develop new microfluidic culturing tools that allow the construction of directional neuronal networks to enhance the understanding
of the changes in brain function at a cellular level in response to disease and injury. The hypothesis underlying this project is that microfluidic devices can be designed and fabricated, and subsequently used in primary culture models, to provide new insight into the mechanisms of axon degeneration and elucidate the pathogenesis of a range of neuronal diseases and disorders.

This hypothesis will be addressed through the following specific aims:

Aim 1) To develop a new microfluidic approach for studying TBI in a fluidically isolated microenvironment (Chapter 2 and 3)

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in children and young adults, making TBI a significant public health problem. TBI may be caused by rapid brain deformation, stretching, compression or shear forces occurring as a result of traumatic incidents such as motor vehicle accidents, falls and assaults. There is currently no in vitro system that is able to allow the study of precise and reproducible mild axonal stretch injury in a fluidically isolated microenvironment. This makes it difficult to investigate the pathological changes within distinct parts of the neuron (soma, axon and dendrites) in response to pharmacological manipulation and therapeutic treatment. This may be particularly important in the investigations of mechanisms of secondary degeneration where the role of retrograde signalling to the soma is unclear. Therefore, newly developed microfluidic devices will be utilised to investigate axonal response to stretch injury in a fluidically isolated microenvironment. In this aim, a device to simulate pressure induced axonal stretch injury of axons by using elastomeric properties of PDMS was designed based on the microfluidic device developed by Taylor and coworkers [8]. The culturing device was separated from the pneumatic channel by a thin PDMS membrane. This membrane was inflated when pressure was applied, resulting in stretch injury of the axons in the
adjacent culturing device. Neuronal responses following stretch injury and repetitive injury were investigated using this new in vitro model of isolated primary cortical stretch injury and assessed by using immunocytochemistry techniques.

Aim 2) To develop a fast method to fabricate microfluidic devices by using a CO$_2$ laser direct machining system (Chapter 4)

With the introduction of hobby laser engravers, the use of CO$_2$ laser micromachining on polymer substrate has the potential for flexible, low cost, rapid prototyping of microfluidic devices when compared with standard photolithography fabrication processes. Unfortunately, the feature size created by most CO$_2$ laser micromachining systems is too large to become a functional tool in neurobiology. Therefore, a novel method was developed to reduce the feature size of the microchannel by passing the laser beam through a stainless steel pinhole. The practicality of the proposed machining process was demonstrated by fabricating a PMMA microchip electrophoresis device. The laser engraver system was then be used to fabricate PDMS devices that were tested for its suitability for culturing primary cortical neurons.

Aim 3) To develop a new microfluidic device with a maze structure for co-culturing of neurons and glia (Chapter 5)

Conventional CNS neuron-glia interaction studies in vitro have been conducted by co-culturing a high density of neurons and glia cells on a polystyrene cell culture plate in a mixed form. It is the most widely used co-culture method and has made a significant contribution in discovering many neurological mechanisms. However, the conventional co-culture method provides no means to spatially control cells during the cultures and makes it extremely hard to study localized neuron-glia interactions. Here, a novel microfluidic compartmentalized co-culture platform that utilizes a maze feature was developed. These maze features enable glia cells to interact with two
isolated neuronal populations, thereby providing more chemically and physically controlled surroundings.
Chapter 2

2 MICROFLUIDIC CULTURE PLATFORM FOR STUDYING NEURONAL RESPONSE TO AXONAL STRETCH INJURY

The content of this chapter has been published in “Biomicrofluidics”. Minor modifications on formatting such as font size, layout etc. have been made in order to have consistency of formatting throughout this thesis.

2.1 INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in children and young adults [100], making TBI a significant public health problem. Widespread axonal damage throughout the brain, referred to as DAI, is one of the most common and important features of TBI [101]. DAI may be caused by rapid brain deformation, stretching, compression or shear forces occurring as a result of traumatic incidents such as motor vehicle accidents, falls and assaults [4]. Primary damage to axons following TBI, progressively develops over a number of days into secondary processes, including axonal cytoskeletal disconnection and neuronal degeneration [53, 102]. The delayed response provides a “therapeutic window” for possible interventions to prevent or reverse these detrimental cellular changes. Critical to the development of such interventions is a complete understanding of the cellular mechanism that comprise the neuronal response to trauma.

Various experimental models have been developed to help understand the effects and the mechanisms of TBI induced cellular dysfunction and death. Both in vivo animal models and in vitro cell culture models have been used to investigate the mechanism and effects of injury [103]. Although in vivo models allow the investigation of the whole organism response to mechanical loading and provide a tool for the assessment of efficacy of therapeutic compounds, these models do not permit an easy assessment
of the effects of mechanical trauma on individual cells. They also do not permit a precise investigation of the cellular and molecular events occurring following traumatic injury. On the other hand, a range of *in vitro* models of axonal injury have been developed. Such models include direct axonal transection [104], transient axonal stretch injury involving pressurized fluid deflection of axon bundles of primary cortical neurons [54], and graded axonal compression[105]. These models allow the study of axonal alterations in real-time and, facilitate investigations of the biochemical mechanisms involved in the progression of axonal pathology, however, the random spatial distribution of neurons limits the effectiveness of these platforms for studying and understanding TBI. There is only a finite distance over which the individual axonal response can be followed and traditional models do not allow for individualised control of the microenvironments of the distinct cellular compartments of the neuron (soma, axon and dendrites), complicating the investigation of pathological changes within distinct cellular compartments of the neuron (soma, axon and dendrites) in response to trauma and pharmacological treatment. Detection of these pathological changes may be particularly important in the investigation of mechanisms of secondary degeneration where the role of retrograde signalling to the soma is unclear.

Smith and co-workers [102] developed a uniaxial *in vitro* model system in which neurons are grown on a flexible silicon membrane. They utilized a controlled air pulse to rapidly decrease the chamber pressure and deflect downward the portion of the substrate that contains the cultured axons, inducing tensile elongation. This model was recently adapted to include axon guiding structures [106, 107]. However, these guiding structures did not allow the fluidic isolation of the soma compartment, limiting the control of the microenvironment in this model, for example, it does not allow for the targeted exposure of the axon or soma to study potential therapeutic
agents that may prevent axons from degenerating and/or may promote regeneration. In addition, studies show that axon degeneration can precede, and sometimes cause, neuronal death in several disorders [25], creating a compelling need to understand the mechanisms of axon degeneration. Microfluidic devices that allow manipulation of fluids in channels with typical dimensions of tens to hundreds of micrometers have therefore emerged as a powerful platform for the study of neuron degeneration and regeneration [108]. The functionality and features such as microscale channels, pumps and valves that can be incorporated into microfluidic devices allow physical and cellular manipulations which are not possible in conventional open cell culture systems [109]. Specifically within the field of neuroscience, a microfluidic device developed by Jeon’s group enabled the physical isolation of axons from their parent soma [7, 8] and the separation of the extracellular microenvironments of these cellular compartments through the incorporation of microgrooves (10 µm wide and 3 µm high) within microfluidic devices. Using such system, Hosie and colleagues [110] demonstrated that glutamate receptors are present in axons and that they can respond directly to excitotoxic concentrations of glutamate independent of the soma. Kim and colleagues [111] adapted this system into their ‘neuro-optical microfluidic platform’ that also consists of a femtosecond laser to enable precise and reproducible axotomy of axons and mini incubator for continuous long term monitoring of post-injury events.

‘Quake valves’[112] are microvalves/microchannel that were originally developed for pumping and valving in microfluidic systems. Within the field of neuroscience, Quake valves have been used to facilitate dynamic neuron and glia co-culture [113]. They have also been utilised in neuronal trauma models to enable complete axotomy [114] and to apply graded compression to a single axon [115]. Here, we have
developed a new device that can apply mild to very mild stretch injuries to individual axons extending from primary cortical neurons by integrating “Quake valves” (also called pneumatic channel) into the bottom of fluidically isolated microfluidic culturing devices. Previous studies [7] used these microfluidic devices in combination with a glass coverslip, taking advantage of the well characterised culturing conditions on glass. Here, we used thin, elastic PDMS membrane as a substrate to apply stretch injury to axons by pressurizing the pneumatic channel. When the pneumatic channel is pressurized, the thin PDMS membrane is deflected upward and stretches the axons. Defined stretch injuries applied using this device resulted in neuronal pathologies typical of those observed in vivo. This new device is unique owing to its ability to apply both mild (5%) and very mild stretch injury (0.5%) and to delineate alterations in response to discrete axonal injury in a fluidically isolated microenvironment. It also allows control over the length of the stretch, in this work set to 90 µm. In the current study, the extent of the injury was quantified using immunocytochemical labelling and assessment of the axonal degenerative index.
2.2 Methods

2.2.1 Characterization of PDMS/PDMS valve

A series of increasing gas pressures were applied to the pneumatic channel and once steady state was reached after 10 s, the membrane deflection was measured with an interference microscope Wyko NT9100 (Veeco Instrument Inc, USA) by dual LED light optical profiling in the vertical scanning interferometry (VSI) through 20X objective (Figure 2.1).

2.2.2 Fabrication and assembly of axonal stretch injury platform

The axonal stretch injury device consisted of two independent PDMS structures separated by a thin PDMS membrane (Figure 2.1B and Figure 2.2). Dissociated rat cortical neurons (harvested at embryonic day 18, E18) were grown in the upper PDMS microfluidic culturing device (Xona Microfluidic, CA), which has 450 µm long, 10 µm width and 3 µm high microgrooves connecting the soma and axon compartments. The small size of the microgrooves prevents migration of cell bodies between the compartments while allowing only axons to pass through [8]. The bottom structure contained the pneumatic channel and was irreversibly sealed with the PDMS membrane using air plasma. In response to a pressure pulse, the pneumatic channel inflated and the PDMS membrane deflected upward, stretching the axons growing on top. The pneumatic channel microfluidic device was fabricated in PDMS (Sylgard 184, Dow corning, Michigan, USA) by soft lithography and replica molding procedure [116]. The template to make the PDMS pneumatic channel device was fabricated by using an office laminator (Peach 3500, Laminator Systems, Australia), similar to the previously described protocol by Kazarian [117]. Briefly, the master template for the pneumatic channel was fabricated by laminating the PMMA substrate (75 mm × 50 mm × 1 mm) with a 17 µm thick layer of dry film (Ordyl 317, Elga Europe, Italy) at
Figure 2.1 (A) Schematic showing experimental setup for PDMS membrane deflection characterization. Gas pressure was applied to the pneumatic channel by using an in-house built valve system which utilized a dynamic pressure regulator, USB-based controller box for the valve manifolds and Labview software. The computer sent the signal to the valve manifold through the controller box. The pneumatic valve opened and applied gas pressure to the embedded microchannel. Thin PDMS membrane deflected upward and deflection was measured with optical profiler system. (B) Photograph of assembled device comprising the pneumatic channel and culturing device. (C) A typical snapshot from Veeco profiler output for membrane deflection data collection.
Figure 2.2 Schematic drawing of microfluidic device used for simulating axonal stretch injury. (A) A thin PDMS membrane separates the pneumatic channel (bottom) from the overlying culturing chamber (top). (B) Application of gas pressure to the pneumatic channel (positioned at 200-300 µm from microgrooves) causes upward deflection of the thin PDMS, which stretches the overlying axon. (C) Rat cortical neurons at 7 days growth in the poly-L-lysine coated culturing device showing adequate axonal extension prior to axonal stretch injury. Scale bar=150 µm
100 °C at a speed of 1350 mm/min. After lamination the substrate was exposed for 90 s through a transparency mask (Kodak Polychrome image setting film Pagi-Set, 4400 dpi, Pagination Design Services, Geelong, Australia) using a UV shark series high-flux LED array (OTLH-0480-UV, Opto Technology, Wheeling, IL, USA) as a light source [118]. The substrate was then baked for 20 min at 110 °C on a programmable hot plate (ECHOthermTM MODEL HS40, Torrey Pines Scientific, USA). The channels were developed and hard baked using the previously described procedure [117]. The template was ready for use as a master after being allowed to cool to room temperature. Curing agent and PDMS elastomer (1:10 ratio) was then poured over the template and degassed under vacuum. It was then cured for 60 min at 75 °C. After curing, PDMS was detached and an air inlet was punched out with biopsy punch (1.5 mm diameter, Huat Instrument, USA). The resulting pieces were cleaned with compressed air and any remaining debris was removed by using a 3M Scotch Brand 471 tape. A thin PDMS membrane was formed on a 1H, 1H, 2H, 2H perfluorooctyltrichlosilane (Fluorochem, UK) coated silicon wafer (100 mm diameter, 525 +/- 25µm thickness, one side polished, test grade, SWI Semiconductor Wafer Inc, Taiwan) at rate of 1500 rpm and 2500 rpm at spinning time of 30 s by using a 8” Portable Precision Spin coater (Model P-6204, Cookson Electronics Equipment, IN, USA), which is a centrifuge-like device with a vacuum chuck and adjustable rotational speed to allow control of the uniformity and thickness of the film. To enable the inflation of the PDMS membrane, the pneumatic channel device was irreversibly sealed with the membrane through the activation of the surface using a handheld corona discharge unit (Electro Technic Product Inc, USA). Pressure was applied to the pneumatic channel by using an in-house assembly valve system developed by Quake and co-workers [112].
2.2.3 Primary cortical neurons preparation

All experiments involving animals were conducted according to protocols approved by the Animal Ethics Committee of the University of Tasmania. Microfluidic devices used in this study were sterilized with 70% ethanol and UV light. Prior to cell seeding, the surface of the PDMS devices was hydrophilized using a handheld air plasma unit and then coated with 0.01% poly-L-lysine (PLL) (Sigma, USA) for three days. After that, they were washed in mili-Q® water and placed in a standard humidified cell culture incubator set to 37 °C and 5% CO₂. The water was removed and the coated devices were filled with pre-warmed initial neuronal growth media consisting of Neurobasal™, 10% heat inactivated foetal calf serum, 2% B27 supplement, 0.5 mM L-glutamine, 25 µm glutamate and 1% penicillin-streptomycin (Gibco/BRL, Life Technologies, USA) (refer section 8.2) and incubated at 37 °C, 5% CO₂ for at least 3 h prior to plating the neurons. Primary cortical neurons were derived from E18 Sprague Dawley rat embryos as previously described[119]. Briefly, after dissection cells were chemically dissociated in 0.0125% trypsin (Life Technologies, USA) followed by washing and gentle manual dissociation. Primary neurons were then loaded into the somal compartment of the microfluidic devices at a density of 9 ×10⁶ cells/mL and incubated at 37 °C, 5% CO₂ for 5 min to enhance cell adhesion to the substrate. After incubation, both soma and axon compartments were filled slowly with pre-warmed initial neuronal growth media to minimise disturbing the cells. Cultures were grown at 37 °C, 5% CO₂. After 24 h, the initial neuronal growth media was replaced with subsequent growth media (initial growth media without the foetal calf serum and glutamate) (refer to section 8.2). The culture media was renewed three times a week (on DIV 3, 5, 7 and 9) to prevent oxygen and nutrient depletion or waste accumulation.
2.2.4 Axonal stretch

A controlled gas pressure was applied to the pneumatic channel to deflect the PDMS membrane, thereby stretching the individual axons located above the air channel. The pressure was applied to using an in-house built valve system that utilized a dynamic pressure transducer (Pneumadyne, Inc, Plymouth, MN), USB-based controller for the 24 solenoid pneumatic valves and Labview software (National Instrument, Texas, USA). The introduction of gas into the valve was gated by a solenoid. A controller box, connected with the computer sent out a signal to a relay circuit. The relay circuit opened a pneumatic valve through the solenoid, and the pneumatic valve then applied a gas pressure pulse to the pneumatic channel on the device. Uniaxial strains were applied 7 days after plating of primary cortical neurons in the somal compartment of the microfluidic device.

