DEVELOPMENT OF MICROSCOPE STAGE INCUBATOR FOR LONG-TERM MONITORING OF NEURONAL CELL CULTURE DURING AXOTOMY

by

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ABSTRACT

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It is imperative to understand the patterns in regeneration of the central and peripheral nervous system after injury. This understanding would provide a fundamental cue towards molecules and other factors involved in regeneration, and how they affect the regeneration pattern after injury. In most culturing techniques currently used for neuronal culturing and observing the neuronal regeneration, it is difficult to determine the regeneration pattern (individual axonal level) though the overall regeneration is observed due to the complexity of neuronal network. Thus a model that provides isolation of individual axons and allowing us to monitor continuously upon injury has been developed. A microfluidic system mounted onto to custom made microscope stage incubator has been demonstrated to provide optimum conditions for neuron survival during axotomy and allowing us to monitor neurons continuously providing better understanding to injury response. In this study mammalian central and peripheral neurons were cultured in the microfluidic platform. The platform consists of a microfluidic device containing micro channels for isolating single axon, a microscope incubator creating a favorable

microenvironment for axons. All components are integrated into the microscope. Embryonic 16 day dorsal root ganglion and embryonic 18 day cortical neurons were cultured in the microfluidic devices. Robust outgrowth of both cortical neurons and dorsal root ganglions were observed, and individual axons growing via the micro channels in the device were observed. Various injury models have been demonstrated before but a system to monitor them closely upon injury to study their response to injury has been demonstrated and the understanding to injury response could help in developing a better treatment.

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CHAPTER 1

INTRODUCTION

1.1 Introduction to the Project

Disorders and diseases caused due to the failure of axons to regenerate after injury in the Central Nervous System (CNS) and Peripheral Nervous System (PNS) have been studied for a long time, however an efficient model to isolate the axons individual and study them individually has not been developed. The individual isolation of axons will provide an insight and better understanding to an axons reaction upon injury. A better understanding about the events and requirements of axon for regeneration upon injury will help develop a better treatment for acute spinal cord injuries and rejuvenation of lost controls. Often after injury due to the inhibitory agents produced in the CNS and PNS axons fail to grow back and thereby leading loss of function. The axon regeneration process by itself is a complex process and thereby requires studying them closely and clearly to understand the entire mechanism to help in providing a robust regeneration of the axons. In the past years axon injury and regeneration has been studied *in vitro,* but has a studied in a culture with thousands of axons and thereby owing to not able to follow each axon individually. In a culture due to high density of axon network, tracing an axon back to the cell body is practically impossible and is often difficult to confirm regeneration as it cannot be traced back. This has led to the need to develop new models which could help in the studying axons and its cell body individually for better understanding. In the past injuries have been made but however a precise injury confined to axon of interest alone has not been impossible. These methods have introduced damage to the surrounding area and thereby introduce nonspecific injury and thereby affect the results. Due to the nonspecific injuries a model that causes accurate injury on the desired axons alone has been needed so that the

results would not be affected. Therefore a model that helps in isolating the axons individually and injuring them specifically and locally has been proposed using microfluidic systems with the integration of femtosecond laser.

1.1.1 Fabrication of Microfluidic Devices

The masks are made using AutoCAD and by the process of soft lithography the mask pattern is impregnated onto the Silicon wafer with the resist on it. The wafer with the photo resist is exposed to UV light and the areas with photoresist exposed to UV light get polymerized and form a hard layer with the pattern in it. The rest of the non polymerized photoresist is washed using a developer leaving behind the desired pattern alone on the silicon wafer. On the master mold PDMS is poured and allowed to cure to fabricate PDMS microfluidic devices.

1.1.2 Fabrication of Microscope Stage Incubator

A microscope stage incubator is fabricated according to the requirements. The incubator is fabricated using antifog acrylic with a CO2 probe and a temperature probe along with a fan installed in it. Optimum conditions with 5% CO_2 and 37℃ is maintained in it. The microfluidic devices are mounted onto this incubator while axotomy.

1.1.3 Extraction of Embryonic Cells

Timed pregnant rats are used for this purpose and the embryos are used at the age of embryonic day 18. The embryos are taken out and the cells are derived the embryos. The obtained tissues are dissociated to get individual cells and these individual cells are seeded onto the microfluidic devices along with little neurobasal medium and is allowed to grow inside the microfluidic devices.

1.1.4 Axotomy of The Cultured Neurons

The axons are isolated in the micro channels and are used as the injury models. The PDMS devices are mounted onto the custom made microscope stage incubator to maintain optimum conditions and to prevent stressing out the cells and thereby eliminating external

factors. The single axons are completely axotomized using femtosecond laser. The axons response to injury is studied and followed over different time points.

1.1.5 Regeneration of Axons

The regeneration of axons is seen after a minimum time requirement of 4hours. After 4 hours an active growth cone is seen at the tip of the axotomized axons. It was observed that twelve out of fourteen cortical neuron axons regenerate on the same path it had grown before. The cortical neurons trace their old track back, however in the case of Dorsal Root Ganglion (DRG) eight out of eight axons regenerated and followed a new path upon regeneration and neglected the old path that they had grown in.

1.1.6 Identification of the Molecule

The identification of molecule is being done by narrowing the various possible molecules that could be involved. The expression of these molecules are blocked by the addition of specific antibodies for them and the regeneration pattern is to be observed if the follow the same path or if the right molecule is blocked they would not be able to recognize their old path and will regenerate in a new path.

1.2 Background and Literature Review

1.2.1. Statistics of Spinal Cord Injury and Significance for an Effective Treatment

In the United States of America 250000-400000 people have their spinal cord injured and 52% of the spinal cord injuries are considered paraplegic and 47% is considered quadriplegic. More than 13,000 additional people are injured each year with the alarming rate of an individual sustaining a spinal cord injury every 41minutes. In this 56% of in the injuries occur between the ages 16-30. Serious injuries at this prime age cause loss of function making them stay with the loss of functions through their life. The cost to maintain the health, accessibility to the community and other direct costs associated with these chronic injuries sums up to approximately $25,213 per annum for an individual. This yearly charge totals to $11,345,850,000 for treating the injury alone. Most spinal cord injuries occur due to vehicular

accidents contributing to 37% of spinal cord injury followed by violence and falls contributing 28% and 21% respectively. The total amount spent towards spinal cord injury per annum sums to a staggering amount of $14,533,466,000. Though with the amount of money spent on spinal cord injuries on 52% of individuals are covered by private health insurance at the time of injury. With the high spending track to cure spinal cord injuries and the rising number to spinal cord injury cases and the lack of treatments to cure spinal cord injuries has made it an important factor to find an effective treatment. Most spinal cord injuries occur at the age of 16-30 years leaving them impaired for the rest of their lives. In order to treat people injured at such prime age and help them recover their lost functions the need to develop an effective treatment has been researched and significant achievements are being made.

1.2.2 Impact of This Project

The successful regeneration study will give an understanding to responses in individual axons and also a novel neuro-optical platform to study the axons individually for different studies along with provision for localized injury. The identification of the molecule will aid in the pavement of a path and guidance of the axon in the direction of our interest and thereby making them reach the target cells to aid in regeneration. The identification of the molecule will also aid in the regeneration of the axons *in vivo* upon injury by providing the molecule invivo will produce a better environment supporting regeneration beyond the other inhibitory environment being presented. This will provide in treating various injuries.

1.3 Neuron

The neurons are the primary functional unit of the nervous system and are involved in all nervous communication and signaling and were first discovered in early 20th century by Santiago Ramon y Cajal [1]. Neurons are excitable cells that are present in the nervous system enabling to process and transmit various messages though electrochemical signaling. Neurons form the core components of the brain, spinal cord and the peripheral nerves. Neurons are of different types and based on their location they have different functionality such as the sensory

neurons which respond to touch, sound along with various other stimuli and theses signals are sent to the spinal cord in the form of electrochemical signals and is processed for appropriate response. The motor neurons receive signals from the brain and spinal cord and cause muscle contractions. Inter neurons are small neurons that connect one neuron to another. Neurons are responsive to stimuli and communicate the presence of stimuli to one another and respond accordingly, which is often the body's response to any actions. Neurons do not multiply and once an acute injury occurs they die and cannot be replaced. As the neurons cannot be replaced the regeneration of neurons is important to maintain all functions. Thus the rejuvenation of injured neurons has been an important aspect in preventing loss of any function due to the death of neurons. Fully differentiated neurons are permanently amitotic [2] but however recent studies show that in the brain new neurons can be formed from the neural stem cells and in relatively higher concentration in the sub ventricular zone (SVZ) and sub granular zone (SGZ) by the process of neurogenesis [3,4,5].

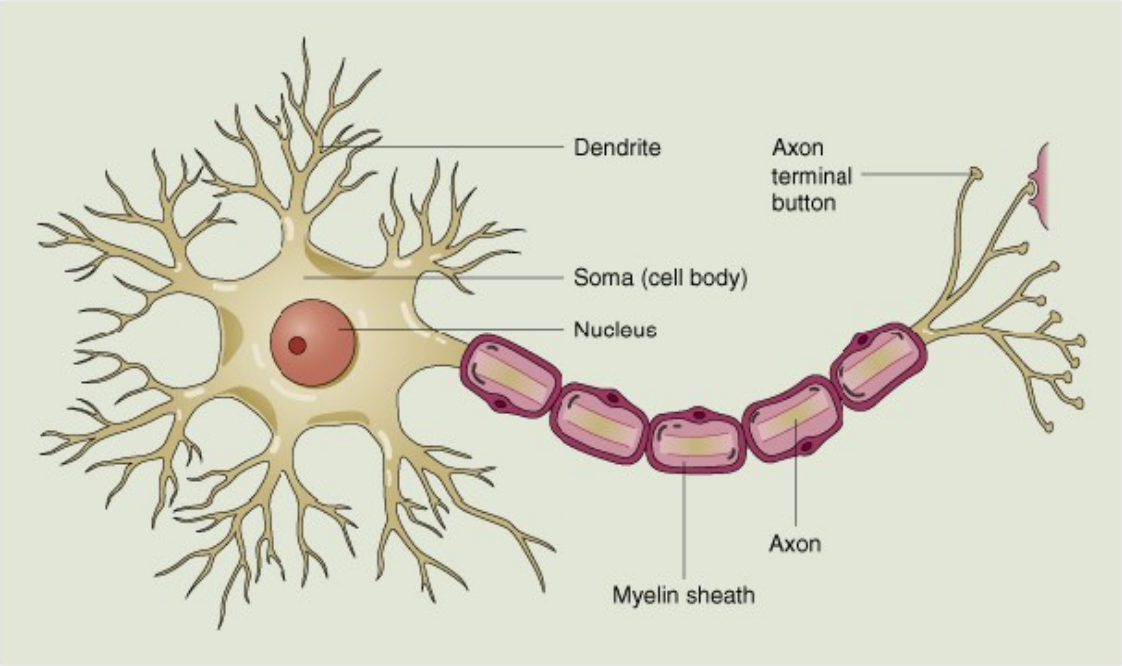


Figure 1.1: Image depicting the structure of neuron and explaining its parts [6]

Neurons exist in various sizes and shapes and are classified based on their morphology and function. A neuron consists of a cell body called the soma and a long fiber like axon which traverses across the body, making contact and functional innervations all over the body and these axons are covered with myelin sheath which aid in the faster transmission of signals. The soma is the central part of the neuron and also comprises the nucleus of the cell. Owing to presence of the nucleus most of the protein synthesis occurs here. The nucleus is normally in the size range of 3-18 micrometers in diameter [7]. From the cell body axons are projected outwards as fine threads. Most neurons have single axons but these single axons will have numerous branches enabling the communication with many target cells. The part of the axon that merges with the soma is called the axon hillock and has the highest density of voltage dependent sodium channels. The end of axons have branched terminals that release neurotransmitters into the synaptic cleft of the other neuron. These few areas secrete transmitter and the remaining areas respond to the transmitters. The neuron membrane has gated ion channels that have fast response sodium channels for the rapid transfer of signals. Closer to the cell body dendrites are present which receive signals from other neurons.

Neurons communicate through the process of synaptic transmission which includes chemical and electrical synapses. Action potential is the key element which triggers synaptic transmission. The synaptic transmission is achieved by the axon potential which is an electrical signal which is generated upon excitation of the electrically excitable membrane of the neuron.

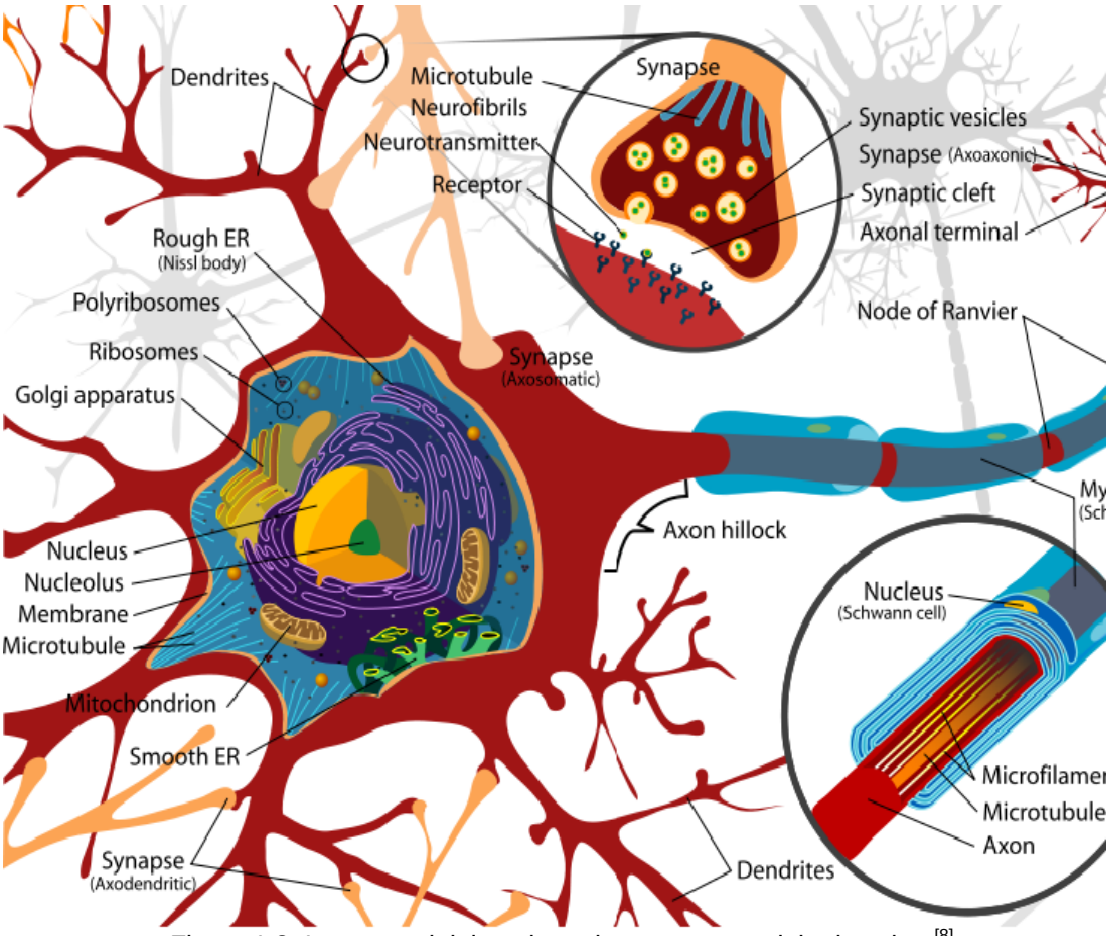


Figure 1.2: Image explaining about the synapse and the junction [8]

1.3.1 Axon Potential

Axon potential is the electrical signal that is sent through the axons. It's a transient alteration in the membrane potential produced across an excitable membrane upon the activity of voltage gated ion channels in the membranes [9].

Electrical signals in biological or living organisms are generally driven by ions and the most important ions involved in action potential are sodium (Na^+) and potassium (K^+) ions [10,11]. Both ions possess a single positive charge but at times calcium (Ca^{2+}) ions are involved in action potential and they possess a double positive charge [12]. Ions cross the cell membrane by diffusion driven by electric fields [13, 14].

Action potentials are initiated at the axon hillock when the excitable membrane is sufficiently depolarized [15]. As the membrane potential voltage is increased sufficiently, the sodium ion channels open and sodium ions start flowing from outside into the cells. When sodium ions enter the cells the potassium ion channels are opened in order to permit the outflow of potassium ions to the exterior surface of the cells [16]. As sodium ions enter the cell it increases the concentration of positively charged cations inside the cell and causes depolarization of the membrane. The charge at the membrane is reversed. The sodium channels close at peak of action potential but however the potassium ions continue to leave the cell and the outward flow of potassium ions decreases the membrane potential or hyperpolarizes the cell [16]. For a small voltage increase the potassium current is stronger than the sodium current and therefore maintains the membrane potential to -70mv or its resting potential [17]. However, if the voltage is higher than the critical threshold the sodium current is stronger than the potassium current and a potential of 15mV higher than resting potential is maintained as sodium current dominates. This is due to the runaway condition where positive feedback from sodium current keeps the sodium channels open owing to the constant inward flow of sodium ions from outside increasing the membrane potential and eventually the action potential is fired by the cell as electrical signals [18,19].

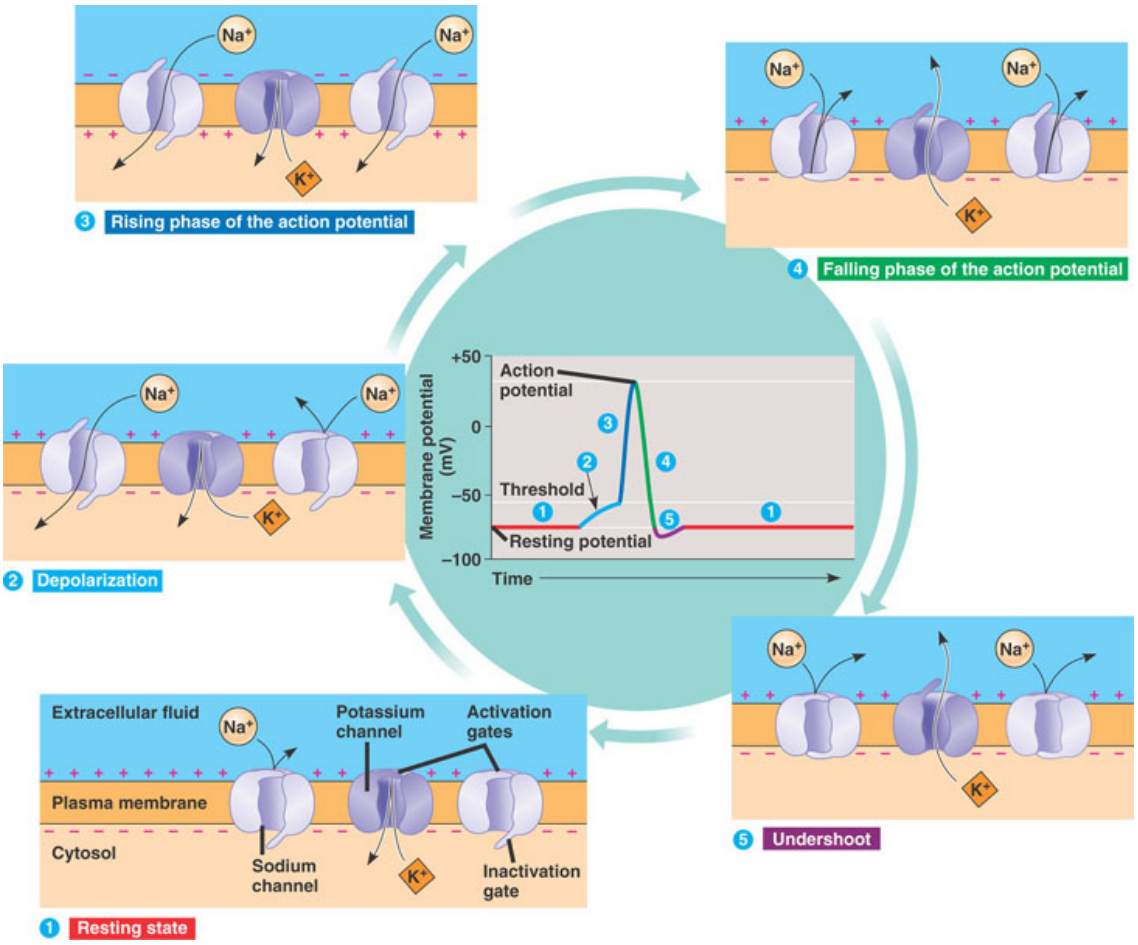


Figure 1.3: Image explaining about the sodium and potential channel activation during firing an action potential [20]

Currents produced on the opening of voltage gated channels during action potential regeneration are larger than the stimulating current. Therefore the amplitude, duration and shape of the action potential are based on the level of excitation of the membrane from its resting state and also on the duration of the stimulus.

