Controlled Guidance of Spiral Ganglion Neurite Growth

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Electrical Engineering: Applied Physics

by

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The following thesis originated from the desire to merge microfabrication with auditory medicine. This graduate student’s interest in “bio-MEMS” commenced during the spring quarter of junior year. Dr. Peter Asbeck was approached among several other faculty from the UCSD Electrical and Computer Engineering department with the question, “How does one combine electrical engineering with biological research?” That question initiated a two and a half year relationship with Dr. Asbeck that started out as frequent discussions with literature reviews and has ended with the research and ideas expressed in this thesis. An independent research project carried out during this student’s senior year consisted of microfabricating a microchannel based MEMs device used to separate bio-molecules.

When deciding between graduate programs, this student explored the possibility of combining the microfabrication techniques acquired as an undergraduate with auditory research. Researchers at UCSD Medical School were contacted and research possibilities were discussed. Dr. Allen Ryan of the Auditory Neuroscience department suggested subjecting spiral ganglion cells to electric fields to affect the growth of their axons and dendrites, or neurites. Controlling the growth of the neurites may eventually be used to create a biological interface to the cochlear implant, allowing for increased resolution of the prosthetic. At the first meeting with Dr. Ryan, the microfabrication of cell culture plates that could be used to study the electric field effects on neurite growth were discussed. In addition, microfabrication
of cell culture plates in which growth factors and media could be delivered selectively through microchannels was proposed.

To consider any masters project that has a time line of one year, the project goals must be realistic. This project qualifies due to the bio-MEMS type microfabrication experience of this graduate student, in conjunction with the established spiral ganglion cell culture method already in practice at Dr. Ryan’s laboratory, and lastly the ambition of the graduate student undertaking the project. The approach and mentality of this graduate student over the course of his undergraduate career has been nothing other than to push the limits. This mentality has lead to the completion of two Bachelor of Science degrees in Computer Engineering and Biochemistry, a handful of rich work experiences, and fruitful relationships such as those made with Dr. Asbeck and Dr. Ryan. Following this graduate student’s undergraduate educational experience, this project has been pursued to the fullest extent, and much more has been gained than just the development of a novel protocol for studying neurons of any type.

This graduate student has gained insights that will be applied to all future research endeavors. These insights range a wide set from how to prepare for a research endeavor to how to persevere in the face of one almost unsolvable problem after the other. Half way through this project, the simple yet powerful understanding that just one search engine isn’t enough to determine the current state of a particular field was learned. Understanding how to properly review literature leads to better experimental construction, as will be addressed in part III of this work. Throughout
the experiments conducted for this project this graduate student found himself dealing
with unexpected problems that did not lie in the areas of expertise of either of the
advisors of this project. This lead to the searching out of other experienced
researchers at both UCSD and via literature reviews. Primarily though, this situation
lead to the arduous iterative trial of combining new ideas with observed results.

Beyond the insights made on how to conduct research, this graduate student
has been fortunate enough to find a research topic that is interesting enough to
dedicate all future research efforts too. As stated previously, the ambition of this
graduate student allowed for a vast sampling of what disciplines and employment
opportunities exist in the worlds of biology, computer science, and electrical
engineering. These experiences have culminated over the last year in combination
with this master’s research project along with courses and instructors such as Dr.
Robert Hecht-Nelsen. The next step for this graduate student is to work towards a
PhD in neuro-engineering at the University of Pennsylvania with the long-term goal of
researching neuroscience in an academic setting.
ABSTRACT OF THE THESIS

Controlled Guidance of Spiral Ganglion Neurite Growth

by

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Master of Science in Electrical and Computer Engineering

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Acquired hearing loss is one of the most wide spread medical afflictions today, afflicting over 50 million people in the world. A current treatment for severe hearing loss due to hair cell damage is the cochlear implant. Users of this implant suffer from poor spectral content as today’s implants successfully stimulate only eight or fewer separate channels or frequencies. Increasing the resolution of the cochlear implant may be achieved by reducing the distance between the electrodes of the implant and the stimulated spiral ganglion cells. This thesis explores the use of two different stimulants to control the growth of spiral ganglion neurites. Electric fields and growth factor concentration gradients are selected as appropriate experimental candidates based on previous results. The technical objective of this project is to determine plausibility of the two stimulants for spiral ganglion neurite guidance, then to harness
the stimulants to control the growth of the neurons in cell culture and in vivo. The technical approach includes modeling of stimulant presentation, microfabrication of specialized cell culture plates, and in vitro biological assays. A fabricated microfluidic cell culture plate has been used to successfully present a concentration gradient to developing rat spiral ganglion cells. Preliminary results indicate preference of the spiral ganglion neurites to grow in a media rich with neurotrophin-3 (NT-3) over the same media absent of NT-3. Preference is indicated by the growing neurites’ point of entrance into the microfluidic structure as well as glial cell growth past the “decision point” in the structure. In addition, a cell culture plate has been fabricated with integrated electrodes for in vitro stimulation with an electric field. Biological assays and modeling have indicated that the current unit requires design revisions.
Chapter I.

Identification and Significance of the Problem
Acquired Hearing Loss

Today more than fifty million people suffer from acquired hearing deficiency (Welch). The major cause of acquired auditory damage is loss of cochlear hair cell function (frequently caused by prolonged exposure to loud sounds). As sound pressure waves travel through the air to the outer ear components, they are propagated as mechanical motion of the malleus, incus, and stapes. The stapes taps on the oval window at the base of the snail shaped cochlea, causing the pressure waves to travel in the fluid contained within (figure I.A.1). The organ of Corti, located within each cochlea, is the place in the auditory system where sound pressure is transduced into electrical signals. The organ is comprised of approximately 15,000 hair cells that are sensitive to sound pressure frequencies from 20 hertz up to 20 kilohertz. Hair cells are tonotopically ordered along the organ of corti in the cochlea such that low frequency tones stimulate hair cells at the apex while high frequency tones stimulate hair cells at the base. The stimulation of hair cells causes the release of neurotransmitters to the dendrites of the innervating spiral ganglion cells (figure I.A.2). Upon achieving an action potential, these sensory neurons then relay the signals to the cochlear nucleus, followed by the superior olivary nuclei, the inferior colliculus, the medial geniculate body of the thalamus, and finally the temporal lobe of the auditory cortex (for review of the auditory system see Purves’s Text Neuroscience).
Figure I.A.1: Artists rendition of the auditory canal complete with middle and inner ear components. (from Neuroscience [et al.] Sinauer Associates, Sunderland, MA)
The Cochlear Implant

Irreversible damage to hair cells means a loss of hearing. The overlap and continuity of frequencies that hair cells are sensitive to allow for loss of some hair cells’ function from different regions of the organ of corti without significant impairment. A hearing aid is a non-invasive tool to help individuals with reduced sensitivity at various frequencies. The hearing aid amplifies selected frequency ranges thereby increasing stimulation above threshold levels for the targeted hair cells. Amplification of the acoustic signal no longer results in the sensation of hearing when too many hair cells are damaged or dead. After hair cell death, the spiral ganglion neurons are no longer able to achieve action potential. This results in retracting of neurites to the cell bodies and eventually spiral ganglion death (figure I.B.2). The current medical solution is to insert an array of electrodes into the cochlea resting upon the organ of corti. When a voltage is applied across a pair of these electrodes, the spiral ganglion neurons may be stimulated to achieve action potential once again. This medical device is known as the cochlear implant (figure I.B.3).
Figure I.B.1-3: Representation of cut away view in the cochlea. The three figures show the hair cell-spiral ganglion relationship in a healthy cochlea with live hair cells (1 - top left), damaged cochlea with some hair cells dead (2 – top right), and a hair cells with the cochlear implant in place (3 - bottom)
Increasing the Resolution of the Cochlear Implant

Controlled guidance of retracted spiral ganglion dendrites to the electrodes of an implanted prosthesis may affectively increase the resolution of the implant. In this work stimulants are assessed for their ability to affect the direction of spiral ganglion neurite growth. Diffusible growth factors and electric fields are chosen as stimulants that may be readily controlled in cell culture models yet have strong potential for \textit{in vivo} studies as well. The work performed for this thesis involves the microfabrication of two types of cell culture plates. The modeling of concentration gradients and electric fields in liquid is used to approximate device characteristics. \textit{In vitro} studies of spiral ganglion cells are performed with the use of the fabricated cell culture plates. The results of the \textit{in vitro} studies may be adapted to study \textit{in vivo} affects of the two stimulants.
Chapter II

Technical Problem Definition
Increasing the Resolution of the Cochlear Implant via Electrode Array Design

The cochlear implant has an external component with a microphone, a signal processor, and a transmitter that sends signals to the surgically implanted component in the skull. The fabrication of up to nineteen source and sink pairs on the electrode array given the size constraints of the cochlea has been achieved (Holmes, et al). Despite this fact, the maximum number of frequencies that a patient can currently differentiate between is eight, while the standard is four (Zwolan, et al). This significant decrease in the spectral distribution of frequency leaves patients with low acoustical sensitivity. The deficiency of today’s cochlear implant is attributed to the significant total power handling limitations imposed by the sensitivity of the biological tissue. The field produced at the electrodes needs to be powerful enough to depolarize a dendrite that is not only on the other side of a dead hair cell but also may have retracted since the hair cell death. The spread of current at such a distance causes stimulation of several of neurons simultaneously (Liang, et al).

The goal of this project is to increase the resolution of the cochlear implant. The two components of the system that may gain from modification are the signal processing unit and the microelectrode array. This project considers increasing resolution by modification of the microelectrode array exclusively. Increasing the number of electrodes in the array has not led to increased resolution based on the power limitations and spread of current through the local biological tissue (Fishman, et al; Zwolan, et al). Decreasing the spread of current at the sensory neurons may be addressed by reducing the voltage applied at the electrodes. In order to continue
stimulating action potentials at a lower voltage, the distance between the electrodes and the spiral ganglion dendrites must be reduced. Ideally, a bioelectrical interface would be created on the electrode surface allowing for direct adhesion of spiral ganglion dendrites to the implanted electrode array of the cochlear implant.

Development of a biologically interfaced cochlear implant involves several components: the fabrication of a microelectrode array addressable by a significant number of neurons, creation of a local biological interface to the array, stimulation of spiral ganglion neurite outgrowth, and control guidance of spiral ganglion neurite growth. A neurite is an extension from a neuronal cell body, either dendritic or axonal. Biotechnology companies, along with university research efforts have addressed two of the developmental components. Microfabrication processes today use sub-micron photolithographic techniques to create sophisticated integrated chips. This technology can relatively easily be adapted to create numerous individually addressable electrodes within the size constraints of the cochlea (Holmes, et al). Both industry and academia have been developing bioelectrical interfaces for “lab-on-a-chip” systems. Contact between the harsh oxidizing environment of an electrode and sensitive biological components has been mediated by various chemical and physical barriers (Chouard, et al).

Bio-medical research has been addressing the later two components of the biologically interfaced cochlear implant. Regeneration of adult spiral ganglion has been stimulated using various growth factors (Lefebvre, et al). Research is also being carried out to understand the pathway of neurite development; what biochemical,
electrical, and mechanical cues are used to guide axons and dendrites to their targets. Chemical cues currently under research include growth factors, collagen gradients, and netrins (Snyder, et al). Neurotrophins such as NGF, BDNF, NT-3, and NT-4/5 have also been shown to affect neurite outgrowth and survival (Snider, et al). Beyond understanding how neurites develop in vitro (cell culture), interfacing the cochlear implant will require manipulation of neurite growth in vivo, in live animals with functioning implants. The problem addressed by this proposal is empirical determination of methods for guiding neurite growth.
The Neuron and Its Development

The neuron is the fundamental unit of the nervous system. It is a specialized cell type that has the ability to receive and send information to and from other neurons, muscle cells, sensory cells, and various other cell types in the body. Although several types of neurons have been identified, ubiquitous are three fundamental component consisting of the cell body, the axons (signal transmission lines), and the dendrites (signal receiving lines). Ion pumps throughout the neuron membrane produce a resting voltage potential of approximately 70mV between the inside and outside of the cell. This voltage gradient is the driving force in neuronal signaling. A neuron is stimulated to “fire” when an incoming signal triggers the opening of ion channels at some point along the membrane. If the opening of the gates depolarizes the resting voltage of the neuron below a threshold value, an action potential is triggered. The action potential is propagated by a series of opening and closing of ion channels sequentially from the site of stimulation (usually at the dendrite) along the cell body and through axon of the neuron. At the end of the axon, the action potential triggers the influx of Calcium ions into the cell, which in turn stimulate the exocytosis of vesicles containing neurotransmitters from the membrane. Neurotransmitters bind to receptors on the extracellular side of the connected neuron, and cause the opening of ion gates that may stimulate an action potential in the adjoining neuron (for review of the auditory system see Purves’s Text Neuroscience).
Figure II.B.1: Diagram of a neuron

(Illustration by Lydia Kibiuk, Copyright © 1996 Lydia Kibiuk.)
The human brain consists of approximately $10^{12}$ neurons. Essential to the functionality of the brain is the development of connections between this expansive numbers of individual units. The developed nervous system contains neurons and various other neuronal cells connected by a combination of the chemical and electrical means described in the last paragraph. Distances from the order of micrometers to centimeters are spanned between connected neurons by the wire like axons and dendrites that propagate action potentials through a neuronal cell. The development process of a neuron therefore consists of two primary phases, differentiation of the cell within the nervous tissue, and connection of the cell’s axons and dendrites. Cues for neuron differentiation include the expression of neuron specific genes triggered by extra cellular matrix and cell placement signaling. Although not completely understood, there exist multiple cues for connection formation, including both diffusible chemical signals and non-diffusible cell adhesion molecules.

