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A microfluidic culture for two populations of dorsal root ganglia for differential staining

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ABSTRACT

A MICROFLUIDIC CULTURE FOR TWO POPULATIONS OF DROSAL ROOT GANGLIA FOR DIFFERENTIAL STAINING

by
Ishnoor Sidhu

The goal of this study was to design and fabricate a microfluidic system that can be used to visibly distinguish the two populations of dorsal root ganglia (DRGs) by differential staining. Polydimethylsiloxane (PDMS) is the most widely used silicon-based organic polymer, and is particularly known for its wide spread use in microfluidics. Various methods have been employed to pump fluids in these channels for applications ranging from patterning of cells and biomolecules to control of local environment factors such as temperature, which requires external pumping or other applied forces. We demonstrated a pump-free device that exploits the surface energy stored in a liquid droplet to pump liquid in the channels. The fluid was pumped by using two droplets of unequal sizes connected via fluid filled channel. The flow was generated from smaller droplet to larger droplet. This passive pumping technique was used to simultaneously stain the two cultured DRGs in connected channels.

The *in vitro* system can be further exploited to study the guided growth in axons. This study provides a cost effective method to detect the influence of the presence of pioneer neuron on the growth patterns of the new generation of neurons. It eliminates the need of using transgenic cells to study the guided growth in axons, thereby giving some insight for the repair of spinal cord injuries and the understanding of the early growth model.

A MICROFLUIDIC CULTURE FOR TWO POPULATIONS OF DROSAL ROOT GANGLIA FOR DIFFERENTIAL STAINING

by
Ishnoor Sidhu

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A MICROFLUIDIC CULTURE FOR TWO POPULATIONS OF DROSAL ROOT GANGLIA FOR DIFFERENTIAL STAINING

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To my beloved family and friends, I never would have been able to do this without your love and support; you make me who I am.

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CHAPTER 1

INTRODUCTION

1.1 Human Nervous System

Human nervous system exhibits bilateral symmetry. It is subdivided into central nervous system and peripheral nervous system, anatomically. Functionally it is divided into somatic nervous system and autonomic nervous system. The CNS comprises the brain and spinal cord, whereas PNS consist of the cranial nerves (nerves emerging from the brain) and spinal nerves (nerves emerging from the spinal cord). The PNS convey neural message from sensory organs to CNS and vice versa. The somatic nervous system is responsible for sensory information (*afferent*) and motor (*efferent*) control of voluntary muscles. The autonomic nervous system, also called as visceral nervous system is concerned with motor control of involuntary and cardiac muscles and of glands of viscera.

1.1.1 The Neuron: Cell of Nervous System

The neuron is the basic unit of the nervous system. There are billions of neurons in human body. Over 100 billion neurons along with many more glial cells are integrated together to form brain. They exhibit various forms and sizes. A typical neuron consists of four morphologically defined regions:

1.1.1.1 Soma or Cell Body. As any other, soma is the metabolic center of the neuron. It contains large nucleus, plasma membrane, cytosol, Nissl bodies and endoplasmic reticulum, mitochondria, lysosomes, neurotubules, and neurofilaments (Noback *et al.*, 1996).

1.1.1.2 Dendrites. Dendrites, or the nerve endings, are the variable number of small branching projections. These allow cell to talk to other neuron or perceive the environment. Dendrites contain same cytoplasmic organelles as the cell body (Noback *et al.*, 1996).

1.1.1.3 Axon. Axons are long, cable-like projections. The axon is specialized for transmission of coded information (nerve impulse or action potential). The axons of CNS are very thin, of the order of 0.2 to 20 microns as compared to diameter of the cell body, about 50 microns (Kandel *et al.*, 1991). It arises from *axon hillock* of cell body and extends from less than a millimeter to as long as 3 meter before diverging into fine terminal branches, telondria.

Depending upon the type of neuron, axons can be covered with a thin layer of myelin, like an insulated electrical wire. Myelin is made of mostly fat (glycolipid called galactocerebroside), and it helps to speed transmission of a nerve impulse down a long axon. Myelinated neurons are typically found in the PNS, while non-myelinated neurons are found in the CNS.

1.1.1.4 Synapse. A synapse is a junction where one neuron meets another neuron's impulse. A synapse has a pre-synaptic component, usually an axon terminal, and a post-synaptic component, part of dendrite, cell body or axonal initial segment (Kandel *et al.*, 1991). A pre-synaptic cell does not actually touch or communicate anatomically with the post-synaptic cell since the two cells are separated by a space, the *synaptic cleft*.

The pre-synaptic cell releases the signal (chemical or electrical) into the synaptic cleft and is received by the post-synaptic cleft.

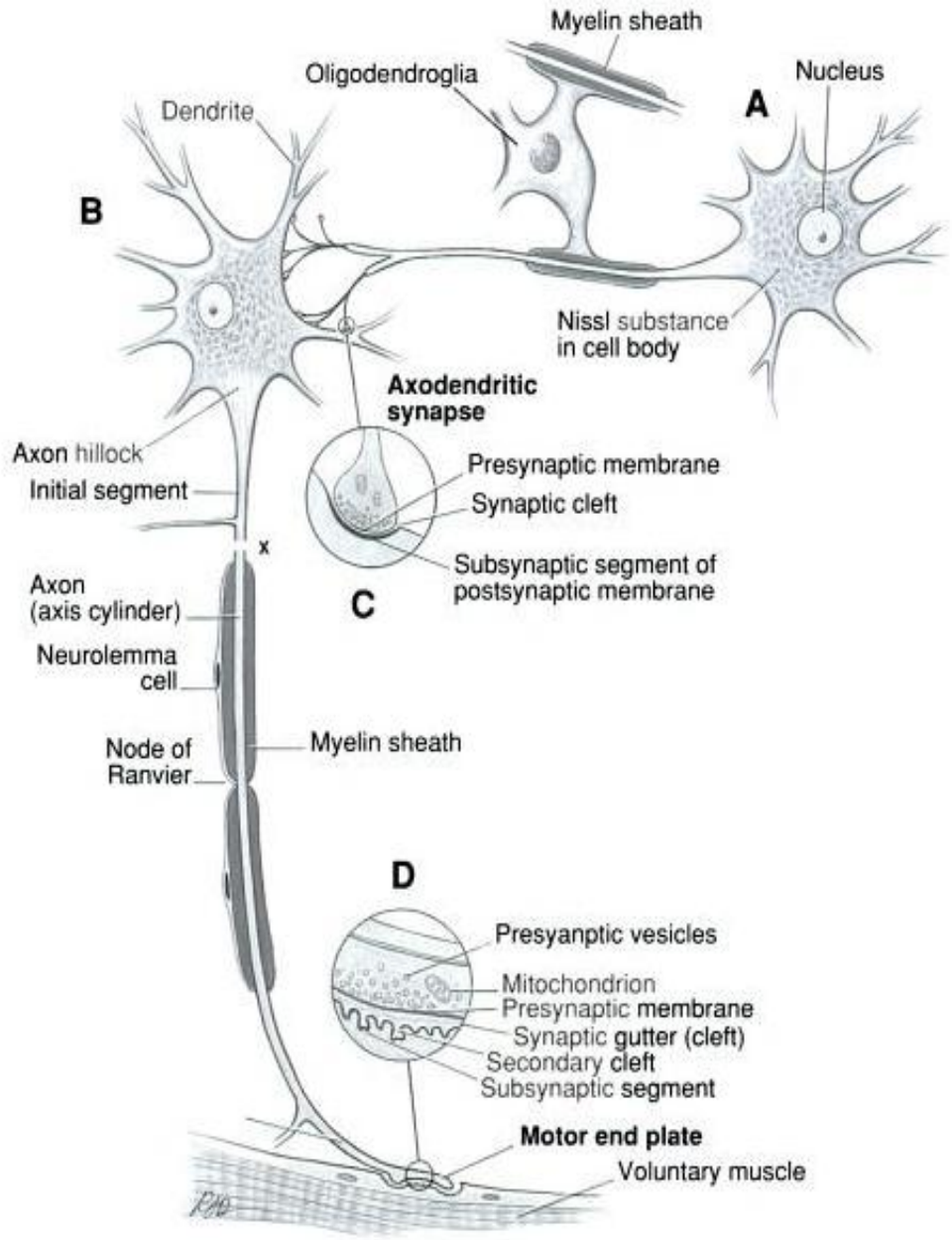

Figure 1.1 Structure of neuron **(A)** CNS neuron, **(B)** a lower motor neuron located both in CNS and PNS, **(C)** Axodendritic synapse between two neurons, **(D)** Motor end plate (synapse), and **(X)** border between CNS (above X) and PNS (below X). The lower motor neuron synapses with a voluntary muscle cell to form a motor end plate.

Source: (Noback *et al.*, 1996)

Figure 1.1 describes the basic morphology of neurons in central nervous system and peripheral nervous system and how the neurons communicate with each other i.e., how the signal is transported from CNS to PNS and vice versa.

1.1.2 The Spinal Cord

The spinal cord is a cylinder that extends from the foramen magnum at the base of skull to a cone-shaped termination (*conus medullaris*), located at the caudal level of the first lumbar centrum. The nonneural filament continues from conus medullaris to its attachment in the coccyx.

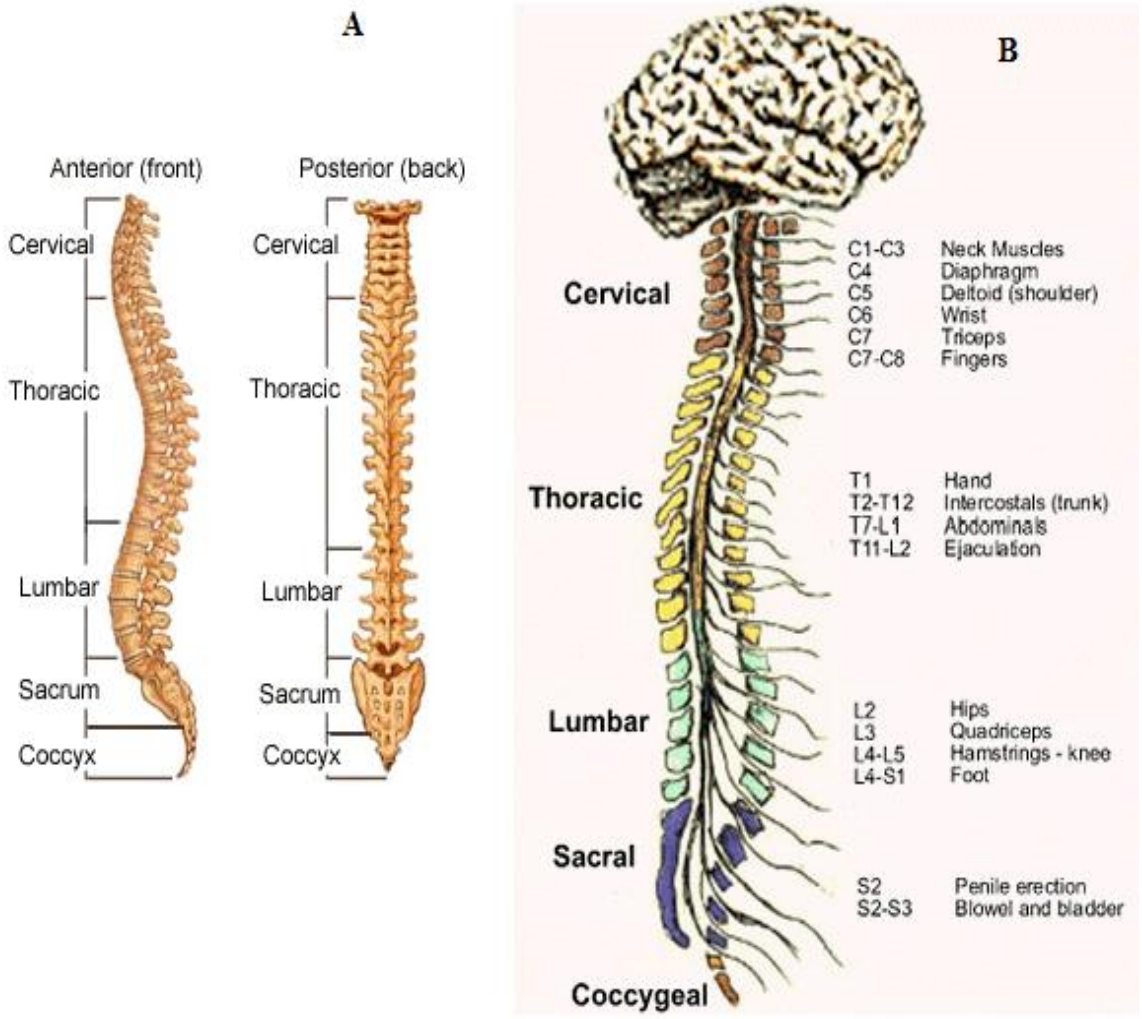

Figure 1.2 Anatomy of the spine. **(A)** The spinal column (front and back). The spinal column extends from skull to pelvis and is divided into five regions: Cervical, Thoracic, Lumbar, Sacrum and Coccyx. **(B)** Spinal nerves. Each spinal nerve controls a specific part of body.