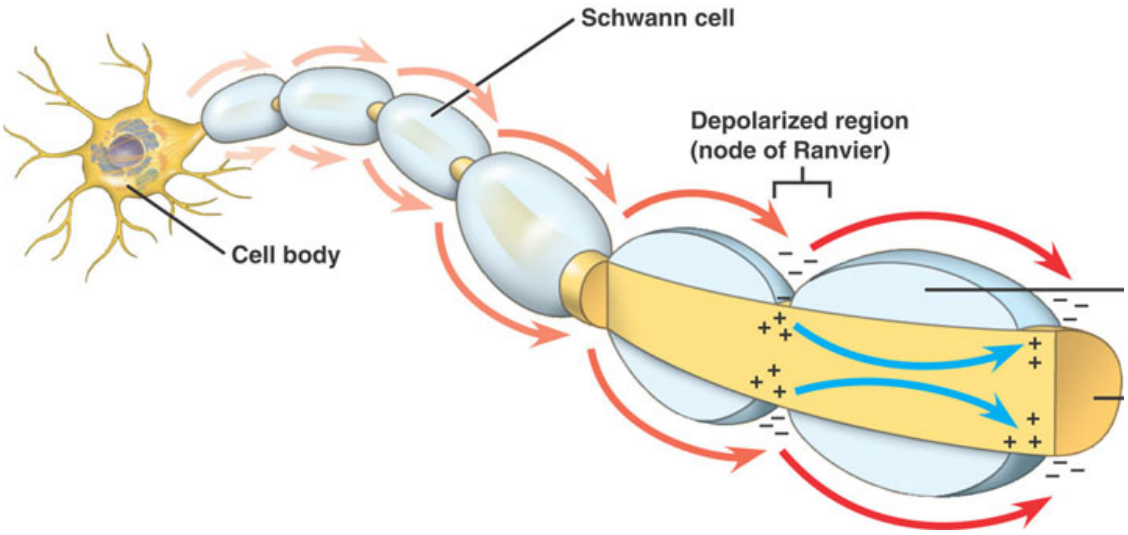


Figure 1.4: Image depicting the conduction of axon potential through axons and axon potential jumping over each node [21]

Axon fibers are covered by myelin sheath which aid in the rapid transfer of signals along the axon fibers. However the myelinated sections of the axons are not excitable and do not produce action potential. The unmyelinated spaces on the axon called nodes of Ranvier generate the action potential [22]. Depolarization of the axon terminal initiate the release of neurotransmitters into the synaptic cleft at the point where they merge with their target cell and thereby transmitting the signals [23].

1.4 Classification Of Neurons

Neurons are classified on the basis of different criteria such as their structure, function, area of presence and their release pattern.

1.4.1 Structural Classification

Unipolar: Both dendrite and axon emerge from the same process

Bipolar: Dendrite and axon emerge from opposite sides of the soma. Eg: Dorsal Root Ganglion

Multipolar: These possess more than one dendrite. Eg: purkinje cell, granule cell

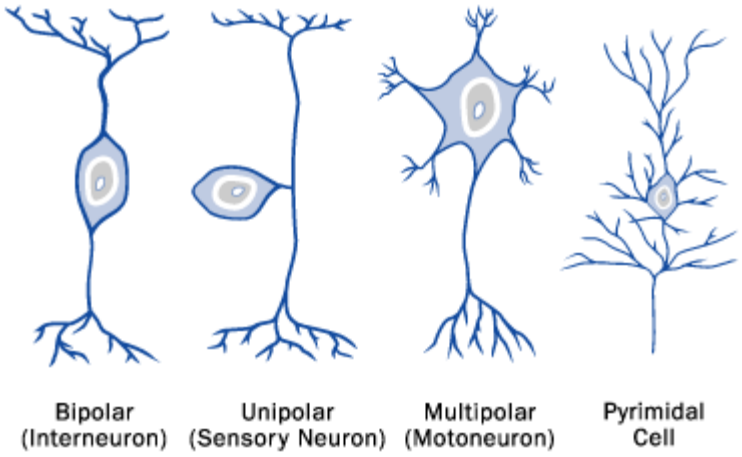


Figure 1.5: Image depicting the different neuron types based on polarity [24].

1.4.2 Function

Afferent Neurons: They convey information from tissues and organs into the central nervous system. These are sometimes called the sensory neurons.

Efferent Neurons: The neurons convey message from the CNS to the effector cells. Eg: motor neurons

Interneuron: These neuron functions in connection one neuron to another and conveying message between neurons.

1.4.3 Discharge Pattern

This is based on the electrophysiological properties of the neuron.

Regular Spiking: These neurons are constantly active. Eg: Interneurons in neurostriatum

Bursting: Neurons that fire axon potential in bursts are called as phasic

Fast spiking: Some neurons have high firing rates and fire action potentials at a rapid rate. Eg: Cortical inhibitory interneurons, retinal ganglion cells [25, 26].

Neurons differ in the type of neurotransmitter they produce. Some examples are

cholinergic neurons - acetylcholine

GABAergic neurons - gamma aminobutyric acid

glutamatergic neurons - glutamate

dopaminergic neurons – dopamine

serotonergic neurons - serotonin

1.4.4 Cortical Neuron

Cortical neurons are the neurons that make up the cortex of the brain. Cortical neurons are important and are a concentrated subject as it forms the cortex. The cortex is involved in the functions like memory, attention, thought, language and all major biological activities. The cortex is 2-4mm thick [27]. Any damage to the cortex affects other biological activities and hence the regeneration upon injury studies in the cortical neurons

1.4.5 Dorsal Root Ganglion

DRG are involved in the sensory system of the body and play a vital role in the transmission of sensory signals. DRG is a nodule on the dorsal root of the spinal cord that is spherical ganglion containing cells inside them and they sprout axons in all directions and make contact with target cells and transmit various sensory signals [28]. The axons of the DRG are called afferents as these axons relay sensory information into the central nervous system and spinal cord. As the DRG play a vital role in the sensory system their regeneration studies have also been concentrated however the regeneration of DRG is aided by Schwann cells and they have a better regeneration mechanism compared to the CNS.

1.5 Axonal Injury

There are different types of injury to the axons depending on the site of injury, intensity of injury and the number of neurons affected by the injury. The injury could either be a nick on the axon, an acute injury or an injury leading to the demyelination in the PNS [29]. In the case of a small damage the axon is repaired and is made functional soon but in the case of an acute damage the regeneration mechanism is often impaired. In case of an acute injury the neuron often dies or the regeneration is inhibited by the inhibitory molecules such as myelin, CSPG and spinal fluid [30]. The complete transection of the adult mammalian spinal cord leads to the irreversible scar formation and the permanent loss of both motor and somatosensory functions

in the PNS below the injury site. The lack of spontaneous anatomical and functional repair after a spinal cord injury is due to the failure of neurons to regenerate their axons beyond the inhospitable environment that is presented upon injury in the mature CNS [30]. These inhibitory environmental factors are associated with the glial cell surfaces and the ECM surrounding the regenerating axon [31, 32, 33, 34, 35, and 36].

Similar to the spinal cord injury, an injury to the adult CNS is devastating due to the inability of central neurons to regenerate the correct axonal and dendritic connections. The consequences not only the loss in communication between healthy neurons but a cascade of events that lead to neuronal degeneration and the destruction of the intricate neuronal network. The axons in the CNS can regenerate if provided the permissive environment for their regrowth [37]. This study proves that the failure in regeneration is not because of an intrinsic property of the neurons but however the conditions in the damaged environment that did not support or prevented regeneration. Based on these findings in the past twenty years progress has been made in identifying the elements responsible for the differences in an adult CNS and PNS environment upon injury and regeneration.The molecular bases of regenerative activity are being researched to provide an effective regeneration beyond the inhibitory molecules and environment.

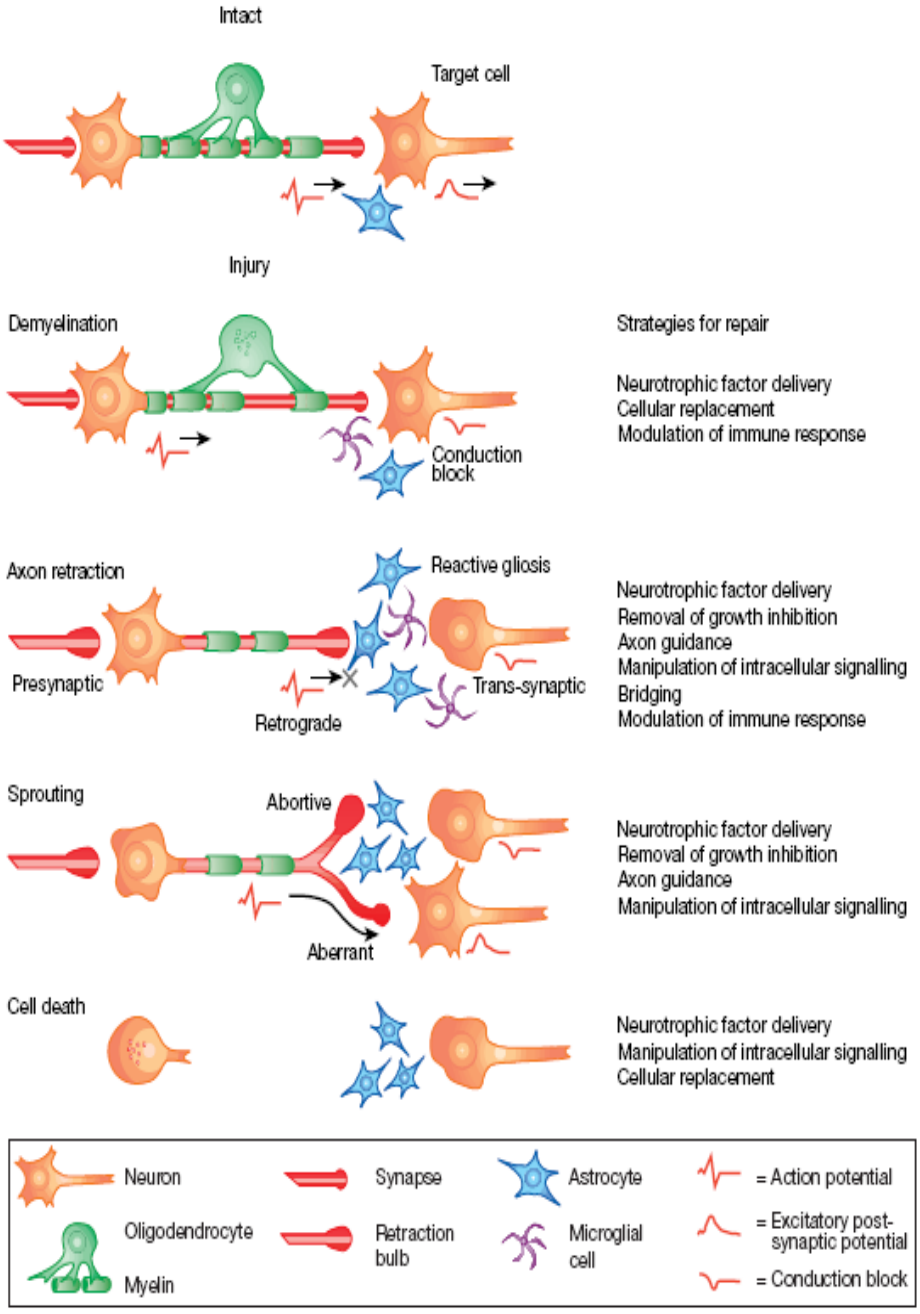


Figure 1.6: Figure explaining consequences of neuronal injury and strategies for repair [38]

From the previous research that has proved that both CNS and PNS neuron could be made to regenerate upon providing the favorable environment various researches are being done to effectively regrow the injured axons over the inhibitory molecules. Various methods

such as blocking the identified inhibitory molecules, cellular replacements, manipulation of intracellular signaling, bridging and usage of artificial substrates and local delivery of growth factors have been implemented to aid in regeneration as it is important in maintaining the neuronal network to retain proper communication throughout the body [39].

1.5.1 Wallerian Degeneration

Wallerian degeneration is the process by which when a nerve fiber is crushed or cut the axons separate out from the soma and start degenerating and this is also called anterograde degeneration [40]. Wallerian degeneration occurs after axonal injury in both PNS and CNS and it occurs at the distal stump from the site of injury. Upon degeneration of the axon the myelin sheath is degradation and macrophages infiltrate the injured area to clear the debris of degeneration along with Schwann cells in PNS [41, 42].

1.5.2 Present Models to Study Injury and Regeneration

The need for a good model to study the replicate *in vivo* neuronal damage and regeneration has been long desired and various models have been developed [43]. These models have been developed to study the axon damage, degeneration and regeneration to help in providing a better understanding to the repair mechanism and other molecules that are involved in inhibiting the axons regrowth.

Models such as *in vivo*, culture of neuronal cells in culture dishes and cover slips have been done in the past to develop a neuronal culture and are damaged using needles; a complete transection using knife, pipette tips, crush injuries, chemicals and other sharp instrument have been used [44]. Using these models the axon injury, its response to injury, regeneration and inhibitory molecules have been studied. However in all the culture models the *in vivo* system had provided the closest insight to the injury, Wallerian degeneration and regeneration but with the complicated network in the *in vivo* system it has been impossible to study the individual axons responses and the axons could not be traced back to its soma [45]. In the *in vitro* culture, where neurons are cultured in a culture dish or cover slips and are used for

injury models, the neurons form a complicated network of axons thereby making it impossible for them to be isolated and traced back. *In vitro* systems are comparatively less complicated when using an animal model but due to the property of axon branching out in open spaces a complicated network is formed eventually and in this various injury ad regeneration studies have been done and the events upon injury have been explained. However it is not fully understood in the individual axon level and at the molecular level. This has been clearly understood in the *in vivo* and i*n vitro* culture systems. But it has been impossible to follow axons individually to study them. Using this system the molecular mechanism involved in regeneration process has been understood but it's imperative to understand the entire process to provide a better system for a robust regeneration upon injury and to overcome the intrinsic barrier posed by the environment upon injury in CNS and PNS. It's important to provide robust regeneration to provide recovery upon injury.

With the injury mechanism such as using needles, knifes, crush injuries, chemicals the injury made is not reproducible and accurate injury cannot be made to the desired axon alone. The injury is not focal and the surrounding axons are injured as well. The injury made in this method cannot be confined and cannot be reproduced. Due to this, different injuries cannot be done, such as ranging from a small nick to a complete transaction. The intensity of the injury cannot be often controlled leading to acute damages and a complete transection of everything and sometimes damaging the soma itself. Due to injury caused to the surrounding it leads to complete destruction of the axonal network and does not provide much information.

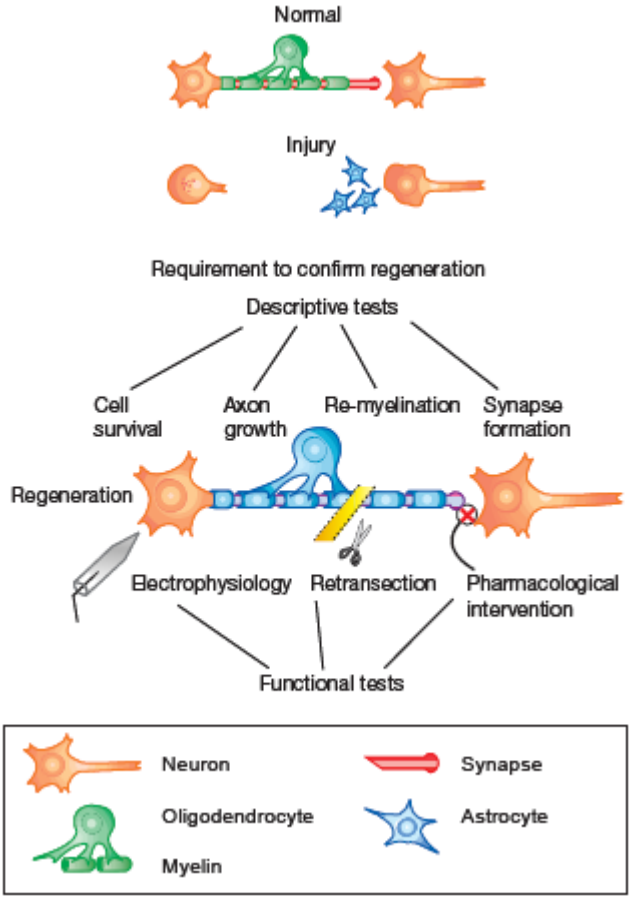


Fig 1.7: Pictorial representation describing the injury and the confirmation of regeneration process [38]

By the given definition on injury and regeneration in the neurons the axons should be completely transected and the degeneration should be observed and followed by the regrowth of the axon back by formation of a new growth cone. The functional regeneration has been checked by implementing electrophysiological studies. By these studies the initial action potential across the axon is measured before injury and in measured again upon regeneration to compare the activity in the axons to confirm regeneration [38].

In order to study the regeneration and understand it completely various models have been developed to individually study axons and specifically damage them without causing injury to the surrounding axons to study the functional regeneration using electrophysiology later. This

need has led to the development of other models to understand the regeneration at a molecular level.

1.6 Microfluidic Devices

Microfluidics deals with behavior, precise control and manipulation of different fluids by geometrically constraining them to a small millimeter scale. The call them microfluidic devices either one of the following properties should be incorporated in them:

- Small volumes
- Small size
- Low energy consumption
- Effects of micro domain
- The internal dimensions should be in micro size

Microfluidic devices have been used for a variety of applications and with the incorporation of micro channels in them with the dimensions in micron size it has been used widely for biological applications. Microfluidic chips have emerged as a powerful platform for cell culture studies [46, 47, 48, 49, 50, and 51]. The functionality and features that can be incorporated into microfluidic chips is enabling investigation of cells and experiment design that is not possible with conventional open cell culture platforms [52, 53, 54, 55, 56, 57]. Moreover, microfluidic chips permit geometries that result in spatial confinement and create biochemical gradients that better mimic the *in vivo* environment. Understanding post injury axonal regeneration processes is fundamental for development of therapies for a range of diseases that involve injury and repair of axons. The complexity of events that follow neural injury and the lack of tools to monitor these events *in vivo* in real time necessitate the use of in vitro models.

The incorporation of the microchannels in the microfluidic devices provides an effective platform to culture neurons and isolate the axons individually in the microchannels providing a better platform to understand the axon mechanism upon injury.

These microfluidic devices are generally made using Polydimethylsiloxane (PDMS) which is a clear material.

1.6.1 PDMS Properties

PDMS belongs to the family of polymeric organosilicone compounds which are commonly known as silicones [58]. PDMS is the most widely used silicon based organic polymer and has been widely used in microfluidics due to its unusual rheological properties. PDMS is optically clear and is considered to be inert, non-toxic and non-flammable.

PDMS is visco elastic, at higher temperatures it acts as a viscous liquid however at room temperature it exists as an elastic solid material upon being crosslinked. If PDMS is left overnight it cures and takes the shape of any imperfections if present on its surface. Upon curing it acts like rubber and has high elastic property. The viscoelastic properties of PDMS can be measured using dynamic mechanical analysis. This involves a specialized instrument to determine the materials flow characteristics over different conditions such as a wide range of temperature, flow rate and deformations. The shear modulus of PDMS varies under different conditions but normally it's in the range of 100kPa to 3MPa and the loss tangent is very low ($\tan \delta \ll 0.001$) [59].