An extension is made in the cell body of a neuron through a series of molecular steps. Cytoskeleton elements called actin monomers assemble into tubules in the cell and eventually build up to the point where force is exerted on the cellular membrane (Hall). The force exerted by the dynamic cytoskeletal formation is coupled with the reapportionment of new membrane phospholipid bi-layer in the region where the force is being exerted. These two mechanisms eventually led to the formation of a projection from the cell body. Various microfilaments, including neurofilament, become structural support beams in the membrane projection as well as aid in the extension of the projection at the tip, or growth cone. Specialized cellular machinery
is localized in this growth cone enabling production of the additive filamentous sub-units. The machinery also provides the ability to sample the local environment, as well as differentiates the process into axon or dendrites with the ability to receive or release neurotransmitter signals.

Vast complexity exists at the growth cone of the developing neuronal extension. Morphologically the growth cone is made up of a sheet-like expansion called the lamellapodium with finger-like extensions dynamically extending called the philopodia. The dynamic movements of the philopodia, which both extend and retract as though sensing the immediate environment, is believed to be mediated by the controlled rearrangement of the actin cytoskeletal elements. As stated above, these same elements are directly responsible for the extension and growth of the neurite as a whole. Growth cone shapes take on different forms according to the cues that are being used to guide the outgrowth of a neurite (Hall). An example is found in the developing optic system, in which some of the retinal axonal projections from each eye need to cross the midline at the optic chiasm while other continue on the same hemisphere of the brain. The axonal growth cones tend to be simple in shape before the axons reach the decision point at the chiasm. These axons are following the led determined by the initial projections from the retinal to the appropriate location in the brain. Once the chiasm is reached, the growth cone slows down, and acquires a complex shape as the decision is made whether or not to cross the midline.
The behavior of the growth cone when approaching the location of an important decision (which direction to grow towards) suggests the presence of specific cues used in the guidance of the neurites outgrowth. These cues have been
categorized into non-diffusible and diffusible signals, although some of the identified
cues have been found to work as both diffusible and non-diffusible signaling
molecules. Non diffusible signals consists of those molecules that are bound to other
cells, held within organized matrices in the extracellular space, or are actually
components of the complex extracellular matrix. Cell adhesion molecules, or CAMs,
are a large group of molecules found on cell surfaces and sometimes held within the
extracellular matrix. Growth cones have been found to have receptors for some of
these molecules that are associated with intercellular signaling molecules such as
phosphatases, kinases, and proteases. The diversity of the set of CAMs found within
the developing nervous tissue means that the developing growth cone is continuously
subjected to a large variety of different signals. One specific type of CAM associated
with the developing nervous system is called the neural cell adhesion molecule, or
NCAM. CAMs play an important role in the “follow the leader” neurite extension
motif in which a few neurites, called pioneer axons, initially span the path connecting
two nuclei (bodies of related neurons within the brain). The subsequent onslaught of
connections between the two nuclei are made as thousands of developing neurites
follow the path defined by the pioneer axons across the neural tissue to the appropriate
target area. Another example of CAMs roles in developmental neurobiology have to
do with “guide post cells”, which are located at crucial decision points in an extending
fascicle (collection of axonal connections). These cells present either repulsive of
attractive molecules indicating the correct direction for developing neurites to grow.
A second class of non-diffusible signaling molecules is found in the extracellular matrix. The most prominent of these molecules for neuronal development are the laminins, collagens and fibroconnectin. The class of growth cone receptors known as the integrins binds specifically to these molecules found in the extracellular matrix. The binding of these molecules to the integrin receptors mediates an intercellular signaling cascade involving fluctuations in inositol triphosphate and the activation of intracellular kinases. Ultimately these cascades led to the extension of axons through developing nervous tissue \textit{in vivo} and \textit{in vitro} cell culture experiments. Experiments have been conducted using micro patterned extracellular matrix component to direct the growth of neurites in cell culture (Matsuzawa).

Diffusible signaling molecules have been found to influence the direction of outgrowth of a developing neurite towards a target cell that produces the molecule. Diffusible signaling molecules have been divided into two groups, tropic molecules that guide the direction of growth of a neurite and trophic molecules that affect the survival and growth of the neurons and their processes once the appropriate target has been reached. The best-characterized family of chemo-attractant (tropic) molecules is the netrins. The netrins have been found to have high homology with extracellular matrix molecules such as laminin and may actually interact with the extracellular matrix to influence axon growth. The growth cone contains receptors for the netrins by which the chemoattractant signals are transduced intracellularly. The netrins have been found to act in at a variety of sights in the developing vertebral nervous system, where axons must decide to cross the midline (as in the retinal development example
above) or to stay ipsolateral. Experiments have been conducted in transgenic mice (with the netrin gene knocked out) and in cell culture that have indicated the importance of netrins in the development of these midline-crossing events (Hopker, et al; Serafini, et al).

The other form of diffusible signaling molecules consists of the trophic factors. Trophic factors are released from a target cell to maintain a newly formed connection. The trophic factor not only promotes the survival of the connected axon, but also the survival of the cell body from which the axon came. This allows for pruning of the vast number of incoming neurites, as there is only a fixed amount of trophic molecules produced and released by a target cell. Several trophic factors have been identified in the developing nervous system, and a family of trophic factors associated with neurite growth and extension are called the neurotrophins. Presently, the primary neurotrophins that have been identified and studied are Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3, (NT-3), and Neurotrophin 4/5 (NT-4/5). These factors bind to the family of Trk receptors, and some of the identified neurotrophins overlap which receptor they bind to (see figure II.B.3 below). When a neurotrophin binds to a specific Trk receptor, secondary messengers are activated and the receptor-neurotrophin complex is phagocytosed. The receptor-neurotrophin complex and the secondary messengers are then retrogradely transported to the cell body, where the cellular response is mediated, and biological material is recycled. This mechanism of neurotrophic signaling response explains the
ability of secreted molecules at the end of an axon to promote the survival of the neuron as a whole.

Figure II.B.3: Neurotrophic receptors

(from Saltiel & Decker (1994) Bioessays 16, 405)

Figure II.B.4: Neurotrophic receptors and signaling

(from Lectures notes of Prof. David J. Tracey, The University of New South Wales)
Guiding Neurite Growth

In order to guide neurite growth in a manner that could eventually lead to increasing the resolution of the cochlear implant, constraints imposed by the tissues involved must be considered. In cell culture, controlling the placement of non-diffusible signaling molecules is possible as exemplified by the work with micro-patterned laminin plates (Matsuzawa). Given the current technology and understanding of how non-diffusible guidance cues work, it seems reasonable to consider coating the electrode array with such molecules prior to implantation. However, this coating will not promote the growth of retracted spiral ganglion neurites towards the electrodes, but rather let them know they are at the right place once they reach the electrodes. The technology does not exist at this point for in vivo positioning of either extracellular matrix components or cell adhesion molecules. This is due in part to a lack of understanding on how to control the rearrangement of the extracellular matrix components in an already developed tissue. Consequently, diffusible signaling molecules are left as a primary means of exerting in vivo control on the guidance of growing neurites based on present understanding of how neuronal connections are developed. In solution, the diffusion of any molecule or particle from a source creates a concentration gradient of the diffused molecule. Modeling of this phenomenon is addressed in Chapter III, Section C.

Previous studies have utilized both the separation of various media and concentration gradients to study the affects of diffusible signaling molecules on
neurite growth. A classic experiment by Camenot in 1981 utilized a specialized cell culture plate that had three separate chambers of media (see figure II.C.1). The neuronal cell bodies were placed in a center well and the neurites projected into adjoining wells that either contained or didn’t contain neurotrophic factors (nerve growth factor in this experiment). This fruitful experiment shows the trophic affect of neurotrophins, as the neurites (and connected neurons) that happened to grow into the wells with trophic factors were sustained. When viewing the experimental results in terms of the project goal, the cell culture model used by Camenot shares similar limitations to those expressed about using non-diffusible guidance cues. The separation of the chambers means that neurites are not actually guided to the appropriate location, but rather survive if the target location happens to be surveyed by the growth cone.

Other experimental models have utilized the source-sink technique to create a concentration gradient of trophic/tropic molecules in solution. This technique involves the controlled release of molecules from one location in solution, and the controlled absorption of the molecules at another point in the solution. The attraction of developing neurites along a trophic concentration gradient is attributed to the receptors found at the growth cone sensitive to both neurotrophic and neurotropic signaling molecules. As the numerous philopodia extend from the end of the lamellapodium, the receptors in each of the finger-like projections interact with the local environment. Philopodia which are randomly extending in the direction of an increasing concentration of some neuro-attractant to which the cell is sensitive will
probabilistically have a higher amount of receptors bound than those philopodia going in another direction. The direction of the philopodia that receives the most intense signal will be followed by the lamellapodium, and eventually the neurite will incorporate this new direction into its overall growth.

Figure II.C.1: Campenot Experiment of trophic affects with separate media

As stated previously, the cochlear implant works by subjecting retracted spiral ganglion cells to pulses of current from the electrodes. Due to the nature of the
implant, the door is opened to a second form of potential guidance cues not yet discussed, electric fields. Previous studies have been conducted on various neuron types to study the affects of electric fields on neurite growth. The majority of this body of literature has focused on the spinal neurons of Xenopus (frog) embryos. The developing embryo has measurable electric fields across the developing spinal cord in the range of 10 to 100 mvolts/cm.

Observation that neurons respond directionally to electric fields in vitro by specific changes in cell shape is called galvanotropism (reviewed by McCaig, et al 1994). Investigations of how electric fields affect neuron growth have resulted in a substantial body of literature. The literature reflects both an increasing understanding of how to create electric field that may stimulate neurons, along with how those fields may be working to cause the observed affect on neurite growth. The first attempts to create an electric field in cell culture media utilized bare wires placed in the media. This technique was modified to incorporate salt bridges to buffer the oxidation and reduction reactions occurring at the electrode-liquid interface. The initial salt bridges took the form of micropipette tips filled with high molar sodium chloride. Platinum wires were placed inside the tips and used to conduct the electric current through the media. The next salt bridge techniques utilize a high salt agar ‘u-bridge’ divider between the cell culture media of interest and the electrode. Electric fields are focused across the neurons through a small fluidic channel between slide cover slips affixed to the cell culture plate. The most recently develop technique for creating an electric field in cell culture media involves the construction of a box coil. This coil induces a field
without using electrodes in the media. This technique is described in Chapter IV, section B.

Various molecular mechanisms have been suggested to describe neuronal galvanotropism. The two primary mechanisms suggested are 1) collection of charged molecules are one region in the cellular membrane (Stewart, et al; McCaig, et al), and 2) electrophoretic migration of growth cone trans-membrane receptors (Erskine, et al; McCaig, et al). Both of these effects are well documented and discussed in the literature.
Chapter III.

Approach and Results: Growth Factor Concentration Gradient
Overview of Concentration Gradient Presentation

This chapter describes one technical approach for controlling the guidance of spiral ganglion neurite growth. The stimulants used to affect growth in this chapter are diffusible growth factors. A microfabricated cell culture plate has been implemented to study the affects of growth factor concentration gradients on spiral ganglion neurite growth. The plate utilizes flow in microfluidic channels to initiate and maintain a concentration gradient in the vicinity of an extending neurite. Section B of this chapter describes the design and modeling of the cell culture plate. A mathematical model of the hydraulic forces necessary to maintain a desired gradient is included. Section C describes the fabrication of the cell culture apparatus that involves patterning and molding the microchannel structure, as well as construction of a fluidic delivery system allowing for reusability of the unit. Experimental results in sections D and E include verification of concentration gradient formation, and visualization of neuronal cells (glial cells) in the microchannel structures. Preliminary results have implicated preferential growth of neurites into media containing NT-3 based on where the neurites entered the microchannel structure (75% of the observed neurites entered on the NT-3 rich side of the channel opening). The validity of this percentage is weak due to a limited sample set of three evaluated experiments. Preferential glial cell growth is also implicated by the preliminary results, as all three control experiments (both sets of media NT-3 rich) yielded glial cells in both delivery channels while the single classifiable non-control experiment only had glial cell
growth in the NT-3 rich delivery channel. Both the preferential growth of neurites based on entrance location and the preferential growth of glial cells based on their presence in a specific delivery channel require more experimental results to be substantiated. The conclusion chapter V, section B describes how this cell culture work may lead to development of an *in vivo* implementation. Optimally the results of both *in vitro* and *in vivo* experiments may lead to increased resolution of the cochlear implant in human patients.
Create Concentration Gradient in Cell Culture

The goal of the biological assay defined in this chapter is to determine optimal conditions for controlling spiral ganglion neurite growth. Subjecting the spiral ganglion cells to different media, the nutrient rich fluid used in cell culture work, is the premise of this chapter. Based on available technologies in microfluidics, a specialized cell culture apparatus has been created with microchannel structures capable of delivering multiple sources of cell culture media. The neurites are presented with a gradient of two different media sources in the “presentation region” of the structure. The microfluidic network has been designed to include a “decision point” for the growing neurites. After extending 200 microns through the presentation region, the extending neurites reach a fork in the microchannel network. The extending neurites may then grow up either of two channels that contain the unmixed versions of the media presented. The preferred media (or the media with a preferred concentration of nutrients) for guiding neurite growth is indicated by the channel into which the neurites extend. This design format allows for easy determination of optimal growth factors as any two media may trivially be compared using this apparatus.
The "decision point" design allows for easy quantification of any soluble molecule's effectiveness as a tropic cue. After a cell culture experiment is run and the neurites have grown past the decision point, the number of neurites found in each channel 'A' or 'B' is summed. These sums are totaled over numerous runs of the experiment to quantify the preference of the extending neurites to the media presented in channel 'A' over the media presented in channel 'B'. Essential to the design
characteristic is the accessibility of both of the media types to the neurites in the presentation region before a decision is made.

