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Axon guided axon growth in DRG neurons from the spinal cord of rat embryos

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ABSTRACT

AXON GUIDED AXON GROWTH IN DRG NEURONS FROM THE SPINAL CORD OF RAT EMBRYOS

by
Neha Jain

Early studies in the area of neuronal physiology and injury have shown that axons are guided by a number of physical as well as chemical cues. Although the guidance effect of initial axons and their processes on later axons has been shown from a development point of view, little light has been thrown on the subject from the injury point of view. This important ability of axons which plays a key role during development and formation of neuronal networks could become crucial in repairing damaged nerves and disrupted neuronal tracts.

In the following study, the guidance effect of an older population of neurons on a younger population was studied using a specialized PDMS well. Embryonic DRGs derived from rats were used for the study and it was found that axons could be guided by other axons. The cues or the signaling mechanism responsible appeared to be contact dependent. However, more studies should be done in this direction to verify these results.

AXON GUIDED AXON GROWTH IN DRG NEURONS FROM THE SPINAL CORD OF RAT EMBRYOS

by
Neha Jain

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biomedical Engineering

Department of Biomedical Engineering

May 2010

APPROVAL PAGE

AXON GUIDED AXON GROWTH IN DRG NEURONS FROM THE SPINAL CORD OF RAT EMBRYOS

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Dedicated to

To the Divine energy

That pervades the entire creation

And to

Mom, Dad and Niku

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CHAPTER 1

INTRODUCTION

1.1 The Human Nervous System

The human nervous system is divided into two parts: central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises of the brain and the spinal cord and the PNS includes all the nerves outside the CNS. The sensory nerves in the PNS receive the various stimuli from the environment and pass on these impulses to the CNS. The CNS decides on how to respond to a particular stimulus and then returns the impulse to the motor nerves which are also a part of the PNS.

1.2 Physiology of a Neuron

A neuron is the cell of the nervous system. Although the nervous system also consists of a large number of glial cells which support the neurons, we will only focus on neurons here. A neuron has four morphologically defined regions:

1.2.1 Cell Body or *Soma*

It is the metabolic center of the cell. It houses the nucleus which stores the genes of the cell as well as the endoplasmic reticulum, where the cell's proteins are synthesized. It comprises of less than a tenth of the neuron's volume.

1.2.2 Dendrites

They are short, branched extensions that protrude from the surface of the soma. They branch out in a tree-like fashion and are the main apparatus for receiving incoming signals from other nerve cells. A synapse is a place where one dendrite meets another neuron's impulse.

1.2.3 Axon

The cell body or *soma* usually gives off a single axon, a thin process that propagates electrical impulses, often over considerable distances (0.1 mm to 3 m), to the neuron's synaptic terminals on other nerve cells or target organs.(Eric R Kandel, 1991) Most axons in the CNS are very thin (0.2-20 micron) as compared to the diameter of the cell body (50 micron). An axon branches distally into a lot of fine branching called the telondria, which synapse to dendrites from other neurons. Once the impulse reaches the synapse, neurotransmitters (chemicals which excite or calm neurons) diffuse into the extra cellular space from one neuron and reach the dendrites of other neuron, once again turning into an impulse.

1.2.4 Pre-synaptic Terminal

The point at which two neurons communicate is known as a *synapse*. The nerve cell transmitting a signal is called the *presynaptic cell.* The cell receiving the signal is the *postsynaptic cell.* The presynaptic cell transmits signals from the swollen ends of its axon's branches, called *presynaptic terminals.* However, a presynaptic cell does not actually touch or communicate anatomically with the postsynaptic cell

since the two cells are separated by a space, the *synaptic cleft.* Most presynaptic terminals end on the postsynaptic neuron's dendrites, but the terminals may also end on the cell body or, less often, at the beginning or end of the axon of the receiving cell.(Eric R Kandel, 1991).

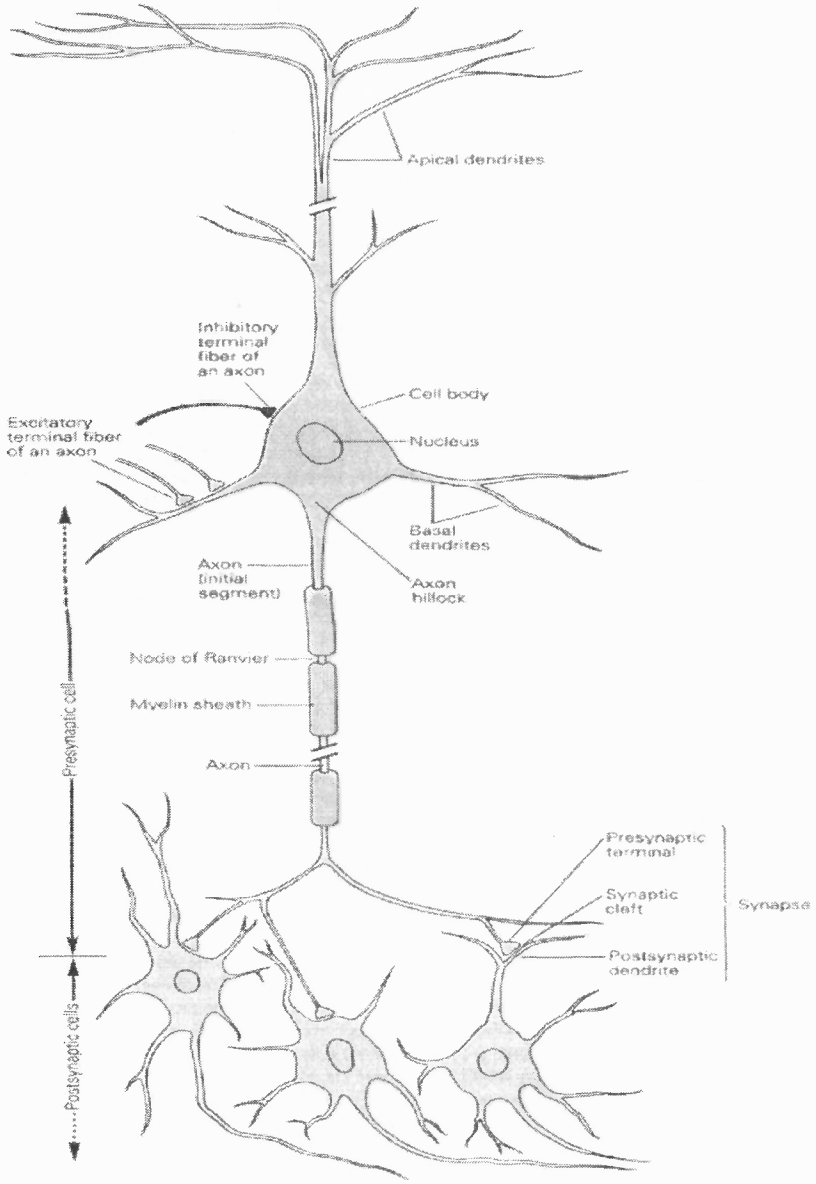

Figure1.1 Structure of a neuron(Eric R Kandel, 1991).

Figure 1.1 refers to the structure of the neuron. Many axons are insulated by a fatty sheath of myelin that is interrupted at regular intervals by the nodes of Ranvier. The action potential, the cell's conducting signal, is initiated either at the axon hillock, the initial segment of the axon, or in some cases slightly farther down the axon at the first node of Ranvier. Branches of the axon of one neuron (the presynaptic neuron) transmit signals to another neuron (the postsynaptic cell) at a site called the synapse. The branches of a single axon may form synapses with as many as 1000 other neurons. Whereas the axon is the output element of the neuron, the dendrites (apical and basal) are input elements of the neuron. Together with the cell body, they receive synaptic contacts from other neurons. (Eric R Kandel, 1991)

1.3 Anatomy of the Spinal Cord

The spinal cord is a highly organized and complex part of the central nervous system.(Vrbová) It extends from the base of the skull to the first lumbar vertebra. The spinal cord receives sensory information from the skin, joints, and muscles of the trunk and limbs and contains the motor neurons responsible for both voluntary and reflex movements.(Eric R Kandel, 1991)