1.7 Objectives Of This Research Project

The goal of the project is to develop a neuro-optical platform using microfludic devices to culture cortical neurons and DRG and isolate their axons individually in the micro channels in the microfluidic devices. To achieve this following specific aims were addressed:

1) Fabrication of microscope stage incubator and imaging chamber
2) Fabrication of silicon wafer and manufacture of PDMS devices
3) Extraction of primary cells and cell culture

CHAPTER 2

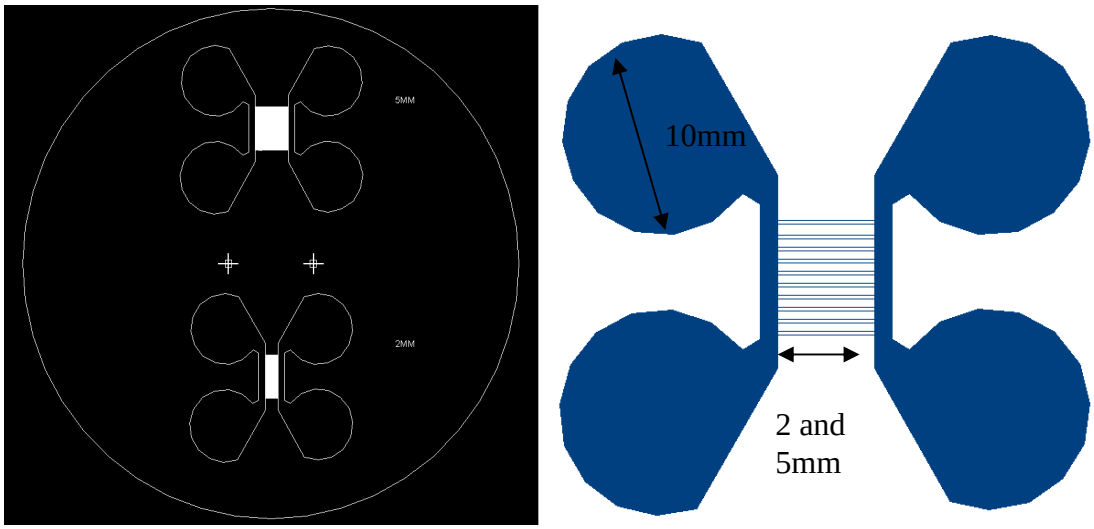
FABRICATION OF MASTER MOLD SILICON WAFERS USING SOFT LITHOGRAPHY

2.1 Preparation of the mask with the pattern

Given the long processes that extend from the neuronal cell bodies in the form of axons and formation of spatially complex network formed in neuronal cell cultures makes it very difficult to study the response to injury of individual axons in isolation. Campenot invented compartmentalized chambers to isolate soma from the axons which has proven to be a very valuable tool to study axonal biology [60, 61, 62, and 63]. Polydimethylsiloxane (PDMS) based microfluidic devices [64, 65, 66, 67, 68, and 69] with compartmentalized chambers connected via micro channels provide an excellent platform for neuronal studies [70, 71]. The advantages of the PDMS based microfluidic devices are flexibility in fabrication with varying dimensions and geometries and an optically clear window for microscopy and optical manipulation. Microfluidic devices are fabricated using soft lithography technique [72].

Before making the master mold in silicon wafers a mask is to be made from which the pattern is transferred onto the silicon wafer with the photoresist on it. The mask is designed using autoCAD software. The mask is designed in accordance to the required dimensions. In this the mask is made channel width of 15µm and the distance between each channel as 50µm. The channel length is made as 5mm in one device and as 2mm in the other device. The different lengths are made due to the ability of axons to grow lengthwise. The circular heads which are punched later for cell seeding have a diameter of 10mm in which an 8mm punch is made. Upon designing the mask in autoCAD it is sent to a commercial vendor for a high resolution reproduction of the design onto a Mylar mask. Each mask has two patterns and

thereby gives a yield of two devices. The device geometry is based on the design developed by Park et al [73].



A) B)

Figure 2.1: A) Image showing the mask designed using autoCAD. This is the final mask that is sent to the vendor for high resolution printing of the designed mask onto a Mylar mask. In the mask two patterns are seen. The mask on the top has channel length of 5mm and the mask at the bottom has channel length of 2mm.

2.2 Master Mold Preparation

The following steps were done on the silicon wafer in order to obtain the master mold

- Etching of silicon wafer using sulphuric acid
- Cleaning of the oxidized wafer
- Dehydration
- Spinning
- Softbake
- Exposure
- Post exposure bake

- Develop and drying
- Hard bake

1) Etching the silicon wafer surface:

   The silicon wafer surface is etched using sulfuric acid. This is done to increase the porosity on the surface of the silicon wafer. The increase in porosity helps in better binding of the photoresist to the silicon wafer.

2) Cleaning of the silicon wafer:

   The silicon wafer is first cleaned using acetone. Acetone is taken in a beaker and the silicon wafer is cleaned using it. Upon cleaning it using silicon wafer it is dried using nitrogen gas and is washed using deionized water at the purity of 18.2 MΩ.

3) Dehydration:

   The dehydration of the wafer helps in better adherence of the resist to the wafer. The dehydration step is important as improper dehydration will affect the process later. The dehydration process is done by placing the silicon wafer at 175℃ for 10 minutes

4) Spinning:

   The spinning cycle is done in two different steps. This is done to ensure the equal distribution of the photoresist throughout the wafer and thereby forming a flat surface. The spin cycle is done in two steps. The two steps are the spread cycle and the spin cycle. The spread cycle is generally done at lower speeds to spread the photoresist all over the wafer and the spin cycle is done at higher speeds. The spin cycle speed is set based on the height of photoresist required. For thicker resist coating lower spin speeds are used and for thin coating higher speeds are used. The spin cycle speed determines the height of the photoresist layer.

5) Soft bake:

This is done to remove the solvent from the photoresist and harden it and to prepare the photoresist for the exposure process. The removal of solvent helps in making the resist solid and thereby accurate design can be transferred on to the wafer. The presence will cause the resist to smudge when it comes in contact with the mask and sharp pattern cannot be printed on it.

6) Exposure:

Using G line OAI806MBA backside aligner the mask in placed on top of the wafer with the resist and UV light with the expose energy of 300mJ/cm2- 550mJ/cm2 is allowed to pass through the mask. The areas onto which UV light is exposed is cross linked and the mask pattern in formed on the wafer

7) Post Exposure bake:

This is done to finalize the cross linking process. This hardens the surface further and thereby the sharp design patterns are maintained accurately

8) Develop and drying:

Upon exposure and post exposure bake, the wafer has to be developed to remove the resist that has not been cross linked and to develop the hardened pattern from the mask. The rest of the resist is washed off using the resist remover and the mask pattern is exposed. Upon developing the wafer is dried using nitrogen gas to remove t he resist remover and is washed with 70% IPA to confirm if all the photoresist is removed.

9) Hard bake:

This is the final step in the wafer fabrication. This step is to end the cross linking process and to harden the resist completely such that it can be used as a mold.

2.3 Method of Fabrication

The 4” silicon wafer is taken and the surface is etched using concentrated sulfuric acid. Sulfuric acid is taken in a glass beaker and the wafer is immersed in it for 10-15minutes allowing the sulfuric acid to etch the surface. The surface etching allows better adherence of the photoresist to the surface of the silicon wafer. After surface etching, the wafer’s oxidized surface is cleaned with acetone to remove any organic materials and impurities present on the wafer’s surface. After the acetone wash the acetone is air dried using nitrogen gas and any leftover acetone is washed using deionized water. The washed and cleaned wafer is left on top of a hot plate at 175℃ for 10 minutes. This is done to remo ve water from the silicon wafer and keep it dry. This step is particularly important as if any water is left over if will interfere in the adherence of the photoresist to the wafer surface. After dehydration the wafer is spin coated for the equal distribution of the photoresist on the wafer. The first layer is required to be made with a height of 15µm and the photoresist for making 15µm height devices SU 85 is used. SU 85 is poured on the silicon wafer placed in the spin coating instrument. The spin coating is a two step process and the spread cycle is done with the speed set to 500 rpm at the ramp of 100 rpm/sec for duration of 5 seconds. This spread cycle ensures the distribution of the resist throughout the surface of the wafer. The spin cycle is done at a speed of 1000 rpm with a ramp of 300 rpm/sec for 30 seconds. The spin cycle speed is the factor that helps in making the resist for the desired height. In this case a first layer with height of 15 µm is required so the resist is spun at 1000 rpm. After spin coating the wafer is soft baked at 65℃ for 2 minutes and at 95℃ for 5 minutes.
Meanwhile in the backside aligner the Mylar mask is mounted onto a glass slide and this is mounted onto to the backside aligner by the use of vacuum. The spin coated wafer is placed on the backside area on the circular disk for placing the wafers. This is below the mask. With the mask on top of the wafer the lamp is turned on and UV light is allowed to pass through the mask and hits the photoresist on the wafer. The wafer is exposed for 14 seconds. The exposed area is cross linked and unexposed area remains as a viscous liquid. After exposure the wafer is

baked by a process called post exposure baking at 65℃ for 1 minute and at 95℃ for 5 minutes. During post exposure bake, the thermally driven and acid initiated reactions cross link the epoxy. In a beaker SU 8 remover or the resist remover (developer) is taken. The wafer is gently placed inside and carefully washed for 3 minutes to remove the viscous resist on the wafer and thereby exposing the design pattern on the wafer.

After the development of the first layer with a height of 15 µm, the second layer with a height of 100 µm is supposed to be made for the cell seeding areas. On top of the wafer with the 15 µm pattern in it SU 85 resist is poured and is spin coated with a spread cycle at 500 rpm and at ramp of 100 rpm/sec for 10 seconds. It is spin coated at a speed of 1000 rpm and at a ramp of 300 rpm/sec for 30 seconds. Due to high viscosity of SU 85 resist at 1000 rpm it forms a layer with a height of 100 µm. This is then soft baked at 65℃ for 10 minutes and at 95℃ for 30 minutes. After soft bake the wafer is taken to the backside aligner and the mask for second layer is also mounted in the aligner. Now the first layer in the wafer is focused using the lenses provided. The first layer is aligned in such a way that it matches with the second layer. After the alignment of the two layers the wafer is brought up and the resist comes in contact with the mask and the resist is pressed against it. Now the wafer is exposed to UV light for duration of 32 seconds in total. But however the exposure is done two times with exposing it for 16 seconds each time. After the exposure it is baked at 65℃ for 1 minute and at 95℃ for 10 minutes. After post exposure bake the wafer is placed in the beaker containing the developer. The wafer is gently washed for 10 minutes with the replacement of new developer at the fifth minute. After developing using SU 8 developer it is dried using nitrogen gas. Finally the wafer is washed with 70% IPA to confirm the removal of all the resist. If in any case some photoresist is left it will turn white when washed with IPA and the resist can be removed by using developer. Finally the wafer is hard baked at 150℃ for 30 minutes to harden the resist thereby providing complete adhesion to the wafer. After hardening this can be used as a mold. After 30 minutes the temperature is brought down to 90℃.

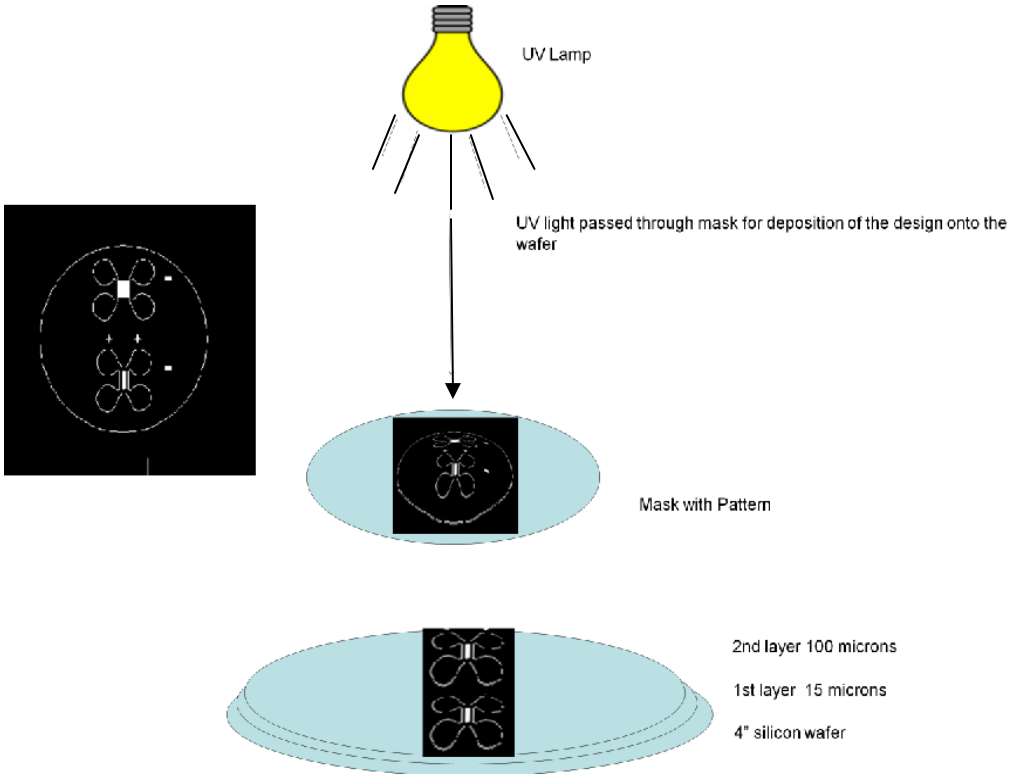


Figure 2.2: A pictorial representation of the exposure process

2.4 Fabrication of PDMS Devices

The microfluidic devices were made using polydimethylsiloxane (PDMS). Sylgard 184 kit was brought from a commercial vendor and was used to make PDMS. The kit consisted of the PDMS pre polymer and its curing agent. The pre polymer and the curing agent were mixed in the ratio 10:1 respectively and this is mixed completely. It's made sure that pre polymer and curing agent was mixed completely and this was desiccated in the desiccators to remove all the air bubbles and a clear solution is obtained. The silicon wafer is placed on the aluminum foil and is kept in the hot plate at 150℃. The desiccated P DMS mixture was poured on top of the mold and any air bubbles formed are removed using a needle and was left to cure at 150℃ for 5 minutes. After it gets cured completely the PDMS hardens and the devices are cut out from the mold using a knife. Due to the presence of two designs in the mold each time we get two

devices. The same process in repeated continuously until the desired number of devices are made.

2.5 Results and Discussions

The wafer was fabricated with the required specifications and the alignment of the second layer to the first layer was crucial for the fabricated devices to work and not be misaligned. Through the second layer of 100 µm the first layer was visualized using contrast and without contrasting the edges it's not possible to visualize the first layer. With the optimization of the technique the mask were fabricated.

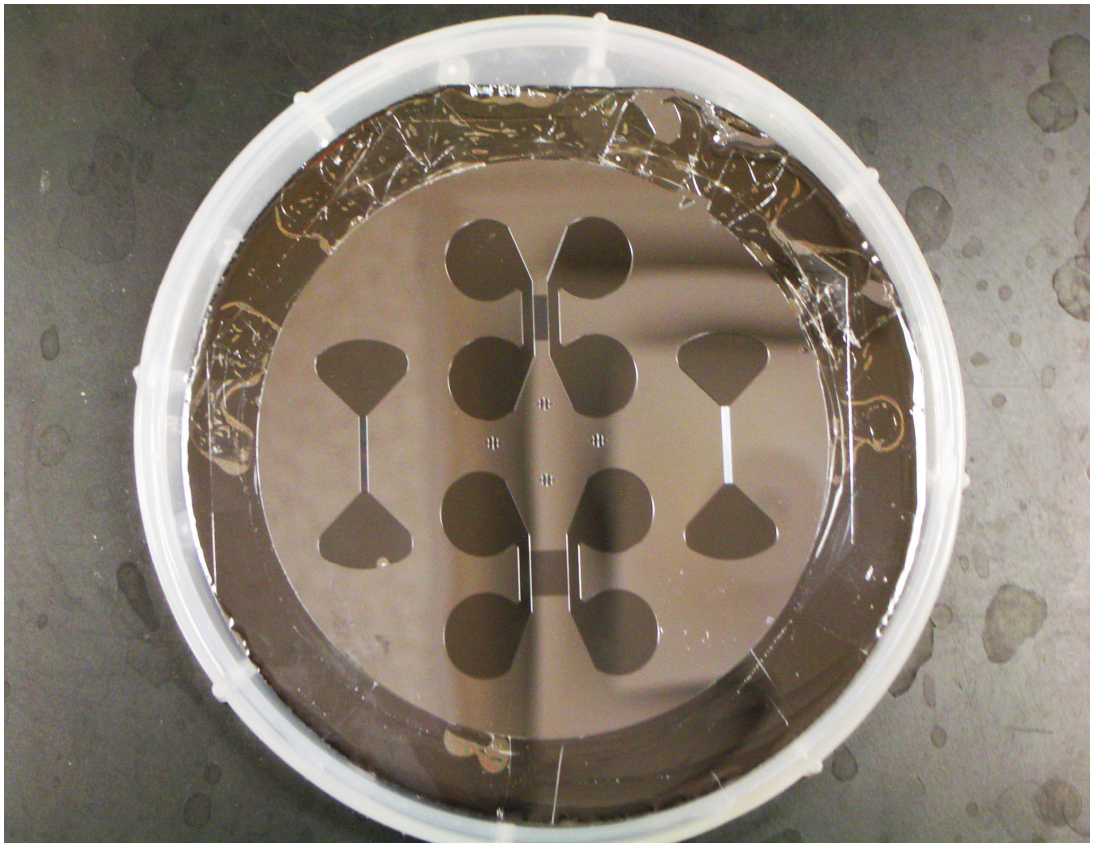


Figure 2.3: Image showing the wafer fabricated using soft lithography technique

In figure 2.3 we see the devices that had been fabricated on the wafer. The pattern on the top has a channel length of 2 mm and the pattern in the bottom has a channel length of 5 mm. In

both patterns the channels have a width of 15 µm and a height of 12 µm. Though the protocol recommended by the resist manufacturer was followed the height of 15 µm was not achieved and only 12µm height was achieved in the first layer. During the characterization of the wafer it was also found that the heights of the channels were fluctuating a little and this was due to the slightly tilted position of the hot plate. Due to the slight tilt the resist tends to move during soft bake.