Large groups of associated spiral ganglion neurons, or explants, are used in the assays performed in this chapter. These explants are composed of hundreds to thousands of individual neurons, and as such usually have several tens of neurites growing from them. Individual neurites have diameters on the order of 2 microns. The collections of neurites growing from the explant come from different directions, and may form bundles of several neurites growing together called neuronal fibers. As such (in addition to other issues discussed in the testing and results section), the width and height of the microchannels are chosen to be 100 microns by 50 microns.

Modeling of the concentration gradient found throughout the apparatus is fundamental to its use in this assay. Based on preliminary experiments and modeling, the apparatus will require flow in order to maintain the appropriate concentration gradient over an extended period. Flow rates may be experimentally controlled down to approximately 0.25 microliters/minute using a digital syringe pump. As discussed in the testing and results section of this chapter, the numerical analysis performed here are confirmed using fluorescent dyes and fluorescent photo-microscopy.

Diffusion is the result of random motion of molecules in liquids and gas. All mobile molecules undergo Brownian Motion due to thermal agitation. Molecules are constantly in a state of motion. The velocity of their motion is proportional to temperature. The distance a single molecule may travel before impacting another molecule may be modeled probabilistically based on the direction of its movement and
the random location of the next molecule to be encountered. In a single dimensional case, a point molecule at any location in space has equal probability of moving in either of two directions. The top of figure III.B.2 depicts a bisected line with five point molecules on one half of the line and no molecules on the other half of the line. As these molecules are allowed to diffuse, or move randomly in either direction, some will continue to stay on their half of the center line while other move towards the empty half (assume they can pass over each other). As the empty half starts out with no molecules, this flux between the two halves is not balanced. Eventually, flux is dynamically balanced when equal numbers of molecules are on each half of the line. Although the molecules are still moving in random directions, the net number of molecules entering the right side is equal to the net number of molecules entering the left side.

Figure III.B.2: Diagram depicting diffusion of point molecules on a line
When more than one molecule is evaluated at a time, the positional location of the molecules are approximated with a Gaussian distribution. Equations describing solute concentration as a function of time and location are well described and will be utilized in approximating the flow rate necessary to maintain a gradient in the presentation region of the microchannel structure. Without flow, the growth factor molecules of interest would saturate the microchannels, and the neurite would no longer have to make a decision, as every direction would have the same concentration.

Flux of diffusible particles undergoing transport in response to hydraulic pressure is given in equation 1 below, where $D$ is the diffusion coefficient of the particle, $c$ is concentration, and $\mathbf{v}$ is the vector describing flow. This equation may be expressed in terms of concentration with respect to time as shown in equation 2.

These equations are used to calculate the expected flow rate necessary to maintain a gradient in the presentation region of the microfluidic structure. The Gaussian distribution may be observed in equation 3, which is used to describe the probabilistic concentration of a body of molecules as a function of location and time. The numerator $n_0$ in this equation represents the molar quantity of the molecule under evaluation. Equation 4 uses the relationship defined in equation 3 to approximately relate the time it takes half the body of molecules ($t_{1/2}$) to diffuse a distance greater than $x_{1/2}$. 


Table III.B.1: Equations used to model diffusion

<table>
<thead>
<tr>
<th>Equation</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation 1</td>
<td>$\Phi = -D \frac{\partial c}{\partial x} + \nu c$</td>
</tr>
<tr>
<td>Equation 2</td>
<td>$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} - \nu \frac{\partial c}{\partial x}$</td>
</tr>
<tr>
<td>Equation 3</td>
<td>$c(x,t) = \frac{n_o}{\sqrt{4\pi D}} \cdot e^{-x^2/4Dt}$ \quad (t &gt; 0)</td>
</tr>
<tr>
<td>Equation 4</td>
<td>$t_{1/2} \approx (x_{1/2})^2 / D$</td>
</tr>
</tbody>
</table>

Determination of the optimal flow rate is a function of several components. A two dimensional rendition of the microchannel structure and the flow vectors are depicted in figure III.B.3. Laminar flow is assumed in the presentation region to simplify analysis of the system. This simplification discounts the mixing effects found at the decision point (labeled time = 0 in the figure) where the two fluids have velocity vectors with both x and y directional components. Throughout the microchannel structures up to the point labeled time = t in figure III.B.3 there is assumed to be no variance in concentration in the z-direction (perpendicular to the plane of the paper). With this assumption, and the addition of flow to the system, evaluation of the concentration gradient may be reduced to a single dimensional evaluation in the x-direction. Modeling of the diffusion of solutes in the presentation region therefore becomes analogous to the initial spatial step of concentration solution shown in figure III.B.4.
Figure III.B.3: Diagram showing direction of flow and diffusion considered in analysis of the microchannel structure.

Figure III.B.4: Diffusion starting from step concentration gradient. (from Cellular Biophysics. The MIT Press. Cambridge, MA)
Two different molecules are separated using the microfluidic system in the experimental section of this chapter; the fluorescent indicator Biodipy (Biorad), and the neurotrophin NT-3. Diffusion coefficients are proportional to the molecular weight of the molecule evaluated, and are a function of the medium of diffusion and temperature. Based on the size of these molecules (much bigger than Oxygen and smaller than DNA) the diffusion coefficient is approximated at $1 \times 10^{-7}$ cm$^2$/sec. The $t_{1/2}$ is defined as the time necessary for half of the molecules of interest to diffuse at least the distance $x_{1/2}$ (Weiss). Using the value 50 micrometers as the $x_{1/2}$ parameter (chosen as half the width of the decision region), the $t_{1/2}$ is approximately four minutes. Table III.B.2 shows a few $x_{1/2}$ and $t_{1/2}$ pairs calculated with the diffusion coefficient approximated for the two molecules studied in this chapter. As depicted in figure III.B.5, this would result in an average concentration of 75% solute in one half of the microchannel slice and 25% in the other. After another $t_{1/2}$ minutes, the gradient would be cut in half again on each side, moving closer to the equilibrium of zero gradient across the channel in the x-direction. The calculation of $t_{1/2}$ will be compared with experimental results in section D of this chapter. Based on the $t_{1/2}$ of approximately four minutes for the decision region width, the decision region flow rate needs to be greater than eight minutes per volume of the microchannel in order to have even a slight gradient at the location marked time = t in figure III.B.3.
Table III.B.2: Diffusion $x_{1/2}$ and $t_{1/2}$ pairs for the molecules in this experiment

<table>
<thead>
<tr>
<th>$x_{1/2}$</th>
<th>$t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(distance diffused by half the molecules at time $t_{1/2}$)</td>
<td></td>
</tr>
<tr>
<td>50 microns</td>
<td>250 seconds $\approx$ 4 minutes</td>
</tr>
<tr>
<td>($\frac{1}{2}$ the width of the decision region)</td>
<td></td>
</tr>
<tr>
<td>200 microns</td>
<td>4000 seconds $\approx$ 1.1 hours</td>
</tr>
<tr>
<td>(length of the decision region)</td>
<td></td>
</tr>
<tr>
<td>5000 microns (0.5 cm)</td>
<td>2,500,000 seconds $\approx$ 29 days</td>
</tr>
<tr>
<td>(distance between decision point and fluidic well)</td>
<td></td>
</tr>
</tbody>
</table>

Figure III.B.5: Depiction of $t_{1/2}$ diffusion times in microchannel structure
Fabricating the Microfluidic Cell Culture Plate

Microfabrication techniques have been used extensively to create microfluidic structures (Dertinger, et al; Duffy, et al; Jo, et al). Interfacing a microfluidic structure with the relatively macroscopic environment necessary for cell culture techniques presents several novel challenges. In addition to the necessity of interfacing macro and micro-structures, the design of the apparatus needs to consider the reusability of all components for multiple experiments. Both of these constraints have led to the implementation of a specialized cell culture plate that has been used in the biological assays found in the results section of this chapter. Following is a description of the fabrication process and the various novel design techniques that went into creating a functional unit.

The microfabrication process utilized to create the specialized cell culture plate builds upon the rapid prototyping of microfluidic structures described by Duffy et al. This process involves the use of a reusable mold master created with the thick positive photoresist SU-8 (1-1000 microns possible in one spin coat). Transparencies defining the microchannels (>10microns thick) are created using an auto-cad program in conjunction with a high-resolution printer. Standard photolithographic techniques yield a positive relief of the microchannel structure on the silicon substrate, which is then molded with a high definition silicon based polymer Poly-Di-Methyl-Siloxane (PDMS). Whiteside’s prototyping technique usually calls for irreversible bonding of the molded PDMS structure to a clean material such as PDMS, treated glass,
polystyrene, or silicon using oxygen plasma. Fluidics are delivered to the structure via insertion of tubing into the PDMS through a punctured or molded hole. In the present work this technique has been used to create a concentration gradient in a microfluidic channel (Dertinger, et al). Adaptation of these techniques was necessary to satisfy the design considerations of this project.

The specialized cell culture plate will be utilized along side standard cell culture plates in the environment already set up in the laboratory. The standard cell culture environment utilizes plates containing a set of individual cylindrical wells 1mm – 1cm in diameter filled with media specific to the tissue being studied. The material of the plates is usually polystyrene, and they are packaged sterile as disposables. Cells are very sensitive to small differences in the environment of the media; therefore care is taken to regulate the media’s pH using buffers that rely on CO₂ to maintain appropriate balance. The plates are stored in an incubator with high humidity to prevent evaporation, and controlled partial pressures of oxygen and carbon dioxide (usually 95% and 5% respectively). Loading of the wells with the cells to be studied involves a transfer from the surgical petri dish into the cell culture well. This usually takes place by using fine dissection tweezers and transferring the tissue in a droplet carefully maintained between the partially opened tweezers tips. The gas exchange taking place in the incubator and the transfer of the tissue under study both require a relatively macroscopic opening into the cell culture well. Therefore, conforming to the set up already used in the laboratory, the specialized cell culture plate utilizes a large opening to the cell culture well for gas exchange and cell loading.
Interfacing the macroscopic cell culture well (approximately 1 cm³) with a precise microchannel environment requires the use of an intermediate layer. The microchannels are created using standard photolithographic techniques to create an inverse mold. To create a cubic centimeter well in the PDMS polymer, aluminum blocks are placed on the flat silicon molding surface. After polymerization is complete, the PDMS that has polymerized under the aluminum block is carefully removed with a scalpel and tweezers. The technique of clearing excess PDMS with a scalpel introduces a significant amount of imprecision. Therefore an intermediate layer of photoresist is created with large dimensions (500 microns thick x 1 mm long x 500 microns wide at the channel interface, 2.5 mm wide at the cell culture well interface) that buffer the inaccuracy of the aluminum block molding technique (see figure III.C.1 and III.C.2). In addition, the trapezoidal area created allows for easy positioning of the explant (group of neurons) close to the microchannel structure.