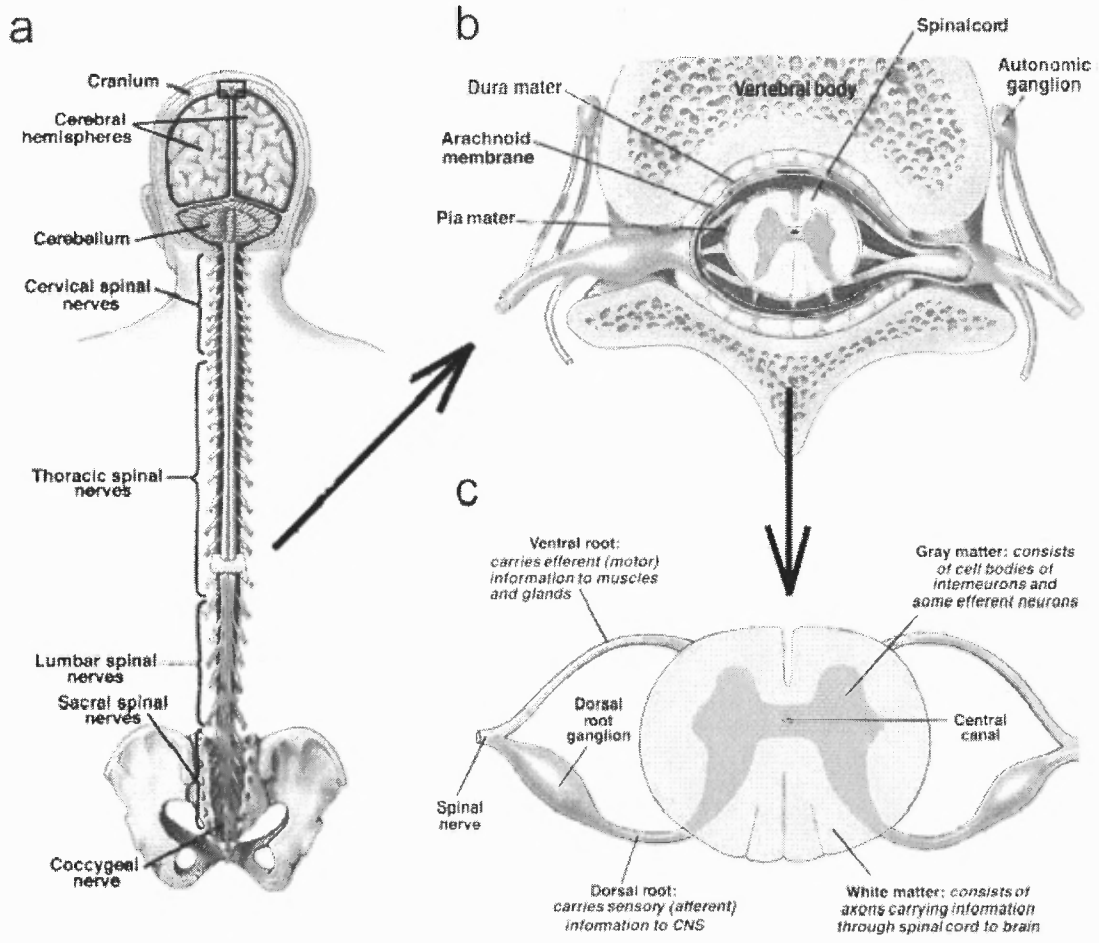

Figure 1.2 Anatomy overview of the Spinal cord.

Figure 1.2 refers to the anatomy of the spinal cord. The spinal cord is composed of the cervical, thoracic, lumbar, sacral, and coccygeal spinal regions. (*b*) Nerves, well protected by vertebrae and protective membranes, project from each side of the cord and connect with innervation sites in the left and right sides of the body. (*c*) The nerves on each side are further subdivided into roots. The ventral root carries motor signals from the CNS to the muscles and glands, whereas

the dorsal root carries sensory signals coming into the CNS. The dorsal root ganglia are located along the dorsal root and contain the cell bodies of sensory neurons. (Leach, 2003)

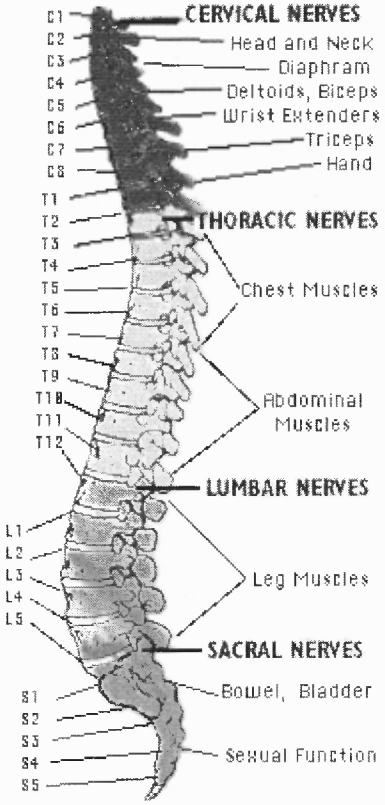

Figure 1.3 Functions associated with the different nerves of the spinal cord.

Source: http://www.neinstein.com/areas-of-expertise/brain-and-spinal-injuries/

The spinal cord is encased by the vertebral column that protects it from damage. Nevertheless, the spinal cord is extremely fragile and damage to it can cause temporary or permanent impairment of function. Figure 1.3 shows how different nerves and thus different organs are associated with the spinal cord.

While the neurons killed by injury and secondary cascades involved in stabilization of the wound contribute to functional deficits, a large proportion of the deficit is caused by the disruption of axonal tracts responsible for neural communication.(Roy M. Smeal, 2008)

Long gaps in the spinal cord are difficult to heal, particularly those longer than 10 mm. Various kinds of bridging devices with specific geometries have been used to treat long gaps in Spinal cord injury. Fibers, meshes and cylinders made of Polypropylene, Poly Lysine and other such polymers have been used. (Skornia, 2004, Roy M. Smeal, 2005, Xuejun Wen, 2005, Roy M. Smeal, 2008) Many materials were used in an attempt to physically guide the regeneration of damaged peripheral nerves, including autologous nerve grafts, bone, metal tubes, fat sheaths.(Leach, 2003) Various natural materials like hyaluronic acid, fibrinogen, fibrin gels, self assembling peptide scaffolds, alginate, agarose and chitosan have been investigated for nerve repair. Also, synthetics like PGA(Poly Glycolic Acid), PLA(Poly Lactic Acid), PLGA(Poly lactic-co-glycolic acid), Polycaprolactones and other polyesters have also shown potential for nerve repair.

But none of the above approaches have shown great success in terms of restoring structure and functionality. Stretch grown axons are mechanically stretched in an elongator at a fixed rate (Pfister BJ, 2006). These axons might be as long as a few cms. Stretch grown axons have been shown to retain their electrical activity (Bryan J. Pfister, 2005, Pfister BJ, 2006).

If we can observe the guidance effect we discussed earlier, then we can use the stretch grown axons (Bryan J. Pfister, 2003) to act as pioneer axons and guide freshly harvested neurons plated close to them. Here, the newly plated cells would behave as

axons and would help bridge the long gaps in spinal cord injuries. Ofcourse, the model would have to be refined a lot for that. Here, we are trying to confirm our hypothesis that axons are indeed guided by pioneer axons. The guidance although has been researched upon a lot, we here bring a new "injury" perspective to the whole problem.

Most of the past work on the same topic has been done using animal models. Although an animal model is a complete system, it is difficult to control the influencing factors in a complex system like that. Also, for studying the molecular mechanisms that are involved in signaling, it becomes necessary to have an in vitro system where the various factors can be individually studied and manipulated.

CHAPTER 3

OBJECTIVE

In the following study we are presenting an in vitro system for studying the guided growth in axons. We did this by plating Dorsal root ganglion neurons from rat embryos inside PDMS stamps. We let these neurons grow and then added more neurons on top of the older cells to see if the presence of the older cells had a guidance effect on the newer/younger cells.

This has important applications in understanding the dynamics of early growth and development and injury. This study can help us understand the early stage developmental process more clearly and the insights gained could be used for solving regeneration problems. We expect to see the newer cells being guided by the older cells meaning the newer cells would preferentially grow towards the older cells or try to align with them.

CHAPTER 4

MATERIALS AND METHODS

4.1 Scratch Experiments

Glass coverslips (Hausser Scientific, 22 x 22 mm, Size 2) were first washed with Sparkleen-water soultion and then rinsed three times with RO (Reverse Osmosis) water. This was followed by a wash of ethanol and a UV radiation exposure for a minimum of 30 minutes. This was done to exterminate any microbial growth on the surface of the material. The coverslips were then placed in sterile tissue culture dishes of 35 mm (Griener Bio-one Cellstar). Later, the surface of these coverslips were scratched using a scratch pen by hand. Attempts were made to keep the pressure same while scratching all the plates. One set of coverslips (Set A) was treated with PLL and collagen and then scratched while the other set (Set B) was first scratched and then treated with PLL and collagen.

4.1.1 PLL and Collagen Treatment

The coverslip-dish assembly was treated with PLL (Poly L-Lysine) diluted 1000 times in water for 4 hrs and then rinsed with autoclaved water three times. The plates were left in the culture hood for overnight drying.

The dishes were then treated with rat tail collagen (BD Biosciences), 25 μl was spread on each coverslip. The collagen was gelled using ammonium hydroxide vapors. The dishes were allowed to sit at least for an hour before they were plated with cells.

4.1.2 Explant Isolation and Cell Seeding

DRGs are found in intervertebral, where the dorsal and ventral roots merge. The DRG ganglion cells are about 10-100μm in size. DRGs are used due to their robust and regenerative nature of the PNS and their potential for nervous system repair (W., 1996)

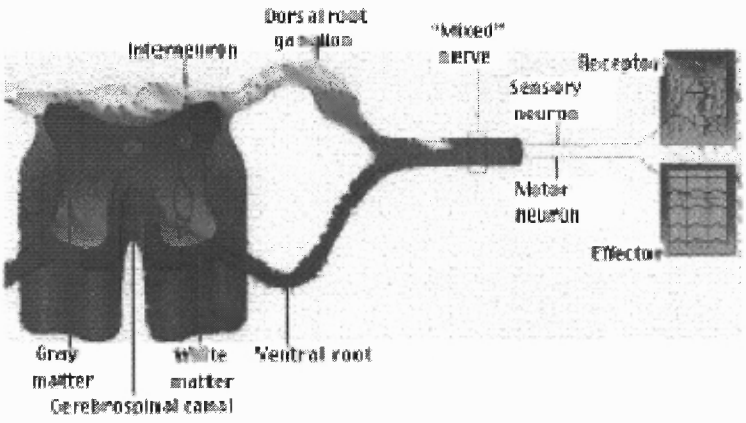

Figure 4.1 Location of DRG explant in the spinal cord.