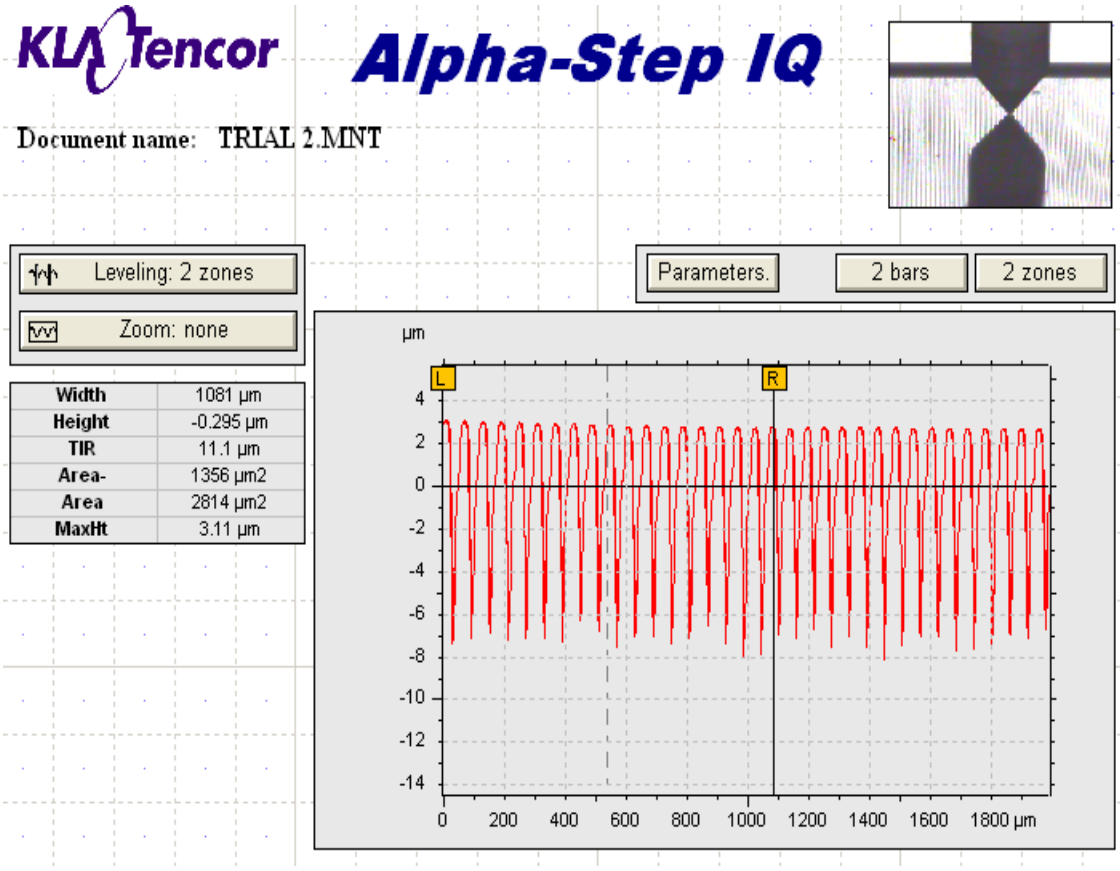


Figure 2.4: Image showing the characterization of the channel height

In figure 2.4 the channel height is characterized and a graph of the channel profile has been provided. When the height of the channel was characterized it was found that the channel height was varying in the range of 11 µm-12.9 µm. This was because of the spin speed and also the tilted surface of the hot plates used for baking.

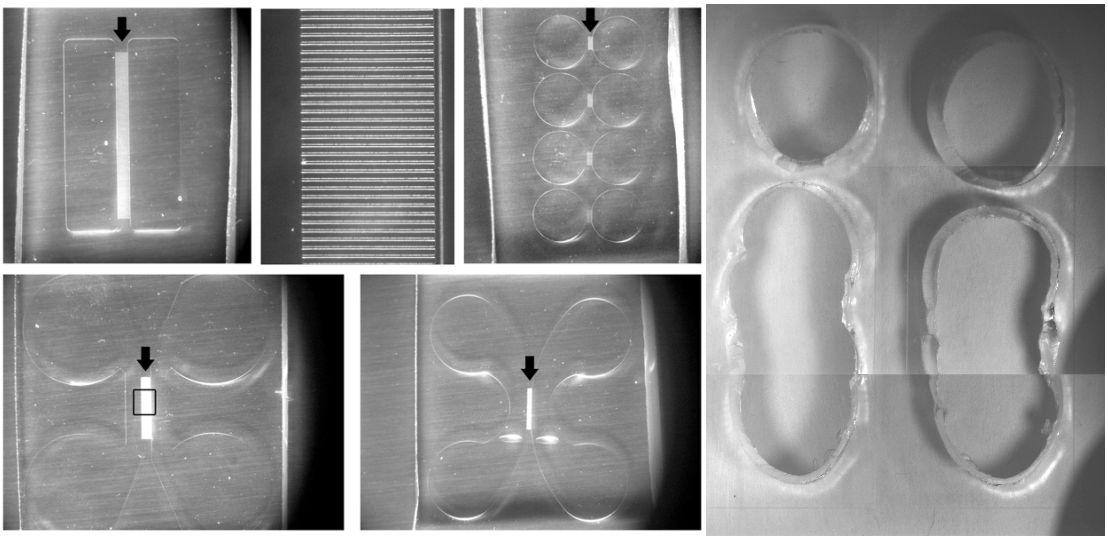


Figure 2.5: Image showing the fabricated microfluidic devices

In figure 2.5 the fabricated PDMS devices are shown which have been cleaned and punched and are ready to be used.

During the fabrication process of the silicon wafer it was found that if the wafer is not dehydrated properly id adversely affects the adhesion of the resist to the wafer. During the soft bake and post exposure bake water still keeps evaporating forming bubbles in the resist. Upon exposure and when the wafer is developed the resist peels off due to the presence of water. As the adhesion is weak the hardened resist peels off and the pattern is lost. So it was found that the dehydration process was crucial for better results in the later part of the process.

It was also found that during exposure if the resist is not soft baked properly sharp pattern are not printed on the resist. When the exposure time is 32 seconds, it is split and is exposed twice with 16 seconds exposure time. When exposed for 32 seconds continuously sometimes the resist in the surrounding areas also polymerize and the sharp features are lost. So in order to prevent this, wafer is exposed twice for 16 seconds and this forms sharp features.

From each wafer on an average around 36 devices could be obtained before the wafer breaks or before the resist peels off.

CHAPTER 3

PREPARATION OF DEVICES, EXTRACTION AND CULTURING OF CELLS IN THE DEVICES

3.1 Preparation of Cover Slips

Normal cover slips are purchased from a commercial vendor and these cover slips are cut using a diamond tip cutter. The cover slips are cut to fit inside a 60 mm Petri dish and to accommodate the PDMS microfluidic devices on top of them. The cut glass cover slips are sterilized in 70% ethanol for 30 minutes. The sterilization process using 70% ethanol also helps in removing other organic materials and impurities from the surface of the cover slip. The sterilized cover slips are then transferred to the inside of the biosafety cabinet. In separate culture dishes de-ionized water is taken. The cover slips from 70% ethanol are taken out and are gently immersed in de-ionized water and are washed in the water for 10 minutes. This step is repeated 4 times. Meanwhile in separate culture dishes Poly-D-Lysine (PDL) solution is taken. After the fourth wash the cover slips are taken and immersed in the PDL solution and left overnight. The following day the PDL solution is removed and the cover slips are transferred to new Petri dishes with de-ionized water. The PDL coated cover slips are washed with DI water for 10 minutes and washing is done four times with new de-ionized water. The PDL coated cover slips are washed thoroughly to remove any unbound PDL which will prove to be cytotoxic to the neurons cultured in it, so care is taken to completely remove any unbound PDL. After the fourth wash the PDL coated cover slips are taken and left to air dry inside the biosafety cabinet for more than 3 hours. The treatment of the cover slips with PDL is generally started the day before collecting cells such that on the day of collecting the cells it would ready to seed cells on it. The glass slides are coated with PDL to make surface of cover slips hydrophilic as they are hydrophobic in nature. If the cover slips are hydrophobic the neurons do not prefer it and they

fail to grow on normal glass. Thus PDL is needed to make the surface hydrophilic and more favorable for the neurons to grow on it.

3.2 Preparation of Microfluidic Devices

After the fabrication of PDMS devices using the mold the devices need to be prepared for usage. The devices are cut out and punches are made to form holes using the appropriate sized biopsy punches and this area is used as the cell seeding area. The surfaces are cleaned using scotch tape to remove any particle on the surface. The surface is thoroughly cleaned and is inspected using a microscope to check for any particles on the surface and to check for any defects in the device. After checking the devices are sterilized by placing them in 70% ethanol for 15 minutes- 20 minutes. They are not kept in ethanol for more than 20minutes as they tend to get bigger in size. After sterilization using ethanol the devices are taken inside the biosafety cabinet and are taken out and placed in new dry dishes and left for drying. They are left for drying for more than 3 hours.

3.3 Assembly of Devices

In this step the microfluidic devices are carefully placed on top of the cover slips and are assembled. Before this step the microfluidic devices are treated with the corona discharger to make the surface of PDMS hydrophilic. The corona discharger deposits free radicals on the channel side of the PDMS device which would be facing the cover slip during assembly. The free radicals introduced make the surface of PDMS hydrophilic. PDMS being extremely hydrophobic in nature needs to be made hydrophilic to enable flow of liquids and the culturing of cells. After corona treating the surface of the PDMS it is placed in sterile aluminum foil and is transported back into the biosafety cabinet and left undisturbed for 15 minutes to reduce the excessive radicals that had been introduced on the surface. After 15 minutes the PDMS devices are assembled on the PDL coated cover slips with the channel side facing the cover slip. The PDMS devices are pressed after placing on the cover slips to ensure proper assembly and it is made sure that there is no air bubble left in between, any air bubble formed is removed and

bonding is irreversible so it cannot be changed later. After assembly medium is fed in one well and is allowed to diffuse to the other connected well and also through the microchannels. This is done to remove air bubbles inside the microchannels. After the medium gets diffused medium is added to the other wells and the devices are left inside the incubator at 37℃.

3.4 Extraction of Cells, Seeding and Culturing

The cells used are primary cells and are collected from rat at the embryonic day 16-18. All procedures were conducted according to IACUC (Institutional Animal Care and Use committee) approved protocols. Time pregnant females were purchased from commercial vendors and the embryonic pups were collected from these animals. The embryos were collected in L-15 medium maintained at 4℃. Both co rtical neurons were collected from these animals. The brain was extracted and was cleaned by peeling off the meninges were kept in L-15 medium. DRG neurons were obtained as individual ganglia from the spinal cord and the roots were removed to clean the DRG. The cells are collected in L-15 medium and to this medium 0.25% trypsin is added to enzymatically dissociate the cells and is left for 20 minutes. After 20 minutes Soy Bean Trypsin Inhibitor (SBTI) is added to the collected cells to inhibit the action of trypsin further and to prevent the cells from any damage. The cortex and DRG ganglion are triturated using fire polished pipette tips to mechanically dissociate the cells. The cells are spun at 1200 rpm for 5 minutes. The supernatant is removed and neuro basal medium with B-27 is added to the pellet at the bottom. The cells are suspended in neurobasal medium and the amount of neurobasal medium us based on the number of devices in which cells are seeded.

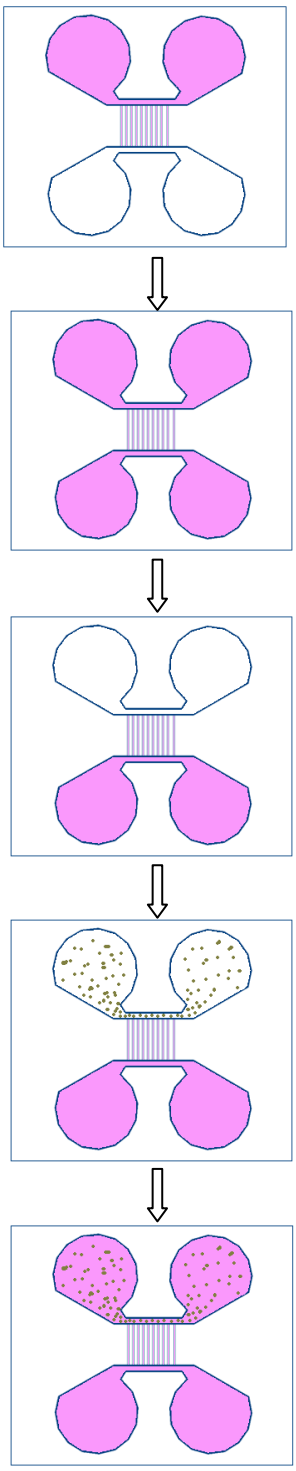


Figure 3.1: Image explaining the cells seeding process and maintenance of pressure gradient

After the assembly of the microfluidic devices on the PDL coated cover slips they are left to attach to the cover slip and neurobasal medium is added in one well and is left to diffuse

through the channels to remove air bubbles. After the medium gets diffused through the channels removing air bubbles the rest of the wells are filled with medium and kept in the incubator. During cell seeding medium is removed from two wells connected to each other and the medium is left on the other two wells to maintain a pressure gradient which inhibits the cells from entering the channels during cell seeding. As the cells are suspended in the medium the cells diffuse through channel in the absence of pressure gradient.

The cell suspension prepared is used for cell seeding. The previously prepared microfluidic devices are taken out from the incubator and the previously loaded medium is drained out with less than 200 µl medium left behind in the device. The cell suspension is triturated again and 60 µl of cell suspension is taken and is loaded into the microfluidic device. Upon loading the devices are placed back inside the incubator for 5 hours to enable cell attachment. After 5 hours Neurobasal medium supplemented with B-27 with growth factors BDNF and NT-3 (10 ng/ ml) was slowly added into the device and all the wells were filled with medium. Each device took around 1ml of medium to fill all four wells. The cells were cultured in the devices until desired number of axons grows inside the channel. Each device took around 1ml of medium to fill all four wells. The cells were cultured in the devices until desired number of axons grows inside the channel.

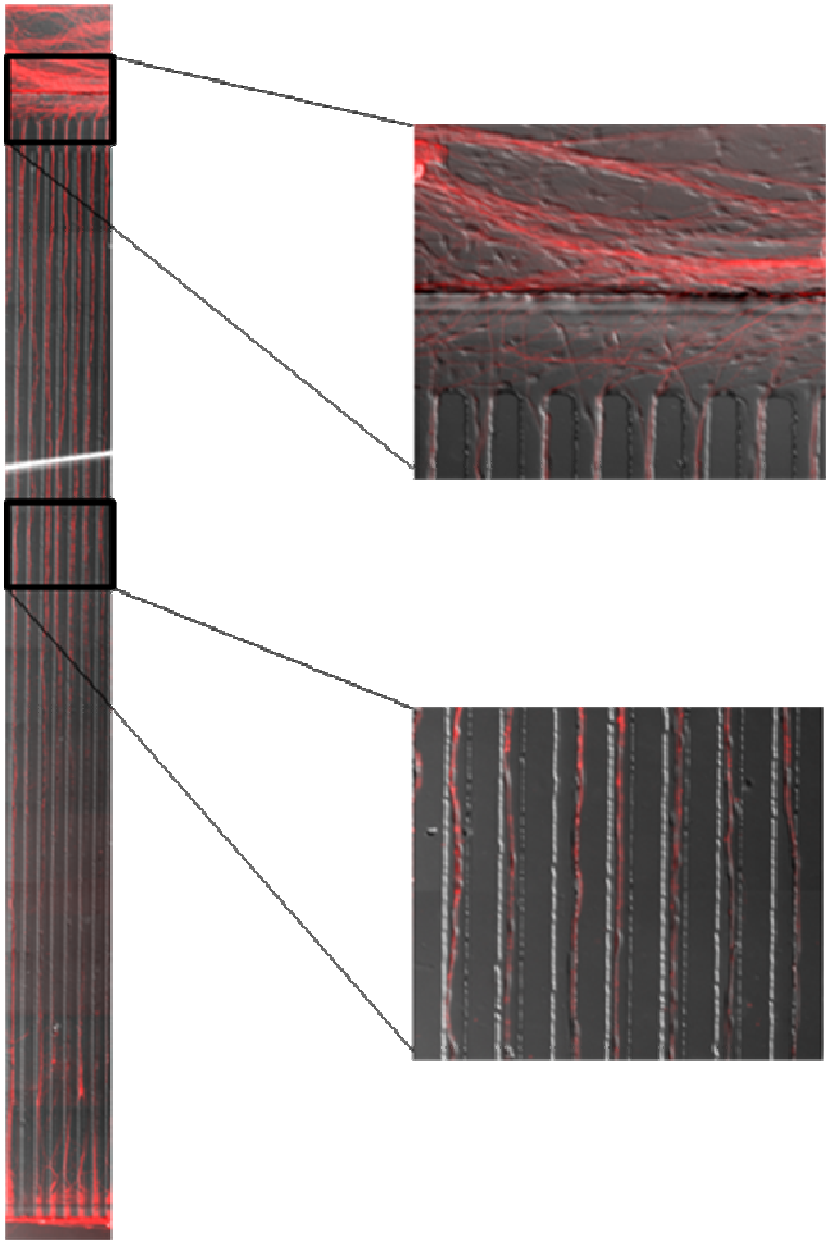


Figure 3.2: shows robust individual DRG neurite outgrowth along with Schwann cells through the 5 mm long microfluidic microchannels. The dissociated DRG neurons seeded in one compartment of the device randomly extended their long neurite processes in the chamber.

Upon seeding and culturing, robust growth of axons was seen. Each axon occupied individual channels and was confined to the inside of the channel and thereby individual axotomy was easily possible. The channels were occupied by axons 6-8 days after seeding the cells. The cell obtained from embryonic neurons showed a faster growth rate. Cells are collected from embryonic day 16-18 as after E18 cortical neurons mature and form other glial cells such as astrocytes and microglia and as to avoid culture of other glial cells cortical neurons were collected from the embryonic day 16-18.

To get high amount of axons a good amount of cells needs to adhered to surface near the channel entrance. In order to have high cell density near the channels the cell seeding method was changed. The cell suspension was seeded and left undisturbed for 5 hours for the cell to adhere to the surface and after 5 hours the medium was gently added ensuring that the cells are not washed away. Using this seeding method high cell density was achieved closer to the channels and made sure that majority of the channels were occupied by axons.

Lifting in the devices was also observed causing the axons to grow outside the channel. The introduction of free radicals on the PDMS surface caused the lifting of devices upon assembly. After corona treatment the devices were left for 15 minutes to remove excess free radicals and upon assembly the devices were left for 10 minutes to adhere properly before adding medium to one of the wells. Ensuring proper bonding between the cover slip and the PDMS device was the key factor in the lifting of the PDMS devices.

CHAPTER 4

FABRICATION OF MICROSCOPE STAGE INCUBATOR AND IMAGING CHAMBER

4.1 Fabrication of Microscope Stage Incubator

Neurons had to be monitored continuously and closely to understand their response to injury done using femtosecond laser and a microscope stage incubator was required. A microscope incubator was also required to maintain the cells under optimum conditions and prevent the stressing out of cells. During the experiment it was found that neurons if left outside without optimum conditions for more than 20 minutes they get stressed and the neuronal network is lost in 4 hours. In order to prevent this, a system that maintains the cells under the optimum conditions was required. Optimum conditions are defined as 5% CO_2, 35.4℃ and pH 7.1-7.4. The incubator had to fit into the microscope and the experiment conditions with hindering the laser entrance.

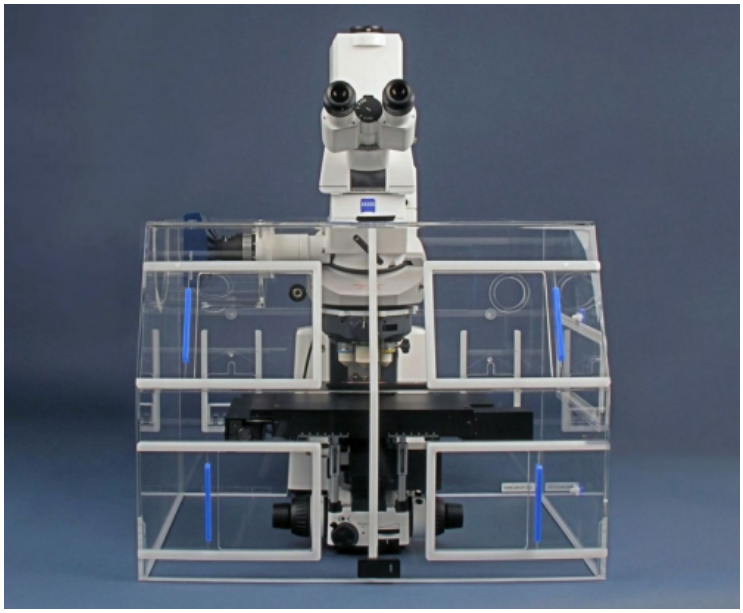


Figure 4.1: Commercially available microscope incubators

Commercially available microscope incubators were not used and a custom made incubator was used. Commercial microscope incubators were not preferred as they are

- Huge in size
- Expensive
- Cumbersome

A custom made microscope stage incubator was made to overcome the above mentioned conditions and was made sure that it fits inside the microscope and does not the hinder the laser port. The incubator was built using antifog acrylic to prevent fogging and had a thermocouple and silicone rubber heaters to measure temperature and maintain set temperature of 35.4℃, CO_2 sensor to maintain 5% CO_2, humidity was maintained using sterile wet gauze.