Source: (http://www.thewellingtonneurosurgeryunit.com/spine-anatomy.asp)

The spinal column is divided into five regions, as shown in Figure 1.2 (A). There are 33 bones which forms the spinal column. The first three regions, cervical, thoracic, and lumbar, are made of number of individual vertebrates; there are seven cervical vertebrate, 12 thoracic vertebrate and five lumbar vertebrate. The sacrum region consists of five fused bones and the coccyx (tail-bone) is a single bone.

Figure 1.2 (B) shows the spinal nerves. The spinal cord receives its input and projects the output via spinal nerves. Each spinal nerve is two components, dorsal root and ventral root, see Figure 1.3. Dorsal root extends from the dorsal surface of the spinal cord to the formation of the spinal nerve. Dorsal root is entirely sensory in function; passes sensation from body to brain. On the other hand ventral root extends from the ventral horn of the spinal cord to the spinal nerve and is entirely motor in function. It carries motor signal from brain to muscles and glands.

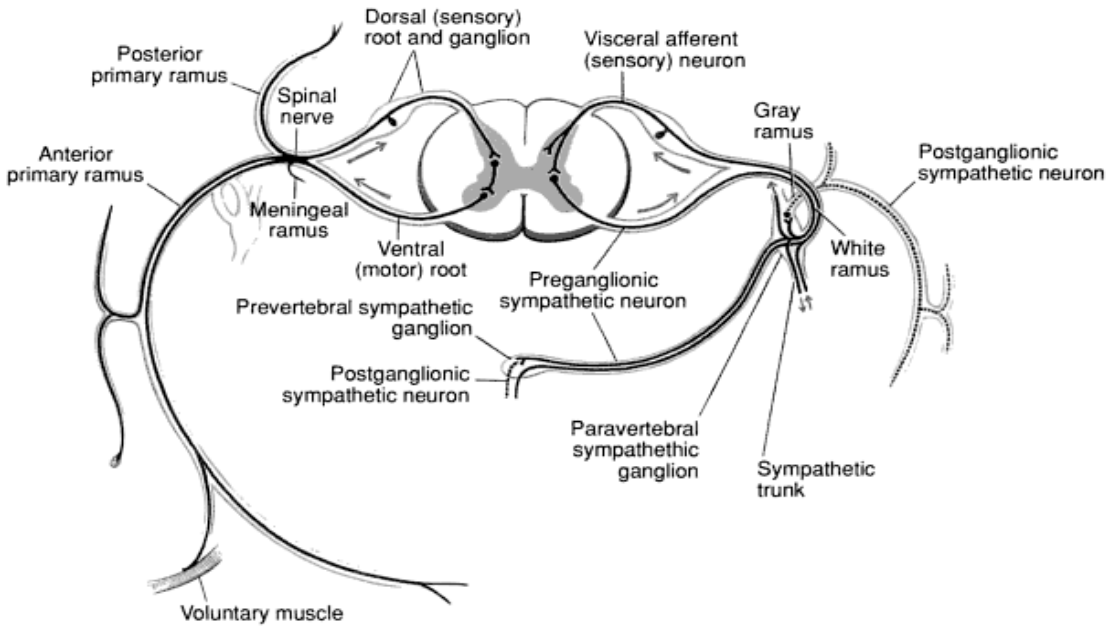

Figure 1.3 The spinal nerve. Each spinal nerve divided into roots, Dorsal (sensory) and Ventral (motor) roots.

Source: (Noback *et al.*, 1996)

The spinal cord consists of gray and white matter. The gray matter consists of neuronal cell bodies, dendrites, axon terminals, synapses, glial cells and is highly vascular. On the other hand the white matter consists of bundles of axons and oligodendrocytes. It lacks cell bodies and is less vascular.

1.1.3 Dorsal Root Ganglion Neuron

Dorsal root Ganglion (DRG) is a sensory ganglion located on the dorsal root. It serves as the location of cell bodies of somatic afferent neurons. The DRG neurons are about 10-100μm in size. DRG neurons possess robust and regenerative nature of the PNS and potential for nervous system repair.

The primary function of the spinal dorsal root ganglion and their cranial equivalent is to serve as afferent conduits to the CNS. Other than signaling, DRG neurons play other important functions, during development and in adult vertebrate body. The absence of DRG neurons, during development, may block the differentiation of certain features of peripheral tissue associated with nerve terminals. In adult body, loss of primary DRG neurons can lead to disappearance of cellular complexes of tissue associated with their peripheral terminals (Perl, 1992).

1.2 Motivation for Using Microfluidics in Neuroscience Research

Human feelings, cognition, emotion, thinking and behavior are all based on activities of the nervous system. The basic and urgent tasks for neuroscience are (Wang *et al.*, 2009):

- to analyze the structure and function of the nervous system,
- to understand the basic rules of nervous system activities,
- to elucidate mechanisms of learning and memory on different levels, and
- to prevent, diagnose, and treat various neurobiological and mental diseases.

With traditional techniques there are still many challenges in studying nerve cell interactions, neural stem cell differentiation, mechanisms of cell attachment to various substrates, neurite growth, myelin formation and the mechanisms of ion channels. Microfluidic systems, with their excellent performance and easy availability of functional components, provide a new means to study these phenomena in a manner impossible on a conventional scale (Wang *et al.*, 2009).

In fact, since the first introduction of microfluidic devices into neuroscience in 1998 (Heuschkel *et al.*, 1998), they have been used to study nerve cell activity and growth on various substrates and, microenvironments, neuropharmacology, neuroelectrophysiology, neural stem cell differentiation, neuron biosensors.

1.3 Microfluidics

Microfluidics- the manipulation of fluids in channels with dimension of tens- emerged as a distinct new field in 1980s. It is the branch of physics that process or manipulate small (10^{-9} to 10^{-18} liters) amounts of fluids, using channels with dimensions of tens to hundreds of micrometers. Microfluidics is closely associated with the lab-on-chip, a termed coined by Harrison et al. in 1992 (Harrison *et al.*, 1992).

The behavior of fluids at microscale differ from their generic behavior in terms of surface tension, rheological properties etc. microfluidics has been applied in many disciplines, including chemistry, biology and medicine, and has shown the significant advantages of low reagent and sample volumes, highly sequential or parallel experimentation, better mimicry of the natural tissue environment, precise experimental control of the cellular microenvironment, and low cost disposable devices (Whitesides, 2006).

Microfluidic-based cell studies present an advantage when compared to conventional in vitro techniques since they have the ability to precisely control the environment around individual cells. This is accomplished by using microfluidic architecture to deposit single cells in dedicated areas and by controlling the quantity of added reagents and factors distributed to these isolated cells via the microfluidic channels. Since the control offered by microfluidic platforms can avoid problems seen with standard in vitro techniques, such as unanticipated extraneous factors, diffusion constraints, and cell population variability, results derived from microfluidic experiments may reveal new details of cellular physiology. Cost savings are another benefit of microfluidic experiments since the volume of expensive media, hormones, and growth factors is orders of magnitude less than that used in conventional culture flasks.

Microfluidic devices and their architectures gives a novel approach to studying the cellular physiology of the nervous system and the pathophysiology of many congenital and acquired neurodegenerative diseases by allowing isolation of cells derived from the nervous system, isolation of neurites and synapses, and analysis of single-cell responses to perturbations of their environment.

1.3.1 Polydimethylsiloxane (PDMS)

Materials employed for fabrication of microfluidic devices include silicon, glass, quartz, polystyrene, PDMS etc. Silicon, glass and quartz are known as hard materials. Although they are chemically robust and solvent resistant, they have disadvantage of being expensive and not flexible. Polystyrene is flammable and degrade easily when subjected to solvent and UV exposure. PDMS belongs to a group of polymeric organo-silicone compounds that are commonly referred to as silicones. It is known for its

unusual rheological properties and widespread use in microfluidics. $(CH_3)_2SiO$ is the repeating unit of PDMS and the polymeric form is shown in Figure 1.4.

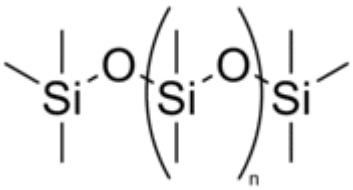

Figure 1.4 Chemical representation of PDMS.

Poly(dimethylsiloxane) has a unique combination of properties resulting from the presence of an inorganic siloxane backbone and organic methyl groups attached to silicon. They have very low glass transition temperatures and hence are fluids at room temperature. These liquid materials can be readily converted into solid elastomers by cross-linking (Xia & Whitesides, 1998).

PDMS is optically transparent down to 280 nm, allowing both optical and fluorescent/chemiluminescent microscopy of contained cells and fluids in the visible spectrum. Other major advantages of PDMS for in vitro cell studies are that it allows respiration of cells that are enclosed within it since it is also permeable to gases. It is nontoxic and autoclavable and, it inhibits cellular adhesion onto its surface if it is not pretreated for this purpose. A final advantage of this material for use on microfluidic chambers is that PDMS has been shown to be a superior protective coating for onboard electronic devices (like surface-emitting lasers) since it is optically transparent yet prevents the corrosive (Gross *et al.*, 2007).

The major drawbacks of PDMS are the limited compatibility with organic solvents and hydrophobic external surface. The PDMS tends to absorb the solvents and

thus become swelled. The hydrophobicity can be compensated by plasma oxidation, adding silanol (SiOH) groups to the surface.

1.3.2 Characteristics of Flow

1.3.2.1 Laminar flow. Laminar flow occurs when fluid flows in parallel layers without lateral mixing. This is also known as streamline flow. A dimensionless parameter, Reynolds number (Re) named after Osborne Reynold, describes the tendency of flowing fluids to develop turbulence.

Equation 1.1 shows the relation of Re with the dimension of channel and the fluid parameters.

$$Re = \frac{Lv\rho}{\mu} \tag{1.1}$$

where, L is characteristic dimension of the channel (m), v is the velocity (m/s), ρ is the density of fluid ($g.m^{-3}$), and μ is the viscosity (Pa.s). Flows with Re >1000 are turbulent i.e., the mixing happens quickly by convection. Re < 1 denotes the laminar flow (Jiang & Whitesides, 2003).

1.3.2.2 Surface Tension and Capillary Action. Capillary action is the tendency and ability of a material to draw another material into it. This is due to adhesive intermolecular forces being stronger in the interface between the liquid and the material than the cohesive intermolecular forces inside the liquid.

Microfluidics is based on the theories of capillary action. When a fluid interacts with the interface of a hydrophilic microcapillary channel, the surface tension induces

advancement and the fluid is drawn into the channel. Specifically, capillary flow is achieved through surface tension equilibrium maintenance (Chakraborty, 2005). The length liquid will travel through a capillary is directly related to the liquid's surface free energy and inversely related to the radius of the capillary. When microchannels with dimensions on the order of microns are used, the lengths liquids will travel based on capillary forces alone are significant. Surface tension is very important to study droplet-based microfluidics. Surface area is another factor that becomes important at the microscale. A very large surface area to volume ratio makes capillary action more efficient in microchannels.

1.3.2.3 Diffusion. Diffusion is the process by which a concentrated group of particles in a volume will, by Brownian motion, spread out over time so that the average concentration of particles throughout the volume is constant. Diffusion is the only force of mass transfer in transverse direction of flow.

$$X^2 = 2Dt \tag{1.2}$$

where, X is diffusion distance, D is the diffusion coefficient ($\mu m^2/s$) and t is time (s).

1.3.2.4 Flow Resistance. The flow rate within a microchannel is given by:

$$Q = \frac{\Delta P}{R} \tag{1.3}$$

where Q is the flow rate, ΔP is the pressure drop across the channel, and R is the channel resistance. For a rectangular microchannel with a high aspect ratio (w<<h):

$$R = \frac{12\,\mu\,l}{h^3 w} \tag{1.4}$$

where μ is the fluid viscosity, l is the length of the channel, w is the channel width and h is the channel height.