Two masks are used for the two photolithographic steps involved. The first mask (red portion of figure III.C.3) defines the microchannel structure. The channels are 100 microns wide and 50 microns deep. At the point where the presentation region meets the intermediate layer, the 50 micron thick channel layer widens from 100 microns to 200 microns. This “channel opening” is designed to create a larger area for incoming neurites to enter the microchannel structure. The widening occurs over 50 microns of length making the total distance from the decision point to the intermediate layer 250 microns in length. As seen in the concentration gradient formation results (section D of this chapter), the widening also decreases the sharpness
of the concentration gradient allowing for more variability in concentration across the width of the opening. The 50 micron layer of photoresist is achieved in one spin coat, using SU-8 10. In general, the process of releasing a PDMS mold from a silicon/SU-8 mold master results in the degradation of the mold master. Parts of the SU-8 photoresist tend to lift and stay in the PDMS upon release, therefore the PDMS molds are examined under microscope before being used. The SU-8 components with the smallest surface area are more likely to come off of the silicon during mold release. In order to extend the life of the mold master, the microchannels split into three beyond the decision point. It has been found that this fanning out of the channels aids in the reusability of the mold master, and also in reducing blockage of the fluidic system by small debris during operation of the device.
Figure III.C.1: Top view diagram of microfluidic cell culture plate

Figure III.C.2: Side view showing the three depths of the specialized cell culture plate
The second mask defines both the trapezoidal intermediate layer described in the last paragraph and positioning elements around the perimeter of the desired aluminum block location. The layer of photoresist exposed through this mask is 500 microns thick, and is formed with a single spin coat of the viscous SU-8 100 photoresist. Use of positioning elements avoids the necessity of an adhesive (such as vacuum grease) to hold the aluminum blocks in place on the silicon substrate during molding. These positioning elements are defined to be an area that is big enough to bond well to the silicon surface, maintaining adhesions after multiple mold releases. If the positioning blocks are too big in area, they crack during high temperature processing cycles (such as hard bake and annealing), and easily come off during mold release.
Figure III.C.3: Overlay of two masks used to produce the microfluidic unit. Red indicated the first mask (microchannels), and white indicates the second mask (intermediate layer and positioning elements).
To develop and maintain a concentration gradient in the microchannel structure, a constant flow of fluid is necessary. Previous work with PDMS based microfluidics (Dertinger, et al; Duffy, et al) injected fluids into the system through a puncture in the PDMS mold. In order to make the PDMS molds created in this project reusable, they are not irreversibly bonded to the cell culture plate surface. Therefore the application of fluidic pressure according to the previously used methods cause a separation of the PDMS mold from the surface to which it is reversibly bonded. The design arrived at in this project entails a set of compression/fluidic delivery plates that sandwich the PDMS mold onto the cell culture plate surface while delivering fluidics through the top of the device (figure III.B.4). Due to the compressibility of polymerized PDMS, application of force via the nuts and bolts clamps indicated in figure III.B.4 is sufficient to create a seal between the top of the PDMS structure and the top compression plate. Aluminum blocks similar to those used to mold the cell culture well (open region in the center of the plate) are used to mold the fluidic wells. Therefore no puncturing of the PDMS mold is necessary to deliver fluids to the system. Having the delivery system built into the compressor plates makes the unit very easy to reuse. Variations on the microfluidic PDMS structure may be tested trivially while using the same compressor plates.
Figure III.C.4: Microfluidic Processing overview

Figure III.C.5: Assembled Microfluidic Cell Culture Plate with Delivery System
Testing and Results of Concentration Gradient Formation

The previous section describes the fabrication of the microfluidic cell culture plate used in the *in vitro* studies. Using the plate successfully with spiral ganglion explants involves experimental assays of both the concentration gradient formation and maintenance within the microchannel structure. Concentration gradient formation and the effects of diffusion have been studied using media containing fluorescent dyes. This section describes the experimental procedure for preparing the cell culture plates for fluidic delivery and presents results from a set of concentration gradient assays.

Both concentration gradient and biological assays are prefaced with a procedure for setting up the microfluidic cell culture plate. In order to maintain constant flow rates in the microchannels, the fluid is delivered using syringes with a high precision digital syringe pump. Fluid is incompressible relative to gas, therefore all space from the syringes to the intermediate layer are flushed with fluid prior to pumping. The microchannels are cleared of bubbles by administering a vacuum at one fluidic well after loading fluid into the adjoining fluidic well and culture area (see figure III.C.5). The fluidic delivery tubes are flushed with media before the clamp is used. The final step before clamping the PDMS structure to the cell culture plate is filling the fluidic wells with the appropriate media so that little to no air is trapped within the system. This is detailed in the experimental procedure found in appendix C. After the structure is clamped, the bolts’ tightness is adjusted such that the microchannels are not deformed due to excessive compressive forces. At the same time, enough
compressive force must be administered to seal the top of the fluidic wells excluding all media is between the top of the PDMS and the top compression plate.

Experiments have been run to assess appropriate fluid flow rates necessary to create and maintain a concentration gradient. The experiments were conducted by filling one delivery line and fluidic well with standard media (Dulbecco's Modified Eagle's Media), and one delivery line and well with standard media plus a fluorescent molecule (0.1 mg/ml Biodipy, Biorad). The plate is then placed on the fluorescent microscope and the Y region of the microchannel structure is observed. Pumping rates of 15, 33, 40 microliters/hour per fluidic well were administered and the formation of a gradient was observed according to table III.D.1. Images of experimental gradient formation and diffusion processes are shown in figure III.D.1 and III.D.2.

<table>
<thead>
<tr>
<th>Flow Rate Pumped into Fluidic Wells (½ presentation flow)</th>
<th>Refresh Rate of Presentation Region</th>
<th>Time for Gradient to Appear</th>
<th>Time for Gradient to Diffuse</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 microliters/hour</td>
<td>11.1 1/sec</td>
<td>None after 12 hours</td>
<td>Not applicable</td>
</tr>
<tr>
<td>33 microliters/hour</td>
<td>18.3 1/sec</td>
<td>14.5 minutes</td>
<td>Not observed</td>
</tr>
<tr>
<td>40 microliters/hour</td>
<td>22.2 1/sec</td>
<td>Observed after 18 hours</td>
<td>~11.5 minutes</td>
</tr>
<tr>
<td>2.25 microliters/hour</td>
<td>1/8 1/sec</td>
<td>Minimum required flow rate (from section B)</td>
<td></td>
</tr>
</tbody>
</table>
Figure III.D.1: Gradient formation pumping 33 microliters/hour (left) and dissipation of gradient (right)
Three flow rates were examined for their ability to form a gradient in the presentation region of the microfluidic network. The total volume of the presentation region is 1 nanoliter based on the length, width, and height of 200 µm, 100 µm, and 50 µm. Therefore the presentation region refresh rate (column 2 in table III.D.1) is determined by summing the flow rates from each of the fluidic delivery channels and converting to nanoliters per second. All images of the microfluidic networks shown in
figure III.D.1 and III.D.2 are oriented such that hydraulic force from pumping is in the upward direction. The bottom of each individual image shows the two delivering microchannels (coming from the two different sources of media). Moving up the image, the channels merge at the decision point, and flow as one channel through the presentation region. The presentation region expands at the channel opening into the intermediate layer, which is trapezoidal in shape, and is 500 μm deep instead of 50 μm deep like the microchannels. The images on the left in figure III.D.1 are a time sequence depicting the formation of a gradient (actually a sharp step function) down the center of the presentation region using the flow rate of 33 μl/hr. The images on the right are a time sequence showing the results of turning off the delivery pumps after a gradient (sharp step function) has been formed.

The first flow rate examined (20 μl/hr per fluidic well) was administered for 18 hours with no visible formation of a concentration gradient. This is attributed to the procedure for setting up the microfluidic assembly and the volume of the fluidic wells (figure III.C.5). The assembly procedure described earlier in this section calls for filling the fluidic delivery tubes, the fluidic wells, and the microchannel structures with media before applying the compression plates. The procedure of filling all four individual wells and tightening the corners of the plate is followed by adjustment of the tension at the four bolts to reduce deformation of the microchannels while maintaining a seal at the top of the fluidic wells. This adjustment procedure invariably causes significant flow in all directions of the microfluidic network. Table III.B.2 shows clearly that the distance between two fluidic wells is much too great for
diffusion to carry molecules from one well to another. Despite this, the fluorescent indicator is observed in both fluidic wells and the cell culture well after the system has been assembled. The flow in all directions between fluidic and cell culture wells is attributed to this observation. The volume of each fluidic well is approximately 425 microliters (6.5 mm x 6.5 mm x 1 cm). At a flow rate of 20 microliters/hour, it would take 21 hours to flush out a fluidic well of this volume. Given the duration of the first experiment was half this time, both delivery channels would still be flowing a mixed version of the media. This offers an explanation to why a gradient was not observed at the slowest flow rate.

A flow rate of 40 µl/hr (with a refresh rate equaling 22.2 l/sec) was administered for 18 hours after which a clear and sharp concentration gradient was observed in the presentation region (top right image of figure III.D.1). This gradient is actually a fairly sharp step function with almost uniform concentration of fluorescent molecules on the left half of the presentation region and almost uniform absence of fluorescent molecules on the right half of the presentation region. The apparent lack of horizontal diffusion (in the x direction of figure III.B.3) in the presentation region is a result of the flow rate versus the estimated diffusion rate of the fluorescent molecule. In section B of this chapter, the t_{1/2} associated with an x_{1/2} taken horizontally across half of the presentation channel was approximated at 4 minutes (table III.B.2). Imagine a window of observation that includes a horizontal slice of the presentation region at the decision point moves upward at the fluid flow rate such that the volume of fluid contained within the window is the same throughout the presentation region.
A refresh rate of 22.2 Hz/sec corresponds to approximately 55 milliseconds of interaction between adjacent fluids in this window that enter from each of the delivery channels before being ejected out to the intermediate layer. The distance half the molecules will travel ($x_{1/2}$) in this time using equation 4 in table III.B.1 is approximately 2.4 µm. As the width of the presentation region is 100 µm, this relatively small $x_{1/2}$ distance correlates with the observed step function concentration distribution at the top of the presentation region.

The image on the top right of figure III.D.1 shows the sharp gradient formed using a flow rate of 40 microliters/hour for 18 hours. The pumping action was started after the fluidic unit was assembled and allowed to sit for several hours without pumping. Based on the diffusion rates in table III.B.2, this waiting period of several hours should have no affect on the concentration of the fluorescent molecule in each of the fluidic wells (the wells are separated by more than 0.5 cm of channel, which takes 29 days to diffuse through). The initial distribution of fluorescent molecules in both wells is again attributed to the flow that occurs during assembly of the apparatus. The pump was turned on and left overnight until observation 18 hours later. Therefore the exact time the gradient appeared is unknown. At a flow rate of 40 microliters/hour, the fluidic well should be completely flushed of its initially mixed solution within 11 hours. Immediately after the upper right image was acquired, the fluidic pumps were stopped. Images were then digitally captured every ten seconds for 12 minutes (samples are positioned on the right side of figure III.D.1). After 6.7 minutes, the sharp change in concentration observed across the midline of the
presentation region at time zero is significantly blurred. This is the best example of lateral diffusion occurring in the set. Figure III.D.2 shows a magnified view of the presentation region in these two images (\(t = 0\) and \(t = 6.7\)) for better observation of the effects of diffusion. The observed degree of diffusion concurs with the approximate \(t_{1/2}\) value of 4 minutes associated with the width of the presentation region channel. The later images, at time 8.3, 10, and 11.7 minutes show a dramatic flux in the location of the fluorescent molecules in the microchannel network in different directions. This flux is associated with residual changes in flow in the system as the distribution of pressure comes to equilibrium. Although the observed flux seems significant, it must be remembered that the entire volume of the presentation region is 1 nanoliter, and as such very small fluctuations in pressure will cause significant amounts of fluid flow in the channels. The decreasing brightness of the fluorescents in the microchannel with respect to time is attributed primarily to mixing fluids in the two channels as a result of this flux. One hour after turning off the pumps, the distribution of fluorescent molecules resembled that found in the top left image.

The magnified images of the microchannel structure in figure III.D.2 enables observation of how the widening of the presentation region at the channel opening reduces the sharpness of the gradient. Throughout the length of the presentation region, the sharpness of the gradient slightly decreases moving in the direction of flow. At the point where widening of the channel opening starts, the gradient slope decreases at a slightly higher rate. The resultant affect is a more continuous gradient formed at the interface between the microchannel and the intermediate layer (see
figure III.C.1 for names of microchannel structures). A gradient may be observed in
the intermediate layer (trapezoidal region) in the bottom left image of figure III.D.1.
Unlike the highly reproducible gradient formed throughout the microchannel structure,
gradients within the intermediate layer fluctuate with time even at constant flow rates.
Turbulent flow in the intermediate layer due to a significant increase in the cross
sectional area from that of the microchannel may lead to mixing in addition to
exchange of fluid with the cell culture well. Therefore, the most continuous gradient
reproducibly formed with this device is found at the “channel opening” end of the
presentation region.

The images on the left of figure III.D.1 shows the formation of a gradient in
the presentation region initiated with a pump rate of 33 microliters/minute. This
experiment was run one hour after the pump was stopped to study the gradient
diffusion shown in the images on the right side of figure III.D.1. Therefore the fluidic
wells were already flushed prior to the initiation of pumping, noting that minor flux
and mixing were observed at times 8.3, 10, and 11.7 minutes in the right column of
figure III.D.1. The top left image shows the state of the microchannel network at time
zero, the moment before the pump was turned on. Images of the microchannels were
recorded every thirty seconds for 15 minutes. No fluorescence was observed in the
channels until 8.5 minutes had passed. The subsequent images showed the flow from
the two delivery channels coming to a dynamic equilibrium over the course of the next
6 minutes. The gradient remained stable after that point.
Testing and Results of Growth Factor Concentration Gradient on Neurites \textit{in vitro}

Biological assays have yielded promising results, although neurite growth has not yet been observed at and beyond the decision point. The biological assays performed test the capacity of Neurotrophin-3 (NT-3) to direct spiral ganglion neurite growth. NT-3 was chosen as it had already been shown to affect the growth of rat spiral ganglion neurites in cell culture (Kim, et al). Based on the background in neuronal development described in chapter II, netrins would be ideal to use in this apparatus. Unfortunately, netrins are not commercially available for purchase at this time. Cell culture media is prepared as described in appendix E such that each explant is subjected to a concentration gradient stemming from one media source with 0% NT-3 and the other source with 100% recommended concentration NT-3 (25ng/ml). Control experiments flow NT-3 rich media to both of the fluidic wells. Both control and non-control experiments have been conducted and results are presented later in this section. The description of additional setup techniques necessary for cell culture experiments with the microfluidic cell culture apparatus is described first.