Source: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/CNS.html

To obtain the required cells, a female rat was exposed to 100% CO2 for 3-5 minutes (until rat ceases breathing for 5 minutes). The counting of pregnancy starts from Day 1, the day after mating. E14 (Embryonic day 14) embryos are difficult to extract due to the delicate tissue and the spinal cord yields easily. Moreover, E16 embryos have more firm spinal cords, but about ½ of DRGs can only be extracted. And, by E17, the DRGs could not be extracted. Therefore, E15 is found to be the optimal because it is the period when the DRGs are matured enough to start extending their axons towards the extremities, but they are soft enough to be easily plucked by the forceps.

To perform the extraction, the rat was placed on its back, and the abdomen was sterilized with 70% ethanol. Scissors and forceps were used to make an incision from the tail to the thorax. Then a thoracotomy was performed to ensure death by puncturing the diaphragm with scissors. Using a clean set of scissors and forceps, the C-section was performed and the uterus was dissected (from both sides) to remove the liters which were then placed in sterile 100mm dish. Then under the dissection hood, embryos were removed, and place in Lebovitz L-15 media.

To extract the DRGs, the head was first cut off the embryo between the skull and the first vertebra. Using a micro knife, the caudal side the pronounced bump on the back of the head, under the ear and under the snout, are cut. This leaves some brain stem visible and from which the cord is pulled out. 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 are pulled straight up. It is placed in 35mm dish with L-15 balanced media. With a fresh pair of #5 Dumont biologine tip forceps, the DRGs are plucked off from the isolated spinal cords. The ganglia were then placed in a 1.5mL centrifuge tube with L-15 medium. About 2 embryos were used for each H plate.

4.1.3 General Plating Procedure

L-15 was removed from the cells and about 500 microliters of fresh media was added to the tube. The cells were gently pipetted and about 100 microliters of media along with 2-3 explants were plated directly on top of the scratched surface of the glass coverslips. The dishes were then left inside the incubator for 3 hrs. This time frame allows the DRGs to adhere to the surface of the dish. After 3 hrs fresh media was poured into the dish, 2 ml for a 35 mm tissue culture dish. The media was changed every two days and the cells were periodically monitored and imaged.

4.1.4 Media Preparation

The media contained: Neurobasal medium with B-27 and 0.4-0.5 mM L-Glut, 1% FBS (heat inactivated), 1 mL 20% Glucose, 10 ng/ml MI (Mitotic Inhibitors) and 10 ng/mL NGF (Nerve growth factor).

4.2 PDMS Channels Experiment

4.2.1 Making PDMS Channels

Poly (dimethyl siloxane) was used to make stamps for the experiments. PDMS was chosen because of its high optical resolution, bio compatibility, high oxygen permeability, and its ability to regenerate surface features. Also, it can be autoclaved to sterilize it.

PDMS base was mixed with the Sylgard 184 Silicone Elastomer curing agent in the ratio of 10:1 in a plastic cup. The two were mixed vigorously using a plastic fork, till the contents turned milky. The cup was then placed in a degassing chamber for about an hour and taken out of the degassing chamber when all the bubbles had disappeared. The

hour and taken out of the degassing chamber when all the bubbles had disappeared. The mixture was then very gently poured in to a 10 mm tissue culture plastic dish. This process if not done gently introduced bubbles into the PDMS, in which case the dish was kept in the degassing chamber for 30 additional minutes. Once, the PDMS inside the dish was free of bubbles the dish was left in the oven at 65 C overnight for curing and then was left outside at room temperature for atleast 24 hrs. Once the PDMS was cured it was gently peeled off using tweezers. The slab could be cut into desired dimension of channels using a sharp blade.

The channels used in this experiment were about 1.5 mm thick with length of 15 mm and height of 2 mm. The channels were carved out from PDMS slabs with a flat blade using hand. The channels were washed with Sparkleen water solution and then rinsed with RO water thrice. The channels were then briefly washed with ethanol to remove any finger grease. Finally they were autoclaved for an hour in a glass jar and then left in the laminar flow hood to dry.

4.2.2 Pre-plating Procedure

Glass coverslips were cleaned as explained in Section 4.1. Once the cover slips had dried they were treated with PLL & collagen as mentioned before. After an hour of collagen application to the coverslips, right before plating the cells, the channels were laid out on a filter paper. This was done to soak up excess water that might have wetted the surface of the PDMS channels. After dabbing them on the filter paper for a minute or two, the channels were carefully placed on the coverslip using metal forceps. The top of the channel was pressed gently with the rear end of the forceps to remove any trapped air between the surface of the channel and the coverslip.

4.2.3 General Plating Procedure

L-15 was removed carefully using a pipette and the explants were suspended in about 100 microliters of fresh media. One explants along with 7 microliters of media was placed in between the channels. The dishes were then left in the incubator for 2 hrs and then more media was added, 2 ml for a 35 mm dish. Figure 4.2 shows the schematic of the dish.

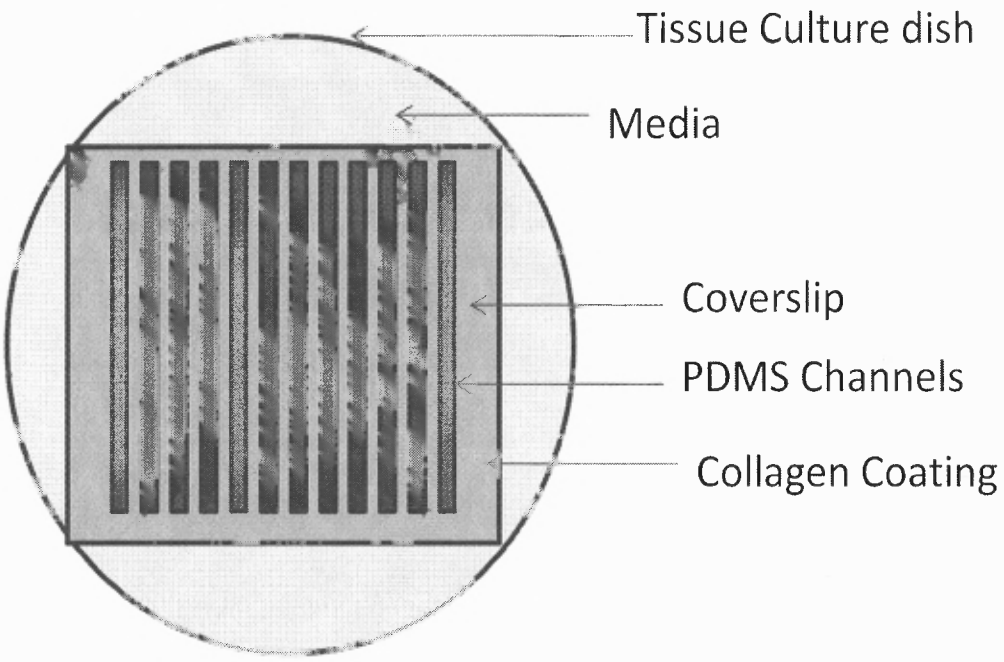

Figure 4.2 Schematic of the tissue culture dish with PDMS channels.

4.2.4 Staining the First Population of Cells

After the neurons had grown for about 14-21 days, the PDMS channels were carefully removed using a pair of forceps. The media was removed from the plate and the cells were stained using the Vybrant green live cell tracker.

The stock solution was made by mixing 90 microliters of DMSO into component A. Warm PBS (with Ca and Mg) containing 0.2% of the stock solution was added to the cells and then incubated for 30 minutes. The PBS (Phosphate buffer saline) was removed from the cells, and they were rinsed with warm PBS four times. The cells were again incubated for 30 minutes and then imaged.

4.2.5 Plating the Second Population

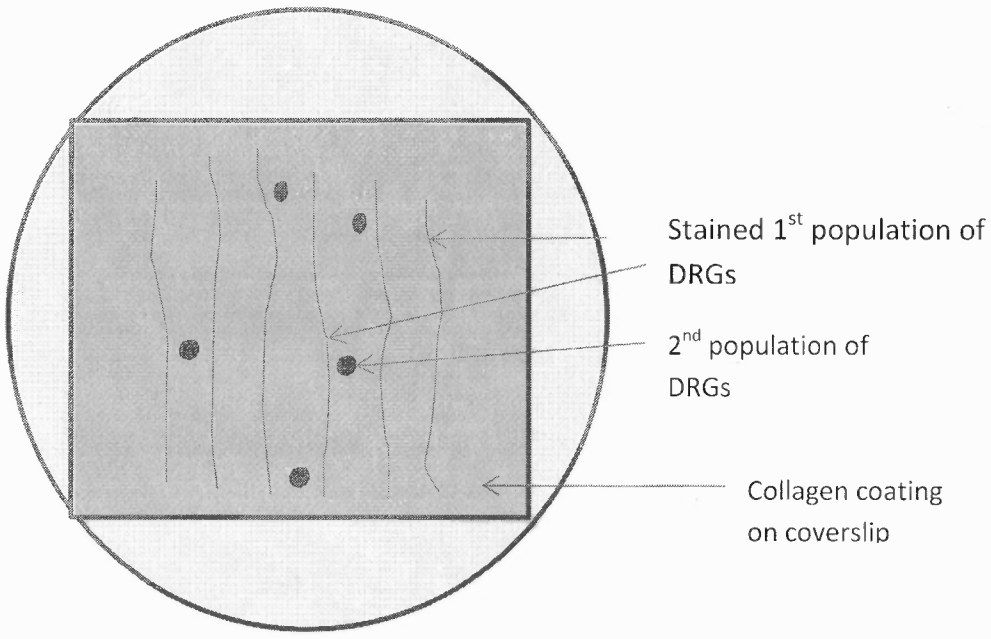

Figure 4.3 Schematic of the tissue culture plate for the PDMS channel experiment after the first population had grown and new cells were plated.