2.2.5 Immunocytochemistry

At 24 and 72 h after stretch injury cells were fixed with 4% paraformaldehyde (Sigma, USA) for 1 h at room temperature. After removal of the fixative, the cells were washed with 0.01 M phosphate buffered saline (PBS). The PDMS substrates were then incubated with primary antibodies in diluent (0.01 M PBS containing 0.3% Trixton X-100) for 1 h at room temperature and at 4 °C overnight. Primary antibodies to the axon specific microtubule associated protein tau (tau; 1:5000, rabbit polyclonal, Dako, Denmark), NFM (neurofilament M; 1:1000, rabbit polyclonal, Serotec, USA) and MAP2 (microtubule associated protein 2; 1:1000, mouse monoclonal, Milipore, USA) were used. Following incubation with the primary antibody, cells were rinsed with 0.01 M PBS and incubated in the dark with isotype and species specific secondary antibodies for 2 h at room temperature. Secondary antibodies (Mouse IgG Alexa Fluor 488 and Rabbit IgG Alexa Fluor 594, Molecular Probes, USA) were
diluted 1:1000 in 0.01 M PBS. The cells were then washed in 0.01 M PBS followed by mili-Q water and mounted on glass slides with Permafluor mounting medium (Immunotech, France). The slides were then allowed to air dry in the dark at room temperature.

2.2.6 Quantification of axon degeneration

Fixed, immunofluorescent labelled samples were visualized with a Leica DMLB2 fluorescent microscope (Leika, Germany) and images were acquired with a CCD camera (ORCA, Japan) and recorded in NIH elements software (Nikon, Japan). To quantify axonal degeneration, we used the method described by Sasaki and colleagues[120]. Here, we analysed axonal degeneration by comparing axonal tau labelling among experimental groups at distal region of axons. Tau is a microtubule associated protein localised specifically to axons and routinely used to visualise these processes [121]. Briefly, each tau labelled image was binarized based on pixel intensity using NIH imageJ software. The total number of detected black pixels was defined as the total axon area. Degenerated axons that were fragmented and beaded were detected as particulate structure. Each particle of contiguous pixels was judged either intact or fragmented based on its circularity, defined as circularity = (area)/(π × radius²), where area and radius are measured in pixels. Using a particle analyser algorithm of ImageJ, axonal regions with circularity more than 0.2 were determined and designated as fragmented. A degeneration index (DI) was calculated as the ratio of fragmented axon area over total axon area. To facilitate the comparison between injuries, we measured the DI relative to control at 24 h and 72 h post injury (PI). For each culture, two randomly selected images (20X) of fluorescently labelled axons were captured. For the quantification of axon degeneration, two images from three different devices from three separate cultures were then analysed,
and the data arising from processing these 18 images is expressed with means ± standard error of the mean (SEM). Statistical analysis was performed using Student’s t-test. p-values <0.05 were considered significant.
2.3 Results

2.3.1 Axonal injury device design and operation

To apply the stretch injury, flexible elastic PDMS was chosen as the substrate for culturing. The isolated axonal stretch injury device contains two independent PDMS structures separated by a thin PDMS membrane (Figure 2.1B and Figure 2.2). The upper structure will be referred to as the culturing device and the bottom structure will be referred to as the pneumatic channel device. The culturing device was a commercially available device used to culture neurons in a fluidically isolated microenvironment [8]. It was positioned 230-300 µm from the microgrooves and sealed with a thin PDMS membrane on top of which the neurons grow. This flexible elastic membrane is deflected upward when the pneumatic channel below is connected to gas, stretching the axons growing on top.

2.3.2 Device characterization

To determine the degree of axonal stretch, the physical extent of membrane deflection was examined by using an optical profiler system. Membrane deflection, and therefore the degree of axonal stretch, depends on the thickness of the PDMS membrane and the applied pressure. In these experiments, PDMS membrane with thickness of ~60 µm (spin speed 1,500 rpm), and ~15 µm (spin speed 2,500 rpm) were used, as determined by an optical profiler system (data not shown). For the experiments presented here, a 35 psi pulse was applied to a 60 µm PDMS membrane, resulting in a 4.3 µm upward deflection (Figure 2.3). The pneumatic channel in this study was 90 µm wide, therefore a mild, 0.5% strain was obtained (estimated by calculating the length of the membrane based on the channel width and deflected height using Pythagoras' Theorem). It is important to note this stretch is significantly smaller than strains previously applied in the literature, for example Dollé et al. reported a deflection of up to 1 mm resulting in
a 11% strain [122]. We also applied a 25 psi pressure to a 15 µm PDMS membrane and thereby obtained 14.1 µm upward deflection which resulted in a 5% strain to axons (Figure 2.3).

2.3.3 **Response to axonal stretch injury**

Primary cortical neurons from E18 embryos were seeded into the poly-L-lysine coated culturing devices and allowed to grow for 7 days *in vitro* (DIV) for adequate axonal extension prior to stretch injury (Figure 2.2C). After 7 DIV, a pressure pulse (10 s) was applied to the pneumatic channel to induce a very mild (0.5%) axonal stretch injury to the overlying axon. Double fluorescent immunolabelling for the dendritic marker protein MAP2 and the axonal proteins tau and NFM demonstrated smooth continuous expression of these proteins in control, uninjured cultures (Figure 2.4 (A) and (B)). Tau and NFM beading was observed along the length of axons 24 h after a very mild (0.5%) injury (Figure 2.4D). In order to examine if axonal injury resulted in signs of retrograde degeneration in the neuronal soma, we examined the somal compartment. At 24 h PI stretched neurons demonstrated dendritic beading and irregular MAP2 expression in the somal compartment (Figure 2.4C). To quantify the extent of distal axonal degeneration at different post-injury time points, we analyzed tau immunolabelled images of fixed axons using a particle analyzer algorithm of ImageJ software (Figure 2.5). After very mild injuries (0.5%), the majority of the axons remained intact and healthy at 24 h PI with no significant difference in degenerative index when compared to the control. However, the degenerative index increased significantly at 72 h following the very mild injury (DI=0.11±0.03). When the injury level was increased (5% injury, mild injury), we observed increased signs of degeneration including beading and fragmentation at both time points with a significant increase in the degenerative index at 24 h PI (DI=0.17±0.02) and 72 h PI.
(DI=0.18±0.01) compared to the uninjured control. Additionally, the degenerative index was increased significantly at 72 h following mild injury (5%), compared to following very mild injury (0.5%).
Figure 2.3 Relationship between applied gas pressure and membrane deflection at steady state for ~60 µm PDMS membranes (1,500 rpm) and ~15 µm PDMS membranes (2,500 rpm). A constant valve opening of 10 s was applied to ensure deflection measurements at steady state.
Figure 2.4 Immunocytochemistry images of uninjured and injured neurons 24 h following 0.5% injury. Cell bodies and dendrites (A, MAP2 labelling), and axons (B, Tau and NFM labelling), in the control chambers were smooth and uniformly labelled for cytoskeletal markers. At 24 h post-injury cortical cultures exposed to axonal stretch injury showed dendritic blebbing, and irregular MAP2 expression (C, MAP2 labelling). The injured axons (D, Tau and NFM labelling) underwent characteristic beading and degeneration, showing punctate accumulation of tau and NFM within the swollen portions of the axon. Scale bar=50 µm
Figure 2.5 Axonal stretch injury to cultured cortical neurons resulted in axonal degeneration. (A) Tau-labeled (microtubule marker) axons following 0.5% injury (very mild) and 5% injury (mild) at 24 h and 72 h time point. Binarized images show fragmented axons defined by the Analyze Particle function in ImageJ software. Stretch injury induced progressive distal degeneration leading to axonal beading and microtubule fragmentation. Scale bar= 100 µm. (B) The degeneration index increased significantly at 72 h following 5% injury, compared to following 0.5% injury. However, there was no significant difference between control and 24 h following 0.5% injury. The degeneration index increased significantly at 72 h following 0.5% injury if compared to the control. Axonal degeneration index is significantly higher than controls at both 24 h and 72 h following 5% injury. The * symbol represents a statistical difference (p<0.05) versus control at each time point. The † symbol represents a statistical difference (p<0.05) versus very mild stretch (0.5% injury) at post 72 h.
2.4 Discussion

Recent studies demonstrated that microfabrication technologies enable the development of powerful platforms to culture and manipulate neurons and allow the study of nerve injuries [123]. However, to the best of our knowledge, no in vitro system is able to study precise and reproducible mild axonal stretch injury in a fluidically isolated microenvironment. Therefore, a novel microfluidic device to simulate mild (5%) and very mild stretch injury (0.5%) of axons was developed in the current investigation by incorporating a microfluidic valve (17 µm high, 90 µm wide) into our devices. This device is capable of applying mild to very mild injuries by stretching only a 90 µm long section of the axons. The microfluidic culture chamber design developed by Taylor and co-workers [8] for fluidic isolation of the axon and soma was used, together with a flexible thin PDMS membrane that can be locally deflected upward using the valve technology. This allows axons growing on top of the PDMS membrane to be discretely stretched, providing a new platform to study stretch injuries and, in later stages, has the potential to test the effects of therapeutic agents in isolated axonal or somal compartments following stretch injury. More recently, Dolle and colleagues [122] developed a new device where uniaxial strains were applied to the elastic PDMS substrate on which axons extend between two organotypic hippocampal slices. This device is similar to our device, where upward deflection is applied to the axons growing on PDMS, however, this device applied stretch injury range from 11-42%, in a non-fluidically isolated microenvironment over a length of approximately a millimeter, whereas, our device applied stretch injury in fluidically isolated microenvironment at a relatively small strain (0.5% or 5%). These significant differences in stretching regimes each provide access to unique scenarios to simulate clinical manifestations.
Chapter 2

Here, flexible elastic PDMS was chosen as the substrate for culturing because PDMS has advantages of being inexpensive, permeable to gas, optically clear and can be simply sterilized by ethanol or UV light exposure [124]. In addition, PDMS has also been widely used in a range of biological applications, providing evidence of its biocompatibility[125]. For example, Dolle et al. [122] proved that organotypic hippocampal slices cultured on elastic PDMS substrate remain healthy over three weeks in culture. In addition, we did not observe any detachment of axons after the application of stretch injury, suggesting that the axons are firmly attached to the surface of thin PDMS substrate and the axons are stretching in the same strain as the PDMS substrate. The pneumatic channel was positioned 200 – 300 µm from the microgrooves. In the same culturing device, Taylor and colleagues [8] observed that axons began to extend into the axonal chamber after 4 DIV with the extensive growth of axons in axon compartment levelling off at 7 DIV. At 7 DIV, we confirmed that all axons that had extended into the axon compartment, past the pneumatic channel, were long, relatively unbranched axons. Based on this information, all the axons in the axon compartment would be subjected to stretch injury when applying the stretch injury at 200 – 300 µm from the axon grooves at 7 DIV, thus these conditions were selected for the stretch injury studies.

Traditionally, only strains over 10% were thought to cause injury [126]. There are a few previous studies that have reported different experimental models for stretching axons in uniaxial field, ranging from 15-65% in a cultured N-Tera2 human neuron cell line [102] to 30-75% in a model of primary cortical neurons [127]. Dendritic alterations following axonal stretch injury were also examined as part of this study. Dendritic beading was observed along the dendrite shaft, a finding consistent with previous studies performing in vitro axonal stretch injury [106]. Changes in dendritic
structural proteins, such as MAP2 and neurofilaments, are also prominent in animal models of TBI [128-130]. Since dendrites are essential to the processes of learning and memory [131, 132], changes in dendritic structure can result in cognitive dysfunction. Evidence from previous studies demonstrated that altered dendritic structure may contribute to the cognitive deficit observed following TBI [133]. Understanding the mechanisms by which axonal stretch injury affects dendrite structure and function may provide additional insight into TBI pathophysiology and lead to novel strategies to improve learning, memory and behavioral dysfunction after TBI.

Most research into axonal stretch injuries has focused on axonal effects, reporting widespread axon swelling and degeneration within 24 h post injury. A 1-6% stretch injury model developed by Staal and coworkers [54] showed neurofilament alterations characteristic of DAI at 48 h post injury, but no distinguishable difference in axonal morphology at 24 hour post injury (PI). In the current study, after mild axonal stretch injury (5%), microtubule fragmentation along the injured axons was observed both at 24 h and 72 h PI. Similarly, axonal degeneration increased at 24 h post 0.5% injury (very mild injury), although there was no significant difference between control and very mild stretch at this time point. However, 72 h post 0.5% injury, microtubule fragmentation was significantly greater in stretched axons compared to the unstretched axons. These findings are considered hallmark pathological features of DAI and have not previously been studied or reported for such small and very mild injuries.

The current study has the ability to reproducibly perform a very small level of strain (0.5% increase in original axon length) as compared to the bundle axonal stretch injury (1-6%) demonstrated by Staal et al. [54] . For comparison, the strain rate applied by Staal and Chung was in the range of 0.8 to 4.2 s\(^{-1}\), while we have applied strain rates of significantly lower magnitude, from \(5 \times 10^{-4} \text{ s}^{-1}\) (0.5% increase in original axon
length) to $5 \times 10^{-3}\ s^{-1}$ (5% strain). Significant changes in both dendritic and axonal structure were observed in our study even after this relatively mild injury. This suggests that even very small stretch injury to axons will induce degenerative changes. The current model is also designed to address single axon responses therefore, circumventing the added variables of investigating bundles of axons of unknown density and varying length that are not attached to the substrate.