In the following study, a device is demonstrated for the culturing two dorsal root ganglion explants in connected channels and simultaneously staining them using the surface energy of liquid droplets and capillary flow.

CHAPTER 2

BACKGROUND

2.1 Neuroscience Study in Microchannels: Literature Review

As described in the previous Chapter, the research in neuroscience aims at culturing the individual neurons and precisely controlling the environment. Various studies have connected microfluidic science to neuroscience for variety of in vitro studies. There exist many reviews on the application of microfluidics in neuroscience research. Pearce et al (Pearce & Williams, 2007), Gross et al (Gross *et al.*, 2007), Wang et al (Wang *et al.*, 2009), and Taylor et al (Taylor & Jeon, 2010) has presented extensive review of the microfluidics in neuroscience.

There are three main reasons for interest in use of microstructures in neuron culture. The first reason is the interactions of microstructures with cell-surface morphology provide more biocompatible surfaces for neuron culture. Previous studies (Turner *et al.*, 2000) showed that neuron culture prefers rough than smooth surfaces. Secondly, microstructures provides useful platforms for studying the responses of neurons and neural networks to physical cues in a controlled environment, since physical cues, including nano- and micrometer-sized ridges and pores formed by cell bodies and cell processes, govern the construction and function of neuronal networks as neural cells connect to one another. Lastly, microstructure can be employed to manipulate neurons, which is very important for research on isolated somata and axons in miniature (Wang *et al.*, 2009).

Researchers in past have extensively studied the neuron culture in the microfluidic devices (Taylor *et al.*, 2003; Taylor *et al.*, 2005; Park *et al.*, 2006; Taylor *et al.*, 2006; Park *et al.*, 2009; Taylor & Jeon, 2010). Taylor et al. (Taylor *et al.*, 2003) demonstrated the culture of rat cortical neurons in microfluidic devices. The device fabricated had two compartments separated by a physical barrier in which a number of micron-size grooves were embedded to allow growth of neurites across the compartments while maintaining fluidic isolation. Cells were plated into the somal (cell body) compartment, and after 3-4 days, neurites extend into the neuritic compartment via the grooves. The viability of neurons was shown to be 50-70 % after 7 days in culture.

In other paper, Taylor et al (Taylor *et al.*, 2005) demonstrated a microfluidic culture platform for CNS axonal injury, regeneration and transport. The platform polarizes the growth of CNS axons into a fluidically isolated environment without the use of targeting neurotrophins. In addition to its compatibility with live cell imaging, the platform was use to isolate CNS axons without somata or dendrites, facilitating biochemical analyses of pure axonal fractions and localize physical and chemical treatments to axons or somata. The platform also served as a straightforward, reproducible method to model CNS axonal injury and regeneration.

The above two studies exploited the less obvious trait of microfluidic devices i.e. laminar flow and capillary action. The microfluidic devices maintain the laminar flow of liquids. This property helps in the fluidic isolation and manipulation of neuron structures independent of the other part.

Based on the previous work Park et al designed novel modified microfluidic platforms for performing biochemical analysis of the pure axonal fraction, culturing

tissue explants, and perform a high content assay on same group of cells. The device demonstrated in the study incorporated a number of microgrooves for isolating axons from the cell body. The design had an open cell culture area in the center and four enclosed channels around open area that made it suitable for multiple drug screening assays. (Park *et al.*, 2009).

A group of researchers in 2005 studied the axonal growth of mouse DRGs on the fabricated PDMS structures with different surface coatings (Kursumovic *et al.*). They studied the influence of surface topography and surface chemistry. They concluded that the influence of topological structures on the guidance of axons depends very much on whether the surface chemistry of the pattern enhances axonal growth or not. For non-favored surface chemistries contact guidance seems to be very important whereas in the case of favored environment axons seem to not care about contact guidance that much.

Lockery et al (Lockery *et al.*, 2008) presented a new class of microfluidic devices for *C. elegans* neurobiology and behavior: agarose-free, micron-scale chambers and channels that allowed the animals to crawl as they would on agarose. One such device mimics a moist soil matrix and facilitates rapid delivery of fluid-borne stimuli. A second device consists of sinusoidal channels that can be used to regulate the waveform and trajectory of crawling worms. Both devices were thin and transparent, rendering them compatible with high-resolution microscope objectives for neuronal imaging and optical recording.

A study done by Botzolakis and group (Botzolakis *et al.*, 2009) demonstrated the microfluidic approach to precisely control the neurotransmitter transient. This study allowed the evaluation of effects of disease-causing mutations. When this system was

used to apply ultra-brief (~400μs) GABA pulses to recombinant GABAA receptors, members of the cys-loop family of LGICs, the resulting currents resembled hippocampal inhibitory post-synaptic currents (IPSCs) and differed from currents evoked by longer, conventional pulses.

Berdichevsky et al. (Berdichevsky *et al.*, 2010) demonstrated the co-culture from cortex and hippocampus in two compartments connected by microchannels. The result showed that the cultures extended and formed functional connections. This research is useful for understanding the development, plasticity and pathologies of neural pathway.

Recently, a group studied the effect of substrate stiffness on the cellular morphology of dorsal root ganglion (Cheng *et al.*, 2011). They found the higher cell densities of DRG neurons and glial cells on semi-rigid PDMS substrate (35:1 base to curing agent ratio) than on more rigid (15:1) and more flexible (50:1) PDMS substrate, demonstrating a localized bimodal response within a very small difference of elasticity on PDMS.

Most of the studies employed PDMS-based microfluidic devices due to their advantages of thermal stability, biocompatibility, low cost, practical scalability, optical transparency, gas permeability, and easy fabrication using standard soft-lithography. In addition, the elasticity of PDMS matrices enables the integration of pressure-driven valves and pumps within microfluidic channels, permitting execution and automation of complex chemical and/or biological processes within a single device.

2.2 Flow in Channels

The flow of liquid in the channels is the key area of interest in microchannels. As said earlier, the microfluidic flow is used to control the microenvironment of the cells. Laminar flow was previously used to pattern different cells, manipulate different parts of a cell (Jiang & Whitesides, 2003), dynamic control of local temperature (Pearce *et al.*, 2004), etc. Various traditional and non-traditional methods have been applied to pump fluids in the channels. The methods to flow fluid in channels includes pressure-assisted and electrokinetic driven pumping (Sia & Whitesides, 2003), thermocapillary pumping (Burns *et al.*, 1996) which is a surface-tension based pump and uses local heating of droplets, and pumping by using centrifugal forces (Duffy *et al.*, 1999). The drawback of almost all the pumping method is the requirement of external equipment

It was also reported in literature that fluid flow can be controlled, closed or switch using pneumatically actuated valve (Unger *et al.*, 2000; Sia & Whitesides, 2003). The elastomeric property of PDMS can be exploited in making these mechanical valves. This is a multilayer system. When pressure is applied to upper channel, it deflects the thin PDMS membrane downwards, thereby closing the channels. This can stop the fluid flow (single layer fluid channel) or can move the fluid to the other channel (multilayer fluid channels).

A simple pumping method had been reported by Beebe et al. The pumping method exploits the surface energy stored in a liquid droplet and requires a simple device, say a pipette, to flow liquid in the channels (Walker & Beebe, 2002).

The amount of pressure in a liquid droplet is given by Young-LaPlace equation, for a spherical droplet:

$$\Delta P = \frac{2\gamma}{R} \tag{2.1}$$

where, ΔP is difference between atmospheric pressure and pressure inside a liquid droplet, γ is surface free energy of a liquid and R is the radius of the liquid droplet. According to the equation the pressure is inversely proportional to radius; the smaller the radius higher is the pressure. Due to the hydrophobicity of the PDMS, liquid droplet tends to make a spherical droplet. The fluid is pumped exploiting the surface tension of the liquid. The two unequal droplets connected via a fluid filled channel have been used to pump fluid, where fluid will flow from smaller droplet towards bigger droplet.

This pumping method can be used to pump fluid in a laminar flow in two connected channels. Using this method of pumping and the knowledge of growing dorsal root ganglion in the PDMS channels from literature, the following study is based on the culturing of two populations of the DRG neurons and simultaneously stains the two explants.

CHAPTER 3

RESEARCH OBJECTIVE

The following study demonstrated simple PDMS devices for the culture of dorsal root ganglion neurons. The study presents a self-autonomous system using micropipette to generate laminar flow in connected chambers.

The study is divided into two parts. The first part focuses on the device for culturing the neurons. The hypothesis tested was that rinsing the channels with fresh medium resulted in better growth of neurites in microchannels.

The second part aims at simultaneously staining the two cultured DRG neurons with different stains. It is of interest to visibly distinguish the two neurons to study the effect of presence of pioneer neuron on the growth rate of the new generation neuron. The presented study also hypothesize that the flow generation by the liquids of unequal sizes (Walker & Beebe, 2002) can be exploited to simultaneously stain the two neurons growing in channels.

CHAPTER 4

EXPERIMENTAL DESIGN

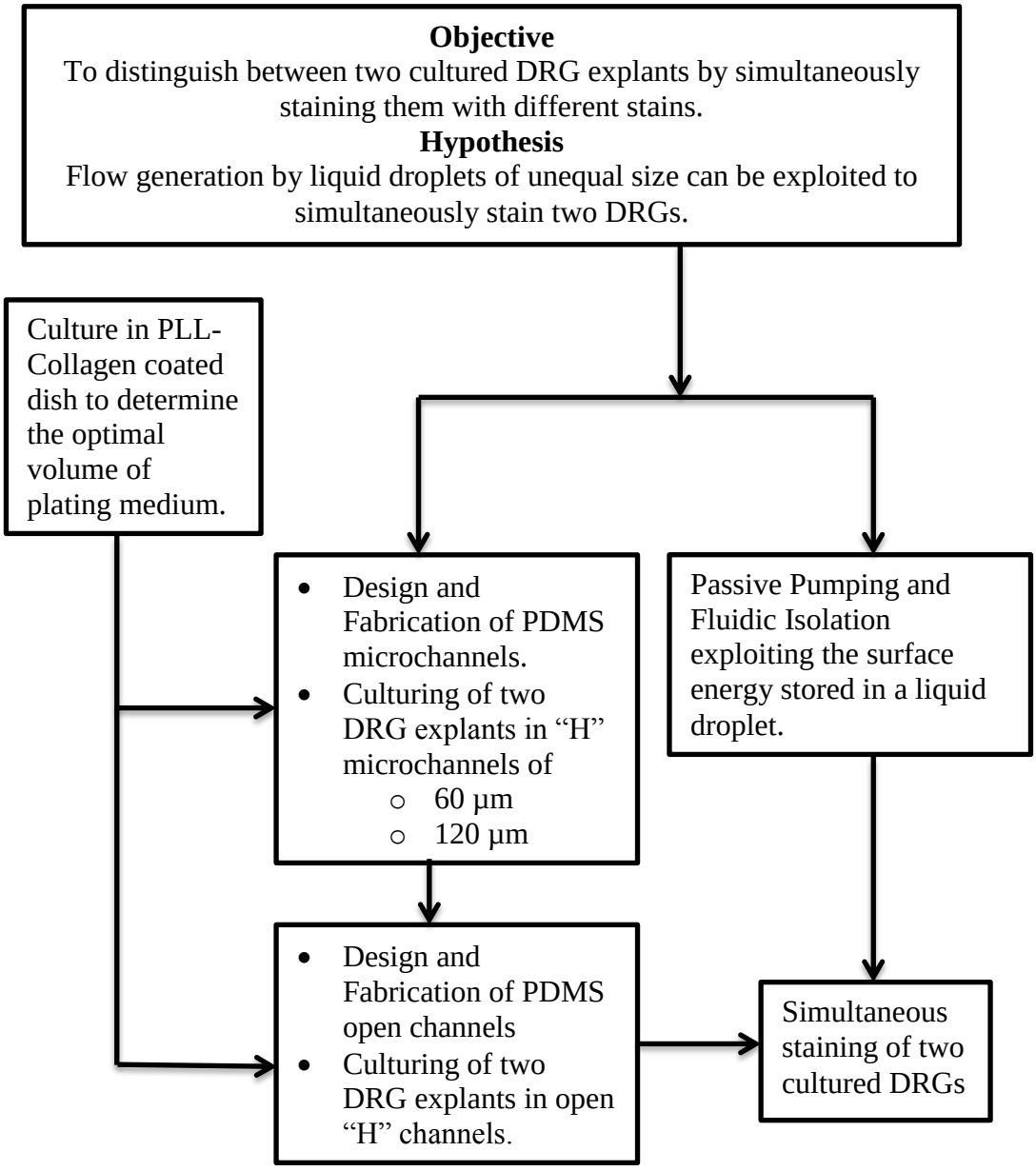

Figure 4.1 Flow chart showing experimental design.