The incubator was fabricated in two parts with the dimensions that allowed the incubator to fit inside the microscope. It had a height of 3 cm, length of 12.3 cm, and a width of 8.5 cm. The length and width was chosen based on the microscope stage insert as the incubator had to be placed on top of the insert and sealed. The height was chosen to be 3 cm such that the incubator fits in-between the lens at the bottom and the light source on the top in the inverted microscope. The incubator had two chambers and the first chamber was used as a premixing chamber for gases and heat provided. This was then blown into the second chamber with the cells along with the temperature and CO_2 sensor. In the second chamber the cells were mounted and were observed.

4.1.1 Materials

The custom designed microscope stage incubator is made of anti fog acrylic and other nontoxic materials with the integration of a timed circuit to control the CO_2 inlet and a real time temperature monitoring probe to maintain the optimum temperature. The lists of materials used are as follows

Table 4.1 Materials Used In Fabrication of the Microscope Stage Incubator

Microscope stage incubator	Description
Walls of microscope stage incubator	Anti fog acrylic
Heaters	Silicone rubber heaters
Temperature probes	Fiber based probes connected to the temperature controller
CO_2 sensor	Vaisala CO_2 controller for real time CO_2 monitoring
Needle valve	To control the flow rate of CO_2 and oxygen
Solenoid valve	Open and closes depending on the CO_2 level
Fan	To ensure proper dissipation of heat and CO_2 inside the incubator
Controller box	Encloses all the temperature regulating components
Bread board	Used for designing the circuit
Power controller	Supplies power to solenoid valve
Sealant	Removable sealant to seal the incubator to maintain sterility and prevent leakage
Temperature controller	The temperature probe is connected to this and based upon the temperature readings regulates the heaters
Filters	0.2µm filters placed in order to filter any particles in the fed CO_2 and air
Wires and adhesive	Wires used for the circuits and epoxy adhesive for fabrication of microscope stage incubator
Sterile gauze and petridish	To maintain humidity

Antifog acrylic was chosen as the building material as it is transparent and provides good optical clarity. The antifog coating prevents formation of any fog and thereby helps in maintaining optical clarity. It also has high operating temperature and operates from 0 ℉-200 ℉. Acrylic also acts as an electrical insulator an d also has high impact strength.

Silicone rubber heaters were used for heating purposes and this was chosen due to the flexibility in their nature and could be placed in the smallest gaps. Nickel alloy wires were embedded inside the heaters to provide efficient heat distribution. The silicone rubber heaters

were sand wedged between two copper strips to provide higher heat radiations. Though silicone is a very good conductor they are poor radiators and in order to overcome this copper strips were used.

Thermocouple were used to measure temperature and they were used as they are durable, can measure temperature from -328 ℉-2282 ℉, has a fast response and are inexpensive. The thermocouple had a sensitivity to measure a 0.1 ℃ temperature difference. The thermocouple was connected to the temperature controller and it had a feedback circuit and to maintain the temperature at the set value of 35.4 ℃.

The CO_2 sensor was purchased from Vaisala and was used as it had high sensitivity to measure 0.01% change in CO_2 level and was also able to work at high humidity. The CO_2 system was connected to a feedback circuit to ensure the opening and closing of the solenoid valve to maintain the 5% CO_2 inside the incubator. A needle valve was installed before the solenoid valve to restrict the flow rate of CO_2 upon opening the solenoid valve. The CO_2 system was connected to two power controllers and one controller provided power for operating the solenoid valve while the other provided power for operating the CO_2 sensor.

A fan was used to ensure circulation of the gases and to prevent the formation of a stagnant environment. The fan was chosen such that it fits inside the incubator.

Removable sealant was used to prevent leakage and to fix the incubator on top the microscope stages insert. A removable sealant was used as the incubator has to be detached from the insert each time when a different sample had to be kept.

0.22 µm filters were installed in the pipes supplying CO_2 and air to the system. It was installed in order to filter any microorganism or other contaminants in the gases and also to maintain a sterile system.

The microscope stage incubator consists of outer walls made of anti fog acrylic mounted on top of the microscope stage insert and is sealed on the outside using the sealant. The microscope stage incubator is divided into two parts. The first part is called as the mixing

chamber and heating chamber and this chamber has the inlet for CO_2 and air. The silicone rubber heaters are attached to the inner walls of this chamber by using thermal adhesive in the ratio 1:1. These rubber heaters derive their power from the temperature controller to which the temperature probe is also connected. Based on the reading from the temperature probes the heaters are either switched on or switched off. The real time monitoring enables to maintain stable temperature. In this chamber a small fan is also installed to ensure proper dissipation of heat throughout the incubator and also ensures the circulation of CO_2 and air. The second chamber is the chamber in which the microfluidic devices are mounted. In the chamber a filter is installed to aid in the exchange of gases and to avoid a stagnant environment and thereby ensures proper exchange and circulation of gases. In this chamber the devices are mounted on top of the optical window present in the center and this covered by using a cover slip. This window enables us to image and axotomize the axons. In this chamber surrounding the device sterile wet gauze are placed to maintain humidity. The CO_2 sensor is also installed in this chamber and gives real time feedback and based on the CO_2 percentage in the incubator the solenoid valve is either opened or closed and thereby 5% CO_2 is maintained.

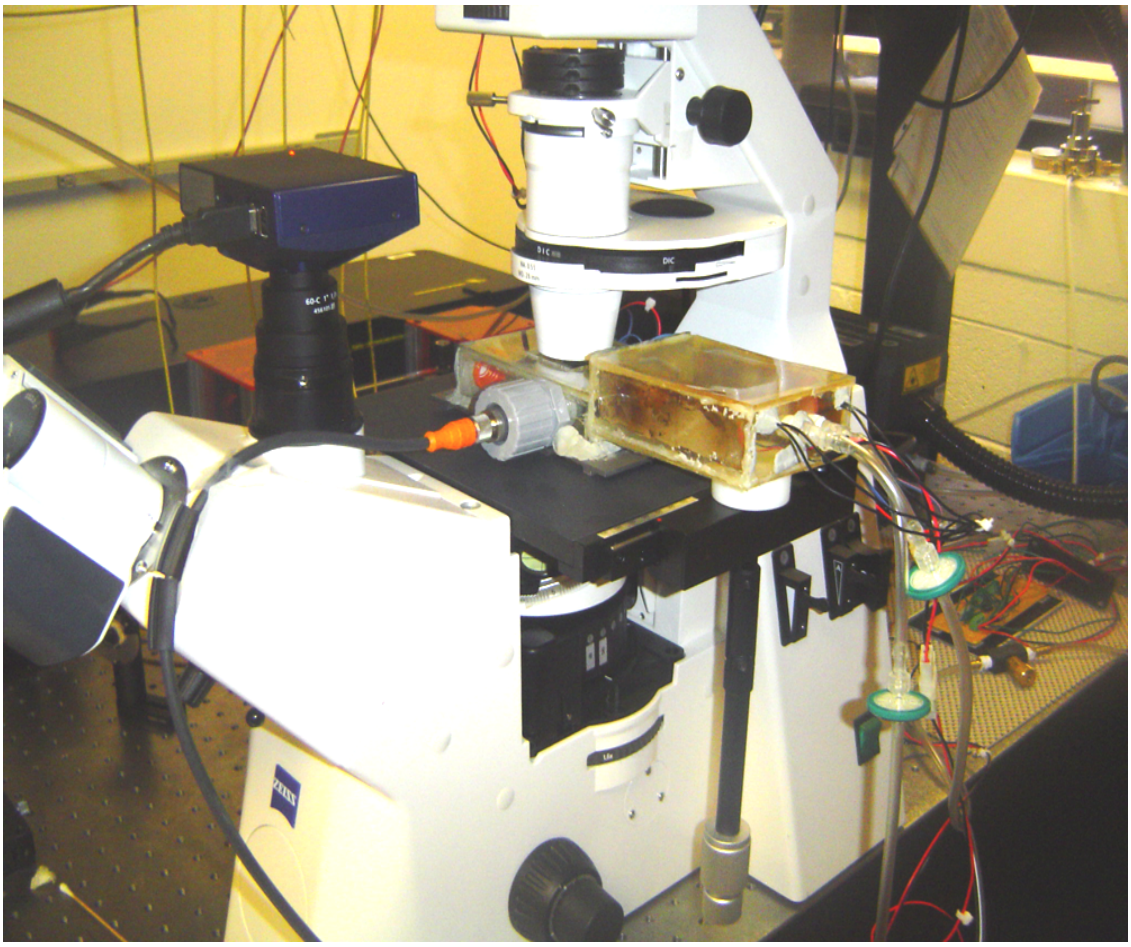
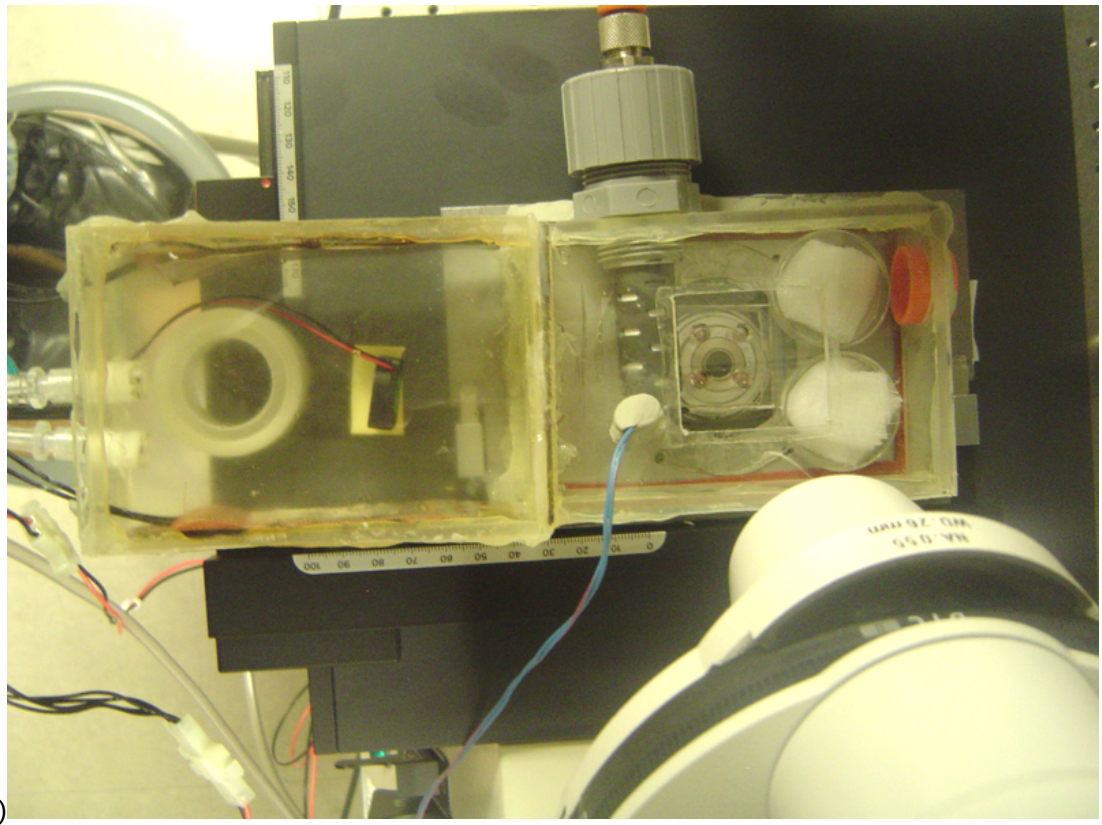


Figure 4.2: Image showing the full assembly of microscope stage incubator mounted on the microscope

A)



B)

C)

Figure 4.3: A) Image showing the aerial view of the incubator showing its parts inside along with a microfluidic device mounted inside in it with neuronal culture. B) Image showing the temperature reading inside the incubator with the set value as 35.0℃ and the value being 35.2℃ and C) Image showing the CO_2 percentage inside the chamber

4.1.2 Control Circuits

To maintain ambient conditions for the cell culture CO_2 level controller was designed (ref). The block diagram of the unit is shown above. The unit includes Feedback circuit, valves, timer module and the tanks of CO_2 gas and air.

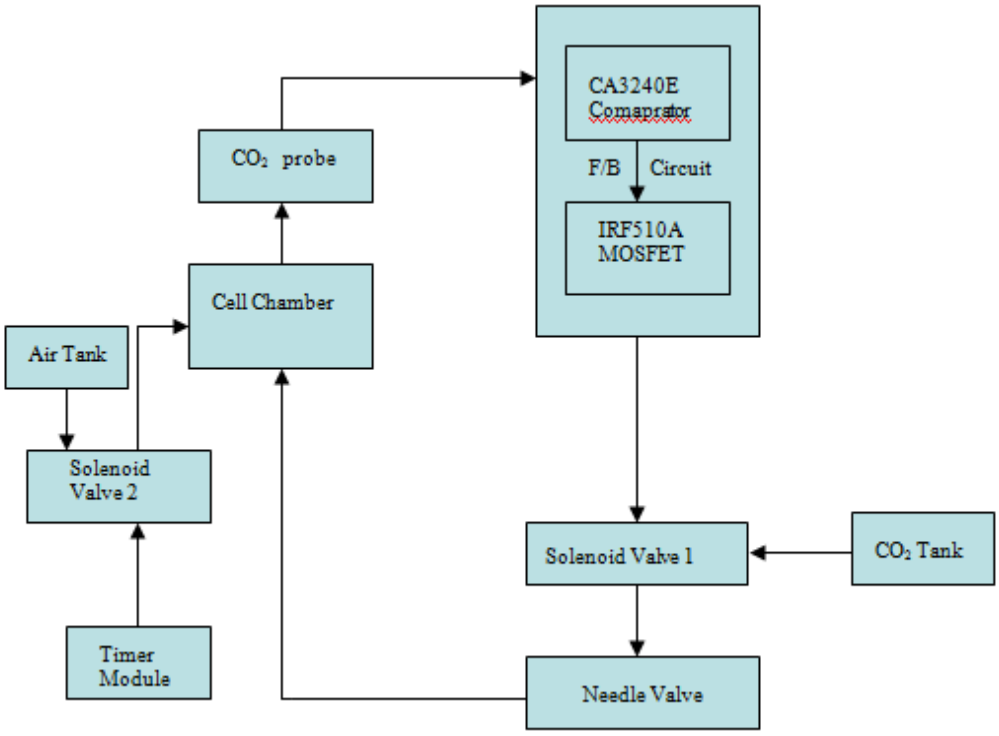


Figure 4.4: Flow diagram describing the circuit controlling the CO_2 integrated with the incubator

4.1.3 Working of the CO_2 control Unit

The unit is composed of CO_2 sensor, the feedback circuit, timer module, 2 solenoid valves, a needle valve and tanks of CO_2 gas and air. The control unit maintains 4.5% concentration of the CO_2 inside the cell chamber.

The CO_2 sensor unit (Vaisala GMT 221) has a probe which was inserted into the cell chamber. The probe continuously measures the CO_2 level inside the chamber. The unit is equipped with a digital display, for the corresponding voltage of the measured CO_2 level at a

particular time. The output signal from the CO_2 sensor is given to the feedback circuit. The feedback circuit is composed of CA 3240E comparator and IRF510A MOSFET. The comparator IC compares the voltage from the CO_2 sensor with a preset value i.e. 4.5 V in this case.

The prefixed value at the non inversible i/p pin of the comparator IC is set with 20k potentiometer. The positive lead of the digital multimeter is connected to pin #2 of the potentiometer and the negative lead to the ground. The screw of the potentiometer is turned till the display shows 4.5 V.

If the CO_2 sensor o/p is less than this preset value, the MOSFET drives the solenoid valve. The solenoid valve opens and the CO_2 gas flows through it to the needle valve. The needle valve controls the surge of CO_2 gas to the cell chamber. This prevents sudden increase in the CO_2 level from 4.5% inside the cell chamber. If the sensor o/p voltage is greater than the preset value the comparator generates 0 V at its o/p pin. The MOSFET does not generate driving o/p at its o/p pin which does not let solenoid valve to open. The CO_2 sensor measures o/p every 20 seconds.

To provide O_2 to the cells the air is fed to the chamber every two hours for 5 minutes. The flow of air is controlled by a Timer Module. The timer opens the solenoid valve every two hours and the air passed through the solenoid valve to the cell chamber for 5 minutes.

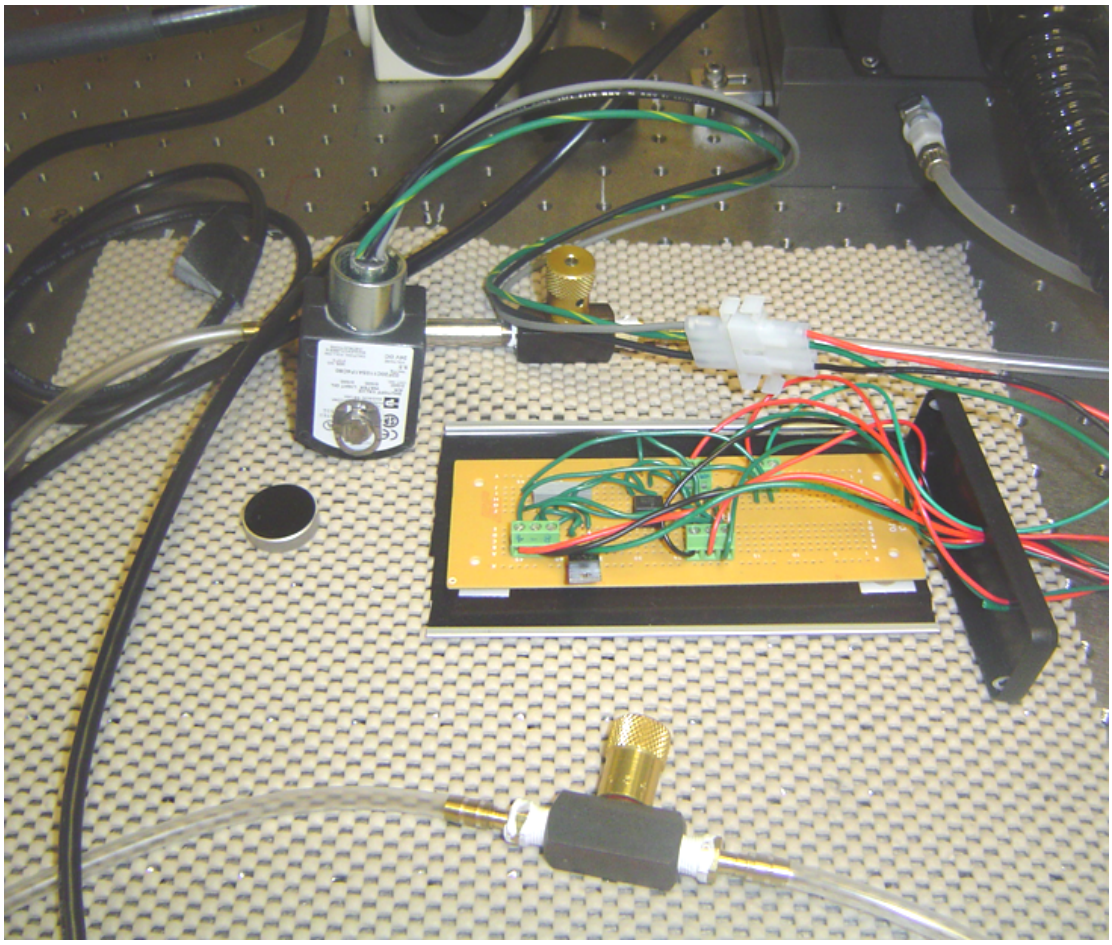


Figure 4.5: Image showing the working circuit controlling the CO2 feed controller along with the solenoid valve and needle valve.

4.2 Sterilization of the Incubator

The incubator was sterilized by gently wiping the surface of the chamber using wipes with 70% Iso propyl alcohol (IPA). After wiping the inner surfaces with IPA the incubator is left upright inside a biosafety cabinet with the UV switched along with the 35 mm Petri dishes, scotch tape, and sealant for 2-3 hours.

4.3 Mounting of Microfluidic Devices in the Incubator

After sterilization of the incubator the microfluidic devices are brought inside the biosafety cabinet and all assembly process is done inside a biosafety cabinet to maintain sterility. The microfluidic device is assembled in the center where the optical window is present.