The surface of the petri dish is coated with various molecules to assist in the adhesion of the neurons to the plate prior to any cell culture work. All cells (other than cancer cells) require surface adhesion to survive. The plates are coated in two steps with chains of the amino acid lysine (Poly-L-Lysine) followed by the extracellular component laminin. Standard neuronal cell culture protocol describes
two stages of media. The first media presented to the freshly excised neurons is serum based (bovine serum albumin was used in the experiments of this project). The media is rich with organic content in order to provide the shocked neurons with all possible nutrients. After the neurons adhere to the coated surface while being submerged in the rich serum based media, the media is changed to one more specific to neuronal growth. This second media is used to test the conditions for guiding neurite growth. As stated above, the components of this media are specified in Appendix C, which clearly describes the protocol for the entire microfluidic/biological assay.

The three standard types of \textit{in vitro} neuronal cell preparations include using previously established cell lines, whole explants from the tissue of interest, or dispersed neurons from the tissue of interest. Cell lines do not display many of the properties of neurons \textit{in situ}, and as such are eliminated from the choices. Although dispersed neurons are reasonable because of the methodology used to differentiate multi-potent cells to the desired cell type, explants are preferred as the cell – cell interactions that will be experienced by \textit{in vivo} spiral ganglion need be considered. Therefore, explants of spiral ganglion are used in the \textit{in vitro} studies presented.

Rat pups are the biological animals utilized for the experiment of this project. Rats are chosen because of the accessibility of their inner ear, along with the complexity and similarity of their inner ear to higher-level primates. Cell culture with rat spiral ganglia explants had previously been established and utilized at the facilities where this research was conducted, therefore the species was natural to select for this work. The age of the rats to be sacrificed for extraction of the spiral ganglion is
determined by the species developmental time line. Rats peripheral sensory system is not fully developed at birth. The spiral ganglion neurites do not complete synaptic connection with the hair cells until day six of life. In order to study how to affect the outgrowth of the neurites, the spiral ganglion are removed on or before day five of life (day three and four rat pups are used in the experiments conducted for this project). This time frame safely guarantees that the developmental window has not been exceeded.

Once the spiral ganglia are removed, they are divided into four pieces (explants) and positioned within the cell culture plate. The size of each explant is chosen so that the piece is small enough to be easy manipulated and large enough to have a significant amount of neurites growing out from it. Once the explants are transferred to the microfluidic cell culture plate under the magnification of a dissection microscope, they are positioned using an inverted microscope. The explants are gently pushed from the macroscopic cell culture well into the intermediate layer. The trapezoidal shape of the layer eases the positioning of the explant next to the microfluidic outlet. Once positioned, care must be taken to not move the fluidic tubing, as this may initiate flow at the microfluidic outlet that will move or damage the explants. As such, at this point the fluidic system is not pumping.

The explants are positioned in the serum based media and given 8 – 18 hours to attach to the coated cell culture plate. After attachment, the syringe pump is activated at a rate of 30 microliters/hour. This provides as total rate of 60 microliters/hour in the presentation region of the microfluidic structure. As seen in the
experimental results of creating a concentration gradient, this flow rate is substantial enough to create and maintain a concentration gradient. The explants are subjected to the gradient for 3 – 4 days before being fixed and stained. The total duration of cell culture growth is therefore 4 – 5 days, which has been shown to be sufficient to achieve neurite lengths of up to 1 mm (Kim, et al). The fixing procedure (using 4% paraform aldehyde) is followed by an immuno-histochemistry fluorescent staining procedure. The primary antibodies react with the neurofilaments found in the neurites, and the secondary antibodies are conjugated with either green or red fluorescent molecules as seen in the resultant images (figure III.E.1 through III.E.6). Figure III.E.1 is an overview of the four experiments yielding neurites in the microchannels, table III.E.1 defines the images in this figure. Table III.E.2 is an assessment of the ten successfully imaged experiments conducted for this project.

Table III.E.1: Definition of images in figure III.E.1 which all exhibit neurite growth into the microchannel structure. Images in the right column of the figure are magnified versions of the images in the left column that are defined in this table. Figure III.E.1 images indexed from top to bottom (top left image = 1, bottom left = 4)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Place in Figure III.E.1</th>
<th>Experiment Date</th>
<th>Experiment Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure III.E.2</td>
<td>Image 1</td>
<td>Exp. 4/18/01</td>
<td>Plate C:a</td>
</tr>
<tr>
<td>Figure III.E.3</td>
<td>Image 2</td>
<td>Exp. 5/22/01</td>
<td>Plate B:d</td>
</tr>
<tr>
<td>Figure III.E.4</td>
<td>Image 3</td>
<td>Exp. 4/25/01</td>
<td>Plate A:b</td>
</tr>
<tr>
<td>Figure III.E.5</td>
<td>Image 4</td>
<td>Exp. 4/25/01</td>
<td>Plate D:b</td>
</tr>
</tbody>
</table>
Figure III.E.1: Neurites growing into channels; High magnification on the right
Figure III.E.2: Neurites growing into channels; control plate C:a on 4/18/01
Figure III.E.3: Neurites growing into channels; plate B:b on 5/22/01
Figure III.E.4: Neurites growing into channels; plate A:b on 4/25/01
Figure III.E.5: Neurites growing into channels; plate D:b on 4/25/01
Table III.E.2: Raw data from properly imaged assays with microchannel unit

<table>
<thead>
<tr>
<th></th>
<th>Growth before flow</th>
<th>Growth during flow</th>
<th></th>
<th></th>
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<th></th>
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<tbody>
<tr>
<td>Data from:</td>
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<td>48 hours</td>
<td>72 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>flow</td>
<td>neurite</td>
<td>neurite into</td>
<td>enter</td>
<td>enter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% NT-3 (µL/hr)</td>
<td>growth</td>
<td>channel (+) side</td>
<td>(-) side</td>
<td>decision</td>
</tr>
<tr>
<td>plate C:a</td>
<td>100</td>
<td>18</td>
<td>yes</td>
<td>YES</td>
<td>1 neurite</td>
<td>control</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>18</td>
<td>yes</td>
<td>YES</td>
<td>1 neurite</td>
<td>control YES-both</td>
</tr>
<tr>
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<td>no</td>
<td></td>
<td>yes-both</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>18</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>yes-both</td>
</tr>
<tr>
<td>plate G:a</td>
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<td>18</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>yes-both</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>18</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>yes-both</td>
</tr>
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<td>no</td>
<td>no</td>
<td></td>
<td>Not clear</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
<td>no</td>
<td>no</td>
<td></td>
<td>Not clear</td>
</tr>
<tr>
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<td>yes</td>
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<td>0</td>
</tr>
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<td>0</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
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<td>yes</td>
<td>1 fiber</td>
<td>0</td>
</tr>
<tr>
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<td>72 hours</td>
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<td></td>
</tr>
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<td>30</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>yes - to (+)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>yes - to (+)</td>
</tr>
<tr>
<td>plate A:b</td>
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<td>30</td>
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<td></td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>no</td>
</tr>
<tr>
<td>plate B:a</td>
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<td>30</td>
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<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>no</td>
</tr>
<tr>
<td>plate B:b</td>
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<td>30</td>
<td>yes</td>
<td>YES</td>
<td>4 neurites</td>
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<tr>
<td></td>
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<td>30</td>
<td>yes</td>
<td>YES</td>
<td>4 neurites</td>
<td>2 neurites</td>
</tr>
</tbody>
</table>

All images are categorized and classified based on the observation of general neurite growth, growth of neurites into the microchannels, what side of the channel the neurites entered into, and if any glial cells are found past the decision point. The apparatus is designed to easily assess what type of media the neurites prefer based on what channel they grow into past the decision point. Over the course of the limited number of experiments performed neurite growth beyond the decision point was not
observed. Potentially longer experimental growth times or decreased flow rates in the microchannels may increase the likelihood of neurite growth beyond the decision point. Based on figures III.D.1 and III.D.2 (previous section), the gradient clearly exists all the way up to the opening of the microchannel structure. The gradient found at the edge of the channel opening of the presentation region before the intermediate layer is relatively invariant under equilibrium conditions of flow. The change in concentration across this area is more gradual than anywhere else in the microchannels. Based on the lack of images of neurites past the decision point, evaluation of the affect of NT-3 on neurite growth is based on neurite location at the channel opening. Although assessment of final neurite growth cone location in the decision region may be a better measure of the affect of NT-3 on neurite guidance, ambiguities in two (of the four) images analyzed makes entrance locations the best choice for evaluation.

Table III.E.1 lists the experimental origins of each of the images seen in figure III.E.1. Figure III.E.1 outlines the results of the four explants that exhibited neurite growth into the microchannel opening. The left column shows low magnification images of the explants and channels labeled with which media (NT-3 rich or NT-3 absent) is flowed into each of the delivery channels. A ‘+’ is used to indicate the delivery channel carrying NT-3 in the media. A ‘−’ indicates delivery channels carrying media absent of NT-3. The right column of the figure shows a high magnification of each image on the right that focuses in on the neurites growing into the microchannels. These images are oriented the same as the images on the left,
therefore the side of the channel carrying NT-3 rich media may be inferred from the ‘+’ and ‘−’ labels on the images to the left.

Table III.E.2 lists the experimental results of an assay of the effectiveness of NT-3 in guiding neurite growth. Although twenty-one explants were subjected to the appropriate experimental conditions, only ten explants were successfully imaged and evaluated. The primary contributors to unsuccessful imaging were biological contaminants in the system and human error following the cell culture or staining procedures (some explants were “sucked up” into the microchannel during the staining). Three of the explants imaged were controls with NT-3 rich media flowing from both delivery microchannels. The other seven explants were subjected to a concentration gradient formed by media with NT-3 and media absent of NT-3.

Plate C:a (from experiments conducted on 4/18/01) is the only control experiment in which neurites were imaged entering the microfluidic network. The top image in figure III.E.2 reveals the distant location of a poorly placed explant from the channel entrance (usually explants are located in the center of the trapezoidal intermediate layer). Despite being a distance of roughly 1.5 mm from the microchannel opening, almost all neurites extend exclusively toward the opening. The label “extending neurites” on the top image is referring to approximately five extensions coming from the explant and growing along the left side of the trapezoidal intermediate layer. Only one or two of these neurites makes it all the way to the top of the intermediate layer in the vicinity of the channel opening. The high magnification version of this image (bottom of figure III.E.2) reveals what appears to be a cluster of
neurites in the upper left corner of the intermediate layer. The arrow indicating “entering neurite” points to the end, or growth cone, of a neurite entering the channel opening. Potentially this neurite-like projection is actually a projection from one of the local glial cells. Sometimes what appears to be a neurite is actually a thin wire like projection between two glial cells. The identification of these glial projections is based primarily on the locality of neurites that are observed to have traveled from the cell bodies of neurons. On the right of this image glial projections are identified as no neurites were observed on that side of the intermediate layer. Observation of the high magnification image from this experiment indicates that the observed projection is actually an entering neurite.

Plate B:b from the experiments conducted on 5/22/01 yielded neurites that entered on one side of the channel opening but apparently grew across the concentration gradient to the other side of the presentation region (figure III.E.3). The low magnification version of this image (top) beautifully highlights the attractive nature of the flowing microchannels, as two or three neuronal fibers (identified because of their thickness) make a sharp turn (labeled “turning point”) and grow directly towards the channel opening. This sharp turn is assumed to take place after 22 hours of growing without pumping of the microfluidic system (experimental times are shown in table III.E.2). The neurites were growing away from the explant in a somewhat random direction until flow introduced fresh media (half of which was rich with NT-3) into the intermediate layer. The remaining 72 hours of the experiment are therefore speculated to result in the growth of the neuronal fibers (individual neurites
that separated from the fibers along the way) directly into the microchannel as seen in
the bottom image. The neurite interactions and staining artifacts seen in this image
make it difficult to accurately assess both the number of neurites entering the channel
and the final growth cone location of these neurites. The growth cone locations are
made final at the end of the experiment with addition of the fixative paraform
aldehyde. Labeled on the high magnification image in figure III.E.3 are six neurites
entering the channel opening. The six neurites selected are the brightest and thickest
neurite-looking objects crossing the interface. Thick looking neurites are frequently
neuronal fibers that are composed of several neurites bundled together. As mentioned
in the previous paragraph, connections between glial cells may sometimes be confused
with neurites. Glial cell projections are usually thin, therefore the thickness and
brightness criterion in neurite selection is in attempt to filter them out of the analysis.
Although the numbers are admittedly not accurate, it is estimated from this image that
4 neurites enter the microchannel on the ‘+’ side of the presentation region and 2
neurites enter on the ‘−’ side of the region.