CHAPTER 5

EXPERIMENTAL SETUP

5.1 General Plating of DRGs

5.1.1 Explant Isolation

The sensory neurons, present in the dorsal root ganglion, were embryonic in nature. Timed pregnant female rat (E15-E17) is generally used for isolation of sensory neurons. Younger embryos are poorly formed, therefore makes it difficult to remove the DRGs. On the other hand, older embryos have more completely formed vertebrae, increasing the difficulty of DRG isolation. Both situations lead to reduced cell yields (Burkey *et al.*, 2004). E15 embryos were found to be optimal for isolation of DRGs from the female rat; the DRGs were matured enough to start extending their axons yet soft enough to be easily isolated.

To obtain the cells, pregnant female rat was sacrificed by exposing to 100% CO_2 for 3-5 minutes or until the ceases breathing. The rat was killed by cervical dislocation. The ventral surface of the rat was sterilized with 70% ethanol. Using scissors and forceps the abdomen was cut open by making an incision from the tail to the thorax. Thoracotomy was performed to ensure death by puncturing the diaphragm with scissors. The uterus was dissected out and placed in sterile 100mm dish in dissection hood. Embryos were removed, and placed in Lebovitz L-15 medium.

To extract the DRGs, the head of embryo was pinched off using a forceps between the skull and the first vertebra. Using a micro knife, the caudal side of the pronounced bump on the back of the head, under the ear and under the snout, was cut and

the cord was dislodged. With the embryo on its side, the anterior portion of the abdomen and limbs were removed with a microknife. The embryo was then placed on its back while the remaining viscera are removed with fine forceps (Dumont #5) until there is a clear view of the vertebral column. Beginning at the dorsal end, a pinch was performed through the vertebral column with fine forceps (Dumont #5). Using #4-#5 forceps, the brainstem was grasped and the ménages were pulled straight up. It was then placed in 35mm dish with L-15 balanced medium. With a fresh pair of #5 Dumont biologine tip forceps, the DRGs were plucked off from the isolated spinal cords. The ganglia were then placed in a 1.5mL centrifuge tube with L-15 medium.

5.1.2 Pre-plating Procedure

The cell culture dishes (35 mm x 10 mm) was treated with 1 ml of 100 µg/ml Poly L-Lysine for 4 hours and then rinsed with autoclaved water three times. The dishes were left in the culture hood for drying overnight. The PLL coated dishes were then coated with collagen 1 hour before plating of DRG neurons. 10 µg of 5 mg/ml Type I rat tail collagen was spread with the back of pipette tip to coat the culture dish completely. The collagen was polymerized using ammonium hydroxide (NaOH) vapors for 2-3 minutes which neutralizes the collagen solubilizing acid.

5.1.3 Medium Preparation

The DRG growth medium contains the reagents listed in Table 5.1

Table 5.1 Reagents for Growth Medium for DRGs (50 ml)

	Reagent	Amount (ml)
1.	Neurobasal with B-27 and 0.4-0.5 mM L-Glutamine	48.00
2.	1% FBS	0.50
3.	20% Glucose	0.50
4.	10ng/ml Mitotic Inhibitor (MI)	0.10
5.	100µg/ml Nerve growth factor (NGF)	0.01

All the reagents were thawed to 37°C and mixed in a 50ml centrifuge tube. The tube was wrapped with parafilm and stored at 4°C.

5.1.4 Plating Procedure

The cells were isolated in L-15 medium. L-15 medium was removed and 100 µl of fresh medium was added and gently pipetted. 5 µl of medium with 1-2 explants was plated in the coated dish using a micropipette. Alternatively, 1 µl of medium with 1-2 explants was also plated to determine the minimum amount of medium appropriate for cell plating without cell death. The dish was kept in the incubator for 1-2 hours for the cells to attach to the dish. To avoid explant dying due to small volume of medium and evaporation, kim wipe balls soaked in phosphate buffered saline (PBS) were kept at the periphery of the dish and dish was wrapped in parafilm. After the cells adhered to the dish, kim wipes were removed and 1ml of fresh medium was added to the dish. The dish was wrapped in parafilm and kept in the incubator. The medium was changed after 24-48 hours and cells were monitored periodically.

5.2 Fabrication of PDMS Microchannels

Poly(dimethylsiloxane) (PDMS) was used to make the device for the experiment. PDMS offers various advantages for in vitro cell culture. It is permeable to gases, is nontoxic, autoclavable, inhibits cell adhesion on the surface (unless otherwise pretreated) and is optically transparent.

The PDMS microchannel devices were fabricated by replica molding. The master for soft lithography was prepared using scotch tape (3M Scotch® Transparent Tape 600) yielding microfluidic devices with uniform height of approximately 60 µm (or multiple of 60, depending on the layers of scotch tape used) (Shrirao & Perez-Castillejos, 2010).

5.2.1 Fabrication of Scotch Tape Master

Using Microsoft Visio Professional 2007, the design of channels was fabricated (Figure 5.1).

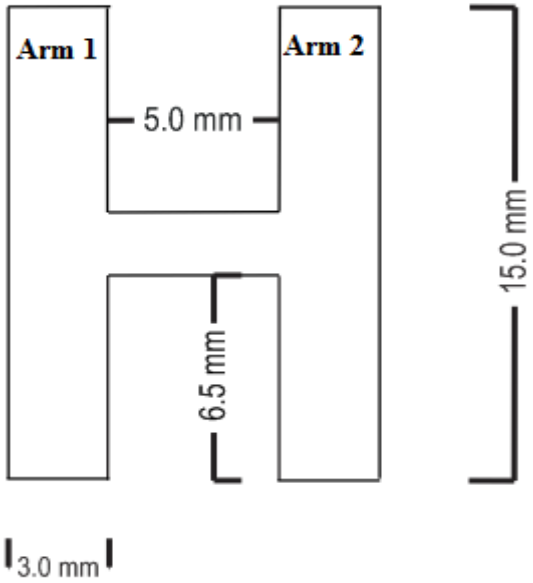

Figure 5.1 Layout of the microchannel. The microchannel is in shape of H. Each arm has length of 15 mm and width of 3 mm. The separation between two arms is 5 mm.

The layout was printed on a paper. Scotch tape was used to fabricate the master. The procedure was adopted from the work of Shrirao et al (Shrirao & Perez-Castillejos, 2010). The thickness of scotch tape used was 60 µm. The glass slide was cleaned using a scotch tape. A strip of scotch tape was attached to the glass slide. To increase the height of microchannel, an additional layer of scotch tape was attached. Two type of microchannels were produced; one with 60 µm and other with 120 µm height. The glass slide was placed on the layout with scotch tape side up. The glass slide was aligned and fixed to the printout using a scotch tape. A sharp scalpel and another glass slide were used to pattern the scotch tape according to the layout. The un-patterned scotch tape was removed from the glass slide. The glass slide with patterned scotch tape was washed with isopropanol to remove any extra adhesive sticking to the glass slide except for the patterned area. The glass slide was then place in oven at 65°C for 2-3 minutes to improve the adhesion of the edges of the patterned scotch tape to the glass slide. The master required no further treatment. The master was kept in a petri dish with patterned side up.

5.2.2 Replica Molding

The PDMS base was mixed with the Sylgard184 Silicone Elastomer curing agent in ratio of 10:1in plastic cup using a fork. The cup was then placed in a degassing chamber for about an hour to remove the bubbles. Once the bubbles had disappeared, the PDMS was poured over the master kept in the petri dish. The PDMS was poured gently to avoid the bubble formation. In case of bubble introduction, the dish was kept in degassing chamber again to remove the bubbles. Once the PDMS is bubble free, the petri dish was kept in the oven at 65°C to cure PDMS. PDMS was cured for at least 24h to avoid the toxicity of the curing agent. This time frame allows the complete polymerization and crosslinking

producing solid PDMS device. Once the PDMS was cured, the scalpel was used to cut the slab of PDMS containing microchannels and was gently peeled off using tweezers. To punch out the reservoirs (Figure 5.2), the PDMS was placed on a clean slab with channels facing up and holes were punched using a sharp punch (diameter = 4 mm).

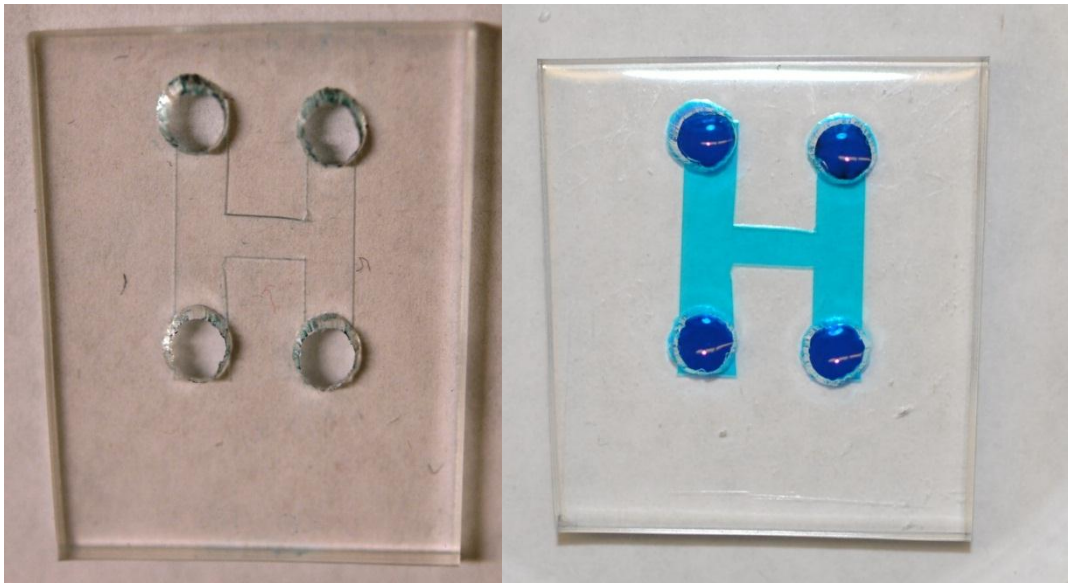

Figure 5.2 "H" microchannels with 4 mm reservoirs punched.

5.3 Cell Culture in PDMS Microchannels

5.3.1 Pre-plating Procedure

The procedure for treatment of cell culture dishes was similar as discussed in section 5.1.2. The cell culture dishes (60 mm x 15 mm) was treated with 2 ml of 100 µg/ml Poly L-Lysine for 4 hours and then rinsed with autoclaved water three times. The dishes were left in the culture hood for drying overnight. The PLL coated dishes were then coated with collagen 1 hour before plating of DRG neurons. 15 µg of 5 mg/ml Type I rat tail collagen was spread with the back of pipette tip to coat the culture dish completely. The collagen was polymerized using ammonium hydroxide (NaOH) vapors for 2-3 minutes which neutralizes the collagen solubilizing acid.

The PDMS device was cleaned using a scotch tape to remove any dust particle sticking to it. The side containing microchannels should be kept dust free, as these particles affect the sticking of PDMS to the substrate and can cause leakage in the channels. The PDMS device should be treated with 70% Ethanol and autoclaved at 121°C for 15 minutes.

The autoclaved PDMS was dried using filter paper in the culture hood. The PDMS device was placed on PLL and collagen treated dishes with microchannels facing the dish.

5.3.2 Plating Procedure

L-15 medium was removed and 100 µl of fresh medium was added and gently pipetted. 5 µl of medium with one explant was plated in the reservoir 1 using a micropipette (Figure 5.3). Similarly the other explant was plated in reservoir 4. The plating was done under the microscope to place the explants properly in the well. 10 µl of medium was added to reservoir 2 and 3. The kim wipe balls soaked in phosphate buffered saline (PBS) were kept at the periphery of the dish and dish was wrapped with parafilm, to minimize evaporation. The dish was kept in the incubator for 1-2 hours for the cells to attach to the dish. After the cells adhered to the dish, 100 µl of medium was added to each reservoir. The dish was wrapped in parafilm and kept in the incubator. The medium was changed after 24-48 hours and cells were monitored periodically.