This window is covered by placind the cover slip on top of it. The placed microfluidic device is secured using sterile scotch tape. Surrounding the device sterile 35 mm culture dish with sterile wet cotton gauze is placed to maintain humidity thereby preventing evaporation. After this the incubator enclosure is placed on top of the metal microscope insert and is sealed using the sealant to prevent any form of contamination. After assembly of the device this is hooked to the circuit and the device is started to run maintaining 5% CO_2 and 37℃ inside the incubator.

4.4 Methods of Fabrication of the Incubator

The fabrication of the microscope stage incubator was done in three steps. The first step was the design of the incubator. The incubator consists of an upper plastic chamber and a lower metal stage insert. The upper chamber maintain sterilized and adequate environment for neuronal cell culture. The chambers were fabricated using anti-fog clear cast acrylic sheet to minimize a condensation. This chamber is made into two different chambers. The first chamber serves as the chamber for mixing the gas that's fed into the incubator. This chamber also encases the heaters and the fan. The other purpose of this chamber is to prevent the direct flow of the inlet CO_2 and air on top of the microfluidic and thereby eliminating any other non favorable factors. From this chamber heat, CO_2 and air are circulated and equilibrium conditions are maintained throughout the incubator by the fan. The second chamber contains the microfluidic device, probes for CO_2 and temperature along with the wet gauzes. The chamber also contained two separate filtered gas inlets (air and CO_2 gas) and a filtered gas outlet. The center of the chamber had an optical window which was covered with cover slip to allow microscopic observation.

The second part is an automatic temperature controlling where the temperature is automatically regulated at preset level using silicone rubber heaters and a temperature regulator. Two silicone rubber heaters were attached on the side wall of the upper chamber by use of thermal adhesive. Thin copper sheet was placed on the heaters using a thermal adhesive to act as a heat sinker and minimize contamination and to provide better heating and

faster dissipation. The temperature was measured by the fiber temperature probe placed on top of the device without touching it. The measured temperature was fedback to the regulator via thermocouple and the regulator controlled the heaters. The heat is circulated by using a fan. The third part of the incubator is the CO_2 and air controller to maintain appropriate CO_2 level (5% CO_2 and 95% air) at the neuronal culture chamber using automatic feedback circuits. The percentage of CO_2 was continuously measured by CO_2 sensor and sent to a feedback circuit (simple comparative IC circuit). If the percentage of CO_2 level was lower than reference value, it opened a solenoid valve to allow CO_2 gas into the incubator. The need to maintain the level of CO_2 at 5% for maintaining the pH at 7.2-7.4 needed a real time monitoring of CO_2 to measure and maintain CO_2 at optimum was provided by the high sensitivity CO_2 sensor. The solenoid valve is supplied 24 volts for operation and when the CO_2 level is below 5% the solenoid valve is opened in order to feed CO2 and once in goes above 5% CO_2 the valve is closed and the CO_2 level is maintained inside the incubator.

4.5 Cell Culture in the Microscope Stage Incubator

Cortical neurons and DRG were isolated from embryonic 16-18 day rat pups. The cortical tissues and DRG ganglions were dissected, cleaned, and enzymatically dissociated (0.25% trypsin) for 20 minutes. The cortical tissues and DRG were triturated and the resulting cell suspension was loaded into the microfluidic devices. The devices consisted of two compartments (100 μm height) bridged by multiple microchannels (15 μm width, 15 μm height, and 2 mm long). After 15 minutes of incubation at 37^{o}C, culture medium (Neurobasal medium supplemented with B-27) with growth factors (BDNF and NT-3, 10ng/ml) was slowly added into the device. The medium with growth factors was changed every two day. The microfluidic device containing cortical neurons or DRG was placed on the center of the incubator and covered with the chamber under sterilized environment. Two humility containers (wet sterilized cotton gauze) were placed next to the device. The chamber was then sealed with a removable sealant and placed on the inverted microscope. The 40x objective was used to continuously

monitor the neuronal activities for 24 hours. The appropriate temperature and gas was automatically controlled by each feedback circuit.

Similarly astrocytes and fibroblasts were collected from postnatal 3-4 rat pups. These cells were extracted and seeded in to the microfluidic devices and were cultured inside the custom made incubator to check their survival and growth inside the custom made incubator to check if optimum conditions prevail for cell survival and its metabolic activities.

4.6 Results

The incubator was first run to characterize the fabricated incubator and to check for its stability in maintaining CO_2%, temperature, pH at optimum levels. Optimum levels are defined as 5% CO_2, 34℃ and pH of 7.1 - 7.4. The microscope stage in cubator was tested for the above parameters and was repeated on different days to ensure their reliability.

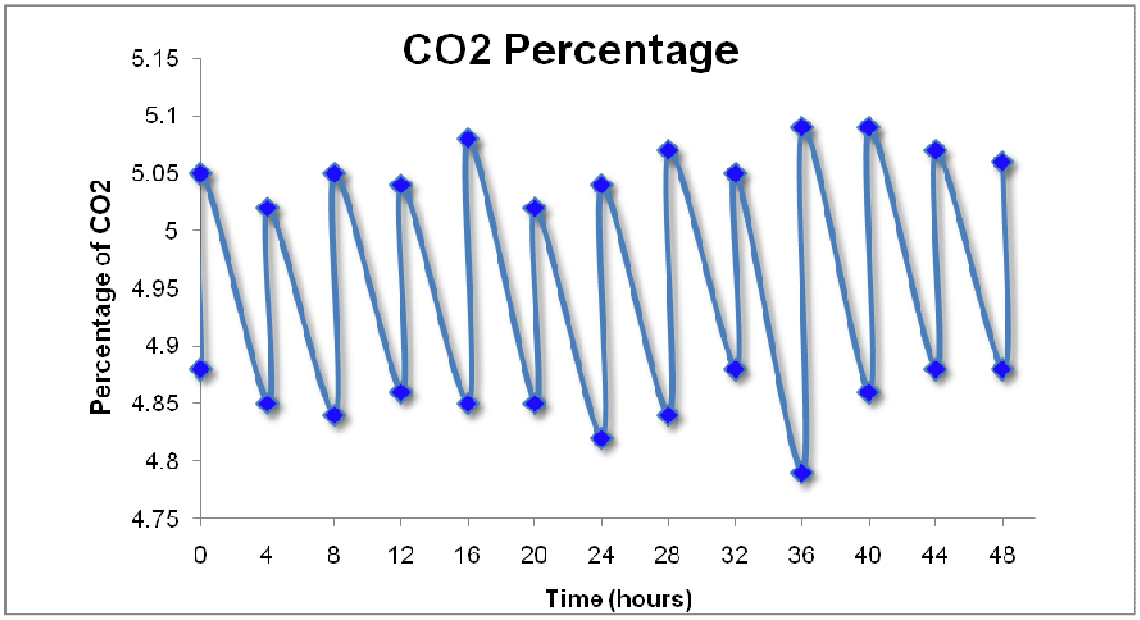


Figure 4.6: Graph showing the CO_2% maintained inside the chamber

Figure 2.5 represents the readings for each cycle in the CO_2. The solenoid valve was set to open once the CO_2 percentage drops below 4.90% and close once the CO_2% crosses 4.90%. In the graph the CO_2 value for each cycle is shown and the cycle is defined as the closing and opening of solenoid valve and the lowest CO_2% reached for CO_2 to increase and the highest

CO_2 value attained each time upon closing the solenoid valve. It was found that CO_2 value fluctuated between 4.82-5.08% CO_2.

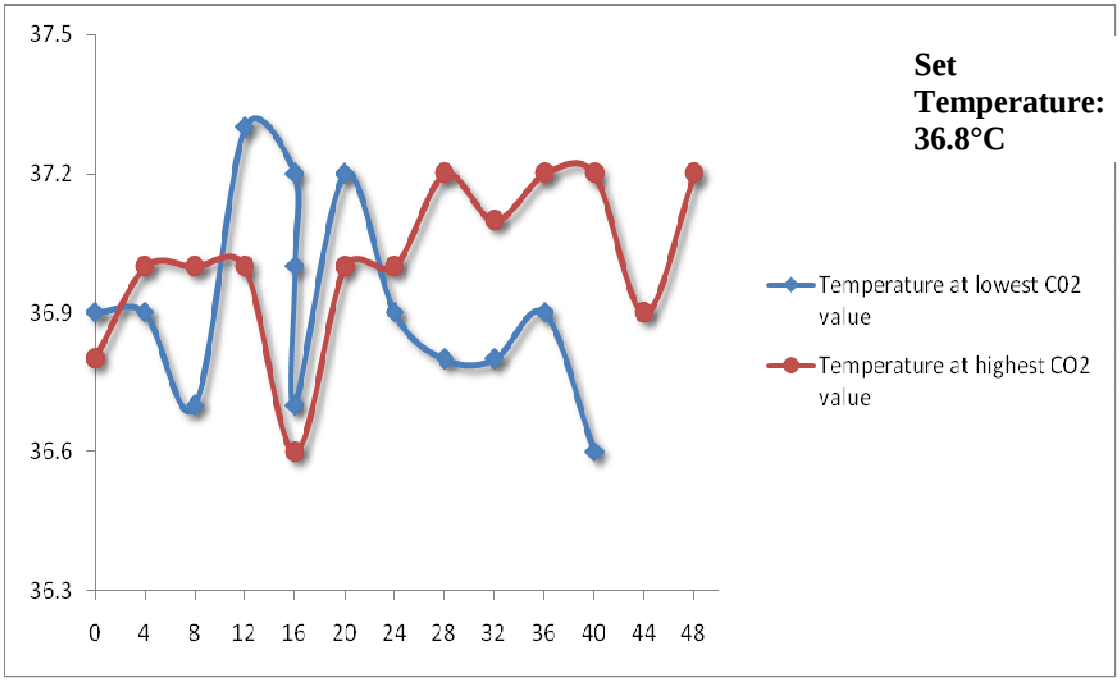
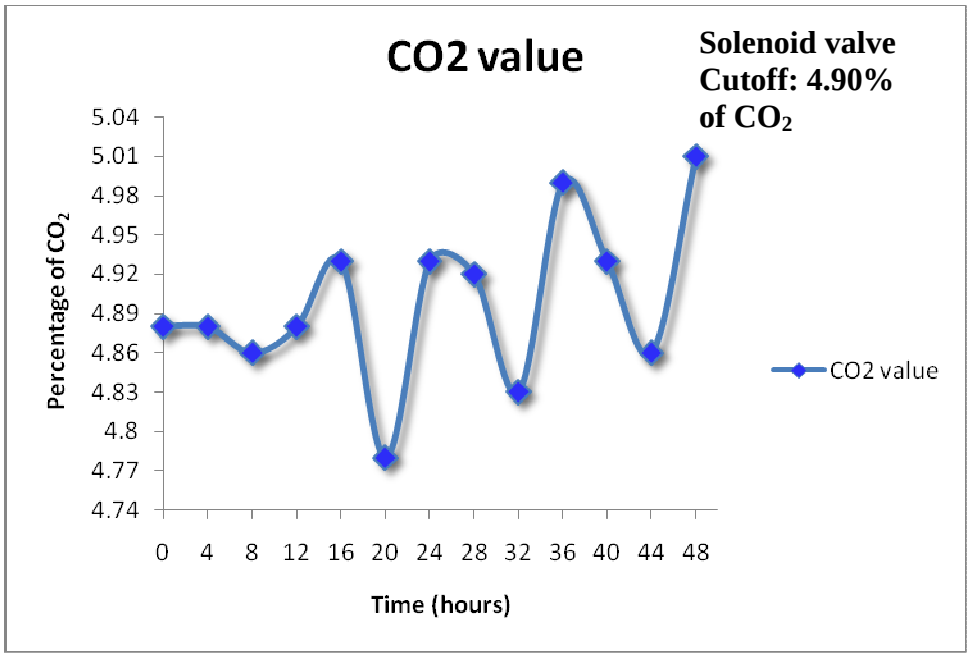
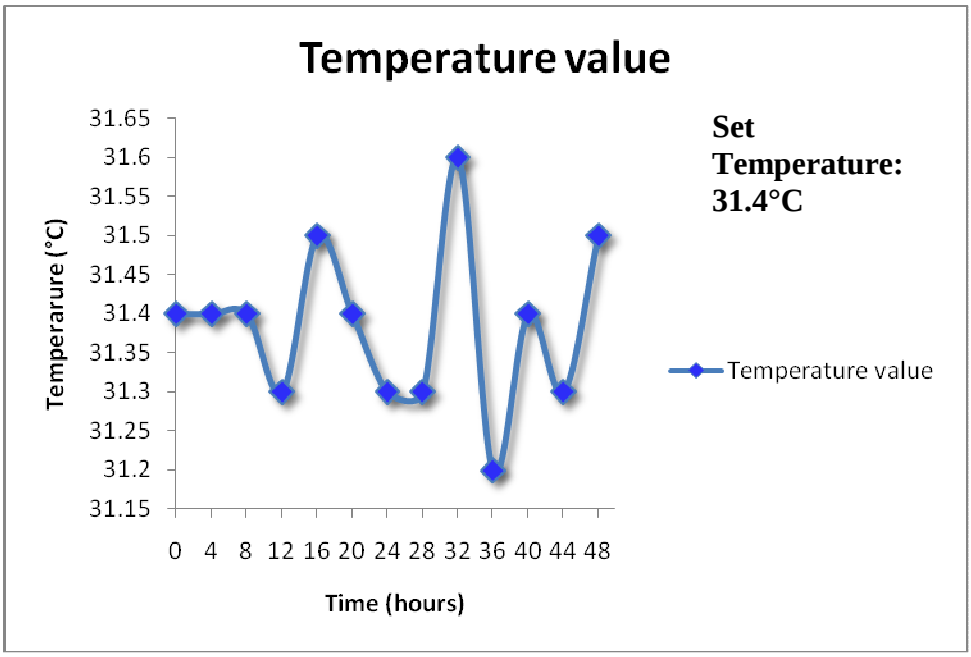


Figure 4.7: Graph representing the temperature value at the lowest and highest $C0_2$ value

The graph in figure 2.6 shows two different sets of temperature reading recorded during the lowest and highest CO_2 value recorded. The temperature values were recorded to their corresponding CO_2 value. The temperature was set to 36.8℃ and was observed to slow mild fluctuation in the range of 36.6℃- 37.3℃.

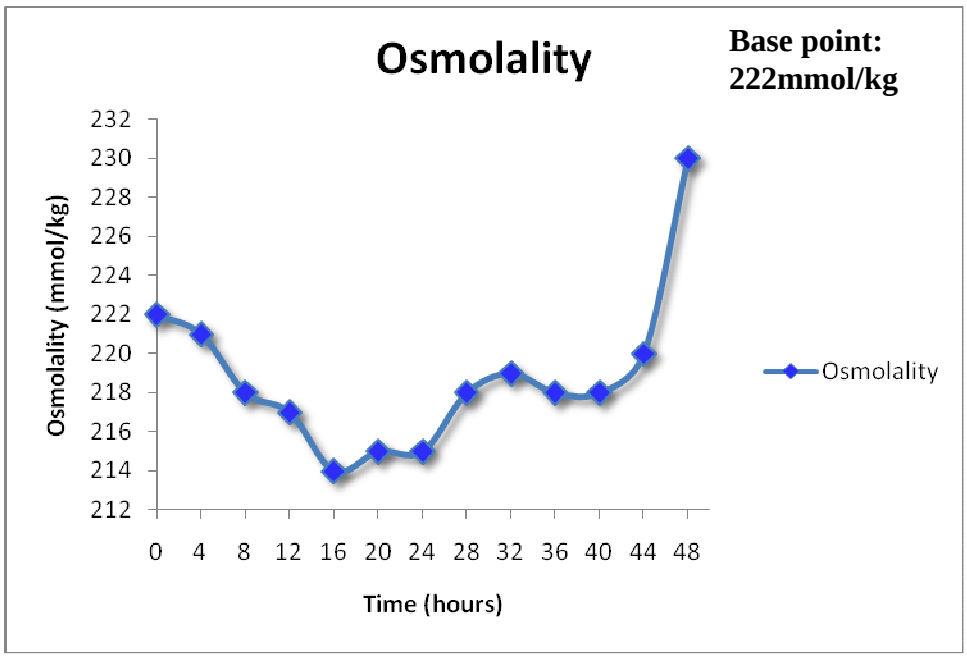


A)

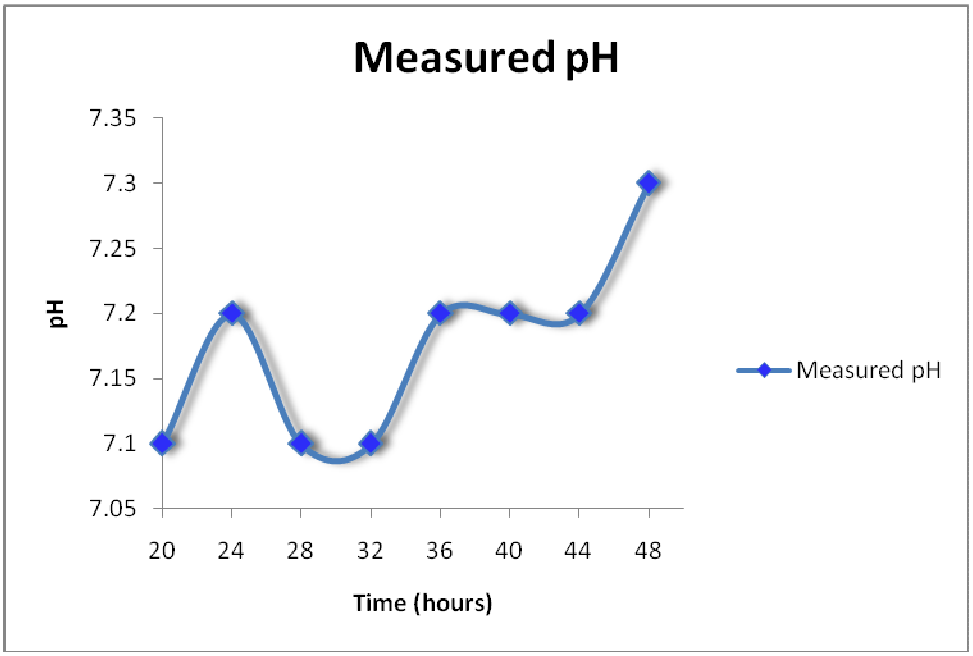


B)

Figure 4.8: A and B show the values of CO_2 and temperature measured every 4 hours for a duration of 48 hours.



A)



B)

Figure 4.9: A shows the value of osmolality measured every four hours for a duration of 48hours. Image 4.9 B represents the pH measured every four hours and for a duration of 28 hours

In image 4.8 A the value of CO_2% percentage recorded for a total of 48 hours is seen. The value was recorded every four hours and it was recorded that the lowest CO_2 value was 4.78 and the highest CO_2 value to be 5.01 but however CO_2 value was maintained between 4.86-4.95 most of the time. The pH was also measured to check for the buffering by CO_2 and if the fluctuations affect the pH of the medium. It was found that pH was fluctuating between the normal value of 7.1-7.3 and was at 7.2 mostly. From the recorded pH values it was inferred that the fluctuation in the CO_2 value did not affect the pH of the system and it was also inferred that this minor fluctuation did not seem to have an effect in the buffering.

The corresponding temperature value and osmolalilty was also recorded and is seen in figure 4.8 B and 4.9 A. The temperature was set to be maintained at 31.4℃ and was found to be maintained at 31.4℃ mostly but however had a ±0 .2℃ fluctuation. In order to check if there is any evaporation that affects the osmolality adversely the osmolality was measured and the base point osmalility was recorded at 222mmol/kg before initiating the experiment. During the experiment osmolality was also measured every four hours with temperature and was found to be dropping initially and dropped to 214mmol/kg. This was found to be due to the condensation inside the chamber. Condensation occurred due to the high humidity inside the chamber and once the humidity was brought down the osmolality was observed to rise again.