The next two figures, III.E.4 and III.E.5 show similar images that are easier to
interpret. In both of low magnification (top) images of these figures the explant may
be readily observed with a single fiber or neurite extending into the microchannel
structure. Based on the thickness and the lack of surrounding neurites, both of these
are actually assumed to be neuronal fibers made up of several neurites. The entrance
of both of these neuronal fibers or neurites is clearly on the ‘+’ side of the decision
region and channel opening. It may be noted that the images in figure III.E.4 were
taken with the compression plates removed from the cell culture plate. This experiment was conducted before holes were drilled through the bottom compression plate for visualization with the inverted fluorescent microscope while under compression. Misalignment may be observed between the edge of the PDMS microstructure and the background stain on the petri dish as a result of the compression plate removal. This misalignment did not affect the entrance location of the neurite visualized in the images, as confirmed by the angle of neurite entrance and the degree of translation/misalignment. The high magnification image on the bottom of figure III.E.5 shows the fiber/neurites clearly entering the microchannel structure from the ‘+’ side. A clearer image showing the neurites inside the microchannel structure was not obtained.

Table III.E.3: Description of Images in figure III.E.6 showing glial cells at various locations in the microchannel structure
Images indexed from top to bottom and left to right. (top left image = 1, top right = 5)

<table>
<thead>
<tr>
<th>Glial Cells in Presentation Region</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Image 1</td>
<td>Exp. 5/22/01</td>
<td>Plate A:b</td>
</tr>
<tr>
<td>Image 5</td>
<td>Exp. 5/22/01</td>
<td>Plate B:a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glial Cells Past Presentation Region in One Channel</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Image 2</td>
<td>Exp. 5/22/01</td>
<td>Plate A:a</td>
</tr>
<tr>
<td>Image 3</td>
<td>Ambiguous: Exp. 4/25/01</td>
<td>Plate A:a</td>
</tr>
<tr>
<td>Image 4</td>
<td>Ambiguous: Exp. 4/25/01</td>
<td>Plate A:b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glial Cells Past Presentation Region in Both Channel</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Image 6</td>
<td>Exp. 4/18/01</td>
<td>Plate C:a</td>
</tr>
<tr>
<td>Image 7</td>
<td>Exp. 4/18/01</td>
<td>Plate C:b</td>
</tr>
<tr>
<td>Image 8</td>
<td>Exp. 4/18/01</td>
<td>Plate G:a</td>
</tr>
</tbody>
</table>
Figure III.E.6: Glial Cells in the microchannels
Table III.E.3 and the corresponding figure III.E.6 show various configurations of glial cells in reference to the microfluidic network. Glial cells seem to pave the way for developing neurites in most of the images observed for this experiment. Therefore the growth of glial cells in various parts of the microfluidic network may indicate the plausibility of successfully using the device. In preliminary structures, the growth of glial cells in the microchannels was the first conformation that the fluidic compartments were interacting with each other and that the cells could survive in the artificial local environment. All images have been classified in reference to glial cell location into three categories; not in the channels, in the presentation region, and past the decision point.

All three successfully imaged control experiments yielded neurites past the decision point. In all three of these experiments, the glial cells grew up both delivery channels (the bottom three images on the right side of figure III.E.6). The observation of glial cell growth past the decision point in the non-control experiments occurred unambiguously once out of the seven successfully imaged experiments. The microchannels from this single experiment (plate A:a from 4/25/01) are show in the second image from the top on the left side of figure III.E.6. The two bottom most images on the left side in figure III.E.6 (plate A:a and plate A:b from experiments on 4/25/01) have ambiguous staining in their delivery channels that seems to result from fluorescing debris rather than stained glial cells. The top two images in this figure are representative of what the remaining four images looked like. Glial cells were observed both in just the channel opening (2 plates), and further within the
presentation region (2 plates). With more than one clear example of glial cells growing into the delivery channel containing NT-3 an argument could be made in regard to glial cell preference. The lack of multiple examples is most likely linked to the duration of the experiment. The experimental conditions during this assay were still being optimized and therefore the experiments have differing durations. The control experiments were run for a total of 120 hours (see table III.E.3) while the non-control experiments were run for 74 and 94 total hours. The one non-control example that had glial cell growth past the decision region came from a 94 hour experiment (5/22/01) which further indicates that time may be the primary factor in having glial cells grow into the delivery channels. The lack of multiple examples of glial cells past the decision point in the non-control experiments weakens any argument in regards to their preferential growth in media containing NT-3.

Table III.E.4: Overall results of assays with microchannel unit

<table>
<thead>
<tr>
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<th>Control: Flow 100%/100% NT-3</th>
<th>Experiment: Flow 100%/0% NT-3</th>
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<tbody>
<tr>
<td>Total Explants Tested</td>
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</tr>
<tr>
<td>Total Properly Imaged</td>
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<td>7</td>
</tr>
<tr>
<td>Explants with Neurites</td>
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<td>6</td>
</tr>
<tr>
<td>Neurite Growth into Channel</td>
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<td>4</td>
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</tbody>
</table>
Table III.E.4 summarizes the results discussed in this section. In the right most experiment column the sum of neurites entering from the NT-3 rich side labeled ‘+’ and the NT-3 absent side labeled ‘−’ are 6 and 2 respectively. This yields a percentage of 75% neurites entering the microchannel structure on the side with NT-3. A comparable measurement cannot be made as only a single neurite entered the microchannel structure. The percentage of neurites showing preferential growth comes from analysis of only three images. Two of those images (figures III.E.4 and III.E.5) have a single, clear extension coming from the explant and entering the microchannel opening on one side. The extension in both of these cases is not clearly imaged within the presentation region. The thickness and lack of approximate neurites indicates that these two extensions are neuronal fibers consisting of multiple neurites.
The third image that contributes to the calculation of 75% is figure III.E.3. The low magnification version of this image impressively demonstrates the tropic affect of flowing media with NT-3 from the microchannel as neuronal fibers and neurites change direction at the indicated turning point after flow began. The high magnification image in this figure is somewhat difficult to assess. The two previous figures were very clear-cut in what location the fibers entered the structure and that they continued to grow up the presentation region without radical adjustments in growth direction. Contrary to those clear-cut images is figure III.E.3, which was approximated to contain 4 neurites entering the ‘+’ side of the channel opening and 2 neurites entering the ‘−’ side of the opening. The other interesting value in table III.E.4 is the percent of glial cells showing preference. The 0% exhibition of glial cell preference in the control experiment and the 100% exhibition of glial cell preference in the non-control experiment suggest glial cells prefer growing in NT-3 rich media. As mentioned in the previous paragraph, these percentages are not valid with such a small data set and should not be considered valid. Overall the data hints that spiral ganglion neurites and glial cells prefer to grow in or towards media that containing 25ng/ml NT-3. With more data the two sets of percentages in this table may become more statistically reasonable assessments of these potential growth preferences.
Chapter IV.

Approach and Results: Electric Field Stimulation
Overview of Electric Field Stimulation

This chapter describes a second technical approach for guiding the growth of spiral ganglion neurites. The stimulant used to affect growth is an electric field. Again, cell culture models are used to determine the effectiveness of this stimulant before \textit{in vivo} experimentation with the stimulant is attempted. Section B of this chapter describes a technique for establishing electric fields in cell culture without the use of electrodes in the cell culture media. Although not implemented through the course of this project, this technique is suggested as a preliminary method for determination of effectiveness of electric fields in controlling guidance of neurite growth. Sections C and D describe the modeling and implementation of a microfabricated cell culture plate that has metal electrodes within the cell culture media. The liquid-electrode interface on this device is difficult to model accurately due to variations in conductivity of fluids over time. Reduction-oxidation reactions occurring at this interface during electrical stimulation may result in deleterious by-products that can contaminate the cell culture media. Despite these facts, studies using metal electrodes \textit{in vitro} are important as ultimately the electrodes of the cochlear implant will be used to stimulate the spiral ganglion neurites. Section E contains the results of the \textit{in vitro} experiments performed using both constant voltage, and oscillating voltage (a sine waveform was used, centered at zero volts). The assays suggest that the survival of a spiral ganglion explant is inversely proportional to the magnitude of the voltage applied and the duration of stimulation. Both modeling and
biological assays indicate, however, that there are major flaws in the design of the current electrode based cell culture plate. The conclusion chapter V, section C describes how modification of this cell culture work may lead to development of an *in vivo* implementation. Optimally the results of both *in vitro* and *in vivo* experiments may led to increased resolution of the cochlear implant in human patients.
Preliminary Technique: Create Electric Fields in vitro Without Electrodes

Various techniques have been developed to isolate the electric field in solution from the metal electrodes that carry current into and out of the solution. These techniques have been developed not only to isolate deleterious electrode products, but also to avoid difficult-to-measure by-products of electric fields in solution such as the ionic double layer. A box coil technique has been developed by Battocletti, et al to non-invasively induce an electric field in solution by pulsed magnetic fields. This technique offers the ability to characterize the effects of electric fields on neurite growth in vitro without deleterious electrode products contaminating the cell culture media. This section describes construction and operation of the box coil device, along with the biological assays used to test if spiral ganglion neurites may be affected by electric fields. The box coil method has not been implemented for this project; therefore no results have been obtained with respect to this non-electrode model. Ideally, the box coil apparatus would be used to substantiate the concept of guiding spiral ganglion neurites with electric fields without subjecting them to deleterious electrode by-products. This technique would then be followed with biologically assays studying how to guide spiral ganglion neurites with an electrode based cell culture model, which has been implemented, and is described in the following section.

The box coil is simply constructed from a large metal sheet that is shaped into a box as seen in figure IV.B.1. The two ends of the sheet, labeled ‘A’ in the figure, are not touching. These are the points at which pulsed current is applied to the system.
The pulses of current create a magnetic field, which in turn induces a parallel electric field following the plane of the top and the bottom of the box. A cell culture dish may be placed on the top or bottom plane of the box, and an electric field will be induced of controlled magnitude based on the dimensions of the box, the amplitude of the voltage pulses, and the duration of the pulses. Figure IV.B.3 shows the waveform used to stimulate neurons in cell culture. A waveform generator driving a power amplifier is used to produce a train of twenty-two rectangular voltage pulses with a duration of twenty microsecond and two hundred microsecond between pulses. A sawtooth current with a typical peak of twenty amps is generated in the coil. The pulse width of twenty microseconds was calculated to induce an electric field of 0.25 V/m.
Figure IV.B.1: Diagram of box coil construction and current flow in the metal sheet

(from Battoceletti, J.H. et. al reference 26)
The box coil is used as a preliminary means to assay of the affects of electric fields on spiral ganglion neurite growth. To prove the functionality of their design, Battacocletti, et al. waited 12 hours after extracting dorsal root ganglion explant for
attachment to the cell culture plate. After attachment, the box coil was active for 18 hours followed by an inactivation period of 18 hours. The explants exhibited galvanotropism as a result of the stimulation from the box coil. Standard cell culture techniques already established for spiral ganglion explants will be used to set up the preliminary experiment using the box coil apparatus. Cell culture plates with attached spiral ganglion explants will then be subjected to electric fields using the protocol dictated by Battacocletti, et al. The ease of development and use of this apparatus makes it an attractive vehicle to study affects of electric field duration and intensity on spiral ganglion neurite growth.
Model and Design Unit for Creating Electric Fields in vitro With Metal Electrodes

Modeling of the electric field produced in the cell culture media is crucial in determining the affects of electric fields on neurite growth. Researching this topic has yielded many questions about the nature of electric fields in liquid. The circuit in figure IV.C.1 models the application of voltage between two metal electrodes submerged in a conducting liquid. Electrons carry current though the conducting metal that makes up the electrodes. In liquid current is conducted via the migration of charged atoms (ions) and charged molecules. Therefore, the conductivity of the fluid is proportional to the concentration of charged species (ions and charged molecules) in solution. At the interface between the electrode and the liquid, electronic current is converted to ionic current by way of oxidation-reduction reactions. The circuit model shown in figure IV.C.1 depicts the liquid electrode interface as a capacitor and resistor in parallel. The capacitance and resistance of these circuit components are used to classify the electro-chemical characteristics of a given fluid.
The conductance of liquid in an electrochemical cell varies with respect to time. This variation occurs primarily as a result of two phenomena. The first is the formation of an ionic double layer at the liquid-electrode interface. This is the collection of charged species in solution at the surface of the electrode of opposite charge. These oppositely charged species develop a voltage drop at each liquid-electrode interface. The result is a decrease in the voltage dropped across the body of liquid, and therefore a decrease in magnitude of the electric field in the liquid. The second phenomenon that causes variation in conduction of liquid over time is the flux of charged species in a finite volume of liquid. When a finite volume of liquid has an electric field applied across it, electrophoresis of the charged species creates a concentration gradient across the electrochemical cell. A concentration gradient of the charged species results in a variation in conductivity between the two electrodes that changes while electrophoreses is occurring.
The simulation program Quickfield4.0 (by Tera Analysis) has been used to model the direction of the electric field in the cell culture wells. Figures IV.C.2 and IV.C.3 depict the voltages and electric fields within a cell culture having metal electrodes. The voltage gradient displayed assumes uniform conductivity of the medium and no formation of ionic double layers. Empirical determination of the voltage drop at the ionic double layers is difficult, as will be discussed in the results section of this chapter. The magnitude of the electric field may be determined by using a constant current source to avoid the adverse affects of charged species migration and ionic double layer formation. The concentration, charge, and mobility of the charged species in solution are used to determine \( \sigma \), the conductivity contributed by individual ions (equation 1 in table IV.C.1). The total current through a well is defined as the integral of the current density over the area of one ‘slice’ of the conducting medium (equation 3). The current density \( J \) and the conductivity \( \sigma \) may then be used to determine the electric field in the medium analyzed (for a review of ohms law for electrolytes, see Weiss’s text *Cellular Biophysics*).