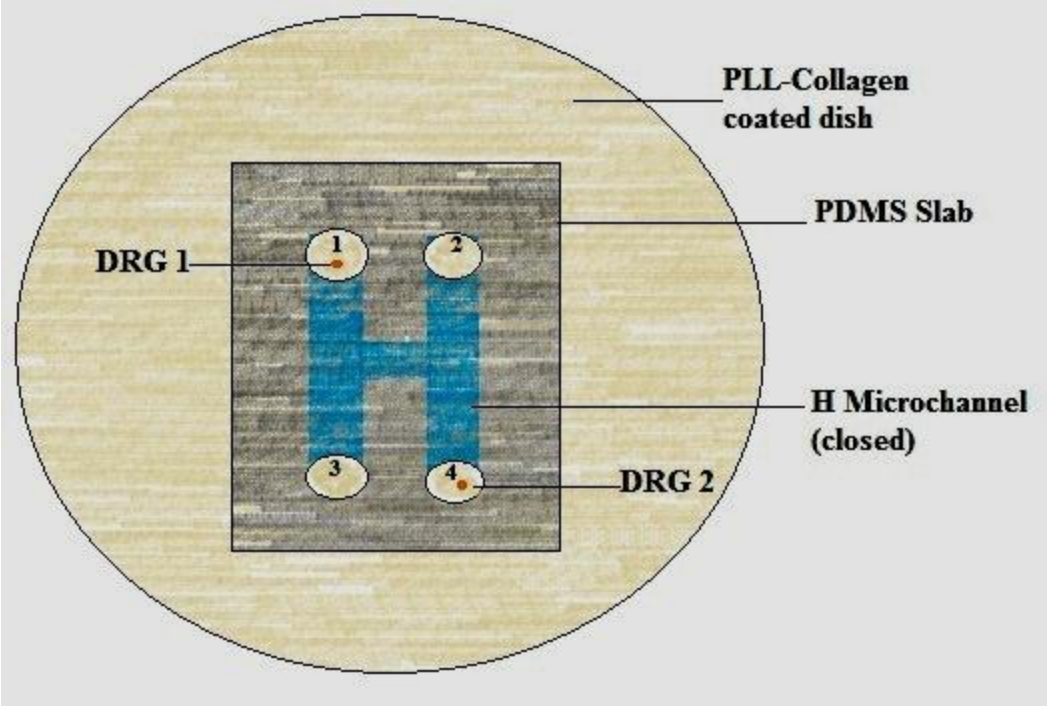

Figure 5.3 Schematics of plating. PDMS device is shown by grey and blue color shows the microchannels facing the plate. 1, 2, 3, and 4 shows the reservoirs of 4 mm diameter each. DRG explants are plated in reservoir 1 and 4.

5.4 Fabrication of PDMS Open Channels

PDMS open channels were also fabricated by soft lithography. The layout of the closed and open channels was same. The open system do not have ceiling to cover the channels as compared to the closed system. The master was designed using ProE and then patterned on Acrylonitrile butadiene styrene (ABS) plastic, which has good impact resistance and toughness, using 3D printer. The master was washed with Sparkleen solution and then rinsed with RO water thrice. This was followed by washing with isopropanol and drying in atmospheric conditions.

Figure 5.4 shows the sketched design of master. The master was 3mm in height and in shape of “H”.

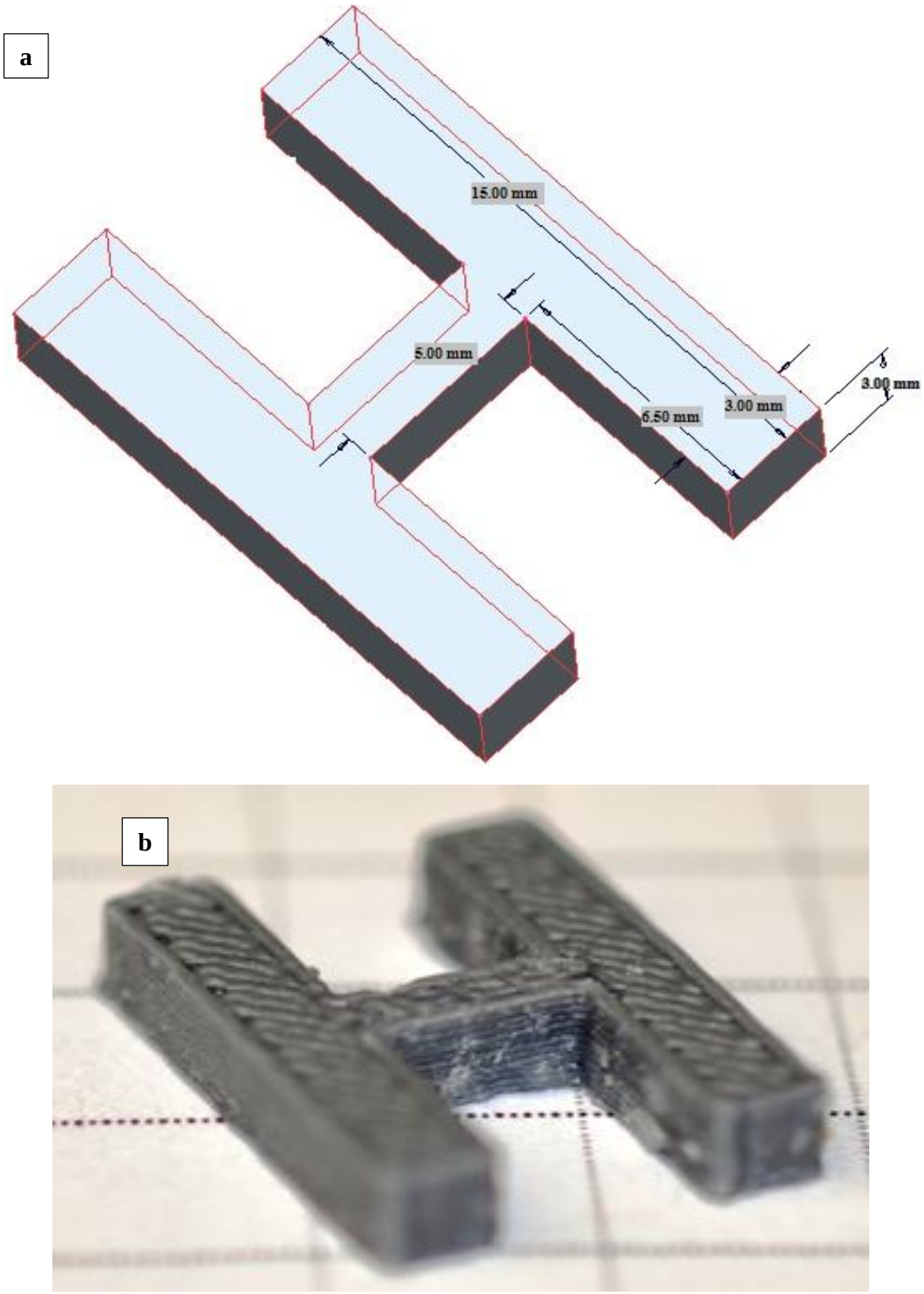

Figure 5.4 3D Master. (a) Layout of the 3D master, and (b) ABS master printed on 3D printer.

The PDMS base was mixed with the Sylgard184 Silicone Elastomer curing agent in ratio of 10:1in plastic cup using a fork and bubbles were removed. A clean glass slide is kept in a petri dish. The master was kept on the glass slide and another glass slide was placed on the top of the master. A glass beaker was kept above the glass slide, and degassed PDMS was poured from the sides. The glass slides and beaker was used to put weight on the H master, so that after curing we get open H channels. The petri dish was placed in degassing chamber to remove any bubble. Once the bubbles were removed, the petri dish was kept in the oven at 65°C for PDMS to cure. After curing (at least 24h), using a scalpel cut the PDMS slab containing the channels. The PDMS channels, thus, resulted were 3mm in height.

5.5 Cell Culture in PDMS Open Channels

5.5.1 Pre-plating Procedure

The PDMS device was cleaned as discussed in Section 5.3.1. The autoclaved PDMS was placed in a cell culture dish (60mm x 15mm). 300 µl of 100µg/ml Poly L-Lysine was filled in the channels and kept for 4h. The PLL was then rinsed with autoclaved water three times. The dishes were left in the culture hood for drying overnight. The PLL coated channels were then coated with collagen 1 hour before plating of DRG neurons. 8-10µg of 5mg/ml Type I rat tail collagen was spread with the back of pipette tip to coat the culture dish completely. The collagen was polymerized using ammonium hydroxide (NaOH) vapors for 2-3 minutes which neutralizes the collagen solubilizing acid.

5.5.2 Plating Procedure

The plating procedure in the PDMS channels was similar as general plating. The schematic of plating is shown in Figure 5.5.

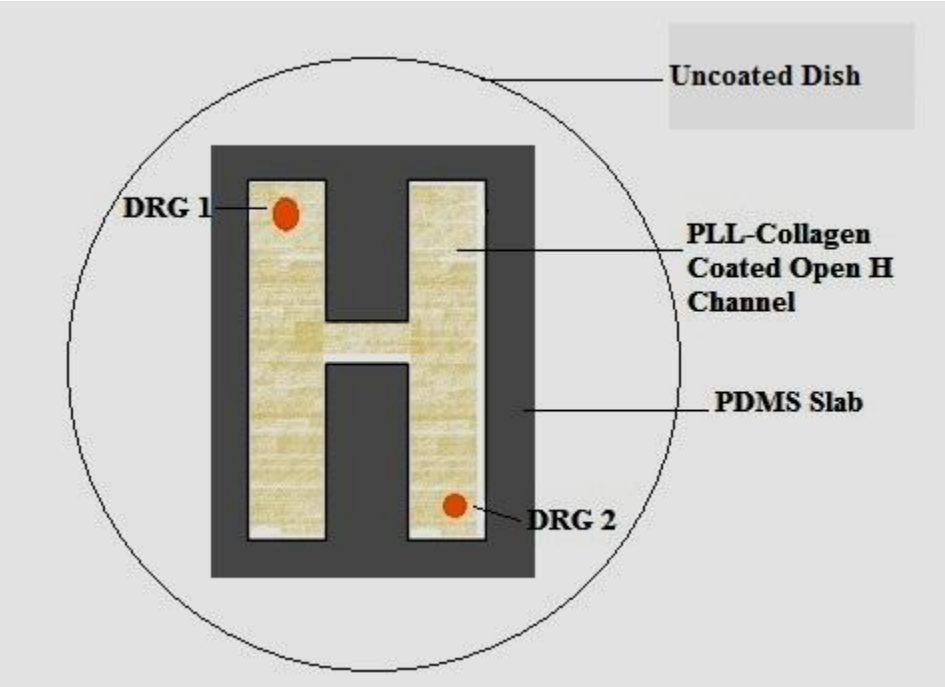

Figure 5.5 Schematics of plating in open channels.

5 µl of medium with one explant was plated in the arm 1 using a micropipette (Figure 5.5). Similarly the other explant was plated in arm 2. The kim wipe balls soaked in phosphate buffered saline (PBS) were kept at the periphery of the dish and dish was wrapped with parafilm, to minimize evaporation. The dish was kept in the incubator for 1-2 hours for the cells to attach to the dish. After the cells adhered to the dish, the channels were filled with 500 µl of fresh medium. The dish was wrapped in parafilm and kept in the incubator. The medium was changed after 24-48 hours and cells were monitored periodically.

5.6 Passive Pumping and Fluidic Isolation

Pumping fluid in the channels, micro or open, is a ubiquitous requirement. The aim was to flow two different liquids in two arms and avoid mixing. This could be achieved by flowing liquid in laminar flow. Walker et al (Walker & Beebe, 2002) suggested semi-autonomous method for pumping fluid in laminar flow in microchannel. The surface energy present in the liquid droplet was used to pump two different liquids in the two arms of the "H".

5.6.1 Fluidic Isolation in Microchannels

The PDMS microchannel device was prepared and cell culture dish was coated with PLL and collagen using procedure described in Sections 5.2.2 and 5.3.1. The cleaned PDMS device was placed on the coated dish with channels facing the dish. The microchannels were filled with water. A larger droplet (100 µl) of water was placed over reservoirs 2 and 3 (Figure 5.3). Smaller droplet (5 µl) of green ink was placed on reservoir 1 and a 5 µl of red ink droplet was placed on reservoir 4. The device was left undisturbed for 30minutes.