L-15 was removed from the cells, and Orange cell tracker solution was added to the cells. The stock solution was made by mixing 900 microliters of DMSO with component A. The staining protocol was similar to the Vybrant green dye except for the fact that these cells were stained in a tube, unlike the first population of cells which was stained in a dish. Figure 4.3 shows the schematic of the dish with the two populations of cells.

Once the cells were stained, they were rinsed with PBS four times and plated with 7 microliters of media on top of the previously stained green cells. The PBS in the dish was removed before the orange stained cells were plated. After incubation for 2 hrs, fresh media was added to the dish. For maintenance, the media was changed after two days in the beginning and later every other day.

4.3 Transfection Studies

The GFP (Green Fluorescence Protein) exhibits bright green fluorescence when exposed to blue light. The GFP gene can be introduced into organisms and maintained in their genome through breeding, injection with a viral vector, or cell transformation. Here, we used a viral machinery to introduce the GFP.

In these experiments, we again used the PDMS channels for growing cells in a straight line. But, instead of using two different kinds of stains, we used the orange cell tracker and the VSVG.HIV.CMV.EGFP (Vesicular Stomatis Virus Glycoprotein Human Immunodeficiency Virus CytoMegalo Virus Enhanced Green Fluorescent protein) to differentiate the two populations. The first population of cells was stained with the Orange cell tracker, and the second population was transfected with the GFP in a tube just

before plating. The transfected cells were then plated on top of the stained orange cells. The GFP being cytosolic is known to mark all of the cytoskeleton.

4.3.1 Transfection Protocol

2.5 microliter of GFP vector was suspended in 40 microliters of transfection media. The transfection media was different from regular media as it did not contain FBS (Fetal Bovine Serum) and PS (Penicillin-Streptomycin). This recipe was used to transfect four DRGs.

4.4 PDMS "H" Stamps

4.4.1 Making the Master

The master pattern was made using the 3D printer located in the Biomedical Engineering Dept. in Fenster Hall. The design was first sketched in Pro E and then patterned on ABS plastic using the 3D printer. ABS stands for Acrylonitrile butadiene styrene plastic. ABS plastic is combines the strength and rigidity of the acrylonitrile and styrene polymers with the toughness of the polybutadiene rubber. It has a fairly good impact resistance and toughness. The mold was gently rubbed on sand paper to make the top patterned surface smooth and to remove the surface deformities, if any. The mold was then washed with Sparkleen-water solution and rinsed with RO water twice. Followed by a wash with ethanol/iso-propanol the mold was allowed to dry at atmospheric conditions. The same procedure was repeated to clean the mold in between experiments. Figure 4.4 and 4.5 show the top view and the close-up of the ABS master.

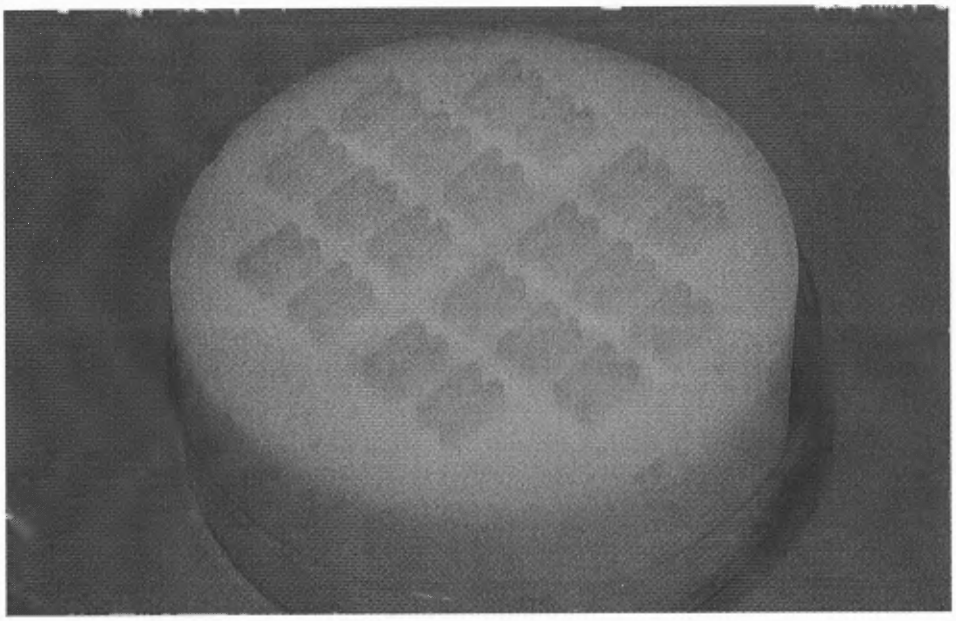

Figure 4.4 Master pattern made of ABS plastic using the 3D printer.

Figure 4.5 Close-up view of the raised "H"s on the surface of the ABS plastic.

4.4.2 Making the PDMS Stamps

PDMS base was mixed with the curing agent as explained before. The mixture was then very gently poured in to a 10 mm tissue culture plastic dish. This process if not done gently enough introduced bubbles into the PDMS, in which case the dish was kept in the degassing chamber for 30 minutes. Once, the PDMS inside the dish was free of bubbles the master was placed on the PDMS with the patterned side facing downwards.

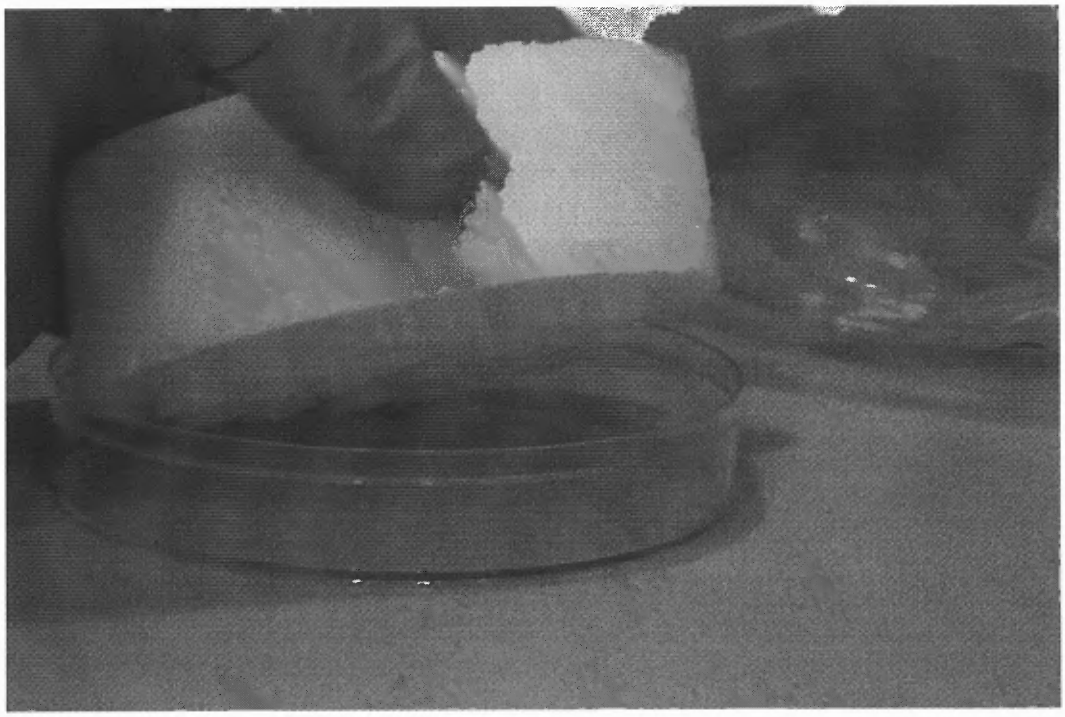

Figure 4.6 Master slowly being placed on the dish to minimize the air trapped inside.

The stamp was placed very gently, one side touching the base first, to minimize the amount of air trapped inside as seen in Figure 4.6. The assembly was again placed into the degassing chamber for about 30 minutes to an hour depending upon the amount of bubbles they have. The PDMS was then taken out of the degassing chamber and left in

the oven at 65 C overnight for curing and then was left outside at room temperature for atleast 24 hrs. before the master was removed. The master was then gently peeled off using tweezers and a thin metal spatula. The thin covering of PDMS on the top of the patterned "H" was then cut with hand using a scalpel blade. This left many micron scale irregularities on the sides of the H pattern.

Later, the process was improvised and all the irregularities were eliminated. This was achieved by applying the 1.5 inch wide scotch tape on the periphery of the master, such that the patterned surface faced up, and was surrounded by a wall of scotch tape. PDMS was then poured into this scotch tape "casing" and the assembly was left to cure, as described before.

The PDMS stamp once extracted from the master was washed with Sparkleen-water solution and then rinsed with RO water thrice. It was then briefly washed with ethanol to remove any finger grease. Finally, it was autoclaved for an hour and then left inside a 10 mm dish coated with PLL inside the laminar flow hood to dry overnight. It was observed that the stamps would stick to the dish better when they were assembled while they were wet.

4.4.3 General Plating Procedure

About 7 micro liter of collagen was spread in each of the "H" using a micro pipette tip. The collagen was gelled using the ammonium hydroxide vapors. The dishes were allowed to sit atleast for an hour before they were plated with cells.