Previous studies [102] observed delayed elastic effects where undulation and waves were observed immediately after injury and then returned to the original axonal length after a period of time. We have not observed this effect on our device, possibly due to the fact that the delayed elastic effect are only observed at high strain rates such as 19 s$^{-1}$ applied by Smith et al. [102] in comparison with the relatively small strain rate magnitudes such as $5 \times 10^{-4}\ s^{-1}$ (0.5% strain) and $5 \times 10^{-3}\ s^{-1}$ (5% strain) reported here. Our small strain rate also explains why we do not observe primary axotomy after application of stretch injury at time duration of 10 s. In addition to the capacity to apply mild axon transient injury, our device also has the capability to investigate the injury-induced sub-cellular alterations because of the fluidic isolation of soma and axon in the microfluidic culturing devices in combination with the discrete and transient stretch of individual axons. Therefore, several aspects of DAI including changes in intracellular calcium levels [134], cytoskeletal structure [104], membrane permeability [135] and axonal swelling formation [136] can be studied through this model. In addition, the fluidic isolation of this device allows independent chemical and/or pharmacological treatments on the axonal side or somal side and will enable insights into signalling between the axon and soma by investigating the site specific neuronal response to blockers of protein synthesis, proteasome degradation, apoptosis or cytoskeletal stabilization after direct axonal stretch injury. This approach has the
capability to investigate a range of different stretch injuries from mild to severe, at
different distances from the soma and over different lengths and at different intervals
due to the ability to precisely control the location and dimensions of the valve, the
thickness of the PDMS membrane and the magnitude and timing of the applied
pressure.
2.5 CONCLUSIONS

A simple, reproducible *in vitro* model of discrete axonal stretch injury of cultured primary neurons is presented, allowing, for the first time, the controlled application of mild to very mild strain injuries. When used for applying very mild (4.3 µm upward deflection, 0.5% strain) or mild (14.1 µm upward deflection, 5% strain) axonal stretch injury to neurons at 7-9 DIV, cellular responses similar to those found in the *in vivo* models of traumatic brain injury were observed. This device is unique in the small size and magnitude of the applied strain, but surprisingly immunostaining and fluorescent imaging still revealed several pathological alterations characteristic of DAI *in vivo* following application of these low level strains. The device allows specific alterations in axons to be investigated. With the fluidic isolation of the axonal and somal compartments enabling targeted exposure to potential therapeutic agents, this system provides a valuable tool for studying the degenerative and regenerative neuronal responses induced by acute axonal stretch injury for testing potential therapeutic agents for traumatic brain injury.
3 NOVEL *IN VITRO* MODEL OF REPETITIVE AXONAL STRETCH INJURY TRIGGERS CYTOSKELETAL MISLOCALIZATION AND GROWTH CONE COLLAPSE

3.1 INTRODUCTION

Over the past few years, public awareness of the consequences of mild traumatic brain injury (mTBI) and concussion has increased. Such awareness has been accelerated by reports of the long term effects on the brain of athletes exposed to multiple concussions in competitive contact sport such as boxing, hockey and various codes of football [137-139] or child abuse victims [140]. These reports show that multiple concussions can result in accumulated brain damage and the development of a post concussive syndrome with persisting cognitive, behaviour and psychiatric problems.

This repeated brain trauma manifests as a slowly progressive neurodegenerative disease known as Chronic Traumatic Encephalopathy (CTE) [141]. It is well known that traumatic axonal injury (TAI), commonly termed diffuse axonal injury (DAI), is one of the most common and important features of TBI [142]. Studies using advanced neuroimaging techniques have identified selective white matter abnormalities in mTBI patients consistent with DAI [143, 144]. Accordingly, it is hypothesized that DAI plays an important role in repetitive mTBI and possible subsequent development of CTE.

In an attempt to identify the abnormal axonal alterations that occur in response to mTBI, researchers have developed animal models such as the controlled cortical impact [145, 146] and weight drop [147] to experimentally induce mechanical axonal stretch or compression injuries. These models have demonstrated exacerbated outcomes of impaired cognitive function and axonal injury with repetitive mTBI.
compared to a single mTBI [145-147]. For example, Huh and colleagues [145] demonstrated that repetitive mild, non-contusive head injury, using a controlled cortical impact model in 11-day old rats, resulted in axonal disconnection at 3 days following single impact, while double and triple impacts produced axonal disconnections at 1 day post-injury. While animal models have yielded considerable insight regarding the changes in animal behaviour and axonal alterations that occur in response to repetitive mTBI, their limitation is that they only postulate the underlying mechanisms of cognitive impairment at the cellular level. In addition, these animal models cannot distinguish if a worse outcome after repetitive injury is simply due to a cumulative effect or reflects a mechanism of exacerbated outcome following first injury.

In vitro models of TBI cannot surpass animal models, but facilitate the investigation of various pathobiological mechanisms at both cellular and subcellular levels and to screen potential neuroprotective agents in a rapid throughput manner. Ellis et al. [135] developed a stretch injury model and defined a mild injury as a 5.5 mm deformation (or 31% membrane strain) of the flexible silicone membrane following the application of a constant air pressure pulse. Recently, Slemmer and colleagues [148] used this model to examine the cellular events following repetitive mTBI. They showed that repeated mild injury resulted in significantly increased apoptosis as compared with a single injury, suggesting cumulative damage to the brain following multiple mild injuries. However, this method cannot be used to study the spatial compartmentalization signals due to the random spatial distribution of neurons on the silicon membrane. Furthermore, the testing of therapeutic strategies that aid in axon protection or regeneration following injury is also limited due to difficulty in isolating axons. Later on, Smith and co-workers [102] developed another in vitro model system
that induces dynamic stretch of isolated axon spanning two populations of the neurons. Subsequently, Yuen et al. [149] utilized this model to examine the effect of repetitive mild axonal stretch injury. When they applied a single, mild strain (3%) at strain rates of 20 s\(^{-1}\) to rat cortical axons in culture, no obvious pathological change was observed, but, the axons were found to display increased sodium channel expression by 24 h. When they applied a second, identical mild injury 24 h after the first injury, a significant increase in intracellular calcium was observed, which then lead to axon degeneration. These findings suggest that initial mTBI triggers a pathophysiological response that makes neurons more susceptible to an exaggerated outcome from a subsequent mTBI.

Growth cones that present on the tips of elongating axons serve as sensory and motor structures and are critically important in axonal guidance and regeneration following injury [150]. Therefore, the axon guidance mechanism and growth cone formation following complete axotomy of axons has been investigated [61, 104, 151]. However, despite accumulating research into axonal pathology of neurons following both mild and repetitive mild stretch injury using in vitro models [127, 148, 149], little is known about growth cone formation and their structure following stretch injury. Here, we have utilized our novel in vitro model of axonal stretch injury [152] to investigate the neuronal response to axonal stretch injury. The microfluidic device physically isolates axons from the soma [8], enabling a focus on the growth cone response in the axon compartment. Very mild (0.5% strain) and mild (5% strain) stretch injuries can be applied to axons by inflating an elastomeric feature [152]. In this study, we investigated the morphology and cytoskeletal profile of growth cones after mild (5%) stretch injury, very mild (0.5%), and repetitive very mild (2×0.5%) stretch injury. We report that the growth cones on the tips of the axons that have received very mild
stretch injury, differ from those on the tips of uninjured axons in their morphology and size although they are similar in cytoskeletal composition. Furthermore, the distributions of actin and microtubules within growth cones following mild and repetitive mild axonal stretch injury cultures were different from those on the tips of uninjured, developing axons.
3.2 METHODS

3.2.1 Microfluidic stretch injury system

The axonal stretch injury was conducted using the novel device which acts as an *in vitro* model of very mild (0.5%) to mild (5%) axon injury with primary cultured neurons [152]. The fabrication and assembly of microfluidic stretch injury system was prepared as described in section 2.2.2.

3.2.2 Primary cortical neuron culture

Cortical neurons were prepared from the cerebral cortices of embryonic day 18 (E18) Sprague Dawley rat embryos as described in section 2.2.3. Briefly the dissected cells were trypsinised (0.0125%) followed by washing and gentle physical dissociation with a pipette. Cells were loaded into the somal compartment of the devices at cell density of $9 \times 10^6$ cells/device and incubated in a humified incubator at 37 °C, 5% CO$_2$ for 5 min to enhance cell adhesion to the substrate. After 5 min incubation, device were filled with pre-warmed initial neuronal growth media (refer section 8.2) and returned to the incubator. After 24 h, the media was replaced with subsequent growth media (refer to section 8.2) and maintained with three times medium change weekly.

3.2.3 Axonal stretch injury

Stretch injury was applied to the axonal compartment at 7 days following plating of primary cortical neurons in the somal compartment of the microfluidic device as described in section 2.2.4. Measurement of strain within the axon is calculated by determining the length of membrane based on the width and deflected height using Phytagoras Theorem [152]. Two levels of cell injury were performed (4.3 μm and 14.1 μm deformations) and defined as “very mild” and “mild”, respectively. These deformations resulted in a stretch of 0.5% and 5% of the axon respectively (refer to section 2.3.2). For sham-injury or control, cultures were grown on the stretch injury
device without applying the gas pressure and fixed and imaged at 8 DIV and 10 DIV. Axons received mild stretch (5%) or very mild stretch (0.5%) at 7 DIV and were then fixed and imaged at both 8 DIV and 10 DIV. For the investigation of repetitive injury, cultures received the double, very mild injury (2×0.5%) stretch injury on day 7 and 24 hours after first injury. Cultures were then fixed and imaged at 10 DIV. (Figure 3.1)

3.2.4 Immunocytochemistry

At 24 and 72 h post-injury (PI), cells were fixed with 4% PFA as described in section 2.2.5. This was followed by incubation with primary antibodies diluted in diluent (0.01 M PBS containing 0.3% Trixon X-100). A primary antibody against βIII-tubulin (mouse) was used at 1: 1000 dilution (Serotec, USA). Mouse IgG AlexaFluor 488 (1: 1000, Molecular Probes, USA) secondary antibody was applied for 90 min at room temperature in the dark. To label filamentous actin (F-actin), cultures were incubated with AlexaFluor 594 phalloidin (1: 200, Molecular Probes, USA) for 30 min after antibody labelling. Fixed, fluorescently labelled samples were visualized with a Leica DMLB2 fluorescent microscope (Leika, Germany) and images were acquired with a CCD camera (ORCA, Japan) and recorded in NIH elements software (Nikon, Japan).

3.2.5 Quantitative analysis

To quantitatively examine the organization of microtubules and actin filaments of the growth cones of uninjured and stretch injured growth cones, 100X images of βIII tubulin labelling were merged with images of phalloidin staining. Growth cones were classified as either “not collapsed” (with filopodia extension and lamellipodia) or “collapsed” (without lamellipodia and with ≤3 retraction fibers) [153]. The total percentage of growth cone collapse was calculated as (number of collapsed growth cones/total number growth cones) × 100. The average area of growth cones in both sham-injury (uninjured) and stretch-injured cultures was measured by framing the
actin positive extension using the freehand modus of NIH Image J software. The amount of colocalization between microtubules and F actin was determined using the Image J-JacoP colocalization plug-in function. Each experiment was repeated three times (n=3 cultures), and data arising from processing images from three different devices were analysed and expressed with means ± standard error of the mean (SEM). One-way ANOVA with post hoc Fisher’s LSD test were performed, with p values less than 0.05 as the level of significance.
Figure 3.1 Timeline for the control, very mild (0.5%), mild (5%) and repetitive very mild (2×0.5%) stretch injury experiments. (i) For the control, neurons were seeded and grown on the stretch injury device without applying any gas pressure, and fixed and imaged at both DIV 8 and DIV 10. (ii), (iii) Isolated axons were stretched at 0.5% strain or 5% strain at DIV 7 and fixed and imaged at both DIV 8 and DIV 10. (iv) For the repetitive injury investigation, isolated axons initially received 0.5% stretch on DIV 7. 24 h later, a second 0.5% stretch injury was applied on the same device and then fixed at DIV 10.
3.3 Results

3.3.1 Compartmentalized culture in a stretch injury model

To further define the sequence of pathological changes that characterise the axonal response to injury, we used the **in vitro** microfluidic device of isolated axonal stretch injury to simulate mild (5%) and very mild (0.5%) stretch injury of axons by incorporating microfluidic valve technology into a compartmented microfluidic culturing device [152] (refer to section 2.2.2). Double immunolabeling verified an extensive network of axons (βIII tubulin immunoreactivity) and growth cones (F-actin immunoreactivity) within the axon compartment of the stretch injury model at 8 DIV (Figures 3.2 (A-C)).

3.3.2 Stretch injured axons form smaller growth cones

To determine whether the size of growth cones was affected by the stretch injury, we determined the size of the growth cones in uninjured and stretched injured cultures by measuring their area, using the marker phalloidin. Our results showed that the area of the growth cone following 0.5% stretch injury \((14.98 \pm 0.78 \mu m^2)\) and 5% stretch injury \((14.86 \pm 0.65 \mu m^2)\) at 24 h PI was significantly smaller compared to the area of the growth cones in the control, uninjured neurons \((18.85 \pm 1.53 \mu m^2)\) \((p<0.05, \text{ Figure 3.3})\). However, there was no significant difference between the size of the growth cones following 0.5% and 5% stretch injury at 24 h PI. Similarly, the area of the growth cones following 0.5% stretch injury \((14.59 \pm 2.03 \mu m^2)\) and 5% injury \((16.25 \pm 1.49) \mu m^2\) was significantly smaller than the area of growth cones in control, uninjured neurons \((22.40 \pm 1.89 \mu m^2)\) at 72 h PI \((p<0.05, \text{ Figure 3.3})\). There was also no significant difference between the size of growth cones following 0.5% and 5% injury at 72 h PI. Taken together, these results indicate that growth cones in cultures following both mild and very mild axonal stretch injury were smaller compared to
Figure 3.2 (A) Axons (green; βIII tubulin) of primary rat cortical neurons extended into the axon compartment through microgrooves (450 µm long, 10 µm width and 3 µm high) at 7 DIV. Dashed lines indicate the location of the pneumatic channel and solid lines show the microgrooves region. (B) High magnification of axons with growth cones immunostained with F-actin (red) and microtubules (green) (white square box in panel A). (C) Higher magnification of growth cone (white square box in panel B). Scale Bars=200 µm (A), 75 µm (B), 40 µm (C)
Figure 3.3 Graphs showing average growth cone area of control, 0.5% stretched, 5% stretched and repetitive very mild (2×0.5%) stretched axons at different time point. There was a significant decrease in growth cone size after a single stretch injury at both 24 h and 72 h PI and after repetitive very mild (2×0.5%) stretch injury at 72 h PI compared to control. *p<0.05. Error bar=mean ± SEM.
growth cones in uninjured cultures at both times point examined. However, at both time points, the size of growth cones in culture following very mild and mild axonal stretch injury were not significantly different.