Different cells such as cortical neurons, DRG, astrocytes and fibroblasts were cultures inside the microscope chamber to check for if optimum conditions prevail for their growth and metabolic activities. The cells were mounted inside the incubator and the device was run to check for it. To compare the results of the cells maintained in the custom made incubator a microfluidic device with cortical neuron was taken and left outside at room temperature to show how non favorable conditions affect the cortical neurons and to know about in how much time they start losing their morphology and axonal network.

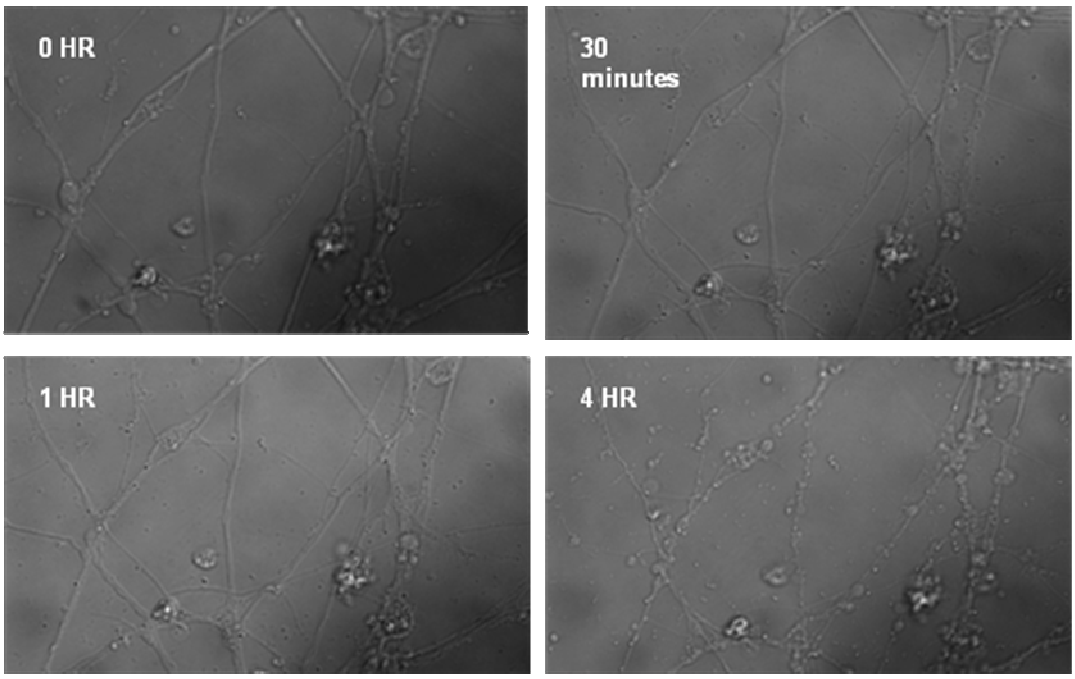


Figure 4.10: Images showing the cell left outside at room temperature and its degradation at 4 hours

From this study by leaving the cells outside the cortical neurons start to lose their morphology and axonal network within 4hours from leaving them outside. Within 4 hours on leaving them outside the unfavorable environment caused the death of the neurons and complete loss of the network. Having this as crucial time period as neuron die if unfavorable conditions are presented within 4 hours the custom made incubator was initiated and was running. Cortical neurons, DRG, astrocytes and fibroblasts were cultured in the custom made incubator and was maintained in it and observed at different time points up to 48 hours.

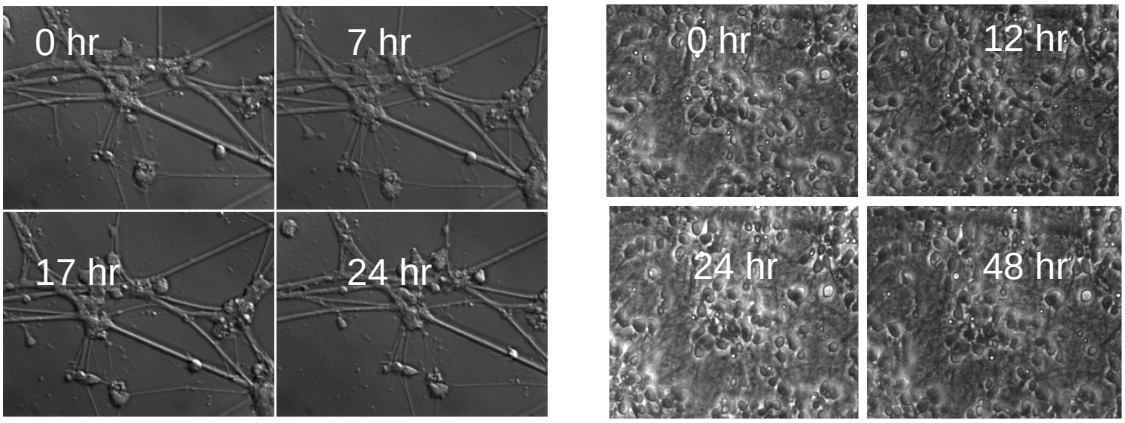


Figure 4.11: A) Cortical neuron maintained inside the custom made microscope stage incubator used to image and monitor axons (Image taken at 63X using oil) for 24 hours. B) Cortical neurons cultured and followed in the incubator for 48 hours (Image taken using 40X objective)

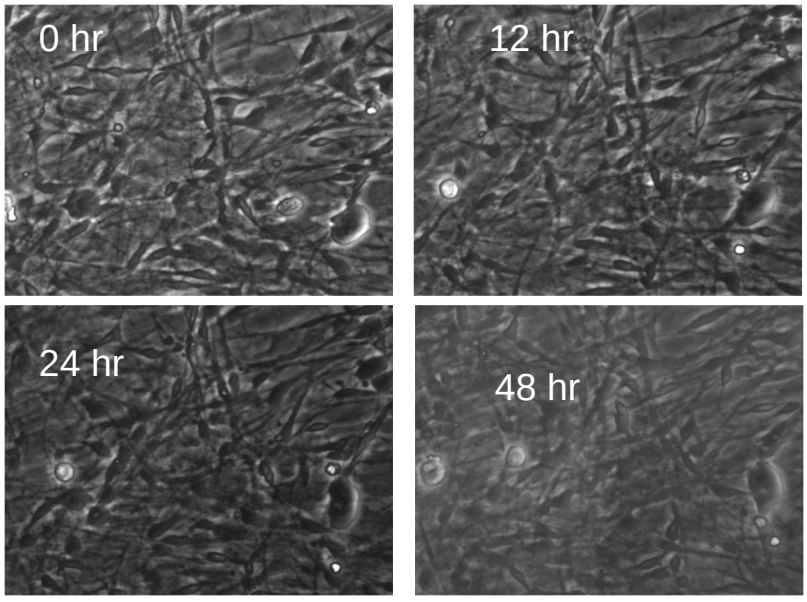


Figure 4.12: DRG neurons grown and cultivated in the custom made incubator and followed for 48 hours

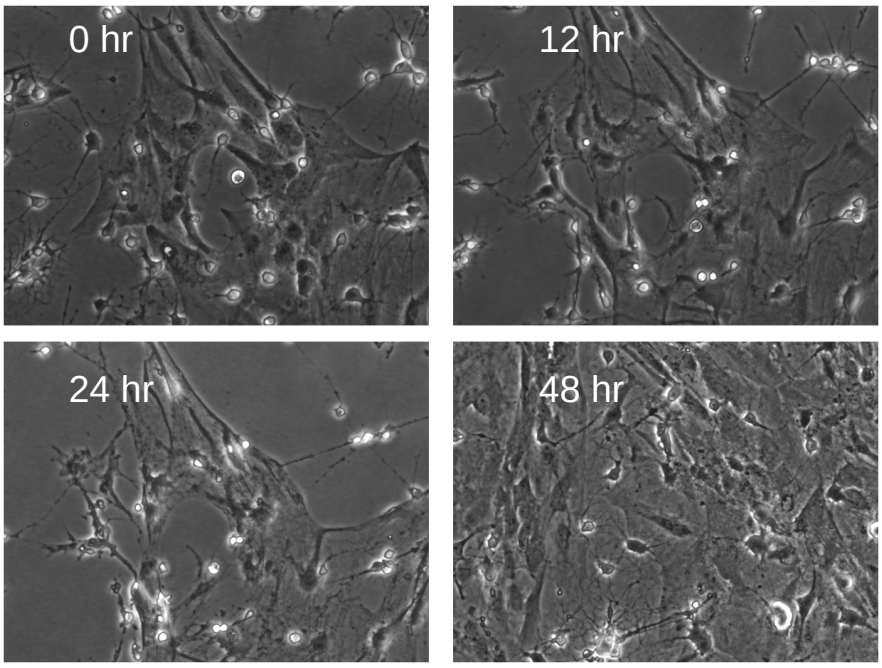


Figure 4.13: Image showing the astrocytes culture in incubator for 48 hours

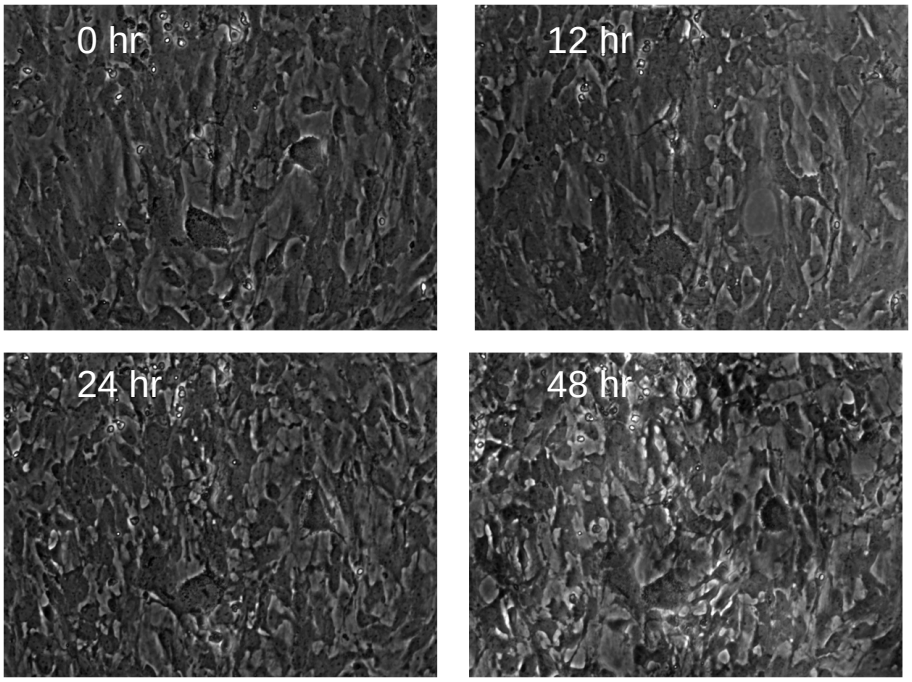


Figure 4.14: Fibroblasts grown in incubator for 48 hours

In figure 4.11 cortical neurons cultured in the microscope incubator are seen. In the two conditions figure A is images using a 63X lens using oil and figure B is images taken using a 40X objective. In fig A the cortical neurons are cultured for up to 24 hours and were observed to remain healthy without any signs of any kind of stress on the cells. In figure B cortical neurons are cultured up to 48 hours and were imaged using a 40x objective. In this also it was observed that the cells were healthy and not a sign of stress were observed either in the cells or in the axons. The cells maintained their normal intact structure and this was seen for the entire observation period of 48 hours.

Figure 4.12 shows the DRG cultured in the custom made incubator for 48 hours. In this healthy DRG cell body and axons were observed from beginning till the end with changes in the morphology in few cells. The morphology changed as its normal phenomenon observed in DRG as their morphology keeps changing along with their growth. In this no stress is seen in the cells for a period of 48 hours with the morphological changes in cells over time. The cells were observed to have an optimum environment for their biological activities to occur along with morphological changes.

Figure 4.13 shows astrocytes culture maintained in the incubator for 48 hours. It was observed that the astrocytes showed some morphological changes leading to confirmation of good biological activities in them. It was also observed that there was an increase in the astrocytes cell number over time confirming the presence of optimum temperature for them to grow. Presence of unfavorable conditions would prevent the cells from growing and would have caused the cells to have stressed out.

Figure 4.14 has fibroblasts culture grown inside the custom made incubator and the cells had increased in number over time. The 48 hour duration for which it was observed it was seen to be healthy and showed normal morphological changes such as growth. On provision of optimum conditions the cell density increased and thereby occupying all remaining area on the glass slide. The normal growth of cell implied that optimum conditions were maintained.

4.7 Discussion

Optimum conditions are maintained inside the custom built incubator and imaging chamber. The temperature and CO2 level was maintained at optimum levels by the feedback circuit. The thermocouple placed on top of the microfluidic device measures the temperature present as is fed back to the temperature controller bought from omega controllers. The temperature is set to 35.5℃ and is maintained in the range of 35.3℃-35.7℃. The temperature is set at 35.5℃ as the size of the incubator is small. Owing to the size of the incubator at higher temperature dry heat is dissipated faster and affects the cells. So a temperature of 35.5℃ was maintained with a variation of ±0.2℃. This was the optimum conditions without high degree of fluctuation in the temperature. At higher temperature due to the dissipation of dry heat the cells are affected and are stressed due to the heat. The real time temperature monitoring by the thermocouple was found to be very effective and there was no delay seen in the feedback circuit. Once the temperature dropped below the set value of 35.5℃ the silicone rubber heaters are turned back on and heats up and this heat is dissipated quickly by means of the fan. Once the temperature reaches the cut off value of 35.5℃ the silicone rubber heaters were turned off. The CO_2 circuit was also a feedback circuit with the CO_2 probe installed closer to the microfluidic device. The CO_2 was fed to the incubator through a pipe with solenoid valve and needle valve in between. The solenoid valve was controlled by the power controller and 24 V was supplied to it in order for it to work. The control of the solenoid valve required two power controllers with one to provide power for the operation of the solenoid valve and the other power controller was used to set the cut off limit for opening and closing the solenoid valve. By means of the feedback circuit the CO_2 level was maintained in an optimum range. The CO_2 sensor relayed a real time reading of CO_2 level inside the incubator and this was seen on the display outside. The cut off percentage of CO_2 was set at 4.87% by using the other power controller integrated to the feedback circuit and its output was set to 14.1V. The flow was controlled by two things: one being the needle valve and the limit to which the valve is opened and secondly

the time for which the solenoid valve is left opened. There was time delay seen feeding the CO_2 in this circuit owing to the length of the pipes used for feeding CO_2.

In the CO_2 circuit the cut off value was set to 4.87% of CO_2 and once upon crossing 4.87% the solenoid valve was closed or if the CO_2% dropped below 4.87% the valve was opened. The CO_2 sensor inside the incubator senses the CO_2% and a read value are fed to the controller outside. This controller based on the CO_2 level inside operates the solenoid valve. Once the percentage of CO_2 drops below 4.87% the solenoid valve is opened and CO_2 is supplied to the incubator, however during this a delay is observed due to the length of the pipe in which CO_2 is supplied to the incubator. The CO_2 flow rate is low due as the needle valve is slightly opened to control flow. Due to low flow rate there is delay as it has to fill the pipe first to reach the incubator. The CO_2 is maintained in the range of 4.72%-5.12%. This fluctuation did not seem to affect the cells and were considered negligible as the cells expressed a healthy morphology. The fluctuation in CO_2 level can further be adjusted by shortening the tubing and also by slightly increasing the needle valve opening and thereby an increase in flow rate will be seen.

Due to the presence of the filter on one side for exchange of gases the CO_2 is never constant and keeps dropping. Due to the exchange of gases to prevent a stagnant environment the CO_2 is constantly leaked out through the filter and drops at the speed of 0.1% every 5 seconds and therefore the solenoid is constantly active and feeds CO_2 every 30 seconds. The time taken to kickback to reach 4.87% CO_2 after dropping is around 20 seconds where it drops till 4.72%.

The presence of sterile wet gauzes and water container inside the incubator helps in maintaining humidity effectively. The dry heat produced by the heaters is moistened by the wet gauzes present and helps in prevention of evaporation or change in osmolarity of the medium.

The fan installed in the incubator was effective in circulating both CO_2 and heat. The dissipation of CO_2 and heat was much faster with the fan switched on when compared to the

fan being switched off and also it ensured equilibrium conditions inside the chamber. The chamber is assembled on top of a metal microscope stage insert and is sealed using a sealant. It was observed that if the sealing is not strong or proper there were leak in the CO_2 and also a drop in temperature. In case of any leak it was found by the rapid drop in the percentage of $C0_2$ inside the chamber and also the sudden drop of temperature. CO_2 generally dropped at the rate of 0.1% every 5seconds but in case of a leak the CO_2 level drop jumps at the rate of 0.7%-0.10% every second. It was ensured that the chamber was sealed properly and adequate amount of sealant was used to seal it completely.

During the fabrication and optimization of the microscope stage incubator we faced different issues regarding maintaining the optimum conditions inside the incubator. While imaging using a 63X lens it was done along with oil immersion technique the lens acted as a heat sink due to constant contact with the cover slip and thereby at the site of contact the temperature was similar to room temperature and led to unfavorable conditions leading to cell death. At the site of contact at the cover slip, room temperature of around 21℃ is maintained and over a period of time the cells are stressed due to the presence of low temperature. This temperature is first at the site of contact and eventually spreads to the surrounding area and thereby lowering the temperature on the cover slips in which the cells are grown and eventually leading to the death of the cells. This was overcome by lowering the 63X objective when not needed and thereby preventing its contact to the cover slip and lowering of the temperature constantly was avoided. In other cases an objective with a comparatively long working range was used and thereby direct contact was avoided. Once the lens is lowered the temperature is constant and cells are provided with optimum conditions

Maintenance of humidity inside the chamber was another concern and top priority. Due to the small area inside the incubator the effect of the dry heat was intense and had a direct effect on the cells. Due to the heat the medium evaporated at higher rates and the devices got dried completely in a day's time. The evaporation of the medium lead to the change in

osmolarity and therefore affected the cells adversely causing them to fragment out and die. To prevent this culture dish with water was placed near the cells. Initially due to space constraints the water dishes was placed after the microfluidic devices and the heat first flowed on top of the devices and the approach did not work that effectively. Then wet sterile gauze was taken and was placed all over the incubator before the devices and near the heaters and thereby making the heat moistened as soon as it is produced. The presence of wet gauzes along with water container solved the problem of maintaining humidity inside the chamber. With the wet gauzes inside a saturated environment was maintained. The gauze placed near the heaters tended to dry up more quickly and this was overcome by adding more gauze and by making the gauzes wetter than the rest of them. The maintenance of humidity helped in preventing the change in osmolarity and also helped in providing optimum conditions for cell culture.

Initially no fan was installed inside the incubator and there was lower rate of heat and CO2 dissipation inside the chamber. It was also observed that CO2 percentage and temperature readings were different at different spots. This was due to the accumulation of CO2 and temperature and the failure to properly dissipate and circulate it in the incubator. Proper circulation and dissipation was ensured by installing a fan inside the incubator. The fan maintained proper circulation of CO2 and dissipation of heat and thereby different conditions inside the incubator was overcome and equilibrium conditions were maintained throughout the chamber.

The chamber design was changed. In the previous design, due to space constraint heater was placed closer to cells and that caused more heat fluctuations and more direct dry heat on the cells. In the old design there was only one chamber into which everything was accommodated and thereby had a higher effect on the cells. In new design a secondary chamber called as the mixing chamber was built for heaters and CO2 inlet. The fan was also placed in this secondary chamber. CO2 and air inlets were in this chamber and after entering this chamber it’s slowly circulated to the chamber with the cells. The direct heating was also

avoided by placing the heaters in the secondary chamber and heat was dissipated passively in order to avoid any direct effect on the cells. The presence of gauze helped maintaining humidity. Thus the secondary chamber acts as a pretreatment chamber from which humidified heat and CO2 is circulated to the chamber containing the cells. The direct flow of CO2 and air, from their inlets, on top of the devices was also avoided by building the secondary chamber. Filters were installed in the tubes in which air and CO2 was fed to maintain sterility and these filters were changed after each use. The filter added to the walls of the chamber provided exchange of gases.