To seed the cells, the L-15 was removed from the explants using a pipette, and 200μL of fresh media was added into the cells. For seeding, about 7μL of the media with a single explant was plated inside the H pattern. With about 20 H's in each dish, there

were about 20 DRGs growing in the same dish. Because the explants are very small, there were times when more than one explants got plated in the same channel. The amount of media was kept to a minimum to ensure the DRGs don't spread a lot. After this the plate was closed with its lid and left in the incubator for 2 hrs. This time allows the DRG to settle down and grab onto the surface of the dish. This time is usually 3 hrs but it was reduced to 2 hrs because the initial volume of the media being very less, it could lead to drying of the dishes after 3 hrs.

After 2 hrs fresh media was added to the plate, 100 microliter for each "H". For maintenance, the media was changed after the first two days and later on alternate days.

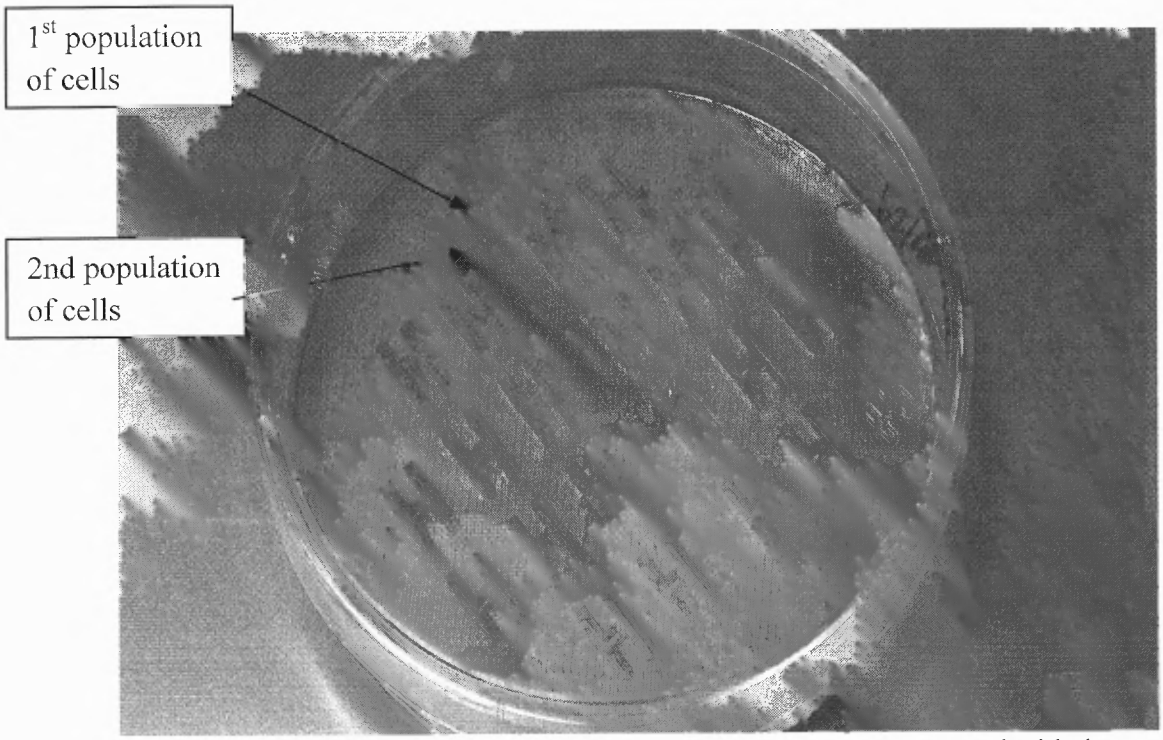

Figure 4.7 The image of tissue culture dish with an "H" PDMS stamp inside it.

Cells were plated inside the pattern, as shown by the arrows in Figure 4.7. The cells were allowed to grow for 14-21 days. Once they were ready for the second plating, the media was removed from the H's and fresh cells were placed on the opposite arm of the "H" at the middle. The cells were allowed to grow for 10-12 days and were monitored every other day.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Scratch Experiments

The objective of these experiments was to see if we could guide the neurons to grow along a particular direction by providing surface cues. It has been shown before that chick DRGs respond to single surface scratch.(StEPie E., 1997)

Figure 5.1 shows that the cells did respond to the scratches on the surface. In Set A, where the surface was first treated with PLL and collagen and then scratched, the guidance effect was quite obvious. In set B, where the scratches were made before PLL and collagen treatment the neurons could not be guided by the scratches. As expected, the neurons growing on the plate scratch system showed more sensitivity towards the scratches. They showed a preference to follow the scratches. The Scratch plate neurons on the other hand grew oblivious of the scratches showing no preferential growth of neurites towards the axis of the scratches.

The idea was not pursued further because the basic requirement of our set up was to be able to provide guidance cues to the first set of cells and then let only the first batch of cells guide the second batch of cells. So, it is of key importance that the second batch of cells does not see any physical cues, except the first batch of cells, which could not be achieved by the given setup. Also, there was difficulty maintaining consistency in making scratches every time the experiment was done because the scratches were made manually.

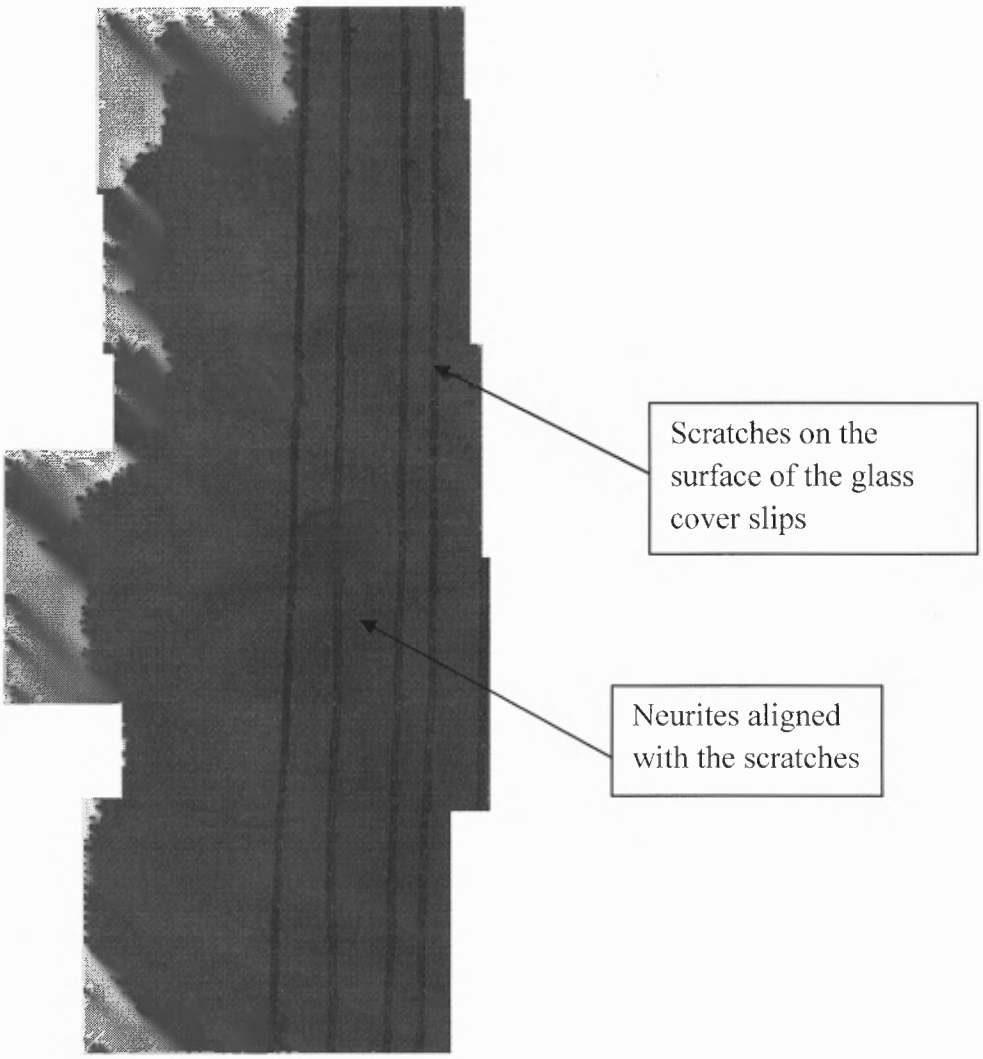

Figure 5.1 Results from the plate scratch experiment.

Here, the coverslips were first treated with PLL and collagen and then their surfaces were scratched.

5.2 PDMS Channels

The PDMS channel experiment was done to guide the growth of neurons along a linear path. By providing physical barriers on the two sides, we constricted the growth of the neuron and made it grow in a linear fashion. This objective was achieved by the channels. Also, as the channels were just placed on the surface of the coverslips they could be removed easily (unlike the scratches that were permanent). Thus, we had a system through which we could use to add or remove physical guiding cues for the neurons. Also, as the PDMS channels are autoclavable, they can be re-used for the different experiments.

Following is a stitched image of two neurons growing inside a channel. The neurons have been stained with Vybrant green. It can be seen in Figure 5.2 how the cells were guided by the channels and grew along the axial direction.