<table>
<thead>
<tr>
<th>Table IV.C.1: Equations used to determine the magnitude of an electric field in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Ion conductivity ( \sigma = u z^2 F^2 c = q \ast N \ast \mu )</td>
</tr>
<tr>
<td>2: Molar conductivity ( \Lambda = \sigma/c = D z^2 F^2/RT )</td>
</tr>
</tbody>
</table>
Design flaws associated with the microfabricated unit discussed in the results section of this chapter have prevented empirical determination of the electric fields produced in solution. Determination of the electric field produced in the cell culture media requires knowledge of the concentration of charged species in the media. Table IV.C.2 lists the charged species that make up the cell culture media and their concentration. The “Charges Contributed” column is determined by multiplying the molar concentration of the species by the sum charge of a single molecule of that species. Table IV.C.3 lists approximate values of the various parameters needed to estimate the magnitude of the electric field (\( \varepsilon \)) in the cell culture media. The resultant estimated magnitude is the last entry in the table.

Table IV.C.2: Determination of Total Charged Species Per Unit Volume (N) based on hypothetical constituents of Cell Culture Media

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration</th>
<th>Charges Contributed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive ions in DMEM</td>
<td>10 mM (Na+)</td>
<td>+1</td>
</tr>
<tr>
<td>Negative ions in DMEM</td>
<td>5 mM (Cl-)</td>
<td>-1</td>
</tr>
</tbody>
</table>
Table IV.C.3: Approximated Values in determination of Electric Field Magnitude (\( \varepsilon \)) in a cell culture well

<table>
<thead>
<tr>
<th>Component</th>
<th>Approximated Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu ): Ion mobility in solution</td>
<td>( \approx 5 \times 10^{-3} ) (S/m / equiv/m(^3))</td>
</tr>
<tr>
<td></td>
<td>( = 5 ) (S/m / equiv/Liter)</td>
</tr>
<tr>
<td>( N ): number of charges per unit volume</td>
<td>((10 \times</td>
</tr>
<tr>
<td>( q ): Value of charge</td>
<td>+1 x q = 1.6 x 10(^{-19}) C/q</td>
</tr>
<tr>
<td>( \sigma ): Ion conductivity</td>
<td>( 75 \times 10^{-3} ) * S/m</td>
</tr>
<tr>
<td>( I ): current through the system</td>
<td>1 micro Amp</td>
</tr>
<tr>
<td></td>
<td>( = 1 \times 10^{-6} ) amps</td>
</tr>
<tr>
<td>( A ): area of cross section of cell culture well</td>
<td>( 1 ) cm(^2)</td>
</tr>
<tr>
<td></td>
<td>( = 0.0001 ) m(^2)</td>
</tr>
<tr>
<td>( J ): current density vector</td>
<td>Assume uniform distribution across area</td>
</tr>
<tr>
<td></td>
<td>( J = I/A )</td>
</tr>
<tr>
<td></td>
<td>( = 0.01 ) amps/m(^2)</td>
</tr>
<tr>
<td>( \varepsilon ): electric field vector</td>
<td>( 0.133 ) V/m</td>
</tr>
</tbody>
</table>
Figure IV.C.2: Side view of cell culture well with electrodes at the bottom. Electric field vectors are displayed as arrow and voltages are color coded according to the legend on the right.
Figure IV.C.3: Top view of cell culture well with electrodes at the bottom. Electric field vectors are displayed as arrow and voltages are color coded according to the legend on the right.
Fabricating the Patterned Electrode Cell Culture Plate

The cochlear implant is constructed of titanium or platinum electrodes and is implanted within the cochlea to stimulate the local spiral ganglion neurites (Chouard, et al; Liang, et al). Therefore, experiments using the box coil to control spiral ganglion neurite growth are not representative of how electric fields will be presented in vivo. To bridge this gap, cell culture experiments are conducted in which electric fields are created in the media using metal electrodes submerged within that media. Microfabrication has been utilized to create a cell culture plate with integrated electrodes for these experiments. Photolithographic processing offers the ability to reproducibly create a wide variety of electrode configurations with accuracy and relative ease. Results of the microfabrication process have yielded several units that have been used in various biological assays. A primary feature in the design of the plates is the ease of applying electrical stimulations to the electrodes, their disposability, and their ability to allow for multiple simultaneous experiments or stimulants.

The fabrication process developed to create the electrode based cell culture units is depicted in figures IV.C.1 and IV.C.2. The process may be described in two parts, the microfabrication of the electrodes on a glass slide, and the molding of cell culture wells in PDMS. Glass slides were chosen as the substrate for three reasons; glass is an established cell culture substrate, its transparence allows for visualization with an inverted fluorescent microscope, and it is relatively inexpensive in the form
The glass substrates utilized are 50 x 75mm slides produced by Dow-Corning. The manufacture of the substrate does not entail rigorous checks on uniform thickness, nor smoothness of the surfaces of the slide. As such, many obstacles have been encountered in the microfabrication of the electrode units.

The mask used in the processing of the metal electrodes was created with an auto-cad program (Canvas 5.0) and printed with a high-resolution printer (linotronic 330) onto transparency paper. Figure IV.D.3 diagrams the mask used to pattern parallel electrodes on the glass substrates with light red shading indicating the location of the twelve cell culture wells. Figure IV.D.4 diagrams a similar design that was not used to create a cell culture plate for this project. The two masks depicted both are designed to create parallel electric fields in each well, so that observed changes in neurite growth could be easily correlated to direction of the electric field. The cell culture plate patterned using the mask in figure IV.D.3 employs constant voltage across the electrodes (and therefore four cell culture wells at a time) to create an electric field. The mask diagramed in figure IV.D.4 would use a constant current supply to maintain the same current levels in the row of four serially connected cell culture wells.

Power is supplied to the electrodes via a female clamping circuit board plug. The masks in figures IV.C.3 and IV.C.4 define connections from all electrodes to the evenly spaced contacts that interface the circuit board plug. This design feature provides for ease of use of the units, as they may be discarded after staining. The circuit board plug and disposable cell culture plate are held in place on a breadboard.
that is fastened to an insulating piece of Styrofoam before being placed into a cell
culture incubator. Figure IV.C.4 diagrams the assembled electric field apparatus.

Titanium and gold are used to create the electrodes on the unit. These metals
are also used in cochlear implants as they are resistant to corrosion and are fairly inert
biologically (Chouard, et al). Cleaning of the glass slides takes place before all
fabrication processes to remove organic and inorganic contaminants. The cleaning
procedure includes sonication of the glass slides in acetone and methanol before a long
bake at 230ºC to evaporate all solvents from the substrate prior to evaporation. An
electron-beam evaporator was necessary to evaporate the titanium and gold layers as
titanium needs to be raised to a high temperature while maintained at low pressure to
become gas. The entire surface of the slide is covered with a 200 Å adhesion layer of
titanium followed by a 2000 Å layer of gold. Standard negative photoresist is then
spin coated over the metal surface. The photoresists is exposed and patterned using
the bright field mask diagramed in figure IV.D.3. The patterned photoresist is used to
protect the metal that will become electrodes while chemical etchants are used to
dissolve the gold and titanium layers respectively. After etching the metals, the
protective photoresist layer is removed with acetone, and the slides are cleaned for
biological use.

The wells that overlay the microfabricated electrodes are molded from PDMS.
Twelve wells that are one cubic centimeter each are molded from aluminum blocks
and align to the electrodes created with photolithography. An assembly has been
constructed that holds the blocks in place during casting of the mold. The molding
process requires 24 hours at room temperature. The molded cell culture wells are visually aligned with the metal electrodes on the glass substrate, and allowed to reversibly bond in place. A silicon-based sealant is applied to the outside edge of the PDMS mold to create a permanent bond between the two components.

Figure IV.D.1: Overview of process used to create electrodes on glass slide
Figure IV.D.2: Overview of process used to create molded cell culture wells to fit over electrodes on glass slide

Figure IV.D.3: Parallel electrode masks pattern used with constant voltage in experiments (black) with position of 12 wells (light red shading)
Figure IV.D.4: Parallel electrode masks pattern for constant current (black) with position of 12 wells (light red shading)
Testing and Results in vitro with Metal Electrodes

The testing of the microfabricated cell culture plate with metal electrodes has yielded poor results. Therefore, although biological experiments have been conducted using the plate, their validity is questioned. This section describes the primary flaws with the fabrication and design of the cell culture plate. The results and discussion of biological assays performed with the plate are also in this section. The conclusions that can be drawn from the biological assays relate the duration and magnitude of an applied voltage to the survival rate of spiral ganglion explants. No directional effects were observed, which is not extremely surprising, as the constant voltage applied did not present an electric field to the explants for any significant duration. Overall, the testing and results of the unit indicate major revisions are necessary in its design. These revisions are discussed in the conclusion chapter.

Testing of the microfabricated cell culture plate with metal electrodes shows significant wear on the metal electrodes with the application of voltages greater than three volts. As clarified in the modeling approach, constant current is needed to maintain an electric field in a liquid. The microfabricated unit has an electrode configuration that doesn’t allow for the stimulation of constant current across multiple cell culture wells simultaneously. Each pair of electrodes connects a row of cell culture wells together in parallel; therefore constant voltage was used in the biological experiments. A constant current source was tested on the plate having fluid only in a single cell culture well. The lowest possible setting on the current source (0.1 microamps) caused rapid (less than ten seconds) destruction of the metal electrodes, as
voltages in excess of five volts were being used by the source to drive the current through the electro-chemical cell. Observation of the bottom of the cell culture plate after an electrode has become destroyed shows the area surrounding the break in the electrode is missing titanium (titanium was found to be missing up to one millimeter on each side of the break). The destruction of the electrode is though to be a result of oxidation-reduction of the titanium adhesion layer, which when gone allows for pealing and breaking of the gold electrode layer on top. This chemical reaction is thought to be so expedient because of the thinness of the adhesion layer, and pores located across the surface of the metal electrodes.

Although the dimensions of the electrodes as observed from the top of the slide are macroscopic (with a width of two millimeters), their total thickness is approximately only 0.22 microns. The titanium adhesion layer alone is only 200 angstroms thick. The thinness of the adhesion layer makes the electrode particularly susceptible to small amounts of the layer going into solution via oxidation-reduction reactions. A potential modification of the unit discussed in the conclusion chapter is the use of significantly thicker layers of metal when making the electrodes.

Inspection of the gold electrodes under the microscope prior to testing with a power supply revealed pores in both the gold and titanium layers. These pores allow contact between the fluid and the titanium adhesion layer resulting in the rapid degradation of the electrodes under stimulation from the center of the electrode instead of just from the side, as would be the case with a complete (without pores) gold covering. Modification of the cleaning procedure for the glass slide substrates before
evaporation of the two metal layers had no effect on reducing the number or size of the pores. Attempts were also made to cover the exposed areas of titanium by electroplating gold onto the electrodes, but this technique did not sufficiently protect the titanium during stimulation while simultaneously introducing additional contaminants to the plate. It is assumed that the pores result from using glass slides that are not polished and flat, as evaporation of two metal layers onto a flat silicon wafer rarely yields such significant blemishes.

Table IV.C.1: Overall results from preliminary biological assays with the metal electrode cell culture plate

<table>
<thead>
<tr>
<th>Assay</th>
<th>Variations Tested</th>
<th>Overall Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant Voltage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>0.68 volts</td>
<td>Survival Common</td>
</tr>
<tr>
<td></td>
<td>1.0 volts</td>
<td>Survival Uncommon</td>
</tr>
<tr>
<td></td>
<td>1.2 volts</td>
<td>No Survival</td>
</tr>
<tr>
<td>Constant Voltage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (at 0.68 volts)</td>
<td>24 hours</td>
<td>Survival Common</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>Survival Common</td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
<td>No Survival</td>
</tr>
<tr>
<td>Sinusoidal Voltage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (10 V p-p)</td>
<td>1000 Hz</td>
<td>Survival Common</td>
</tr>
<tr>
<td></td>
<td>100 Hz</td>
<td>Survival Common</td>
</tr>
<tr>
<td></td>
<td>50 Hz</td>
<td>Survival Common</td>
</tr>
</tbody>
</table>
Biological assays with the device tested variation in duration of exposure to a constant voltage, magnitude of a constant voltage, and oscillatory frequency of a sinusoidal waveform with 10 volts peak-to-peak amplitude. An overview of the assays results are shown in table IV.C.1. The four documented experiments conducted all tested the variation of a different parameter. Only two of the four experiments even had a control experiment running in parallel. Detailed discussion of these results is therefore avoided on the basis that the data set was poorly constructed, and the realization of major design flaws in the device described below. At the time of the first biological assays, literature regarding previous work on galvanotropism had not been properly reviewed. With no understanding of what voltages or waveforms to use as a stimulant, each experiment conducted was designed to screen possible stimulants by exposing the spiral ganglion to a new set of variants. If a stimulant were found to have an interesting effect on the explants, research would have focused on the voltage or frequency used in that experiment.