### 3.3.3 Distribution of actin and microtubules are significantly altered in growth cones after axonal stretch injury

We have demonstrated that the size of growth cones following axonal stretch injury was smaller than the growth cones in the uninjured neurons. In order to examine whether these smaller growth cones exhibit different cytoskeletal profiles, we investigated the cytoskeletal changes of the growth cones in the uninjured, control culture and also cultures after axonal stretch injury by examining the distribution of actin and microtubules. The growth cones were labelled with both Phalloidin (F-actin marker) and βIII tubulin (microtubule marker). βIII tubulin was confined to the central domain, while F-actin was localized to the peripheral domain and the distal tips of filopodia throughout control, uninjured growth cones at 10 DIV (Figures 3.4 (A-C)). However, we observed that the central region of microtubules of growth cones at 72 h following very mild stretch injury (0.5%) was organized in a prominent loop and short microtubule fragments invaded the peripheral ‘actin containing’ region (Figures 3.4 (D-F)). At the same time point, 72 h after mild stretch injury (5%), many of the growth cones at the proximal tip of the axon had a collapsed morphology where the growth cones were oval in shape and lacked filopodia extensions (Figures 3.4 (G-I)).

To further examine the changes in microtubule and actin organization following stretch injury, we quantitatively measured the extent of colocalization of F-actin and βIII tubulin in growth cones of control cultures and in cultures after mild (5%) and very mild (0.5%) axonal stretch injury. At 24 h PI, the colocalization of F-actin and βIII tubulin throughout the growth cones of axons following 0.5% (Pearson’s
coefficie\textit{nt}= 0.55 \pm 0.02, n=35 growth cones) or 5% (Pearson’s coe\textit{fficient }=0.58 \pm 0.02, n=33 growth cones) stretch injury was not significantly different to the control, uninjured axons (Pearson’s coe\textit{fficient }=0.54 \pm 0.02, n=21 growth cones). In addition, we also observed that colocalization of βIII tubulin and F-actin in growth cone following 0.5\% stretch injury (Pearson’s coe\textit{fficient}= 0.57 \pm 0.03, n=9 growth cones) was similar to the growth cones in control culture (Pearson’s coe\textit{fficient }= 0.53 \pm 0.04, n=13 growth cones) at 72h PI, with no significant difference. However, the extent of colocalization between βIII tubulin and F-actin in growth cones following 5\% stretch injury was significantly higher at 72 h (Pearson’s coe\textit{fficient }0.65 \pm 0.02, n=14 growth cones) compared to the control (p<0.05, Figure 3.5B). Futhermore, the colocalization of βIII tubulin and F-actin in growth cones following 5\% stretch injury was significantly higher than in the growth cones following 0.5\% stretch injury at 72 h PI (p<0.05, Figure 3.5B).

3.3.4  Collapsed growth cones are increased following stretch injury

Trauma such as axotomy is known to cause growth cone collapse [61]. In order to investigate the number of collapsed growth cones following stretch injury, we quantitatively measured the proportion of collapsed growth cones in all conditions. We found that the proportion of collapsed growth cones following 0.5\% (50.29 \pm 4.98\% at24 h and 68.33 \pm 6.31\% at 72 h) and 5\% injury (64.09 \pm 7.01\% at 24 h and 93.89 \pm 3.09\% at 72 h) was significantly higher compared to control cultures (24.09 \pm 5.43\% at 24 h and 32.38 \pm 3.72\% at 72 h) at both time points examined (p<0.05, Figure 3.5A). There was no significant difference between the percentage of collapsed growth cones at 24 h following 0.5\% stretch injury and 5\% stretch injury. However, the 5\% stretch injury resulted in a significantly higher proportion of collapsed growth cones compared to 0.5\% injury at 72 h PI.
Figure 3.4 Immunocytochemistry images of growth cones of control (uninjured), 0.5% stretched and 5% stretched axons at 10 DIV and 72 h PI. (A-C) In the control, growth cones distinct filopodia were apparent (arrow). The βIII tubulin labelling (green) was distributed predominantly within the central domain of the growth cone while F-actin (red) was confined to the peripheral region. (D-F) In the 0.5% stretched axon, microtubules formed a prominent loop in the central region of the growth cones and F-actin was confined to the peripheral and transition region only. (G-I) In the 5% stretched axon, F-actin was most abundant in the axon tip, forming bulb like accumulations. Scale Bars=20 µm
3.3.5 Repetitive very mild stretch injury aggravates growth cone collapse

Knowing that the growth cones following mild and very mild stretch injury exhibited different cytoskeletal profile, smaller size and increased proportion of collapsed profiles when compared to the growth cones in the uninjured cultures, we investigated the response of axons to repetitive very mild (2×0.5%) stretch injury. Axons that received a repetitive insult were stretched again 24 h after the first stretch injury and evaluated at 72 h post the first injury (Figure 3.1(B)). We found that the size of the growth cones following single injury (14.59 ± 2.03 µm²) and repetitive injury (16.54 ± 0.93 µm²) were both significantly smaller compared to the control (22.40 ± 1.89 µm²) (Figure 3.3). However, there was no significant difference between the size of growth cones following single injury and repetitive injury. We then determined the percentage of collapsed growth cones following repetitive injury and compared these with both single injury and uninjured control. Our results show that the percentage of collapsed growth cones in cultures following repetitive injury (94.10 ± 3.02%) was significantly higher compared to the control, uninjured cultures (32.38 ± 3.72%) (p<0.05, Figure 3.5A). Most importantly, the percentages of collapsed growth cones in cultures following repetitive injury was significant higher than following a single injury (68.33 ± 6.31%) (p<0.05, Figure 3.5A). Additionally, there was a significant increase in the colocalization of F-actin and βIII tubulin in growth cones following a repetitive very mild insult (Pearson’s coefficient= 0.67 ± 0.03, n=10 growth cones), as compared with both the single, very mild insult (Pearson’s coefficient= 0.57 ± 0.03) and uninjured control (Pearson’s coefficient= 0.53± 0.04) (Figure 3.5B).
Figure 3.5 (A) Graphs show the mean percentages of collapsed growth cones in control, 0.5% stretched, 5% and repetitive very mild (2×0.5%) stretched axon compartments at different time point. Stretch injury induced increased axonal growth cone collapse at both 24 h and 72 h PI compared to the control. In addition, repetitive very mild (2×0.5%) stretch injury induced more collapsed growth cones when compared to single 0.5% stretched axon at 72 h PI. (B) The growth cones in 5% stretched axon had a significantly higher Pearson’s Coefficient value of βIII tubulin and F-actin compared to both the growth cones in control and 0.5% stretched axon at 72 h PI. However, there was no significant difference between the growth cones in control, 0.5% stretched or 5% stretched axons at 24 h PI. The growth cones in 2×0.5% repetitive stretched axons has significantly higher Pearson’s Coefficient value of βIII tubulin and F-actin if compare to both the growth cones in control and single 0.5% stretched axon at 72 h PI. *p<0.05. Error bar=mean ± SEM.
3.4 DISCUSSION

*In vitro* experiments are important to achieve a better understanding of the cellular mechanisms that contribute to repetitive concussion related cellular dysfunction. Previous studies show that growth cones play an important role in axonal regeneration following axotomy [150]. Developing growth cones normally exhibit a characteristic morphology where actin filaments form thin bundles of filopodia and flattened, veil like lamellipodia [154] and microtubules extend into the peripheral region where they overlap with actin bundles at the bases of filopodia [155]. Here, we investigate the alterations in the growth cone morphology and cytoskeleton after single and repetitive stretch injury using our *in vitro* model.

We found that growth cones on the tips of axons following mild and very mild stretch injuries were smaller compared to the growth cones of unstretched axons. This observation is similar to other studies where the growth cones of the tips of regenerative sprouts after axotomy were smaller compared to growth cones of developing axons [156]. In addition, we found that both very mild and mild axonal stretch injury resulted in an increase in the percentage of collapsed growth cones compared to the control, suggesting that stretch injury even at very mild levels of strain can trigger growth cone collapse.

Differences were not only noticeable in the size and the percentage of collapsed growth cones, but also in their morphology. In the current study, we observed that following very mild stretch injury most growth cones were characterized by the presence of central loops of tubulin immunoreactivity at 72 h PI. Previous studies demonstrated that microtubules in slowly growing growth cones become bundled and form loops before developing a new axon branch or new growth cone [60, 157]. Taxol treatment (microtubule stabilizing agent) has also been shown to trigger the
formation of microtubule loops following axotomy [157]. Therefore, we suggest that following very mild stretch injury axons have the capacity to repolymerize microtubule elements at 72 h after injury, leading to the formation of loops. Most importantly, growth cones on the tips of axons that have received very mild stretch injury have similar localization of microtubule and F-actin at both 24 h and 72 PI as growth cones on uninjured, developing axons, indicated by phalloidin staining and βIII tubulin labelling. This suggests that growth cones formed following very mild axonal stretch injury possess the cytoskeletal capacity for motility and extension similar to normal developing growth cones [104].

Conversely, we observed the formation of swellings at the axon tips after a mild stretch injury. These structures lacked the actin rich filopodia and lamellipodia hence lost the ability to detect guidance cues [61, 158]. These collapsed growth cones are known as “retraction bulbs” and are considered important hallmarks of failure to regenerate in TBI, as well as in other neurodegenerative diseases such as multiple sclerosis, Alzheimer’s disease and Parkinson’s disease [39, 61, 159]. These bulbs were typically round or oval shaped and lacked any kind of extensions. Previous studies show that stretching induces damage to microtubules and as such causes failure of axonal transport and leads to axon degeneration [107, 127]. Here, we found that a mild stretch injury resulted in greater growth cone collapse compared to controls at 24 h and 72 h PI and also compared to the axons that had received very mild injuries at 72 h PI. Therefore, we suggest that in the current study, mild axonal stretch injury triggers the formation of retraction bulb, possibly due to the damage to the microtubules.

A number of clinical and experimental investigations have described the behaviour, physiological, pathological sequelae of mild repetitive head injury on macroscopic and microscopic levels [145, 148, 160]. Recently, in a study by Shitaka et al. [161] using
an *in vivo* controlled cortical impact model, two mild injuries were administered to mice 24 h apart and significant increased cognitive deficits were demonstrated after repeated injuries. Another repetitive injury study using an *in vitro* axonal stretch injury model also observed significant increased intracellular calcium that led to degeneration of axons after the application of a second, identical mild stretch injury (3%) 24 h following an initial mild injury [149]. Here, we used a similar “double insult” timeline by stretching the axons 24 h after their initial insult. After a single, very mild injury (0.5%), we found that the extent of colocalization of βIII tubulin and F-actin in growth cones was similar to the growth cones in normal developing distal axons. When after 24 h a second, very mild injury is applied, both the extent of colocalization of βIII tubulin and F-actin in growth cones, and the proportion of collapsed growth cones becomes significantly increased at 72 h PI when compared with the control. These results demonstrate that even at a very mild strain level the growth cone response in repetitive very mild stretch injury was greatly increased compared to single injury. Previous experiments demonstrated that the animals that receive repeated injuries seven days apart do not exhibit cognitive deficit, suggesting that the brain can recover from first injury if given sufficient amount of time [162]. Our results suggest that 24 h is not sufficient for the axons to recover and re-establish cytoskeletal structures and processes such as axon transport or calcium signalling even though the injury was very mild.
3.5 **CONCLUSIONS**

In summary, our results suggest that growth cones exhibit a different cytoskeletal profile and characteristics following very mild and mild stretch injury. Particularly following mild or repetitive very mild stretch injury, the tip of distal axons formed abnormal and dysfunctional retraction bulbs. Experiments were conducted using a novel model of axonal stretch injury that is highly adaptable and therefore amenable to revealing further insights into the cellular changes triggered by axonal injury. Investigations adapting levels of strain, altering the age and anatomical sources of the neurons and the position of the pneumatic valve relative to the soma as well as co-culturing with glial cells will all likely contribute to a much greater understanding of the complex cascade of cellular mechanisms underpinning the response to TBI. Such a model has a great potential for high throughput screening of potential interventions in such a cascade.
4 Fast Fabrication of Microfluidic Devices by Using CO₂ Laser Engraver System

The content of this chapter has been published in “Analytical Chemistry”, minor modifications on formatting such as font size, layout etc. have been made in order to have consistency of formatting throughout this thesis. New data regarding the biocompatibility of primary neurons grown on laser engraved devices has also been integrated into this chapter.

4.1 Introduction

Polymer microchips have been championed for the production of low-cost and disposable microfluidic devices because of the ability to fabricate by mass replication techniques such as hot embossing [163] and injection molding [164]. These are however very expensive in a research intensive environment, as the master die can cost tens of thousands of dollars per iteration, making it a costly and time consuming process in design and development stages. Furthermore, these approaches are often beyond the budget of research laboratories in universities and small companies.

In the search for cheaper, more flexible fabrication strategies, laser machining, also called laser ablation, is an attractive method for microchip fabrication. Laser ablation provides a high flexibility in design in a short time frame as devices can be generated from a simple CAD diagram and printed like a conventional printed image on paper. This cost effective, rapid fabrication technique is also desirable for the development of both 2D and 3D complex integrated systems because it has the capability to create microchannels of different widths and depths in a single device without the precise alignment required for photomasks in multistep photolithography.
Laser machining is typically undertaken with low wavelength excimer lasers (248 nm)[165]. These are expensive with a low ablation rate. PMMA, amongst a number of other substrates, can be ablated using infrared radiation from a CO$_2$ laser at a wavelength of 10.6 µm [166]. CO$_2$ lasers are typically much cheaper than excimer lasers, and have been used for many years for cutting/engraving various substrates with logos and other designs. Since the first report by Klank et al. in 2002 [167], CO$_2$ laser machining has been used as a very cost effective method for rapid fabrication of PMMA devices for a range of applications including a microfluidic co-culture system [168], a cytometer and a methanol detection chip [169], applications where wide microchannels are applicable. A number of groups have evaluated CO$_2$ ablation systems for patterning capillary electrophoresis (CE) microchannels. Forgaty et al. [170] made 33 µm deep and 110 µm wide microchannels in PDMS for the separation of the neurotransmitters aspartate and glutamate in 60 mM boric acid, pH 9 buffer. Baker et al. [171] fabricated 35 µm deep, 162 µm wide microchannels in glass and compared the separation performance with wet etched devices. They found that the laser ablated devices had efficiencies 3-6 times lower than the ablated channels of similar size, with the best efficiency of 2,500 plates (~60,000 plates/m) obtained for fluorescein.