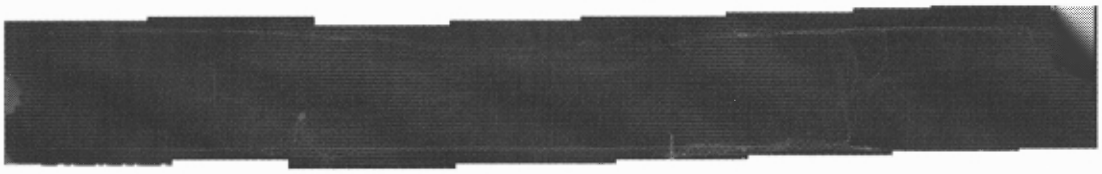

Figure 5.2 Stained DRG neuron growing linearly inside the PDMS channel.

This is the image from the first population of cells, which were grown axially before the new cells were plated on them. The new or rather the second population of cells was stained in a tube and then plated on the already stained vibrant cells.

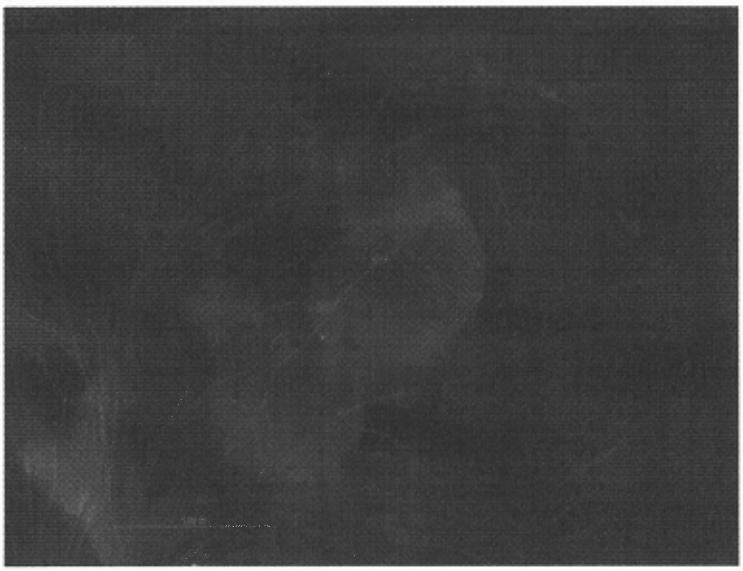

Figure 5.3 Second population of cells stained with orange cell tracker placed on top of the first population of cells which were stained with Vybrant green live cell tracker.

The image showing second population of cells in Figure 5.3 was captured just after plating. The neurites from the first population of cells are visible here. A lot of background was seen in these images, even when the imaging was done while the cells were in PBS.

The objective of using a double stain was to differentiate the two populations of cells in culture. We were unable to achieve this, because the stain from the second population would fade very fast. This left us with a culture where everything was green and nothing would show up for the orange stain. So, the two populations of DRGs could not be differentiated.

To verify whether it was the stain or the method of staining, we stained one batch of cells with orange cell tracker before plating, and the other after plating. The results are shown below in Figure 5.4.

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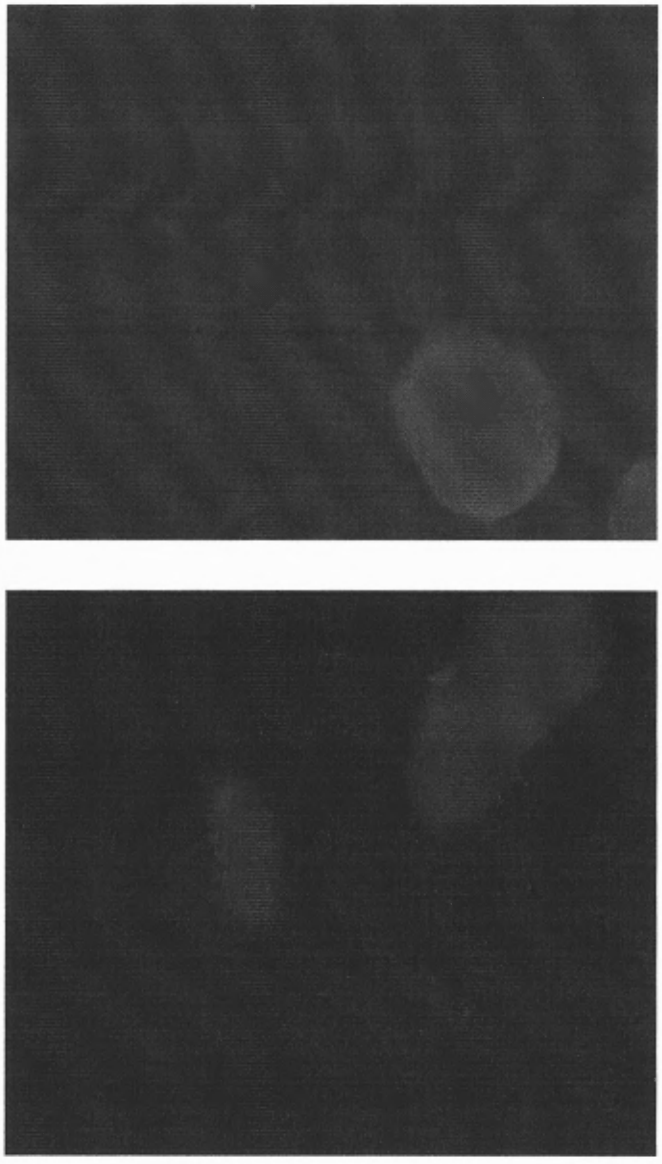

Figure 5.4 Comparison of a DRG neuron (a) stained after plating (b) stained before plating.

The above figure shows the difference observed when the cells were stained at the two different time points. It was observed that the cells metabolized the stain much better when they were spread out and well attached to the surface of the dish as opposed to being suspended in a tube. It also could be due to the fact that the attached cells absorb more stain and thus shine for a longer period of time compared to the freshly harvested cells.

To eliminate this problem we decided to use GFP viral vector along with the orange cell tracker to differentiate the two populations of cells. In this case, the freshly harvested cells were transfected with GFP inside a tube and then were plated on the first population of cells which was stained with the orange cell tracker just hrs before. The following are the pictures of the transfected DRGs.

(a) (b)

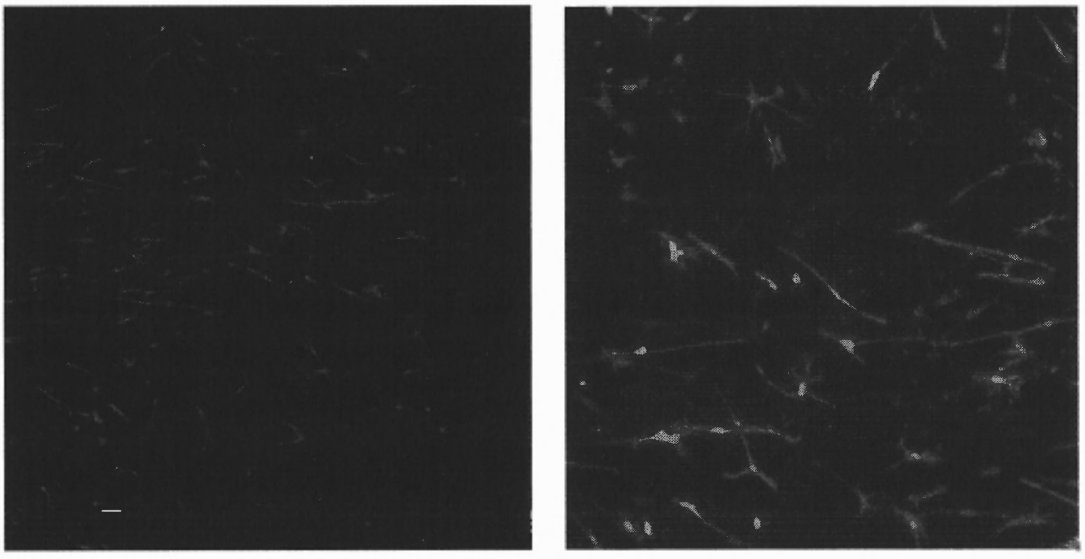

Figure 5.5 Confocal images of GFP transfected DRGs (a) 10X (b) 20X.

In the Figure 5.5, we observe that not all of the neurites are transfected by the GFP. There are a lot of neurites that don't show up in the images, which might in make it difficult for us to interpret the results. Also, we were unable to rinse of the GFP completely. It was observed that the second population of cells that was plated also got transfected over time and this made it very challenging to differentiate between the two cell populations.

Upon more thought we also realized that while we remove the PDMS channels before plating the second population of cells, we might be actually removing some cues from the surface e.g. we might be peeling of the collagen beneath the PDMS channels. Also, it was seen that some of the neurites got peeled off while removing the PDMS channels, which might also contribute in some way towards the results. These neurites were attached to the cell body, but were detached from the surface.

5.3 PDMS "H" pattern

The images below in Figure 5.6 show an image of a single DRG neuron growing inside the H pattern. The surface irregularities of the H pattern that we talked about earlier are quite apparent here.

Figure 5.6 Single rat embryonic DRG neuron growing inside the PDMS "H" pattern (Day 12).

The figure below shows the two populations of cells growing inside the same "H" pattern. The cells have been labelled and it can be seen how the two populations are interacting with each other.