At the beginning as the filters was installed no air was fed to the cells assuming that due to the gas exchange air will be provided. But however it was found that it was not sufficient and we started feeding air at regular intervals. Air was fed at regular intervals of 3 hours apart from the air provided by exchange of gases via the filter. Every 3 hours air was fed until the CO2 level dropped down to less than 1% or was fed for 2 minutes continuously. This provided the air required for the cells and also imitates to opening the door of a commercial incubator and draining the CO2 inside. This helped in preventing a stagnant environment.

To compare if cells are provided with optimum conditions, cortical neurons, which are sensitive to slightest changes, was placed outside at room temperature. At the room conditions degeneration of axons and cells was seen in 4 hours. The cells started to spike up after 2 hours and degeneration started in less than 4 hours. This was considered as the maximum threshold period that takes for cells to degenerate if not provided with optimum conditions.

Cortical neurons were chosen to be cultured in the microscope stage incubator due to their high sensitivity to the surrounding and expression of stress in their axons upon facing unfavorable conditions. Due to their high sensitivity their response was faster to surrounding temperature and this was used to detect any unfavorable conditions inside the incubator. Other cells such as DRG, astrocytes and fibroblasts were also cultured to confirm their growth and metabolism inside the incubator. The first aim was to culture the cells inside the incubator and

check if they survive. Neurons if left outside at room temperature starts to fragment out in 4 hours and therefore this was the critical time point to check if the cells survive in the incubator. If any unfavorable conditions are presented to the cells in the custom made incubator they should behave in the same way as they fragment out in less than 4 hours if kept outside. The first four hours the cells were observed closely to check for any sort of stress if shown by cells. In this aspect cortical neuron were the most sensitive to the surrounding and expresses stress soon after any unfavorable condition is present. This property of cortical neuron made it the important cells to be cultured in the microscope incubator as if would show if unfavorable conditions are present and the survival and growth of cortical neurons in the custom made incubator showed the presence of optimum conditions for cells to survive and perform its metabolic activities. The cortical neurons were grown for 24 hours in one set and in the other they were grown for 48 hours. In the set grown for 24 hours cortical neurons was imaged using a 63X objective using oil. In this the axons were focused and no signs of any stress were seen on the axons. The regular beading structures seen in axons if stressed were not observed and the axons maintained its morphology implying the optimum conditions present inside. In the set that was cultured for 48 hours it was imaged a 40X objective and was focused on the cells to check if any stress is seen on the cells. However the cells did not show any signs of stress and were maintained healthy inside the chamber.

In the case of DRG cultures if optimum conditions are not present the stress was first observed on the Schwann cells. The Schwann cells lost their bipolar structure and become a spiky spherical structure and start to die. In the DRG cell cultures Schwann cell were the most sensitive to unfavorable conditions followed by the loss of smooth morphology in the axons leading to the spiking in axons and finally leading to fragmentation of the axons and the entire network is lost once they are stressed. After they are stressed once even if optimum conditions are restored the cells do not recover and all the cells follow apoptosis. The DRG culture grown in the custom build incubator did not show any form of stress and were maintained healthy for

up to 48hours. They were cultured for 48 hours to check if optimum conditions are present only initially or throughout the experiment.

Astrocytes and fibroblasts were also cultured in the system for 48 hours and were observed to be healthy with normal morphology and biological activity. It was found that both astrocytes and fibroblasts were growing in the culture and astrocytes increased in their size and occupied the empty spaces. Fibroblasts also increased in number and showed no signs of stress on them. Astrocytes showed a robust growth inside the chamber and when compared to cells cultured in commercial incubators the morphology was similar. The increase in cells and area occupied by them inferred that optimum conditions for cells survival and growth was maintained. Cells fail to grow when adverse conditions are presented and from the growth of the cells it was found that optimum conditions were prevalent inside the chamber.

A custom made incubator was built with incorporating all the required conditions for providing optimum conditions for cell culturing was build and the different issues related to maintaining optimum conditions were rectified. A custom made incubator based upon our requirements was designed, fabricated and used to culture cells. This custom made incubator gives us the flexibility of fabrication based on our needs and applications. Different variations based on the applications can also be added. The custom made incubator has a relative ease in fabrication and allows for a do-it-yourself project.

CHAPTER 5

FUTURE DIRECTIONS AND CONCLUSION

5.1 Future Directions

The effect of humidity in osmolality changes will be studied to improve the incubator design. The continuous reaction of cells to injury will be studied using the system. Neurons reaction upon injury and the pattern exhibited in them will be studied over time. Migration of cancer cells on different substrates will be studied to check for their migration speed and proliferation. The proliferation will be recorded continuously and the system will provide the optimum conditions for cell survival.

5.2 Conclusion

A system with microfluidic devices to isolate axons and also to provide them with optimum conditions has been developed. A seeding method has been developed to prevent migration of cells into the channels by maintenance of a pressure gradient. A system that fits into the microscope has been developed to provide optimum conditions for cell survival during axotomy has also been demonstrated. The incubator fabricated provides all the required conditions for healthy cell survival and also fits into the need of the experiment by fitting in the microscope and also by not hindering the laser port.

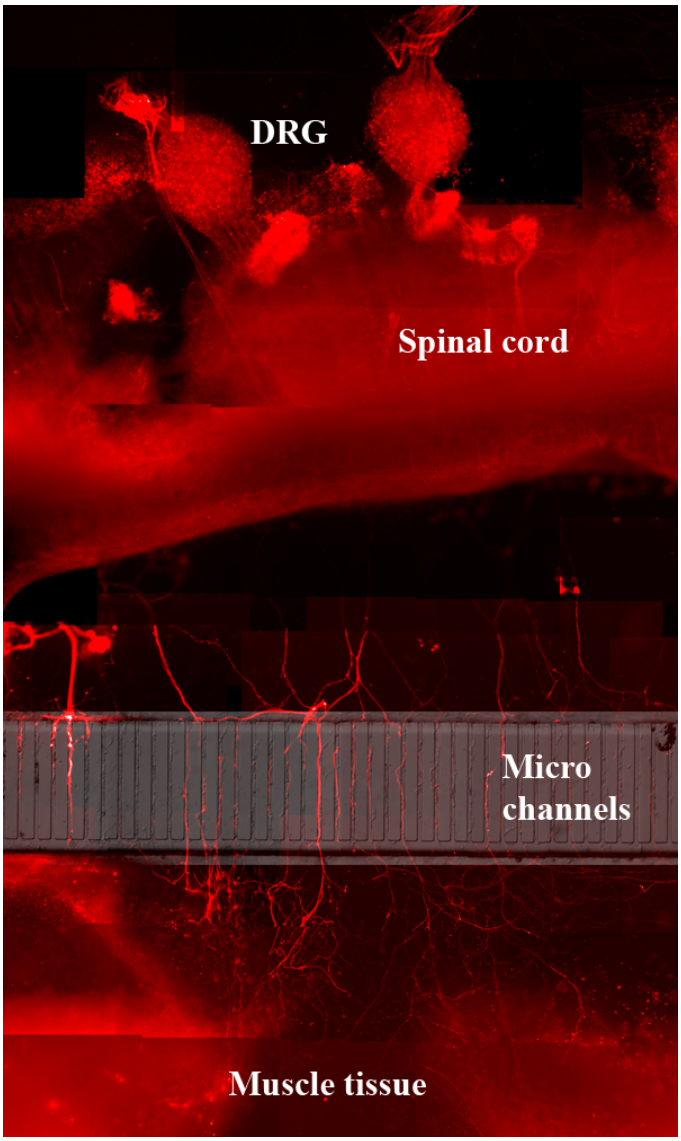


Figure 5.1 Image showing an organotypic culturing of spinal cord and muscle slices and the spinal cord axons traversing thorough the channel and reaching muscle tissue

Shown in Figure 5.1 is an example of an in vitro model of neuronal network connection between spinal cord and muscle tissue. In a microfluidic chip consisting of rectangular reservoirs bridged via microchannels, explanted spinal cord tissue and micro-dissected hindlimb muscle tissue (14–16 day rat embryos) were seeded. A high number of NF160+ axons from spinal cord tissue grew via individual microchannels and innervated into muscle tissue. Neuronal connection between attached DRGs and spinal cord tissue is also clearly observed.

REFERENCES

1. Lopez-Munoz, F., J. Boya, et al. (2006). "Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramon y Cajal." Brain Res Bull **70**(4-6): 391-405.

2. Herrup, K. and Y. Yang (2007). "Cell cycle regulation in the postmitotic neuron: oxymoron or new biology?" Nat Rev Neurosci **8**(5): 368-78.

3. Roy, N. S., S. Wang, et al. (2000). "In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus." Nat Med **6**(3): 271-7.

4. Gould, E., A. J. Reeves, et al. (1999). "Neurogenesis in the neocortex of adult primates." Science **286**(5439): 548-52.

5. Alvarez-Buylla, A. and J. M. Garcia-Verdugo (2002). "Neurogenesis in adult subventricular zone." J Neurosci **22**(3): 629-34.

6. www.utexas.edu/courses/bio365r/Images/neuron.JPG

7. Goldenring, J. M. (1985). "The brain-life theory: towards a consistent biological definition of humanness." J Med Ethics **11**(4): 198-204.

8. www.chemistry.ucsc.edu/.../images/neuron.png

9. Berlucchi, G. (2009). "Chapter 13 The contributions of neurophysiology to clinical neurology an exercise in contemporary history." Handb Clin Neurol **95**: 169-88.

10. Hodgkin, A. L. and A. F. Huxley (1952). "Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo." J Physiol **116**(4): 449-72.

11. Huxley, A. F. and R. Stampfli (1951). "Effect of potassium and sodium on resting and action potentials of single myelinated nerve fibers." J Physiol **112**(3-4): 496-508.

12. Biales, B., M. A. Dichter, et al. (1977). "Sodium and calcium action potential in pituitary cells." Nature **267**(5607): 172-4.

13. Adelman, W. J., Jr., Y. Palti, et al. (1973). "Potassium ion accumulation in a periaxonal space and its effect on the measurement of membrane potassium ion conductance." J Membr Biol **13**(4): 387-410.

14. Hodgkin, A. L. and B. Katz (1949). "The effect of sodium ions on the electrical activity of giant axon of the squid." J Physiol **108**(1): 37-77.

15. Colbert, C. M. and E. Pan (2002). "Ion channel properties underlying axonal action potential initiation in pyramidal neurons." Nat Neurosci **5**(6): 533-8.

16. Strong, M., K. G. Chandy, et al. (1993). "Molecular evolution of voltage-sensitive ion channel genes: on the origins of electrical excitability." Mol Biol Evol **10**(1): 221-42.

17. Fishman, H. M. (1970). "Direct and rapid description of the individual ionic currents of squid axon membrane by ramp potential control." Biophys J **10**(9): 799-817.

18. Haspel, D., P. Krippeit-Drews, et al. (2005). "Crosstalk between membrane potential and cytosolic Ca2+ concentration in beta cells from Sur1-/- mice." Diabetologia **48**(5): 913-21.

19. Brink, P. R., K. Cronin, et al. (1996). "Gap junctions in excitable cells." J Bioenerg Biomembr **28**(4): 351-8.

20. http://kvhs.nbed.nb.ca/gallant/biology/action_potential_generation.html

21. http://frashdi.files.wordpress.com/2009/07/saltatory_conduction.jpg

22**.** Dugandzija-Novakovic, S., A. G. Koszowski, et al. (1995). "Clustering of Na+ channels and node of Ranvier formation in remyelinating axons." J Neurosci **15**(1 Pt 2): 492-503.

23. Gasnier, B. (2000). "The loading of neurotransmitters into synaptic vesicles." Biochimie **82**(4): 327-37.

24. http://static.howstuffworks.com/gif/brain-neuron-types.gif

25. Keller, E. L., B. T. Lee, et al. (2008). "Frontal eye field signals that may trigger the brainstem saccade generator." Prog Brain Res **171**: 107-14.

26. Freeman, D. K., W. F. Heine, et al. (2008). "The maintained discharge of rat retinal ganglion cells." Vis Neurosci **25**(4): 535-48.

27. Zecevic, N. and P. Rakic (2001). "Development of layer I neurons in the primate cerebral cortex." J Neurosci **21**(15): 5607-19.

28. Huang, C. W., J. N. Tzeng, et al. (2007). "Nociceptors of dorsal root ganglion express proton-sensing G-protein-coupled receptors." Mol Cell Neurosci **36**(2): 195-210.

29. Agius, E. and P. Cochard (1998). "Comparison of neurite outgrowth induced by intact and injured sciatic nerves: a confocal and functional analysis." J Neurosci **18**(1): 328-38.

30. Ramon-Cueto, A., M. I. Cordero, et al. (2000). "Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia." Neuron **25**(2): 425-35.

31. Navarro, X. (2009). "Chapter 27: Neural plasticity after nerve injury and regeneration." Int Rev Neurobiol **87**: 483-505.

32. Liuzzi, F. J. and R. J. Lasek (1987). "Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway." Science **237**(4815): 642-5.

33. Bovolenta, P., F. Wandosell, et al. (1992). "CNS glial scar tissue: a source of molecules which inhibit central neurite outgrowth." Prog Brain Res **94**: 367-79.

34. Schwab, M. E., J. P. Kapfhammer, et al. (1993). "Inhibitors of neurite growth." Annu Rev Neurosci **16**: 565-95.

35. McKerracher, L., S. David, et al. (1994). "Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth." Neuron **13**(4): 805-11.

36. Silver, J. (1994). "Inhibitory molecules in development and regeneration." J Neurol **242**(1 Suppl 1): S22-4.

37. Richardson, P. M., U. M. McGuinness, et al. (1980). "Axons from CNS neurons regenerate into PNS grafts." Nature **284**(5753): 264-5.

38. Horner, P. J. and F. H. Gage (2000). "Regenerating the damaged central nervous system." Nature **407**(6807): 963-70.

39. Stoll, G. and H. W. Muller (1999). "Nerve injury, axonal degeneration and neural regeneration: basic insights." Brain Pathol **9**(2): 313-25.

40. Serrano-Pozo, A., J. R. Gonzalez-Marcos, et al. (2005). "[Wallerian degeneration of the corticospinal tract following spontaneous brain haematoma]." Rev Neurol **41**(8): 501-2.

41. Megdiche Bazarbacha, H., R. Jeribi, et al. (2005). "[Wallerian degeneration]." Presse Med **34**(17): 1264.

42. Miyai, I., T. Suzuki, et al. (1998). "Wallerian degeneration of the pyramidal tract does not affect stroke rehabilitation outcome." Neurology **51**(6): 1613-6.

43. Albensi, B. C. and D. Janigro (2003). "Traumatic brain injury and its effects on synaptic plasticity." Brain Inj **17**(8): 653-63.

44. Krassioukov, A. V., A. Ackery, et al. (2002). "An in vitro model of neurotrauma in organotypic spinal cord cultures from adult mice." Brain Res Brain Res Protoc **10**(2): 60-8.

45. Fehlings, M. G. and R. Nashmi (1997). "A new model of acute compressive spinal cord injury in vitro." J Neurosci Methods **71**(2): 215-24.

46. Sia, S. K. and G. M. Whitesides (2003). "Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies." Electrophoresis **24**(21): 3563-76.

47. Hong, J. W., V. Studer, et al. (2004). "A nanoliter-scale nucleic acid processor with parallel architecture." Nat Biotechnol **22**(4): 435-9.

48. Vilkner, T., D. Janasek, et al. (2004). "Micro total analysis systems. Recent developments." Anal Chem **76**(12): 3373-85.

49. Foley, J., H. Schmid, et al. (2005). "Microcontact printing of proteins inside microstructures." Langmuir **21**(24): 11296-303.

50. Zhong, J. F., Y. Chen, et al. (2008). "A microfluidic processor for gene expression profiling of single human embryonic stem cells." Lab Chip **8**(1): 68-74.

51. Gomez-Sjoberg, R., A. A. Leyrat, et al. (2007). "Versatile, fully automated, microfluidic cell culture system." Anal Chem **79**(22): 8557-63.

52. George, A., D. H. Blank, et al. (2009). "Nanopatterning from the Gas Phase: High Resolution Soft Lithographic Patterning of Organosilane Thin Films." Langmuir.

53. GrandPre, T., S. Li, et al. (2002). "Nogo-66 receptor antagonist peptide promotes axonal regeneration." Nature **417**(6888): 547-51.

54. King, J. A. and W. M. Miller (2007). "Bioreactor development for stem cell expansion and controlled differentiation." Curr Opin Chem Biol **11**(4): 394-8.

55. Kuypers, P. D., J. M. van Egeraat, et al. (1995). "Loss of viable neuronal units in the proximal stump as possible cause for poor function recovery following nerve reconstructions." Exp Neurol **132**(1): 77-81.

56. Liu, D., H. B. McIlvain, et al. (2007). "Screening of immunophilin ligands by quantitative analysis of neurofilament expression and neurite outgrowth in cultured neurons and cells." J Neurosci Methods **163**(2): 310-20.

57. Heredia, A., C. C. Bui, et al. (2007). "AFM combines functional and morphological analysis of peripheral myelinated and demyelinated nerve fibers." Neuroimage **37**(4): 1218-26.

58. Hardy, B. S., K. Uechi, et al. (2009). "The deformation of flexible PDMS microchannels under a pressure driven flow." Lab Chip **9**(7): 935-8.

59. Kim, S. J., J. K. Lee, et al. (2008). "Surface modification of polydimethylsiloxane (PDMS) induced proliferation and neural-like cells differentiation of umbilical cord blood-derived mesenchymal stem cells." J Mater Sci Mater Med **19**(8): 2953-62.

60. Campenot, R. V. (1977). “Proceedings of the National Academy of Sciences of the United States of America,” **74**, 4516–19.

61. Bertrand, J., M. J. Winton, et al. (2005). "Application of Rho antagonist to neuronal cell bodies promotes neurite growth in compartmented cultures and regeneration of retinal ganglion cell axons in the optic nerve of adult rats." J Neurosci **25**(5): 1113-21.

62. Ivins, K. J., E. T. Bui, et al. (1998). "Beta-amyloid induces local neurite degeneration in cultured hippocampal neurons: evidence for neuritic apoptosis." Neurobiol Dis **5**(5): 365-78.

63. MacInnis, B. L. and R. B. Campenot (2002). "Retrograde support of neuronal survival without retrograde transport of nerve growth factor." Science **295**(5559): 1536-9.

64. Wu, M. H., J. P. Urban, et al. (2006). "Development of PDMS microbioreactor with well-defined and homogenous culture environment for chondrocyte 3-D culture." Biomed Microdevices **8**(4): 331-40.

65. Mi, Y., Trau, D.., Huang, P., Chen, E. (2006). “Micromolding of PDMS scaffolds and microwells for tissue culture and cell patterning: A new method of microfabrication by the self-assembled micropatterns of diblock copolymer micelles.” Polymers **47**(14):5124-5130

66. Ng, J. M., I. Gitlin, et al. (2002). "Components for integrated poly(dimethylsiloxane) microfluidic systems." Electrophoresis **23**(20): 3461-73.

67. Makamba, H., J. H. Kim, et al. (2003). "Surface modification of poly(dimethylsiloxane) microchannels." Electrophoresis **24**(21): 3607-19.

68. Recknor, J. B., J. C. Recknor, et al. (2004). "Oriented astroglial cell growth on micropatterned polystyrene substrates." Biomaterials **25**(14): 2753-67.

69. Park, J. W., B. Vahidi, et al. (2006). "Microfluidic culture platform for neuroscience research." Nat Protoc **1**(4): 2128-36.

70. Taylor, A. M., M. Blurton-Jones, et al. (2005). "A microfluidic culture platform for CNS axonal injury, regeneration and transport." Nat Methods 2(8): 599-605.

71. Gattass, R.R., Maxur, E,. (2008). “Femtosecond laser micromachining in transparent materials**.**” **Nat Photonics** 2: **219-225.**

72. Whitesides, G. M. (2006). "The origins and the future of microfluidics." Nature **442**(7101): 368-73.

73. Park, J. W., B. Vahidi, et al. (2006). "Microfluidic culture platform for neuroscience research." Nat Protoc **1**(4): 2128-36.

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