One of the reasons for the poor performance of these microchips for electrophoresis is that the channel dimensions (in the range of 100-300 µm) are typically larger than is ideal for efficient CE separations [167, 172, 173]. A few groups have proposed methods to reduce the channel feature size and the bulging of PMMA substrates caused during the CO$_2$ ablation process [174, 175]. Recently, Chung and colleagues [176, 177] fabricated a PMMA chip by scanning the CO$_2$ laser across a lithographically patterned metal film (a proximity mask), leaving only the exposed
PMMA for ablation. The feature size could be reduced to a width of 58 µm when using 50 µm patterns on the film and the bulges were reduced to less than 0.2 µm.

Here, we present an alternative and novel method to fabricate small microchannels (width <100 µm) in PMMA by using a low cost commercially available hobby laser system ($2,500). We applied the near field scanning optical microscopy (NSOM) concept to narrow the size of the laser beam by passing the CO$_2$ laser through a stainless steel disk containing a small aperture – a pinhole. NSOM imaging [178] relies on an aperture physically smaller than the wavelength of the light to achieve sub-diffraction imaging. However in our study, we used 35 µm or 50 µm pinholes, which is 2.5-5 times larger than the CO$_2$ laser wavelength (10.6 µm). By mounting a stainless steel pinhole on the movable laser head very close to the substrate, the laser beam is narrowed and collimated from the pinhole at the near field region, ablating a narrower structure, while still retaining the ability to pattern in a single step direct from a CAD file. We show that by using a 50 µm pinhole, channels with a width of 60 µm could be made in PMMA, and as narrow as 25 µm when using a 35 µm pinhole. Furthermore, the size of the bulges on the rim of the engraved channel was reduced to less than 0.8 µm, increasing the bonding success rate. The analytical significance of this method was demonstrated by comparing the quality of the electrophoretic separation of fluorescent dyes in devices made with and without a pinhole. This approach offers the capability to rapidly prototype rigid plastic microdevices thereby bridging the gap between soft lithography and hot embossing/injection moulding. The additional advantage of this approach is its ability to engrave channels with two different dimensions in a single chip without the need of expensive mask alignment system, thereby simplifying the fabrication process when compared with the two step photolithography. Laser engraved PDMS devices was
evaluated for culturing of primary cortical neurons using methods established in previous chapters of this thesis.
4.2 METHODS

4.2.1 Materials and reagents

Fluorescein isothiocyanate (FITC), Fluorescence sodium salt (FI), sodium tetraborate, PLL and hydroxypropylcellulose (HPC) were purchased from Sigma Aldrich (St. Louis, MO). Cast PMMA plates (2mm) were received from Global 372 Arcylic Pty Ltd (Queensland, Australia). PMMA film (50 µm) was purchased from Goodfells (UK). Sylgard 184 PDMS was purchased from Dow Corning Company (Michigan, USA). Neuronal culture reagents and trypsin were obtained from Life Technologies (USA).

4.2.2 PMMA chip fabrication using laser engraver system

A commercial hobby laser system (Full Spectrum, USA) equipped with a CO₂ laser with an output of invisible infrared beam at wavelength of 10.6 µm, and a maximum output of 40W was used to engrave the cast PMMA sheets. Note: Caution should be taken when operating the laser system. The laser beam contains sufficient energy to damage eyes and skins. Therefore, direct exposure to the laser beam must be avoided. Never operate the laser with the presence of combustible materials, explosive or volatile solvent such as ethanol as the laser may cause ignition of these materials. The laser frequency was 100 MHz and the maximum cutting speed of this system is 20 mm/s. The system comprises the CO₂ laser source, a reflective mirror, a XY stage, a focal lens and a table for positioning the substrate. The focal length of the lens was about 38.1 mm and according to the manufacturer specifications guideline, the smallest beam spot size of the lens could reach approximately 76 µm. Microchip designs were created using the free CAD software, Draftsight (Dassault Systèmes SolidWorks Corporation, USA) and were sent to the Retina engraver software (Full Spectrum, USA) as a print job to drive the laser system for the ablation process.
Experimental parameters of laser power, scanning speed and number of laser beam passes were set by using the Retina engraver software. After ablation, fluidic access holes were drilled using a drill press and a 3 mm drill bit and the device was sealed by lamination bonding of the patterned PMMA substrate with a PMMA film at 150 °C at speed 2 in an office laminator (Peach 3500, Lamination Systems, Australia).

4.2.3 Integration of pinhole into the laser engraver system

The pinhole was mounted between the focusing lens and the substrate, as illustrated in Figure 4.1. An in-house made holder was designed to screw onto the lens mount of the laser engraver to suspend the pinhole just above the surface of the substrate such that it was free to move across the substrate without touching the surface. Three screws allowed accurate positioning of the pinhole in the centre of the IR beam. Alignment of the pinhole with the laser was verified using thermal paper. The 20 mm disc pinholes were produced from 1.5 mm thick stainless steel (grade 304) by the LaserXperts Pty Ltd (NSW, Australia). Two different pinhole discs were made, one with an exit hole diameter of 35 µm and another with an exit hole diameter of 50 µm. The substrate was positioned at the focus point of the lens, which is 38.1 mm from the focusing lens and the pinholes were mounted at position of 0.5 mm from the substrate.

4.2.4 Channel characterization

After laser ablation, the structures were characterized by using UHR field emission scanning electron microscopy (SEM) (Hitachi SU-70, Japan) and non-contact 3D optical profiler system (Wyko NT 1100, Veeco Instrument inc, USA). The optical profiler data were analysed using Wyko Vision 32 software (Wyko Corporation, USA).
Figure 4.1 Illustrative diagram of the integration of pinhole into the CO$_2$ laser engraver system. The pinhole was mounted between the focusing lens and PMMA substrate. The engraver software of computer was connected to the laser engraver system to drive the system for ablation process. When the CO$_2$ laser passing through the pinhole, the size of laser beam was reduced and consequently reduced the dimensions of the ablated features.
4.2.5 Electrophoresis

All channels were flushed with separation buffer prior to injection and separation. The separation buffer for the microchip fabricated with the laser system without using the pinhole (chip A) was 2 mM sodium tetraborate buffer, pH 9.0 with 0.75 % (w/v) HPC to increase the viscosity and reduce hydrodynamic effects and to also suppress the electro-osmotic flow (EOF). For the microchip fabricated using the 50 μm pinhole (chip B) the electrolyte concentration was increased to 10 mM sodium tetraborate buffer, pH 9.0, again with 0.75 % (w/v) HPC. The sample solution contained a mixture of 0.5 mM FITC and FI. The chip design comprised a cross geometry with a distance of 15 mm from cross to sample, sample waste and buffer wells, and 45 mm to the buffer waste. The chip was placed in a custom-made chip holder (CSL mechanical workshop, University of Tasmania, Hobart, Australia) containing platinum electrodes for application of the voltages using an in-house built four channels high voltage power supply (CSL electronic workshop, University of Tasmania, Hobart, Australia). For separation, the following voltages were applied: sample (+320 V), sample waste (+320 V), buffer (-200 V) and buffer waste (+2400 V). For injection the following voltages were applied: buffer (-120 V/-240 V), buffer waste (-1060 V/-800 V), sample (-320 V), sample waste (+320 V) for the microchip produced with and without the pinhole.

4.2.6 Laser-induced fluorescent system

All microfluidic separations were performed on the stage of a Nikon Eclipse Ti-U inverted fluorescence microscope (Nikon Instruments Inc., USA) operated with NIS-Elements BR 3.10 software (Melvile, USA). A filter cube (Semrock, Rochester, USA) consisting of an excitation band pass filter (488 ± 10 nm), emission filter (520 ± 10 nm) and dichroic mirror to deflect the broadband light source to a 20x
objective were used for all experiments. Fluorescence images were acquired by using a high definition colour charge-couple device camera (Digital Sight DS. File, Nikon, Japan). Electropherograms were recorded using a photon multiplier tube (PMT) (Hamamatsu Photonic KK, Hamamatsu, Japan) connected to the microscope. The PMT was interfaced to an Agilent 35900E A/D box allowing collection of data with the Chemstation software (Agilent Technologies, Waldbronn, Germany).

4.2.7 Fabrication of PDMS microfluidic device using laser engraver system

To prepare flat surface PDMS with thickness of 2 mm, 10:1 mixture of PDMS prepolymer and crosslinking agent was left to cure at 70 °C overnight in a covered plastic petri dish. The cured PDMS was then removed from the petri dish using a razor blade, cleaned with isopropanol and dried under a stream of pressurized air. Microchip designs and channel length were created using the Draftsight software. The laser mode was set to raster or vector mode. The laser was then focused and ablated on the surface of PDMS. The ablation process involved only single pass over the surface of the PDMS. In this study, raster mode of 250 x 250 dpi (the laser sweeps 259 times per vertical inch) and vector mode of 10% power, 100% speed were set to fabricate the PDMS device that used for culturing neurons.

4.2.8 Primary cortical neurons and biocompatibility of engraved PDMS devices

Primary cortical neurons were derived from E18 Sprague Dawley rat embryos as previously described in section 2.2.3. Briefly, after dissection cells were chemically dissociated in 0.0125% trypsin followed by washing and gentle manual dissociation. Primary neurons were then seeded onto engraved PDMS microfluidic devices at density of $9 \times 10^6$ cells/mL and incubated at 37 °C, 5% CO$_2$ for 5 min to enhance cell adhesion to the substrate. After incubation, both soma and axon compartments were filled slowly with pre-warmed initial neuronal growth media to minimise disturbing
the cells. Cultures were grown at 37 °C, 5% CO₂. After 24 h, the initial neuronal growth media was replaced with subsequent growth media (initial growth media without the foetal calf serum and glutamate). The culture media was renewed three times a week to maintain neuronal viability.

### 4.2.8.1 Microscopy analysis

Live, non-fluorescent cell cultures were observed on a Leica DMIRB inverted fluorescent stereomicroscope with images taken using a CCD camera (ORCA, Hamamatsu, Shizuoka, Japan). Individual still images were captured with NIH elements software (Nikon, Japan).
4.3 RESULTS AND DISCUSSION

The mechanism of CO₂ laser micromachining is photothermal melting and evaporation due to the long wavelength (10.6 µm) of the laser [167]. It leads to the formation of heat affected zone (HAZ), molten liquid and hot evaporated gas with high pressure in the laser focusing area. Bulges are formed on the edge of the channel at the heat affected zone when the molten polymer is resolidified by cooling in atmospheric air. These bulges can disrupt successful bonding of microchip components and therefore a number of efforts have focused on reducing the bulge size, including the use of cast instead of extruded PMMA [172]. Snakenborg et al. [172] investigated the effects of power, number of laser passes and the linear velocity on the width and depth profile of the channel. They noticed that slow ablation rate and higher number of laser passes generated wider microchannel and higher bulges on the PMMA due to the softening intensity caused by the infrared irradiation.

Based on these findings, we used a single pass at maximum speed to achieve a fast ablation rate and minimal bulging in combination with the targeted smaller channel size. The width and depth of laser-ablated microchannels can be varied by changing the intensity and movement speed of the laser, and in general a lower power and higher speed produces narrower and shallower channels. However, as shown previously, even at fast speeds and low powers, there is a limitation to the width of the channel that can be obtained – defined by the physical size of the focused laser spot. The novel, but logical way to produce smaller features, is to physically shape the light such that a smaller beam reaches the substrate. Here, we used both 35 µm and 50 µm pinholes mounted just above the surface of the cast PMMA substrate on the moveable laser mount to investigate the capability of pinholes to reduce the physical size of the laser beam and consequently the dimensions of the ablated features. It is important
that the substrate is positioned at the focusing plane to obtain minimum spot size. If the substrate is outside the focal range, the spot size can increase due to beam divergence, resulting in a wider channel. In addition, pinhole reduced the laser beam and hence more laser power is needed in order to have enough laser energy to apply on the substrate surface for the ablation process. Therefore, range of laser power of 0.4 W to 12 W was used during the ablation without pinhole. However, range of laser power of 16 W to 40 W was used during the ablation using a 50 µm pinhole and 20 W to 40 W was used during the ablation using a 35 µm pinhole.

Figure 4.2 shows SEM images of microchannels produced without the pinhole and with the 50 µm and 35 µm pinholes. Using the same write speed, without the pinhole at a power of 0.4 W, the channel had a width of 306 µm. With the 50 µm pinhole and a power of 16 W the average width was 54.2 µm while with the 35 µm at 28 W this decreased to 36.4 µm. These microchannels had a depth of 18 µm to 35 µm, see optical profiler images in Figure 4.3, clearly indicating that the pinhole is capable of reducing the channel width. One of the unexpected benefits of using the pinholes was a decrease in the bulge height at the HAZ. This was reduced from 1.8 µm without the pinhole to approximately 0.4 µm (or 0.8 µm) when the laser passed through a 50 µm (or 35 µm) pinhole during ablation process. This may be explained by the fact that high conductivity 304 stainless steel has low absorption to CO₂ laser [176] hence the pinhole can reflect/block the laser output and conduct and dissipate the surplus heat during ablation to reduce the bulge height. The increase in bulge height with the 35 µm pinhole is currently not understood.