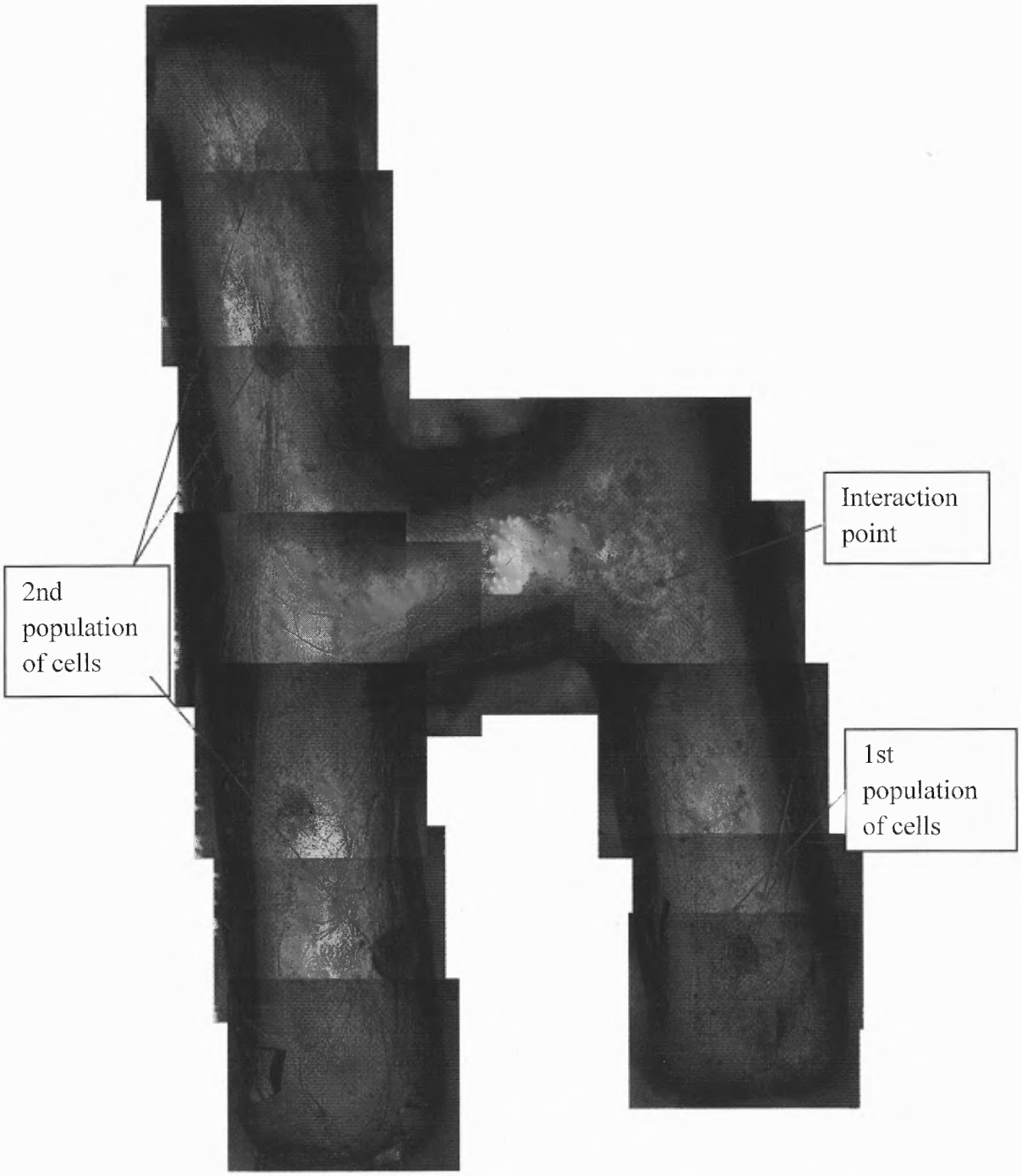

Figure 5.7 Two population of rat embryonic DRGs growing inside the PDMS "H" Pattern (2^{nd} population: Day 20; 1^{st} population: Day 44).

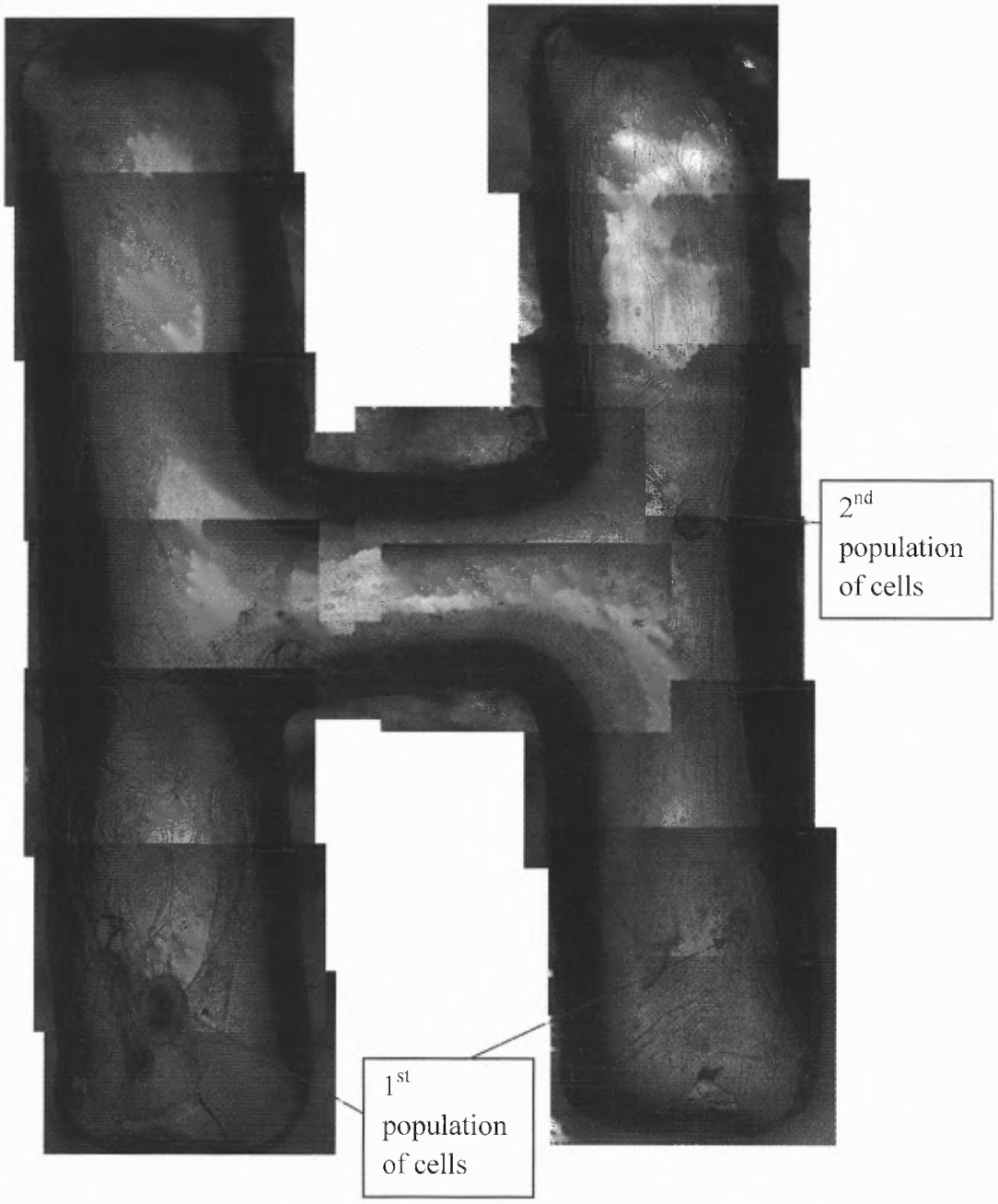

Figure 5.8 Two population of rat embryonic DRGs growing inside the PDMS "H" Pattern (2^{nd} population: Day 20; 1^{st} population: Day 44).