The same procedure was repeated using larger droplets of 100 µl of water and smaller droplets of 1µl of green and red ink. Photographs were taken using Nikon camera.

5.6.2 Fluidic Isolation in Open Channels

The PDMS open channels were fabricated as described in Section 5.4. The channels were coated with PLL and Collagen (Section 5.5.1). Additionally, the PDMS base was mixed with the Sylgard184 Silicone Elastomer curing agent in ratio of 10:1in plastic cup using a

fork and bubbles were removed. A thin layer of PDMS was poured in to the petri dish, taking care that no bubbles are formed. The PDMS was cured at 65°C. After PDMS is cured, a rectangular slab of PDMS is cut with scalpel and gently peeled off using tweezers. Four holes were punched of 1.50 mm diameter each using a sharp punch. The holes were punched such that when this slab was used as a ceiling to cover the channels, the holes are on the ends of the two arms.

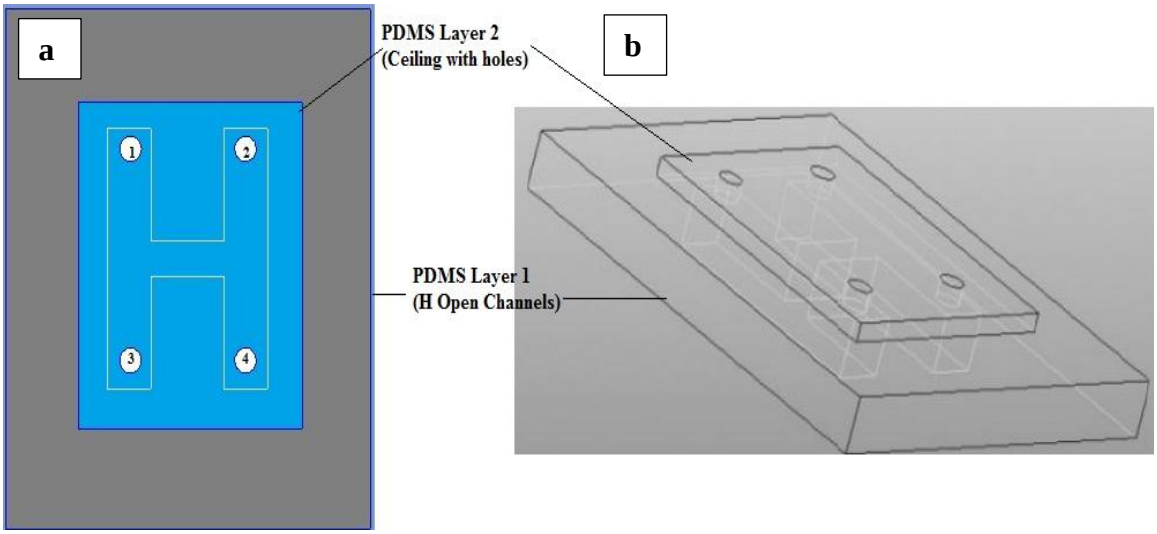

Figure 5.6 Schematics of open channels with ceiling. (a) Top view, grey shows the PDMS slab with channels and blue shows the ceiling on top of channels with four holes of 1.5 mm diameter each, and (b) 3D view.

The open channels were filled with water. The ceiling was placed over the fluid filled channels, as shown in Figure 5.6. 100 µl of water droplets were placed over holes 3 and 4. 5 µl droplet of red ink was placed on hole1 and a 5 µl of green ink droplet was placed on hole4. The device was left undisturbed for 10 minutes. The same procedure was repeated using larger droplets of 100 µl of water and smaller droplets of 1µl of green and red ink. Photographs were taken using Nikon camera.

5.7 Staining of Two DRGs

The research aims at staining the two DRGs with two different stains so that the growth of DRGs can be followed with respect to each other. The concept of passive pumping and fluidic isolation described in Section 5.6 was applied to stain the two DRGs. The stains used were CellTrace™ calcein green AM (Invitrogen) and CellTracker™ Orange CMRA (Invitrogen). Calcein green AM is a cell-permeant dye. In live cells the nonfluorescent CellTrace calcein green AM is converted to a green-fluorescent calcein after intracellular esterases remove the acetoxymethyl (AM) esters. CellTracker Orange CMRA is a rhodol-based fluorophore and remains primarily in the cytoplasm instead of being sequestered inside actively respiring mitochondria. Once inside the cell, these mildly thiol-reactive probes react with intracellular components to produce cells that are fluorescent. The two dyes are light sensitive, so the staining was done in dark.

5.7.1 Calcein AM Stock Solution

The frozen calcein aliquot (50 µg) was thawed at room temperature for 20-30 minutes in dark. The stock solution was prepared by adding 10 µl of dimethyl sulfoxide (DMSO). The mixture was gently pipetted in and out for proper mixing.

5.7.2 Cell Tracker Orange CMRA Stock Solution

The aliquot (50 µg) of orange cell tracker was thawed at room temperature for 20-30 minutes in dark. The stock solution of orange cell tracker was prepared by adding 900 µl of DMSO. The mixture was gently pipetted in and out for proper mixing.

5.7.3 Staining in Open Channels

After the DRGs had grown for about 5-10 days, the neurons in the channels were stained using calcein and orange tracker. The medium was removed from the top of the PDMS slab and the surface is dried using vacuum pump. A thin PDMS ceiling as described in Section 5.6.2 was cleaned and sterilized.

To stain cells, the channels were filled with approximately 300 µl of PBS with calcium and magnesium. The sterile ceiling was placed over the channels. 100 µl droplets of PBS were placed over holes 2 and 3. 1 µl of calcein stock solution and 1 µl of orange cell tracker stock solution were placed over holes 1 and 4 respectively. The dish was placed in incubator at 37°C for 10-15 minutes. The channels were washed with PBS three to four times to wash away any unabsorbed dye. The channels were filled with fresh PBS and kept inside the incubator for 30 minutes. The cells were imaged using fluorescence microscope.

CHAPTER 6

RESULTS AND DISCUSSION

This chapter focuses on the neuron growth in microchannel and open channel PDMS devices, the fluidic isolation results and staining results.

6.1 General Plating

The neurite growth in PLL and collagen coated cell culture was considered as control. This was to determine that the plating medium and the quality of medium are appropriate for the neuron growth. The minimum amount of plating medium that doesn't cause any cell death was 5 µl. The cells adhere to the collagen in one and a half hour at 37ºC. The cells plated with 1 µl mostly die. If the cell was alive, it showed minimal growth. Figure 6.1 shows the neurite growth in control plates with 5 µl and 1 µl plating medium on Day3.

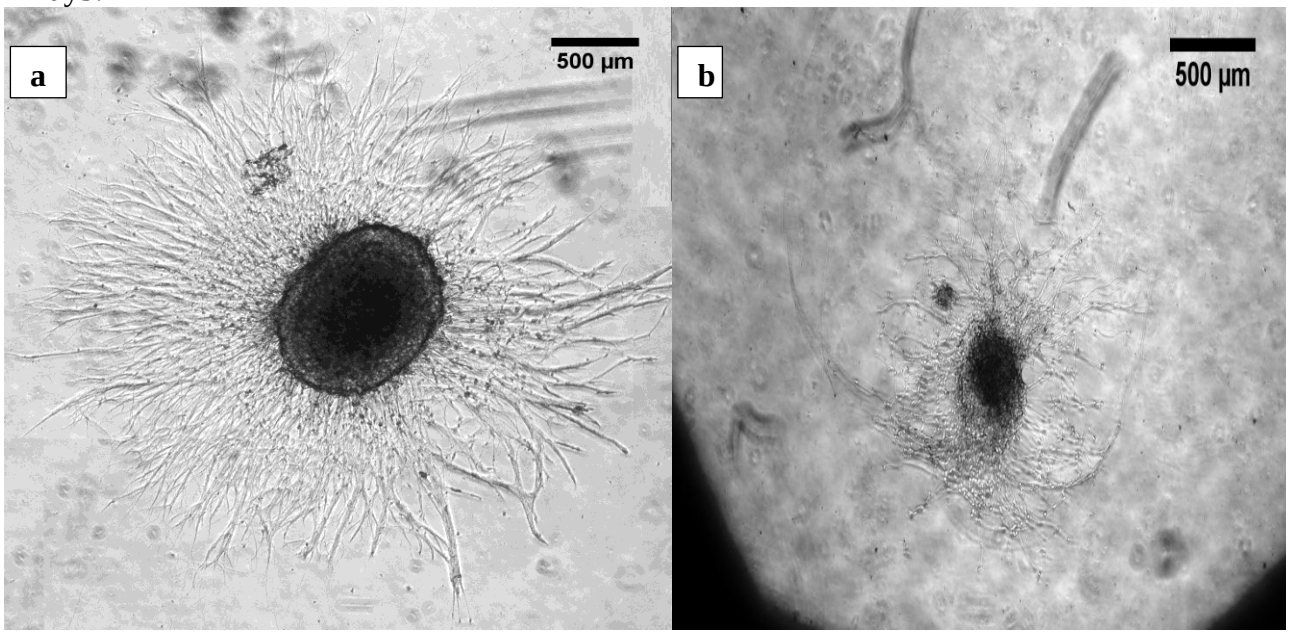

Figure 6.1 Day 3 images of DRGs grown in control plates with (a) 5 µl, and (b) 1 µl of plating medium.

The controls were also kept to test the medium. There was no contamination seen in the plate. The results suggested that 5 µl of plating medium was at least required for avoiding cell death while the cells were adhering to collagen, provided evaporation is minimized by keeping PBS soaked kim wipes at the periphery and wrapping the dishes with parafilm.

6.2 Neurite Growth in Microchannels

Two types of microchannels were used to plate cells: one with 60 µm and other with 120 µm of channel height. The neurites in the microchannel with 60 µm were growing only in the reservoir and not in the channels. The neurites follow the wall of the reservoir.

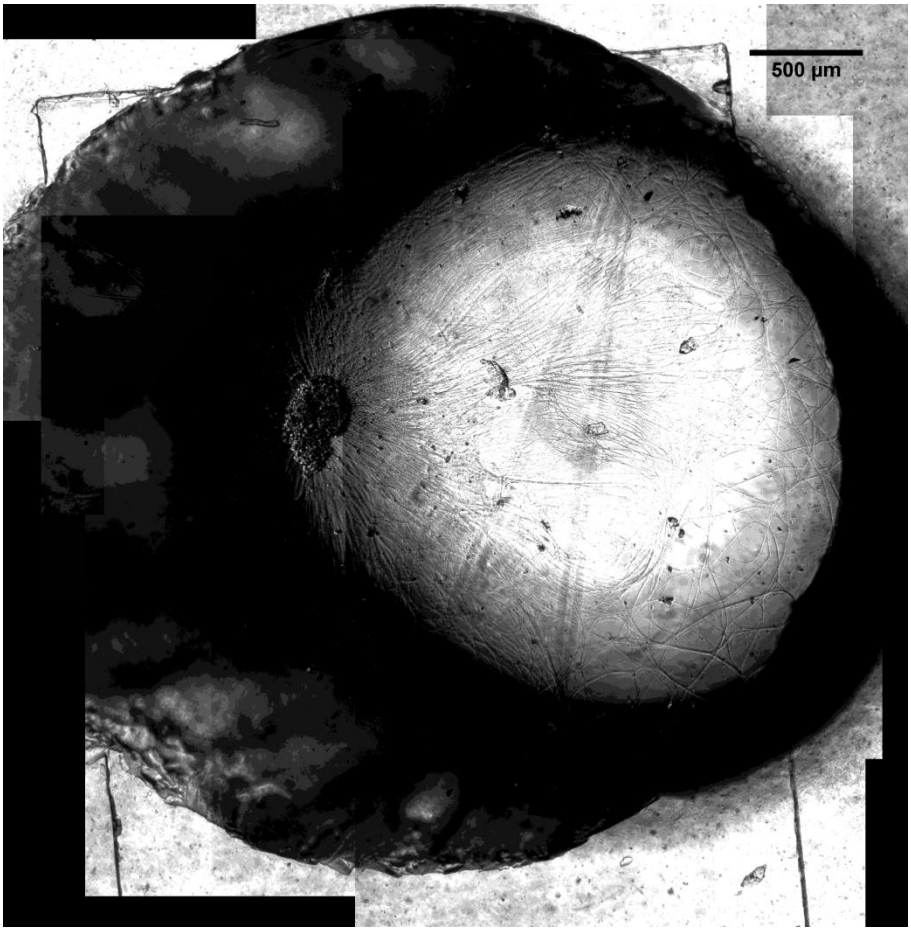

Figure 6.2 Day 20 image of DRG of reservoir 1 in 60 µm high microchannel device.