Figure 4.4 shows a plot of the width and depth of the microchannels as a function of the laser power with and without using the pinholes. As expected from previous work,
Figure 4.2 SEM image of PMMA microchannel fabricated at the maximum speed with and without the pinhole in a single pass. (A) Laser power of 0.4 W without using pinhole. (B) Laser power of 16 W with 50 µm pinhole. (C) Laser power of 28 W through 35 µm pinhole.
Figure 4.3 The profile of the channels on PMMA. The laser processing at maximum speed for single scan. (A) ablation without using the pinhole. The laser power was set at 0.4 W. (B) laser processing through the 50 µm pinhole during machining. The laser power was set at 16 W. (C) laser processing through the 35 µm pinhole during ablation. The laser power was set at 28 W.
Figure 4.4 The geometry of the microchannels fabricated at different laser power in the presence and absence of a pinhole using a 40 W hobby laser system. Maximum laser cutting speed (20 mm/s) was used. The data was measured by using an optical profiler system. (A) The width of the microchannels ablated at different laser power. (B) The depth of the microchannels fabricated at different laser power.
the channel width and depth increased with laser power due to more energy applied to the surface for the ablation process. Without the pinhole, the channel width increased from 309 µm to 363 µm, and depths increased from 39 to 250 µm when increasing the laser power from 0.4 W to 12 W. When using the 50 µm pinhole, the channel width increased from 55 to 100 µm; the depth from 25 to 65 µm, when increasing the laser power from 16 to 40 W. When using a 35 µm pinhole, the channel dimensions were further reduced to 25 to 50 µm wide and 10 to 20 µm deep, with power ranging from 24 to 40 W. This is an important finding because it demonstrates that the flexibility to create different width and depth channels by changing the parameters within the software remains, all be it with a reduced size range. The performance of the pinhole fitted to the CO₂ laser used here is compared with published literature using CO₂ systems for PMMA in Table 4.1. In this study, a cheap hobby CO₂ laser system (~$2500) was used to fabricate smaller feature size microchannels on PMMA by scanning the CO₂ laser across a stainless steel pinhole (30 or 50 µm diameter). These pinholes were commercially produced within 3 days at cost of ~$300 and the in-house made holder that mounted the pinhole between the focusing lens and substrate was ~$200. Compared to a previous study by Klank et al. [167] our study fabricated a smaller feature size microchannel (<60 µm) on PMMA because of the ability of the pinholes to reduce the physical size of the laser beam and subsequently the width of the ablated microchannels. Recently Chung and colleagues [176, 177] fabricated a PMMA chip with width approximately 58 µm by using a CO₂ laser. They patterned a 50 µm wide channel in a metal foil mask by using wet etching and then fabricated the PMMA chip by scanning the laser across the lithographically patterned metal film. While effective for the fabrication of small feature size microchannels (<60 µm), this method loses the flexibility and simplicity of the laser ablation process because the
Table 4.1 The comparison between the performances of the pinhole fitted to the CO$_2$ laser used here with published literature CO$_2$ system for PMMA.

<table>
<thead>
<tr>
<th>CO$_2$ laser system</th>
<th>System cost</th>
<th>Fabrication Conditions</th>
<th>Minimum Width (µm)</th>
<th>Bulge Height (µm)</th>
<th>Disadvantage compared to this work</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 W Full Spectrum Hobby Laser</td>
<td>$2500 + $500 for pinhole and mount</td>
<td>Passing laser through 50 µm pinhole at 16 W power</td>
<td>60</td>
<td>0.4</td>
<td>-</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Passing laser through 35 µm pinhole at 24 W power</td>
<td>25</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65 W Synrad Inc</td>
<td>$4000</td>
<td>6.5 W power</td>
<td>100</td>
<td>-</td>
<td>Large feature size</td>
<td>Klank et al. [167]</td>
</tr>
<tr>
<td>30 W Universal Laser Systems</td>
<td>$15000</td>
<td>Scanning laser across the metal film with 50 µm wide pattern at 1.5 W power</td>
<td>58</td>
<td>0.2</td>
<td>Expensive, need to deposit and pattern a metal layer prior to engraving</td>
<td>Chung et al. [176, 177]</td>
</tr>
</tbody>
</table>
metal film needs to be patterned by conventional lithography and wet etching prior to laser ablation, thus losing the ability to directly and simply pattern from CAD diagram only. In addition, this method also involves traditional lithography and wet etching which require handling of corrosive chemicals/solvents, a photomask and of course significant disposal cost. Our approach with a pinhole requires a one-off cost for the pinhole and mount, which can be used for the production of numerous chips and retains the ability to change design and rapidly fabricate new microchips. In summary, the use of a pinhole allows for the fabrication of much narrower channels at a significantly reduced cost and time while also preserving the flexibility of the CAD – print capability of the laser ablation system.

4.3.1 Fabrication of PMMA based microchip capillary electrophoresis device

The hobby laser was used to fabricate PMMA microchips for capillary electrophoresis (CE) with a standard cross-geometry used commonly for electrophoresis with pinched injection. Figure 4.5A shows the separation of FI and FITC on PMMA devices fabricated without the pinhole, with the microchannels 300 µm wide and 36 µm deep in 2 mM borate buffer, pH 9.3, with 0.75% HPC. The separation efficiency for the FI and FITC in these wider microchannels was 4,215 plates (140,000 plates/m) and 3,600 plates (120,000 plates/m), respectively, and the separation was completed within 30 s. Using the 50 µm pinhole, a PMMA chip with 60 µm wide and 27 µm deep microchannels was created. The 50 µm pinhole was selected for the fabrication of the CE microchip because of the prevalence of 50 µm capillaries used for electrophoresis. Using these narrower microchannels, an electrolyte of 10 mM borate buffer (instead of 2 mM), 0.75% w/v HPC were used for the separation. This allowed separations in both chips to be performed with the same separation current of 30 µA at same electric field strength (520 V/cm). The separation in the narrower microchannel was
completed within 50 s, approximately 20s slower than the separation in the wider microchannel due to the higher ionic strength electrolyte used which decreased the electrophoretic mobility of the analytes (Figure 4.5). As anticipated, the separations are much more efficient in the narrower microchannel, with separation efficiencies of 9,276 plates (310,000 plates/m) and 8,000 plates (260,000 plates/m) for FI and FITC respectively. These separations are better than the 66,300 plates/m reported for the fluorescein peak separated in 10 mM sodium borate buffer at 470 V/cm by Koesdjojo et al. [179] in a hot embossed PMMA microchip and the ~60,000 plates/m reported for fluorescein separated with 20 mM Tris at 313 V/cm in a laser ablated glass microfluidic device[171]. The most likely reason for the improved performance of our microchip is the smaller channel dimensions thereby significantly reducing the Joule heat and increasing the separation efficiency. One should keep in mind though, that during the ablation of simple cross designs, the laser passes twice over the intersection, resulting in a larger sample volume at the intersection [170]. In our work, we did not observe significant tailing or fronting for the separation of FI and FITC (Figure 4.5), indicating the system was not overloaded. The larger volume at the intersection might be an attractive way to improve the detection sensitivity by increasing the injected sample volume. The excellent performance of these laser ablated PMMA microfluidic devices in CE clearly demonstrates the applicability of the use of an economical laser ablation system with a pinhole for rapid prototyping of PMMA devices.
Figure 4.5 (A) Electrophoretic separation of FI and FITC in 0.75% w/v HPC, 2 mM borate buffer, pH 9.3 at 520 V/cm on microfluidic device that ablated without using a pinhole. (B) The same analytes were separated on a laser ablated device at the presence of a pinhole during ablation, in 0.75% w/v HPC, 10 mM borate buffer, pH 9.3 (electric field same as above).
4.3.2 Neurons cultured on PDMS device

In this experiment we compared the growth of the primary cortical neurons derived from E18 rat embryos on bare, raster engraved and vector engraved PDMS devices. Devices were prepared for culturing as described in sections 4.2.7. and 4.2.8. We observed that neurons cultured on a bare PDMS device were healthy and consisted of intact neurites and cell bodies that were smooth and circular to oval in shape at 5 DIV (Figure 4.6A). In comparison, neurons plated at the same density but cultured on raster engraved PDMS devices were unhealthy, with only a few non-viable neurons attached on the PLL-coated surface at 5 DIV (Figure 4.6B). No viable neurons were present on the vector engraved PDMS at 1 DIV (Figure 4.6C). The poor viability is possibly due to the high surface roughness (a few µm) in the laser engraved channels, significantly influencing the fluidics and hence cells adhesion and unselective absorption of proteins such as PLL [180, 181]. Our results were also consistent with previous study by Fan and colleagues [65] who found that the surface roughness affected cell adhesion and viability of central neural cells (substantia nigra) cultured on wet etched silicon wafer. They demonstrated that cells grown on surfaces with average roughness (Ra) above 70 nm have reduced cell viability when compared to cells grown on surfaces with Ra ranging from 20 to 50 nm. Therefore, the laser engraving method developed here requires some further modifications before the practical implementation for the fabrication of PDMS devices for use in neuroscience research can occur. Surface modification of the PDMS after patterning, for example, could be trialled to reduce the surface roughness.
Figures 4.6 Live, non-fluorescent images of primary cortical neurons cultured in PDMS microfluidic device. (A) At 5 DIV, neurons were healthy and intact with high viability when grown on bare PDMS. (B) When grown on raster engraved PDMS device, neurons were unhealthy with reduced viability. (C) When grown on vector engraved PDMS device, no viable neuron was observed within the channel. Scale Bar= 100 μm
4.4 CONCLUSIONS

We have developed a novel, low cost approach for fast prototyping of rigid PMMA microchips by inserting a pinhole in the optical pathway of a commercial hobby CO₂ laser engraver. The pinhole significantly reduced the diameter of the laser spot, decreasing the ablated feature width from around 300 µm to around 60 µm by using 50 µm diameter pinhole. When a 35 µm pinhole was used, the width was reduced to around 25 µm. Integrating a pinhole into the laser system also obtained a low bulge height on the rim of the channels, which is less than 0.8 µm in this study. The practicality of the proposed machining process has been demonstrated by fabricating a microchip electrophoresis device. PMMA devices with 60 µm x 27 µm microchannels ablated with the help of the pinhole allowed for a 2.2 fold increase in separation efficiency when compared with devices of the same design made by laser ablation without the pinhole. The separation efficiency also compared favourably with literature reports of similar separations in embossed PMMA and laser-ablated glass devices. This illustrates the great potential of upgrading a $2,500 hobby laser with a $500 pinhole for affordable and fast microfabrication. However, neurons cultured on laser ablated PDMS device were unhealthy and therefore further modifications of the laser engraving process are required before these devices can be applied on the investigations of neuronal cells.
Chapter 5

5 MICROFLUIDIC MAZE FOR STUDYING THE ROLE OF GLIA IN NEURONAL NETWORKS

5.1 INTRODUCTION

The mammalian nervous system is composed of several highly specialized cell types, including many neuronal subtypes as well as glial cells, and is the most complex part of the human body. The human brain alone contains roughly 12-15 billion neurons that communicate with each other via dendrites and axons, which respectively are responsible for integrating synaptic input and providing output. These neurons are supported and maintained by an approximately equal number of glial cells, which have an increasingly appreciated diversity in form and function. Interaction between different cells populations such as neurons and glia are particularly important in maintaining the metabolic balance of brain [182]. In addition, there is now compelling evidence that axon glia interactions play a role in the pathogenesis of a growing number of neuroinflammatory and neurodegenerative diseases, but the underlying cellular and molecular mechanisms have yet to be determined [5, 39].

Elucidation of the mechanisms involved in axonal injury and axon-glia interactions can be difficult in the complex in vivo setting and thus many investigators have turned to in vitro approaches. However, standard cell culture technologies, where neurons are plated on petri dishes using glia as feeder layers make it difficult to delineate interactions of neuron and glial specific mechanisms. To address this problem, microfluidic technologies, that use channels and structures with typical dimensions of tens to hundreds of micrometers, have been applied to provide unique solutions to study cellular and molecular interaction between neurons and glial cells due to their
ability to provide excellent and precise control over the extracellular microenvironment[9, 11, 183].

For example, Hosmane and colleagues advanced existing microfluidic devices design (device with 10 µm wide, 3 µm high, 450 µm long microchannels that are capable of passively guiding axons of both CNS and PNS neurons as described in section 1.2.2) by manually punching larger access ports (≥3 mm) near the microchannels and introducing PDMS microstencils with circular structures (500-1000 µm in diameter and 500-1000 µm in thickness) within the axonal compartment [11]. They utilized this device to position the cells close to the microchannel interface and directly placed microglial cells within areas of interest in the axonal compartment via standard direct pipetting techniques. Utilizing this compartmentalized circular axon-glia microfluidic culture platform, they found that a two-fold preferential accumulation of microglia specifically near degenerating, injured CNS axons as compared to healthy axons. However, the error introduced through alignment problems with the manual punching process made it unsuitable for high throughput testing of drugs or growth factors.

More recently, Park and colleagues[183] utilized a “Micro-macro Hybrid Soft-lithography Master Fabrication” technique to fabricate a multi-compartment neuron-glia co-culture platform. This technique combined bench top computer numerical control (CNC) milling machining, acid etching and hot embossing techniques to allow the fabrication of the macroscale access port (3.5 mm high, 3-7 mm wide) and microscale axon-guiding channel (3 µm high, 20 µm wide) on a single PDMS master template. The final PDMS microfluidic devices could be easily replicated from the master template using the replica molding technique, without any manual punching processes. They successfully co-cultured oligodendrocytes progenitor cells with the isolated axons and differentiated them into mature myelin basic protein expressing
oligodendrocytes.

These microfluidic approaches are, however, limited by the inability to control the direction of connectivity of the cell types. Here we present a novel platform that consists of two individual neuronal compartments connected with one glia only compartment through a maze like structure (n=10). The maze structure consists of multiple offset walls (50 µm wide, 22 µm high, 250 µm long) spaced at regular intervals (50 µm) with 150 µm gaps in between to stop axonal growth while being permissive to glia. To our knowledge, this is the first platform that has constructed a neuronal network with directional connectivity between different populations of neurons and glia, thereby allowing the investigation of the interaction between neurons and glia in the absence of neuron to neuron signalling. Ongoing investigation utilizing this platform will help to establish whether glial cells can facilitate signalling and/or spread of pathogenic protein between distinct neuron populations.
5.2 METHODS

5.2.1 Fabrication of co-culture platform

The co-culture platform is composed of three cell culture compartments, two for neuronal cells and the other for glial cells, as shown in Figure 5.1. The platform was fabricated in PDMS, a biocompatible polymer, following well-established replica molding procedures [88]. Briefly, a master mold was created on a PMMA substrate (75 mm × 50 mm × 2 mm) using a two layer-photolithography process. PMMA was first coated with a thin photoresist layer (SU-8 2005, Microchem, USA), soft baked, over exposed and then post-baked to form the adhesive layer for second layer. The process was immediately repeated with a thicker photoresist (SU-8 3025, Microchem, USA) and exposed with a high resolution transparency mask, and developed to generate a second layer that consisted of a maze array of 10 offset banks (dimensions: height= 22 µm, width=50 µm, length =250 µm and spacing between bank=150 µm), a glia compartment (length=3.5 mm, width=1.5 mm, height= 22 µm) and two soma compartments (length=1.5 mm, width=1.5 mm, height= 22 µm). Reproducible replication of the device was done by soft polymer casting using PDMS (10: 1 mixture, Sylgard 184, Dow Corning, Inc, USA), followed by curing at 70 °C overnight. Once replicated, the reservoirs to hold culture media were formed using a biopsy punch (3 mm diameter, Huat Instrument, USA).