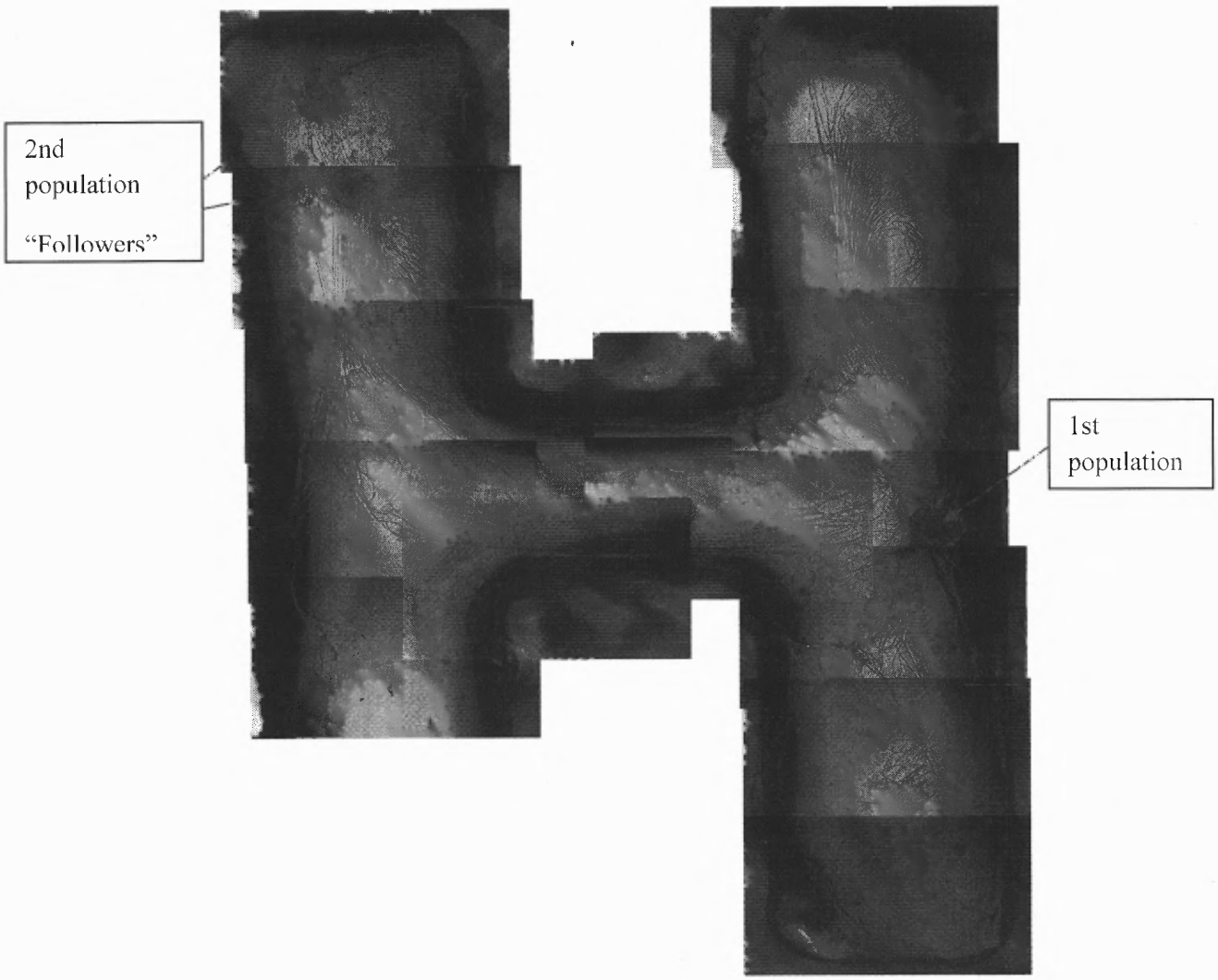

Figure 5.9 Two population of rat embryonic DRGs growing inside the PDMS "H" Pattern (2^{nd} population: Day 20; 1^{st} population: Day 44).

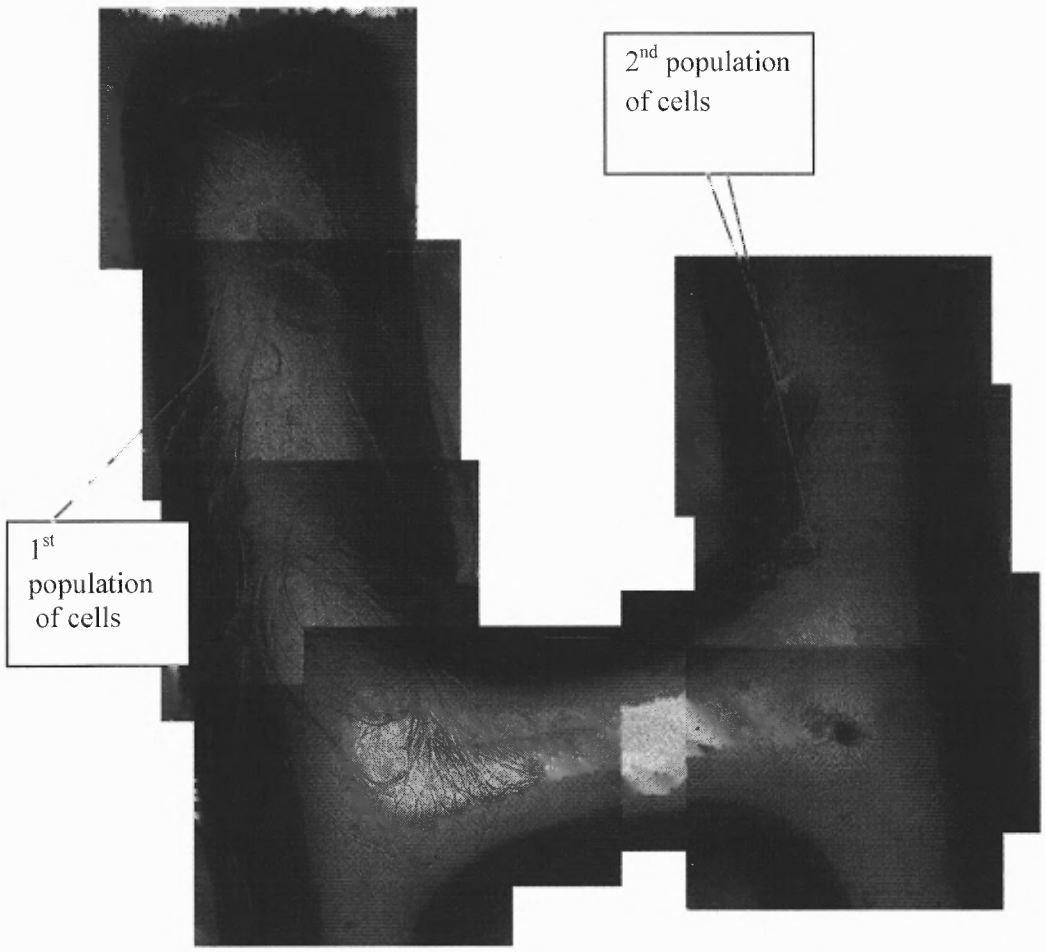

Figure 5.10 Two population of rat embryonic DRGs growing inside the PDMS "H" Pattern (2^{nd} population: Day 6; 1^{st} population: Day 23).

It was observed in Figure 5.7-5.10 that there was some form of communication happening between the two cell populations. If the neurites of from one population came into contact with the neurites from the other population, they would preferentially grow towards them. But, there were some cases when the neurites did not come into contact with each other, and grew as they would in a normal culture, oblivious of the presence

of the other population. This indicates that the cues that govern guidance might be in place i.e. contact dependent and not diffusive in nature. But, more studies need to be done to verify this fact.

There was also one other interesting observation about the growth rate of the two cell populations. The 2nd population of cells showed a faster rate of growth compared to the 1st population. This might be due to the fact that the 1st population was releasing some molecules that helped accelerate the growth of the 2nd population. It has been known that the DRGs secrete NGF when grown in cultures. The reason can also be attributed to the change in solvophilicity of the PDMS substrate.

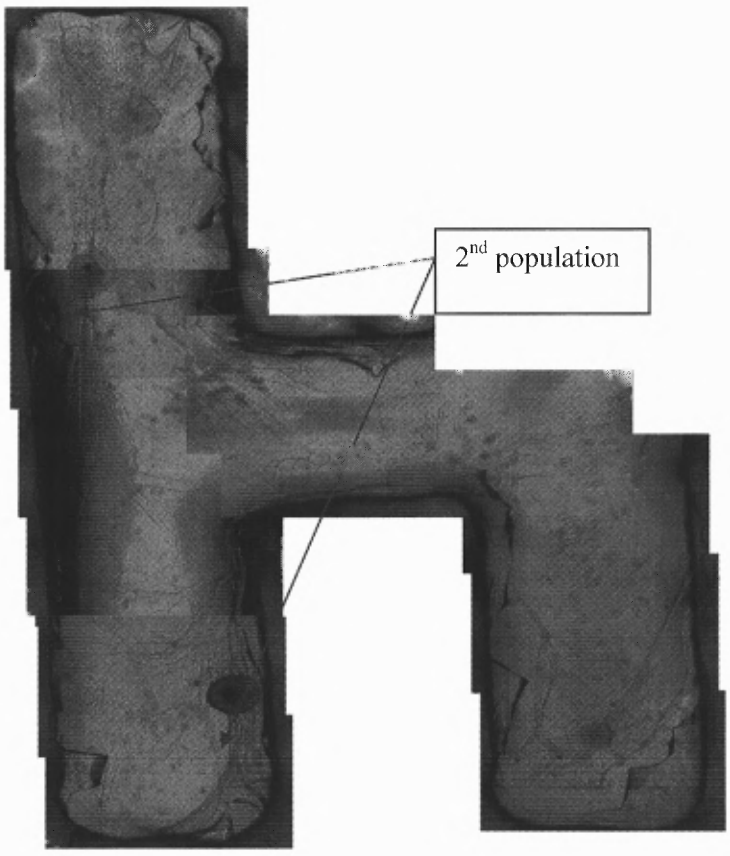

Figure 5.11 DRG neuron at day 13.Compare the growth rate with the neuron in Figure 5.6.

After about about 44 days in culture with constant contact with the medium, the PDMS might have turned more solvophilic and thus might have led to the improved growth rate of the neurons.

This might also be the result of the surface of the dish getting conditioned by the media. It does seem that the 2nd population was guided by the first, but there were results that didn't show the guidance. The results showed that the DRGs prefer to communicate with each other. It might be the 1st population of cells that reaches out to the 2nd or the other way around.

The plating methods can be improved to get better results. There were some lanes where the DRG wasn't placed exactly at the same location where it was supposed to because the dish when used for the 2nd plating was already wet. This led to movement of the plated DRG explant. This could be eliminated by aspirating the media and making the dish dry, but would result in the cells peeling off and dying for lack of media.

CHAPTER 6

CONCLUSION

The above results show that the axons are guided by other axons. It seems from the results that these guidance cues might be more contact dependent than diffusive. The neurites from the two populations when touched each other, were observed to grow more neurites towards each other. But, there were cases when the neurites hardly came into contact with each other. And perhaps due to that they continued growing as they would in a normal culture dish oblivious of the other population's presence.

It would be of interest to repeat the same experiments using cells harvested from transgenic animals so as to eliminate the problems in differentiating the two cell populations and to be able to quantify the guidance effects. Also, it would be interesting to see if there is some critical distance beyond which the neurites cannot sense each other's presence. These studies can help answer important questions in regeneration.

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