To test whether the channel height was the reason for no neurite growth in the channels, DRGs were cultured in microchannels with 120 µm height. Figures 6.3, 6.4 and 6.5 show the DRG1 and DRG2 in reservoir 1 and 4 on day 3 and day 12. Few neurites were seen in the channels on day 12.

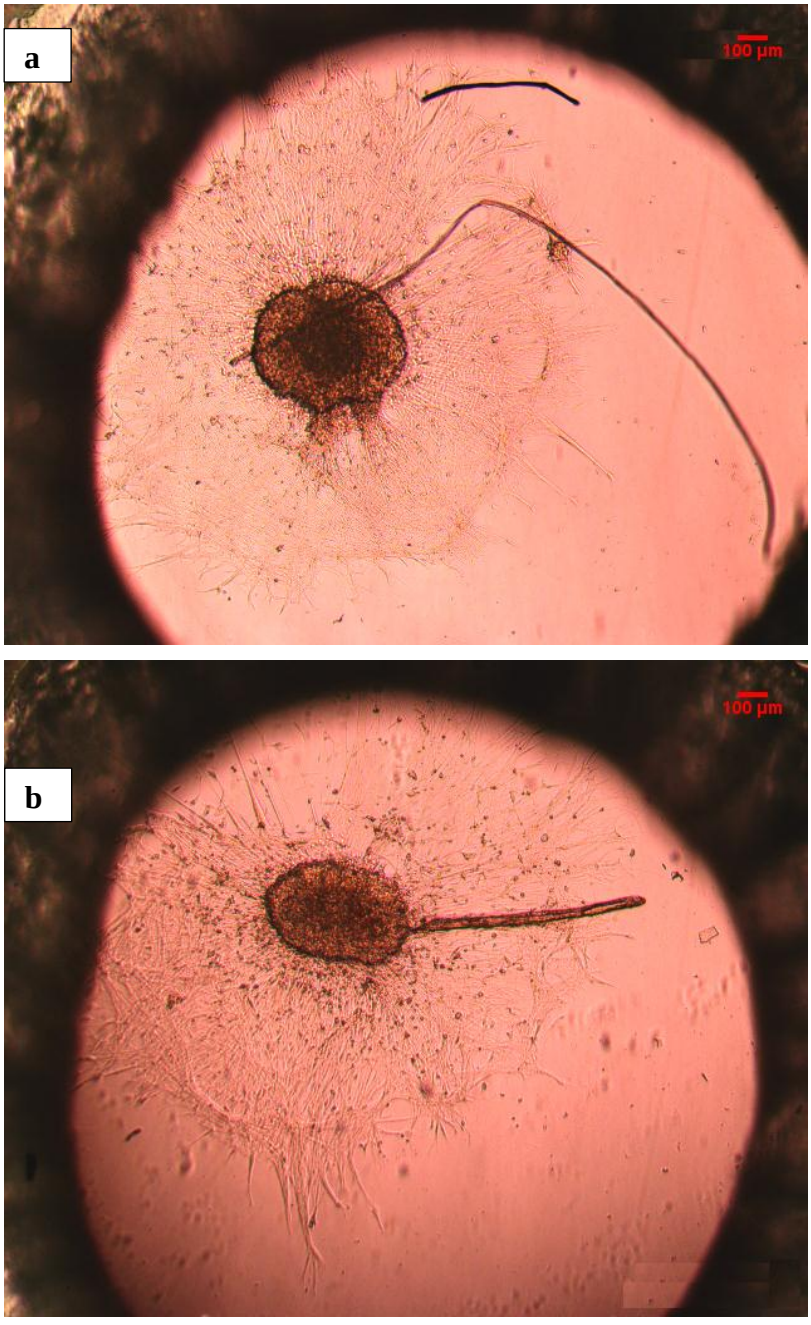

Figure 6.3 DRG in microchannels with 120 µm height. (a) DRG1 in reservoir 1 on day 3, (b) DRG2 in reservoir 4 on day 3.

Figure 6.4 DRG 1 in reservoir 1 in microchannels with 120 µm height on day 12.

Figure 6.5 DRG 2 in reservoir 4 in microchannels with 120 µm height on day 12.

The neurite growth was not seen in the channels may be due to less amount of medium or the restriction to grow. Additional research is required to determine the reason. To enhance the growth of neurites, the culturing was done in open H channels.

6.3 Neurite Growth in Open Channels

The DRGs were grown in opposite arms as discussed in Section 5.2.2. Figure 6.6 show the day 5 culture of two DRGs.

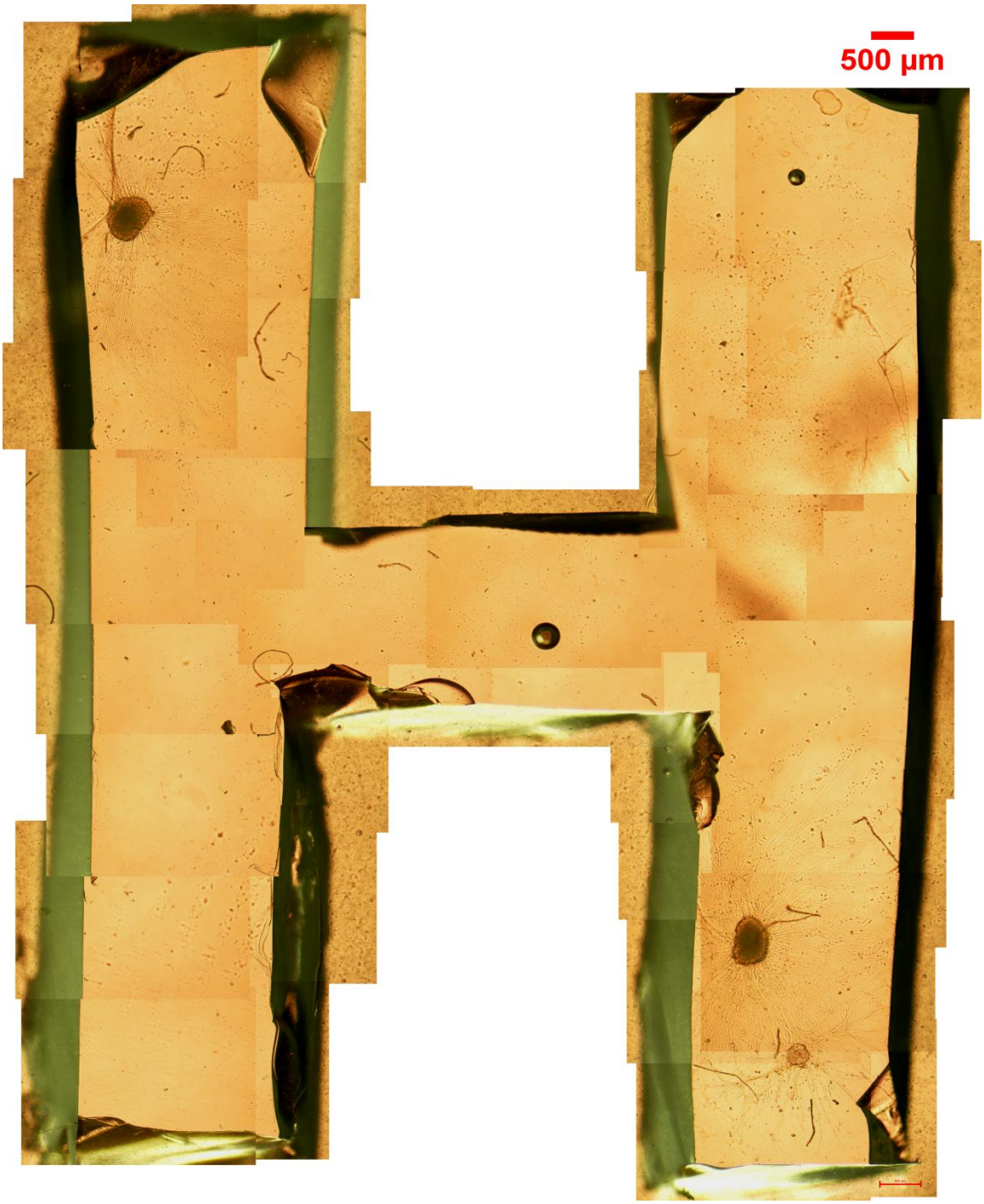

Figure 6.6 Day 5 culture of DRGs in open channels.

The neurites in open channels grow faster than the neurites in microchannels. The reason may be the amount of medium is more and the neurites have no physical barrier such as wall of reservoir in microchannels.

6.4 Fluidic Isolation

The surface energy present in liquid droplet was exploited to flow the liquids in the channels without mixing. This was done in microchannels as well as the open channels. The aim for fluidic exploitation was to determine the volume of liquid droplets and time required for liquid to flow without mixing.

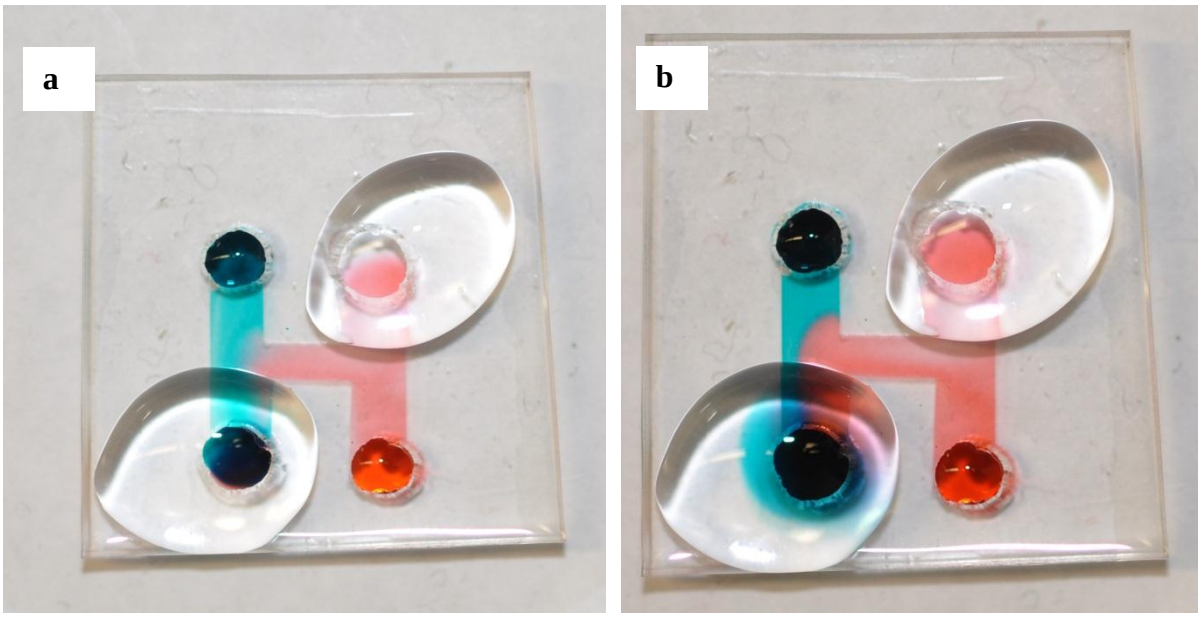

Figure 6.7 Fluidic isolation in microchannels with 100 µl of water droplets and 5 µl of green and red inks. (a) after 10 minutes and (b) after 30 minutes.

The droplet of 5 µl flows through the channels. The ink start mixing after 10 minutes and there was complete mixing around 30 minutes. The same experiment was repeated with larger droplet of 100 µl and smaller droplets of 1µl. Figure 6.8 demonstrates the flow of two liquid without mixing.