5.2.2 Testing of fluidic isolation

We tested fluidic isolation in triplicate using microfluidic devices without cells by creating a fluidic level difference between the reservoirs that resulted in a difference in hydrostatic pressure. Initially, the bottom “glia only” compartment and the top neuronal compartments were filled with PBS and 0.5 mM fluorescence sodium salt (FI) (Sigma, USA) respectively, with the glia compartment having a slightly higher
Figure 5.1 (A) Schematic illustration of the microfluidic compartmentalized CNS cell co-culture platform. The two top compartments are for the growth of two neuron populations (N₁ and N₂) and are connected to the bottom “glia only” compartment for co-culture through arrays of a maze like structure. The maze restricts the growth of neurites into the glia compartment but permits the entrance and growth of glia throughout the neuronal compartments. (B) Photography of assembled devices comprising the glass coverslip and the microfluidic co-culture platform.
fluidic level. The slightly higher volume on the glia side caused a slow net flow of liquid from the neuronal to the “glia only” compartment that acted against leakage or diffusion of fluorescein from the neuronal to the “glia only” compartment.

5.2.3 Preparation of microfluidic device

Microfluidic devices used in this study were sterilized with 70% ethanol and UV light. Prior to cell seeding, the sterile device was assembled onto the glass coverslips (24 × 24 mm²) and then coated with 0.01% PLL (Sigma, USA) for two days at room temperature. After that, PLL was removed by washing with sterile mili-Q® water and the device was placed in a standard humidified cell culture incubator set to 37 °C and 5% CO₂. The water was removed and the coated devices were filled with pre-warmed culture media for at least 3 h prior to plating the cells.

5.2.4 Cell preparation

All experiments involving animals were conducted according to protocols approved by the Animal Ethics Committee of the University of Tasmania.

5.2.4.1 Co-culture of glial and cortical neurons in microfluidic device

Primary mixed glial cultures were derived from postnatal rat pups (1-3 days) with minor modifications of previously described protocols [184]. Briefly, cortices were stripped of the meninges and then trypsinised in 0.0125% trypsin (Life Technologies, USA). The Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS was then added to inactivate the trypsin and the cell suspension triturated and filtered through a 70 µm gauze filter.

Cells were then plated into T75 mL flasks pre-coated with 0.01% PLL (Sigma, USA) with 10 mL DMEM/10% FCS/1% penicillin/streptomycin (Gibco/BRL, Life Technologies, USA). Mixed glia cultures were then maintained at 37 °C in humidified
air containing 5% CO$_2$ and media was changed every 3 days. When grown to confluency (8-9 DIV), mixed glia were harvested from flasks by trypsin/EDTA and loaded into the “glia only” compartment and the neuronal compartment of PLL-coated microfluidic devices through middle and bottom reservoirs respectively at cell density of 6 x 10$^6$ cells to ensure more glial cells can be plated within the microfluidic device and can be proliferated throughout the device faster. Glia cells were refreshed with the DMEM/10% FCS/1% penicillin/streptomycin media every 3 days. After the glia reached confluence in the microfluidic devices, primary cortical neurons were loaded into two neuronal compartments of the microfluidic device at a cell density of 8 x 10$^6$ cells/compartment.

Cortical cell cultures were prepared as described in section 2.2.3 [152],[110]. The bottom reservoirs were filled with 20 µl of prewarmed initial neuronal growth media (section 8.2) and then 8 x10$^6$ mL cortical neurons, suspended in 10 µl of media were seeded into the top reservoirs. By keeping the media level (20 µl) of the bottom reservoirs of the “glia only” compartment higher than the top reservoirs of the neuronal compartment (20 µl) while emptying the middle reservoirs, hydrodynamic pressure was created from the “glia only” compartment and hence prevented the neurons from fluidically entering the maze. After seeding, the neurons were allowed to adhere for 10 min in an incubator at 37 °C, 5% CO$_2$. After this incubation, all the compartments were filled slowly with pre-warmed initial neuronal growth media and returned to the incubator. After 24 h, the media was replaced with subsequent growth media (initial growth media without the glutamate). The cell culture was then monitored at regular intervals and the culture media was replaced three times a week (on DIV 3, 5, 7 and 9) to prevent oxygen and nutrient depletion or waste accumulation and to maintain neuronal and glia cells’ viability.
5.2.5 Immunocytochemistry

Cells were fixed and permeabilised as described previously (refer to section 2.2.5). The cells were then incubated with primary antibodies diluted in PBS for 1 h at room temperature and at 4 °C overnight. Primary antibodies to the axon specific beta III tubulin (1:5000, Promega, Denmark) and glial fibrillary acidic protein (GFAP) (1:1000, Dako, USA) were used. Following incubation with the primary antibody, cells were rinsed with 0.01 M PBS and incubated in the dark with secondary antibody (refer to table 8.2) for 2 h at room temperature.

5.2.6 Microscopy

The PDMS microfluidic devices produced were examined under an optical profiler microscope Wyko NT9100 (Veeco Instrument Inc, USA) by dual LED light optical profiling in the vertical scanning interferometry (VSI) through a 5X objective. Live, non-fluorescent cell cultures were observed on a Leica DMIRB inverted stereomicroscope (Leica, Germany) with images taken using a CCD camera (ORCA, Japan) and NIH elements software (Nikon, Japan). Fixed, immunofluorescent labelled samples were visualized with a Leica DMLB2 fluorescent microscope (Leika, Germany) and images were acquired with a CCD camera (ORCA, Japan) and NIH elements software (Nikon, Japan).

5.2.7 Statistical analysis

For each condition, a minimum of five coverslips from at least three separate cultures (derived from different animals), were used for quantification or otherwise stated. For each coverslip, three images were acquired (20 X lens). All graphical data is presented as means ± SEM. One-way ANOVA with post hoc Fisher’s LSD test were performed, with p values less than 0.05 as the level of significance.
5.3 **RESULTS**

5.3.1 **Microfluidic device design**

Neuron-glial co-cultures normally performed in close cell to cell contact, where glia act as a feeder layer to support neural cell survival through direct supportive interaction and secretion of metabolic products. As seen in Figure 5.1, the device contained three compartments, with the two neuronal compartments (N1 and N2) connected to the “glia only” compartment by a maze structure (10 banks) with height-22 µm, width-50 µm, length -250 µm, with spacing of 150 µm between each bank. The purpose of the microfabricated maze was to prevent axons from entering the “glia only” compartment, whilst permitting the glia to link between N1 and N2 cells. It can also be seen from the optical profiler data and optical image that the maze structure connecting the neuronal compartment and the “glia only” compartments was successfully transferred to the final PDMS device without any noticeable distortion (Figure 5.2).

5.3.2 **Fluidically isolation**

To verify that the maze structure completely separated the two neuronal compartments from the “glia only” compartment, we tested the efficiency of fluidic isolation in our novel platform by creating a minute fluidic level difference between the reservoirs that resulted in difference in hydrostatic pressure. We observed fluidic level pressure from the bottom reservoirs of the “glia only” compartment (red color, higher fluidic level, 20 µL) counteracted diffusion to the top reservoirs of neuronal compartments (blue color and green color, lower fluidic level, 10 µL) due to the 20µm high maze array (Figure 5.3A). Fluidic level differences of 177, 354, 708, and 1416 µm were achieved by creating volume differences of 5, 10, 20, 40 µl between reservoirs, respectively (see
Figure 5.2 (A) Optical image of the multicompartment PDMS neuron-glia co-culture device fabricated by photolithography process. Scale bar = 125 µm. (B) 3D reconstructed optical profilometry image of the maze microchannels connecting the neuronal and “glia only” compartments.
Figure 5.3 Fluidic isolation in the neuron glia co-culture platform. (A) Photographic image of the neuron glia co-culture platform (20 x 20 x 4 mm$^2$) filled with three different color dyes for visualization. (B) Dotted white lines in the fluorescence microscopy image show the boundary between the fluorescence dye and PBS, indicating that fluidic isolation was achieved when the volume of top reservoirs (neuronal compartment, 10 µl) were smaller than that in the bottom reservoirs (glia only compartment, 20 µl) while the middle reservoirs were kept empty.
appendix D). Successful fluidic isolation between the compartments was achieved with a height difference as little as 354 µm, as shown in (Figure 5.3B). We observed FI does not diffuse or flow to the opposite compartment, with a sharp boundary after 10 min indicating that the hydrostatic pressure created across the maze structure by the height difference between the two compartments fluidically separated the neuronal and “glia only” compartments, at least for this length of time.

5.3.3 Glia cell growth throughout the compartments

Prior to co-culture with neurons, we examined the migration and replication of glia throughout all compartments. Cells were harvested from 8 DIV mixed glia cultures in 75-cm flask and then plated into the PLL-coated microfluidic device at cell density of 6 x 10^6 cells/mL. Cells were loaded through the bottom and middle reservoirs into the two neuronal compartments and the “glia only” compartment. Preliminary data demonstrated that the glial cells grew into the maze structures and extended throughout the whole microfluidic device by 20 DIV, as shown in Figure 5.4 (A-C).

5.3.4 Restricted neuronal growth

In order to isolate the neuronal populations to their neuronal compartments and prevent the neurons from entering the “glia only” compartment, a maze structure with arrays of 10 banks was integrated into this novel platform. Prior to trialing co-culture in this device we confirmed that the maze structure was capable of preventing axonal extension into the “glia only” compartment. We first filled the bottom reservoirs of the “glia only” compartment with 20 µL media and then loaded the 10 µL neuron cell suspension (cell density=8 x 10^6 cells/mL) into the neuronal compartment through the top reservoirs. Phase contrast imaging of cell bodies within the neuronal compartment at DIV 10 confirmed that the hydrodynamic confinement had been successful (Figure 5.5A). To quantitate the capacity of the axons to penetrate the maze structure, we
Figure 5.4 (A-C) Live imaging of mixed glia cells within the neuronal compartment, maze like structures and “Glia only” compartment at DIV 20 in microfluidic device demonstrated that glia replicated and extended throughout all compartments. Scale Bar= 100 µm.
Figure 5.5 (A) Phase contrast image of neurons cultured at DIV 10 within the neuronal compartment demonstrated that the cell bodies were confined within the neuronal compartment. (B) Live image of axons within the maze like structures at DIV 10 demonstrated that the axons were stopped at bank 9. Scale bar= 50 µm
counted the number of individual axons present between banks from images acquired at DIV 10. The average percentage of axons between banks was calculated as (number of individual axons present between banks/total number of axons within banks) × 100. After 10 DIV, the average percentage of axons between bank 1 to 2 relative to total axons (38.31± 2.68 %) was significantly higher than the percentage between bank 2 to 3 (25.32± 1.51%) (Figure 5.6, p<0.01, n=8). Most importantly, we found that at DIV 10, no neurites extended beyond bank 9 (Figure 5.5B) of the maze and therefore no axon/dendrites extending into the “glia only” compartment.

5.3.5 Loading and co-culturing of neurons and glia in the microfluidic device

Prior to cell loading, microfluidic device were coated with PLL as above. The glial cells were first loaded into the bottom reservoir of “glia-only” compartment and middle reservoirs of neuronal compartment at the cell density of 6 x 10⁶ cells/mL. After the glial cells proliferated and reached confluence throughout the microfluidic device at 20 DIV, we seeded the non-proliferating cortical neurons at 8 x 10⁶ cells/mL suspended in 10 µL media in the top reservoirs of neuronal compartment while keeping the bottom reservoirs of the “glia only” compartment filled with 20 µL media. This created a small hydrodynamic pressure required to prevent the suspended neurons from flowing into the “glia only” compartment, and hence retained all neurons within neuronal compartment. After the neurons attached to the substrate, all compartments were filled with media. Figure 5.7 shows neurons (stained for βIII tubulin, green) and glial (stained for GFAP, red) inside the neuronal compartment at 20 DIV. No βIII tubulin staining (green) was observed in the glial compartment, confirming the absence of neurites in the glial compartment (Figure 5.7B).
Figure 5.6 Graph shows the average percentage of individual axons between banks per total axons at 10 DIV. There was a significantly larger percentage of axons between bank 1 and 2 compared to bank 2 and 3. *p<0.01. Error bar=mean ± SEM, n=8. In addition, no axons were observed between bank 9 and 10.
Figure 5.7 (A) Immunostaining image demonstrated neurons grown with glial network layer inside the neuronal compartment. Axons were stained for βIII tubulin (green) and glial cells were stained for GFAP (red). (B) Immunostaining image of “Glia only” compartment demonstrated that only glial cells (red; GFAP) present within the compartment. (C-D) Immunostaining images within the maze like structures demonstrated that axons (Green; βIII tubulin) were stopped at bank 7 however the glial cells extended and replicated throughout all compartments. Scale bar=50 µm
5.4 Discussion

It is well known that glia are important, active participants in the formation of the complex neuronal circuitry found in the brain [185]. Previous studies also show that glia enhance the formation and stabilization of synaptic contacts [186]. Although traditional culture platforms have led to important findings regarding glia biology and their interactions with neurons, these are limited in their capacity to investigate communication in the absence of neuron to neuron signalling.

To this end, a multiple compartment system that allows for segregation of different cell types and isolated manipulation of individual environments was developed by Mingjian and colleagues [186]. They utilized a pressure enabled valve barrier (4 mm wide, 12 mm long, 1 mm high) to separate the compartments to isolate and culture different cell types. When the valve activated, the compartments were separated and while the valve deactivated, the neuron population in one of the compartments was connected at close proximity to the glial population in the adjacent compartment through the connecting channels (100 µm wide, ~50-100 µm long, 5 µm high). Hence, this microfluidic device is able to reversibly isolate the neuron and glial population both physically and chemically, thus allowing control over communication between these cell types and also permit the simultaneous independent chemical or physical treatment of both populations. In this model, after deactivation of the valve, the axons extend into the glia compartment or the glial cells proliferate or migrate into the adjacent neuron compartment through the connecting channel. Recently, Hosmane and colleagues developed a valved based compressive injury model for single CNS axons utilizing independently controlled “push down” PDMS injury pads [115]. They specified the severity of injury as follows by applying different pressure such as mild (<55 kPa), medium (55-95 kPa) and severe (>95 kPa) estimated using finite element
modelling (FEM). Therefore, although the independent chemical or physical treatment was permitted through the activation of valve in the co-culture device developed by Mingjian and colleagues [186], this device has a possible drawback in that the axon or glia in the connecting channels could be compressed by the valve.