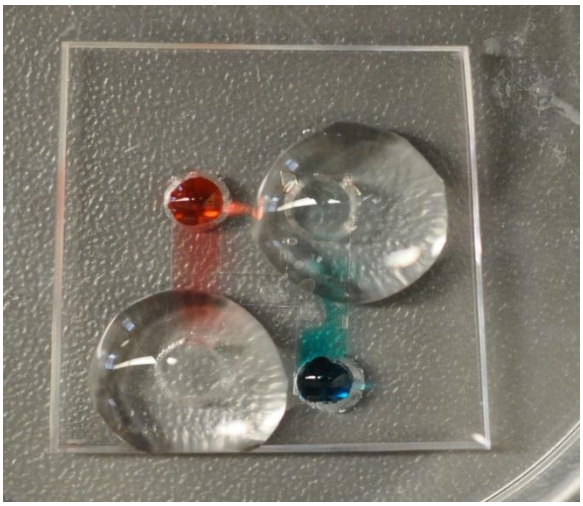

Figure 6.8 Fluidic isolation in microchannels with 100 µl of water droplets and 1µl of green and red inks after 30 minutes.

The flow was successfully driven by unequal droplets. The pressure presented in liquid droplet is inversely proportional to the radius of the droplet. The liquid with smaller radius exert more pressure whereas the pressure exerted by bigger droplet is negligible. The resulted pressure gradient causes the flow of smaller droplets toward bigger droplet (Walker & Beebe, 2002).

Similarly the concept of unequal droplets was applied to the open channels with ceiling place separately.

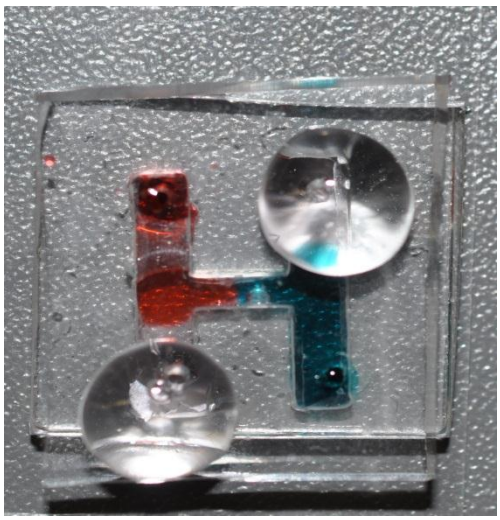

Figure 6.9 Fluidic isolation in open channels with 100 µl of water droplets and 5 µl of green and red inks after 10 minutes.

Figure 6.10 Fluidic isolation in open channels with 100 µl of water droplets and 1µl of green and red inks after 20 minutes.

The results of fluidic isolation experiments in microchannel suggested that the smaller droplets of 1 µl can drive the flow towards bigger droplets without mixing for 10-20 minutes. This experiment demonstrates a simple autonomous method of pumping liquid in laminar flow without using any external pumping apparatuses.

6.5 Staining of Two DRGs

The results of fluidic isolation were used as a base to stain the two DRGs with two different dyes (calcein AM and orange cell tracker). Calcein AM is a non-fluorescent dye that is hydrolyzed by cell intracellular esterases and produce calcein which has absorbance maxima at 490 nm (blue filter) and maximum emission at 520 nm (green filter). The orange-fluorescent CellTracker Orange CMRA is a rhodol-based fluorophore with absorbance and emission maxima at 548 nm (green filter) and 576 nm (orange filter).

The fluorescence microscopy images of two DRGs in open channels are shown in Figures 6.11 and 6.12

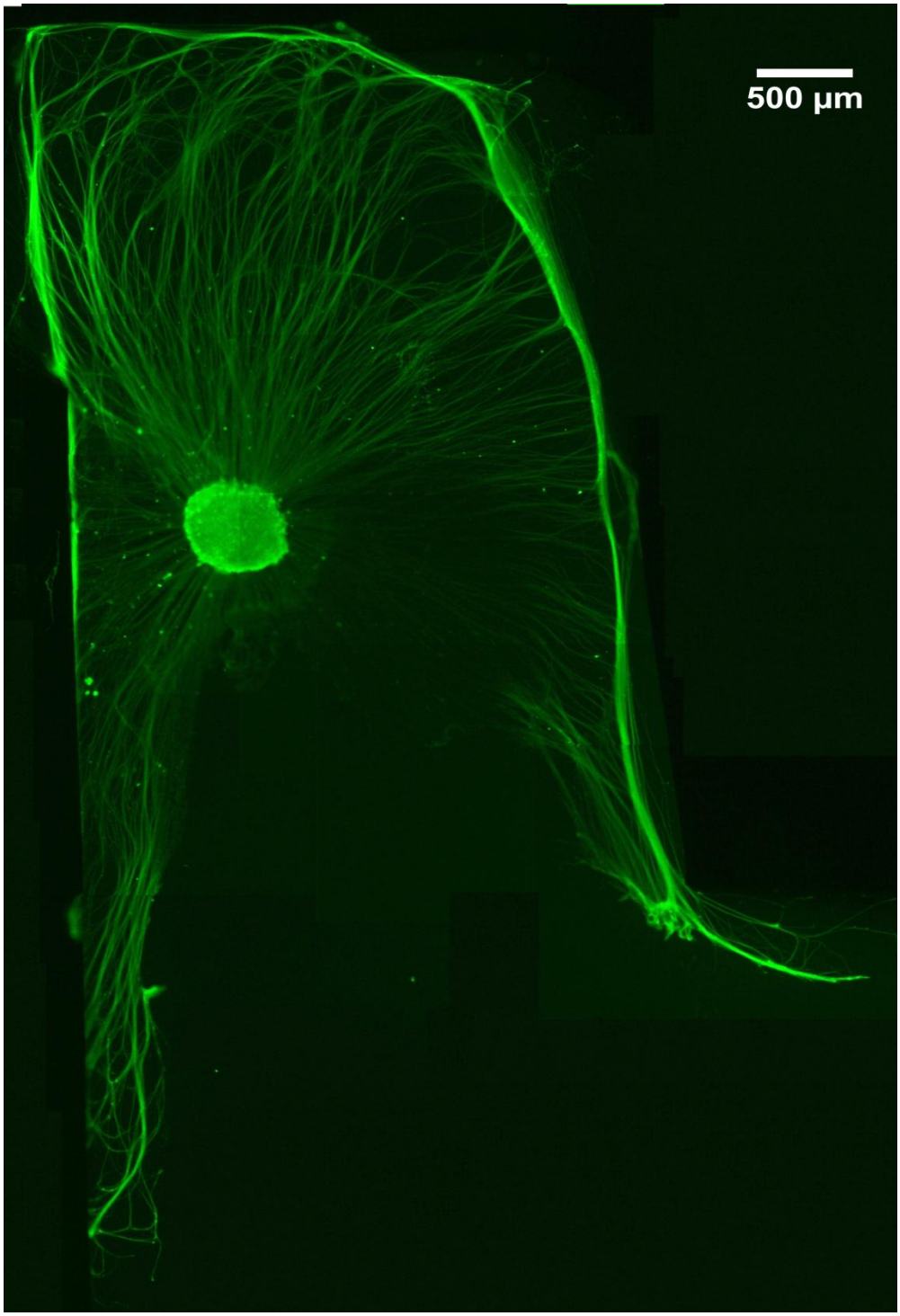

Figure 6.11 DRG1 stained with calcein in open channel arm1.

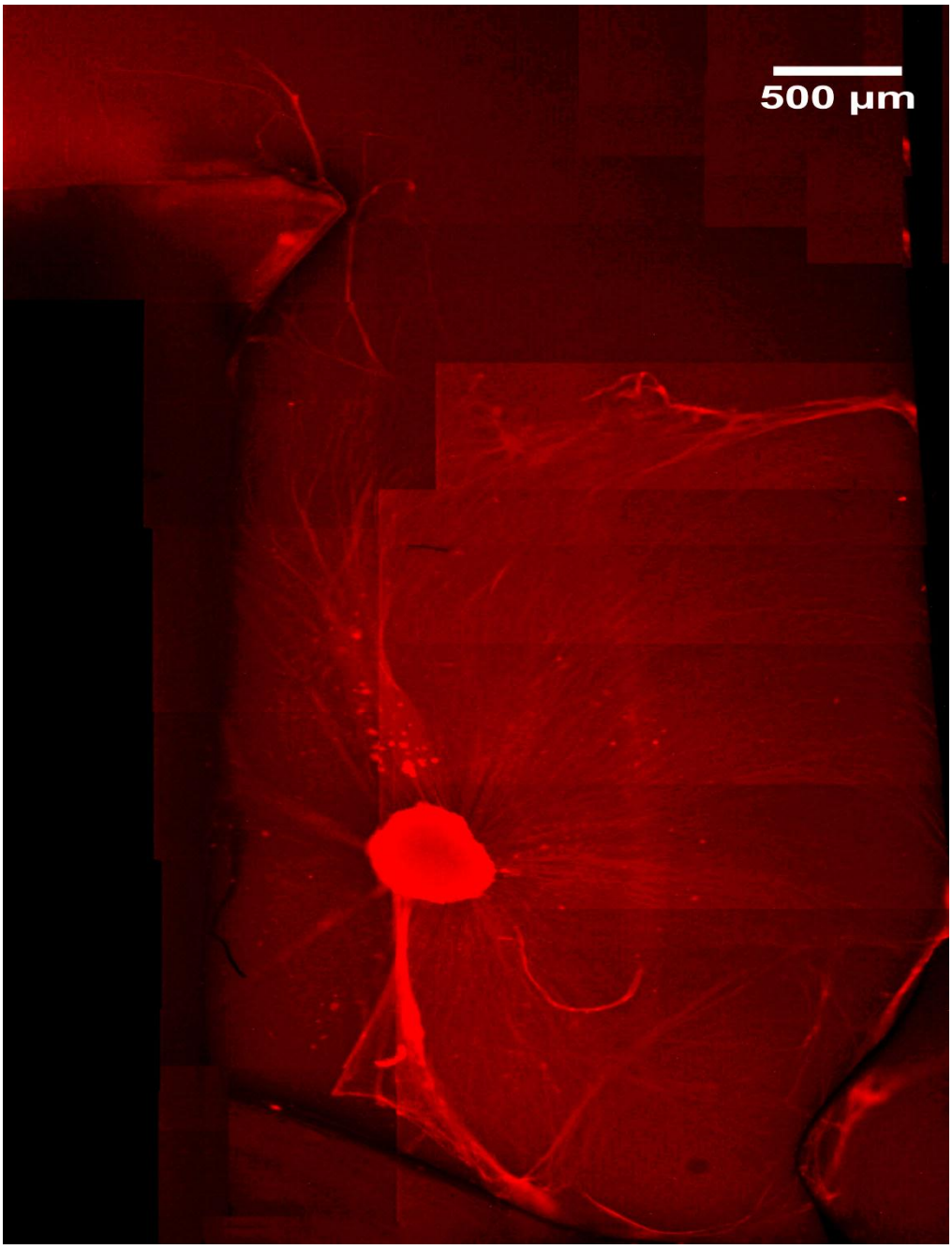

Figure 6.12 DRG2 stained with orange tracker in open channel arm2.

The successful staining of the two DRGs was achieved without mixing of stains both in microchannels and open channels. Calcein stained neurons and neurites images are sharper than those of orange cell tracker stained neurons. There is much background seen around the neurons stained with orange cell tracker even after three-four washings.

The fluorescence microscopy images show that the neurons when culture in open PDMS channels show much faster neurite growth than the microchannels. The neurites in the microchannels follow the trajectory of the reservoir's wall. Due to medium volume difference in reservoir and microchannels, the neurites preferably grow in the reservoirs. Although the neurites in open channels also follow the trajectory of the "H" walls, the neurites can be much better seen as growing in all directions.

CHAPTER 7

CONCLUSIONS

The presented study demonstrated a simple PDMS device to grow dorsal root ganglion neurons. Single explants were successfully grown in microchannels and open channels. However, the growth rate of the neurites differs in the two types of channels due to difference in volume of maintenance medium and physical barrier for the growth of neurites in case of closed channels. Although the closed system was not very successful for the presented study, it could be used to culture the other types of cells that can be grown in the channels and not in the reservoirs only. Device optimization could be achieved by growing other type of cells (dissociated DRGs or other smaller cells) in the channels; thereby the reason for slow and confined growth rate in closed channels could be identified.

The study also reported a simple self-autonomous method of isolating fluids exploiting the surface tension of a liquid droplet, as suggested by Walker et al. (Walker & Beebe, 2002). The method requires no expensive apparatus. This method can be further exploited to provide the two cells with different environments, as required by any research.

The simultaneous staining of two DRGs in culture was successfully achieved. This method is cost effective as compared to the use of transgenic animals. The simultaneous staining of two DRGs can be further used in distinguishing the two populations and studying the effect of presence of pioneer neuron on the growth pattern of the new generation.

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