Here, we overcame this drawback by developing a novel three-compartment microfluidic device for co-culturing two neuron populations with one glia population utilizing an array of maze like structures. This device was capable of physically separating two neuronal populations connected by a common glial population whilst also providing fluidic isolation between the compartments. We demonstrated that the maze channels with arrays of 10 banks allowed the glia to grow throughout the device but prevented the axons/neurites from entering the “glia only” compartment at DIV 10. However, ongoing experiments are required to investigate if axons will be extended further than bank 9 if allowed to remain in culture beyond DIV 10. If axons extend further than bank 9 when culture beyond DIV 10, further modification on the device’s design are required, for example, modification of the number of banks from 10 to 20. Therefore, the ongoing investigations utilizing our novel platform, will establish whether glial cells can facilitate communication between different neuron populations. In addition, the neuron-glia co cultures in our devices can also be tested with different therapeutic agents simultaneously in all populations due to fluidic isolation between three compartments.
5.5 CONCLUSIONS

In this study, we designed a novel microfluidic co-culture scheme with a three chamber configuration that can be used for the long term culture of primary central nervous system cells such as neurons and glia and for studying communication between these cells. The inclusion of maze-structures in our platform design permits the fluidic and physical isolation of the two neuronal populations and facilitates the glial cell growth throughout the cell culture compartments. The microfluidic devices described here can also be easily adapted for the co-culture of different populations of other cell types such as muscle cells, motor neurons etc. Overall, this novel three-compartment co-culture platform enables distinct modes of neuron-glia co-culture and will become a powerful platform to study neuron-glia specific cellular and molecular events implicated in neurological disorder and diseases.
6 General Conclusions and Future Directions

Therapeutic pathways for the treatment of TBI and other neurological disorders are still unclear and new experimental models that mimic specific aspects of injury and disease are required to define underlying mechanisms and trial different therapeutic agents. This thesis focused on the development of new microfluidic platforms for studying neuronal responses to trauma and disease. The first goal was to develop a new neuron culture platform with a high spatially and biochemically controlled microenvironment, for studying localized axonal stretch injury. Working towards this goal, a novel microfluidic device was developed, evaluated and applied to the study of singular and repetitive stretch injury of axons. Subsequently, an economical method for high resolution manufacturing of microfluidic devices, by using direct laser patterning technique, was developed to simplify the fabrication method. The devices fabricated using this approach were then evaluated for neuronal culture applications. Finally, a new device for controlled neuron-glial co-culture was developed, allowing for the study of the role of glia in CNS communication pathways.

The study of axonal stretch injury has been hampered due to the lack of *in vitro* models that can be used to apply reproducible and spatially localized stretch injury. Performing localized manipulation of the biochemical microenvironment as well as physical guidance of cell compartments cannot be achieved through the use of conventional cell culture methods. The two CNS neuron culture platforms developed here use microfabrication techniques to overcome these limitations of conventional culture methods.

Microfluidic based experimental platforms can provide a novel methodology to direct and isolate neuronal processes and to mimic the *in vivo* microenvironment of injured
CNS neurons and their degeneration. In this thesis, we have developed a novel *in vitro* microfluidic model to simulate very mild (0.5%) and mild (5%) axonal stretch injury in a fluidically isolated compartmentalized culturing device. We integrated pneumatic valve technology within the microfluidic neuronal cell culture platform to deflect a flexible PDMS membrane and therefore stretch the axons growing on top. Fluorescent imaging of immunostained cultures revealed several pathological alterations characteristic of DAI such as growth cone collapse, significant abnormal cytoskeletal rearrangement, delayed microtubule fragmentation and a degenerative response. These alterations were similar to those observed *in vivo* and hence confirmed the suitability of this system for studying the neuronal response to discrete axonal stretch injury. Results also suggested that repetitive very mild stretch injury significantly exacerbated these responses.

Future investigations using this device could perform drug-screening assays that aim to find suitable chemical candidates that promote the regeneration of stretch injured axons. Taxol (Paclitaxel) is a complex diterpene alkaloid drug that is known to bind with beta tubulin and thus stabilize the polymerization of tubulin [187]. It has been proposed as a potential therapeutic for axonal pathology that may involve destabilization of microtubules and has been reported to reduce beta amyloid toxicity in AD [188] and calcium mediated neuronal death [189]. Furthermore, Adlard et al. [190] demonstrated that in the short term, taxol also has beneficial effects in preventing microtubule loss and neurofilament accumulation in an *in vivo* model of cortical injury in the rat. Therefore, future studies can utilize this platform for the investigation of the effects of pharmacological agents such as the microtubule stabilizing agent-taxol in preserving and protecting neuron function as well as promoting the regeneration of axons following stretch injury. Combining these
platforms with single molecule evaluation such as optical microscopy or by AFM force spectroscopy techniques could also provide valuable data on the response of receptors and ion channels to these agents. In addition, the position of the pneumatic valve relative to soma, as well as the age and anatomical sources of the neurons, can be altered to provide further understanding of the complex cascade of cellular mechanisms underpinning the response to both TBI and repetitive TBI.

A novel, low cost approach for fast prototyping of rigid PMMA microchips by passing the laser beam through a stainless steel pinhole during CO\textsubscript{2} laser ablation was also developed. This direct laser machining process retains the high flexibility in design and fabrication of microfluidic devices, thereby overcoming the limitation of the photolithography fabrication process that requires a photomask. Results show that the pinhole significantly reduced the diameter of the laser spot and hence reduced the feature sizes of the microchannels during CO\textsubscript{2} laser micromachining. Separations of fluorescent dyes on devices ablated with and without the pinhole were compared. Results show that devices fabricated with the pinhole allowed for a 2.2 fold increase in separation efficiency when compared with devices made by laser ablation without the pinhole. Furthermore, efficiencies compared favorably with literature reports of similar separation in embossed PMMA and laser ablated glass devices. Finally, however, results demonstrated that neurons cultured on the laser ablated PDMS device were not healthy and therefore further modifications of the laser engraving process are required before this technique can be used for the fabrication of novel neural culturing devices. For example, surface modification such as solvent treatment on PDMS could be included to reduce the surface roughness of the ablated features.

Finally, a novel three compartment CNS neuron-glia co-culture microdevice has been developed using a one step photolithography process. Preliminary data demonstrated
that this unique design utilizing an array of maze like structures, allowed the growth of glia throughout the device, but restricted axonal outgrowth in a fluidically isolated microenvironment. In addition, this innovative design also has applicability for culturing other cell population such as muscle cells, motor neurons, interneurons and hippocampal neurons. Using this device, we can determine whether chemical damage (such as the application of neuronal cytoskeleton destabilizing agent-nocodazole) to one part of the network affects the other part, and we can also investigate if glial cells are required for the propagation of normal and/or compensatory or degenerative signals. This co-culture neuronal network device could also be combined with flexible PDMS membrane to study the effect of stretch events on the network and the role of glia in the transmission of signals to other parts of a network. Fluorescently labeled neurons can be utilized to visualize the dynamic changes to cellular morphology and dendritic spines following stretch injury to discrete sections of the neuronal network. Live calcium imaging can also be achieved through the addition of cell-permeable fluorescent dyes [191], such as Fura-2, to the culture chamber to determine if a wave of signals passes through neuronal or glial networks. Therefore, future studies can utilize this platform to study neuron-glia specific cellular and molecular events implicated in TBI and other neurological diseases and disorders.

Studies of neuronal cells within microfluidic platforms represent a new approach to experimental investigations that may reveal critical new information about the cellular and biochemical physiology of the central nervous system in health and in disease. In the future, we believe that microfabricated devices and microfluidics will become increasingly common tools for neurobiology, because of their spatiotemporal control and their ability to address biologically relevant questions that traditional techniques have been unable to address. The development of microfluidic tools for studying
neurons in an innovative and practically useful manner requires close collaboration between the neurobiologist and fabricator, making cross disciplinary communication and training quite essential in the development of a better understanding of neurological disorders and trauma.
7 References


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References


References


References


8 APPENDICES

8.1 APPENDIX A

Common laboratory reagents:

0.01 M Phosphate Buffered Saline (PBS) – 1.0L

850 mL MilliQ® water
100 mL 90.0 g/L Sodium chloride (NaCl; BDH, USA)
40 mL 28.0 g/L Di-sodium hydrogen orthophosphate (Na$_2$HPO; BDH, USA)
10 mL 31.2 g/L Sodium di-hydrogen orthophosphate (NaH$_2$PO$_4$.2H$_2$O; Ajax, Australia)

4% Paraformaldehyde – 1.0L

40 g granulated paraformaldehyde (PFA)
500 mL MilliQ® water
400 mL 28.0 g/L Na$_2$HPO
100 mL 31.2 g/L NaH$_2$PO$_4$.2H$_2$O
1.0 M NaOH & 1.0 M HCl to pH

Heat MilliQ® to 80 °C, add granulated PFA and 5 drops of NaOH. Stir until PFA dissolved, add NaH$_2$PO$_4$.H$_2$O and Na$_2$HPO. Filter and pH to 7.4.
8.2 Appendix B

Reagents for cell culture:

**Neuron Initial Growth Medium**

500 mL Neurobasal Medium (Gibco)

50 mL (10%) Fetal Bovine Serum (Invitrogen), heat inactivate at 65°C for 1 hour

10 mL (2%) B27 Neuronal Supplement (Invitrogen)

5 mL (1%) Antibiotic/Antimycotic (Invitrogen)

0.5 µM Glutamic acid

25 nM Glutamine

Combine aseptically. Stores 1 month 4°C.

**Neuron Subsequent Growth Medium**

500 mL Neurobasal Medium (Gibco)

10 mL (2%) B27 Neuronal Supplement (Invitrogen)

5 mL (1%) Antibiotic/Antimycotic (Invitrogen)

25 nM Glutamine

Combine aseptically. Stores 1 month 4°C.

**Glial Cell Growth Medium**

500 mL Dulbecco’s Modified Eagle Medium (DMEM; Gibco)

50 mL (10%) heat-inactivated Fetal Bovine Serum (Invitrogen)

5 mL (1%) Antibiotic/Antimycotic (Invitrogen)

Combine aseptically. Stores 1 month 4°C.

**0.001% Poly-L-lysine**

450 mL 0.01M PBS

50 mL (10%) Poly-L-lysine
0.25% Trypsin

20 mL 0.01M PBS
0.05 g Trypsin

Combine and filter-sterilise. Store 1 mL aliquots -20 °C.

0.05% Trypsin EDTA

100 mL 0.01M PBS
0.05 g Trypsin
0.0186 g EDTA

Combine and filter-sterilise. Store 10 mL aliquots -20 °C.
8.3 Appendix C

Reagents and Antibodies for immunocytochemistry:

0.3% Triton-X-100 Diluent – 200mL

200 mL 0.1M PBS

600 µL Triton-X-100 diluent (Sigma, USA)

Table 8.1 – Primary antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Immunogen</th>
<th>Species*</th>
<th>Dilution</th>
<th>Fixation</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-III tubulin</td>
<td>Neuron-specific cytoskeletal marker</td>
<td>Mm</td>
<td>1:1000</td>
<td>4% PFA</td>
<td>Promega</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>Rp</td>
<td>1:1000</td>
<td>4% PFA</td>
<td>Dako</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2 – dendrites</td>
<td>Mm</td>
<td>1:1000</td>
<td>4% PFA</td>
<td>Millipore</td>
</tr>
<tr>
<td>NFM</td>
<td>Neurofilament medium chain</td>
<td>Rp</td>
<td>1:1000</td>
<td>4% PFA</td>
<td>Serotec</td>
</tr>
<tr>
<td>Tau</td>
<td>Axon specific microtubule associated protein</td>
<td>Rp</td>
<td>1:5000</td>
<td>4% PFA</td>
<td>Dako</td>
</tr>
</tbody>
</table>

*Mm = mouse monoclonal (IgG unless specified), Rp = rabbit polyclonal

Table 8.2 – Secondary antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Fluorophore</th>
<th>IcC dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse IgG</td>
<td>Alexa 488</td>
<td>1:1000</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>Alexa 594</td>
<td>1:1000</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>
Table 8.3 – Stains

<table>
<thead>
<tr>
<th>Name</th>
<th>Labels</th>
<th>Fluorophore</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phalloidin</td>
<td>Filamentous actin</td>
<td>Alexa 594</td>
<td>1:200</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>
8.4 Appendix D

The calculation of the fluidic level differences in the three compartment microfluidic device described in Chapter 5 as following:

For example, the fluidic access port= 3 mm radius

When the volume differences=10 µL or mm³

Fluidic level differences

\[=\frac{10 \text{ mm}^3}{(\pi \times 3^2 \text{ mm}^2)}\]

\[= 0.354 \text{ mm}\]

\[= 354 \mu\text{m}\]
8.5 **Appendix E**

The separation efficiency and resolution were calculated based on the equation

\[ N = 5.54 \left( \frac{t_R}{w_{0.5}} \right)^2 \]

Where \( N \) = number of theoretical plates,

\( t_R \) = retention time

\( w_{0.5} \) = half of the width of the peak at its base

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Molar Mass (g/mol)</th>
<th>(^a) Migration Time (s)</th>
<th>(^a) Half of the width of the peak (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein sodium salt (FI)</td>
<td>376.27</td>
<td>24.66</td>
<td>0.894</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate (FITC),</td>
<td>389.38</td>
<td>27.36</td>
<td>1.068</td>
</tr>
</tbody>
</table>

\(^a\) Data of the separation electrophograms were recorded with photomultiplier tube and then collected in Chemstation software of the computer.

Table 8.5- Retention time, half of the width of the peak and properties of the analytes separated on microfluidic device that ablated using a pinhole.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Molar Mass (g/mol)</th>
<th>(^a) Migration Time (s)</th>
<th>(^a) Half of the width of the peak (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein sodium salt (FI)</td>
<td>376.27</td>
<td>40.02</td>
<td>0.978</td>
</tr>
</tbody>
</table>
Fluorescein isothiocyanate (FITC),

| Fluorescein isothiocyanate (FITC), | 389.38 | 45.24 | 1.188 |

A) Data of the separation electrophograms were recorded with photomultiplier tube and then collected in Chemstation software of the computer.

For example, when the migration time = 24.66 s, half of the width of the peak = 0.894 s

Number of theoretical plates, N

\[ N = 5.54 \left( \frac{t_R}{w_{0.5}} \right)^2 \]

\[ = 5.54 \left( \frac{24.66}{0.894} \right)^2 \]

\[ = 4215 \text{ plates} \]

The separation channel is 30 mm, hence number of theoretical plates per meter

\[ = 4215 \text{ plates/30 mm} \]

\[ \approx 140500 \text{ plates/m} \]