After approximately two months of biological assays (and four months of development of the unit) a body of literature pertaining to the topic was discovered. The techniques used in the literature involved non-electrode electric field stimulation to isolate the electrode by-products from the cell culture media. In addition, 90% of the literature utilized the spinal neurons of Xenopus embryos to research the galvanotropic effect. Although the fabricated plate seemed poorly designed one more experiment was conducted before submitting to a compete redesign. A collaborative experiment with Professor Spitzer, a developmental neurobiology researcher at UCSD
who uses Xenopus embryos, was set up to test the microfabricated cell culture plate using neurons documented to be affected by electric fields. The first attempt to plate Xenopus neurons on the microfabricated cell culture plate with metal electrodes after coating with PLL and Laminin resulted in lack of differentiation and complete cell death before subjection to any stimulation. The media used by the Spitzer lab is minimal, and the neurons are dissociated rather than explants. The lack of a rich media and neighboring neurons makes the interaction between the neurons and the cell culture plate direct without a buffer. As a result of this first experiment, the current microfabrication process was deemed toxic and revisions in the fabrication technique became apparent.

The literature findings suggested problems associated with metal electrode based stimulation. This in conjunction with the shortcoming of the biological assays conducted discouraged the continuation of this component of the project. The recommended approach to continuing this research is suggested in the conclusion chapter V.
Chapter V.

Conclusions
Project Overview and Implications

This project introduces two microfabricated cell culture plates designed to assess if various stimulants can be used to affect the growth of spiral ganglion neurites. The ultimate goal of directing spiral ganglion neurite growth is to increase the resolution of the cochlear implant, as explained in chapter II. Chapter III describes the technical approach of presenting a concentration gradient of diffusible growth factors to extending neurites. In the chapter the design and fabrication of a microfluidic cell culture plate for gradient presentation to spiral ganglion neurites is explained. Preliminary experimental results with the microfluidic plate suggest the preferential growth of spiral ganglion neurites and glial cells into media containing the neurotrophin NT-3. Chapter IV describes the technical approach of subjecting electric fields to extending spiral ganglion neurites. A non-invasive electrode-free design by Battocletti, et al. is discussed as a recommended preliminary technique. The design and microfabrication of a cell culture plate with patterned metal electrodes in the cell culture media is found in sections C and D of chapter IV. Experimental results with the fabricated plate suggest a relationship between the survival of a spiral ganglion explant and the magnitude of the voltage differential across the electrodes on either side of the explant.

This conclusion chapter summarizes the design and biological results obtained from each of the two fabricated cell culture plates. Each of the plates is critiqued as its strengths and weaknesses are evaluated. Suggested alterations in the design of the
plates and experimental protocols associated with the plates are detailed in this chapter. Adaptation of *in vitro* experimentation and results to the *in vivo* problem of increasing the resolution of the cochlear implant is also described. Future work based on the plates and protocols created for this project may eventually lead to increasing the resolution of the cochlear implant. Additionally, the techniques develop to study spiral ganglion neurites may be applied to studying other neurons. Therefore this work may potentially assist in the future repair of other nerve damage. Basic research in developmental neurobiology may too benefit from the successful implementation of the devices designed for this project.
Microfluidic Cell Culture Plate

A cell culture plate has been designed and used to present a concentration gradient of diffusible molecular cues to extending spiral ganglion neurites. Chapter III details the design, fabrication, testing, and biological assays associated with the microfluidic cell culture plate. The unit has been designed to assess an extending spiral ganglion neurite’s preference to grow towards one of two media sources. Hydraulic pressure is applied to fluids in the microchannel network to create a concentration gradient in the vicinity of the cultured neurons while overcoming the effects of diffusion and mixing. Experiments using a media source with a fluorescent dye have confirmed the formation of such a gradient. Preliminary results of these biological assays using these plates suggest that spiral ganglion neurites and glial cells grow preferentially towards media rich with NT-3 (concentration of 25ng/ml).

A network of microchannels 100 microns wide and 50 microns deep are arranged to deliver two sources of cell culture media into a single 200 micron channel in the immediate proximity of a spiral ganglion explant. The distance from the explant to the opening of the microfluidic network is roughly 500 microns with reasonably good explant positioning during the procedure. This 200 micron channel is called the presentation region, as neurites and glial cells growing within are presented with media from the two sources. The decision point occurs where the two delivery channels come together forming the presentation region, and an extending neurite is forced to enter only one of them. This decision point design characteristic allows for
easy assessment of the preferred media for directing neurite growth. This statement raises the question of what the desired effect of the optimal concentration of growth factor on an extending neurite is. In the presence of the optimal concentration of a growth factor neurites should either exhibit proliferation while extending and growing throughout the volume containing the optimal concentration, or termination of growth as they sense that they have reached their final destination. These two possible results are reminiscent of the described effects of tropic versus trophic factors in chapter II. Once again, tropic growth factors guide neurite growth while trophic factors sustain the life of neurites (and therefore neurons) subjected to them.

Experiments conducted using the fluorescent molecule Biodipy have visually confirmed the formation of a sharp concentration gradient down the center of the presentation region (see figure III.D.1 and III.D.2). Based on an approximated diffusion coefficient of $1 \times 10^{-7} \text{ cm}^2/\text{sec}$ the predicted time for half of the molecules in the presentation region to diffuse half the channel width is roughly four minutes (this is the $t_{1/2}$ value for a $x_{1/2}$ value of 50 microns, see table III.B.1 and III.B.2 for details). This approximation is qualitatively confirmed by the images in figure III.D.2 that show the sharp concentration gradient while pumping (left image) and the blurred gradient formed after allowing diffusion with out flow for 6.7 minutes (right image). The flow rates of 33 and 40 microliters/hour that successfully initiated a concentration gradient (listed in table III.D.1) are substantially higher than was approximated necessary. This is primarily attributed to the significant volumes (approximately 425
microliters) of the fluidic wells that need to be flushed free of mixed cell culture media before the desired gradient may be formed.

Biological assays have been conducted to study spiral ganglion neurite preference for the neurotrophin NT-3. Control experiments used media with equal concentrations (25ng/ml) of NT-3 in both of the fluidic delivery channels. Non-control experiments presented media with 25ng/ml NT-3 in one channel and media with no NT-3 in the other channel. The results summarized in this paragraph are table III.E.4 come from a small set size of only ten successfully imaged explants (three of which were controls). These preliminary results have shown four out of six (75%) of the spiral ganglion neurites entering the microchannel structure have done so on the side containing NT-3 (control neurite not counted in this measure). In addition, three out of three of the control experiments resulted in glial cells inside both delivery channels (past the decision point). Only one out of seven of the non-control experiments clearly showed glial cell growth past the decision point, but in this plate glial cells were found only in the delivery channel containing NT-3 rich media.

The assembled microfluidic cell culture apparatus and protocol used in the experiments conducted for this project were a result of an iterative design process. Ten total experiments were successfully imaged and evaluated as stated in the previous paragraph. This small set size is in contrast to the twenty-three total experiments conducted. Contamination and human error are the primary contributors to the low yield of successfully imaged explants. The contamination was usually in the form of fungal growth within the cell culture area as penicillin was present in the
cell culture media to prevent bacterial contamination. This was especially a problem during experiments that had total incubation times of more than five days. Gas sterilization in ethylene oxide of the entire microfluidic assembly including the PDMS mold and compression plates is an important preventative measure to reduce the likelihood of contamination. Additionally, sterile handling of the syringes during the media loading process contributes to avoiding contamination.

The proper adjustment of the compression of the microfluidic unit onto the coated petri dish was frequently a point of human error in execution of the protocol. In order to avoid significant deformation of the microchannel structures, the tension of the compression bolts was relieved before loading the explants. After pumping the media for more than 24 hours, it would become apparent that the force acting on the PDMS mold was not great enough to seal the top of the PDMS and the top compression plate (see figure III.C.5 for diagram of assembled apparatus). Therefore media would not be forced into the microchannels but instead flow into each of the wells in the space between the top compression plate and the top of the PDMS mold. These experiments were lost, as the application of force to tighten the bolts would cause significant flow of media out of the microchannels frequently dislodging explants properly positioned in the intermediate layer.

Implementation of the staining protocol too was a source of human errors. Detailed in appendix E, the staining procedure calls for the application of vacuum to the fluidic wells in order to bring the various reagents into the microfluidic structure to stain and fix the biological tissue inside. If the media level inside the cell culture well
became very low during application of a vacuum, sometimes the flow into the fluidic wells would bring the meniscus of the media into the intermediate layer. The force of the fluids meniscus and subsequent air bubbles rushing by the explant would frequently dislodge it. These and other various human errors in the execution of the protocol contributed to the lack of properly imaged experiments.

Assessment of the success of the microfluidic cell culture plate design is difficult due to the limited set of imaged experiments. None of the successfully imaged experiments showed neurites extending through the presentation region past the decision point. Despite this, there was an apparent attraction of the extending neurites towards the channel opening, as seen in figures III.E.1-5. This indicates that the media being freshly pumped out of the microchannels is acting as a chemo-attractant to the growth cone of the extending neurites. Another set of control experiments in which both fluidic wells were loaded with NT-3 deficient media would help to clarify that the attraction of the neurites was to the NT-3 versus another component of the fresh media. The lack of neurite growth past the decision point is possibly contributed by three facts discussed in this and the following three paragraphs.

The lack of neurite growth past the decision point may be related to the length of the experiment. Increasing the duration of fluidic flow to 72 or 96 hours will increase the probability that a neurite will have enough time to grow up the channel. Eventually transgenic mice or rats may be used that have spiral ganglion cells that express fluorescent molecules. Experiments conducted with such neurons would allow for accurate determination of the necessary duration of exposure to flow, as the
extending neurites can be visually inspected without fixation. This use of transgenic spiral ganglions cells promises not only to give information as to the proper duration of flow to use, but also the real time decision making process may be observed.

The second possible contribution to the lack of observed neurite growth in the presentation region is the excessive flow rate in the microchannels moving in the direction opposite that of the desired growth (a presentation refresh rate of 16.6 \(1/sec\) for a flow rate of 30 microliters/hour into each fluidic well). As discussed in the modeling of diffusion found in section B of chapter III (and shown in table III.D.1), the flow rate should be kept above roughly 2.25 microliters/hour in order to maintain a horizontal concentration gradient at the end of the presentation region. Empirical verification of the flow rates necessary to create and maintain a sharp concentration gradient (33 microliters/hour was the minimum confirmed flow rate) throughout the presentation region are more than ten times greater than the theoretical rate suggested from modeling (approximated in chapter III section C). As addressed in section D results of the concentration gradient experiments, the observed requirement for increased flow rates to initiate a concentration gradient is most likely due to the necessity of flushing the fluidic wells free of mixed media constituents (mixing occurred primarily during assembly of the apparatus). In order to decrease the hydraulic force acting against the extending neurites, experiments should be conducted with two phases of flow rates. An initial phase of 30 microliters/hour may be used to initiate the concentration gradient by flushing the volume in the microfluidic wells for roughly 18 hours. A subsequent phase of decreased flow on the order of 3 to 10
microliters/hour may then be used to maintain the gradient while subjecting the neurites to less harsh conditions. Experimentation using fluorescent molecules to confirm the concentration gradient formation and maintenance would need to occur before biological assays with the two-phase system could be conducted.

The third possible contribution to the lack of neurite growth past the decision point may be the lack of a vertical concentration gradient along the length of the presentation region (a gradient in the y direction of figure III.B.3). As seen in the concentration gradient experiments (left image of figure III.D.2), there is not a differentiable vertical gradient of fluorescence in the presentation region. The fluid within the widened channel opening does appear to have a slight vertical gradient in addition to the horizontal gradient addressed in section C of chapter III. Therefore neurites that have entered the presentation region are already surrounded by the concentration of growth factor that is being supplied by the corresponding delivery channel. Due to the lack of a vertical concentration gradient in the presentation region, growth beyond the decision point would indicate the growth factor being tested causes a proliferative effect with the concentration tested rather than a terminal effect (these two effects are described earlier in this section). An optimal concentration of growth factors that causes a terminal effect would instead results in neurites extending only to the opening of the presentation region, as at that point the concentration does not appreciably change as a function of position moving towards the decision point. Therefore the apparent lack in neurite growth beyond the presentation region in this limited set of successfully imaged experiments suggests that NT-3 exhibits exactly the
response expected from a trophic guidance cue. The primary argument against this
claim is the observation of increased spiral ganglion neurite numbers and length in cell
culture media containing NT-3 over the same media without NT-3 (Kim, et al;
Lefebvre, et al). These results suggest that extension of the spiral ganglion neurites is
mediated by the presence of NT-3, which would indicate that presence of the molecule
in the decision region should lead to proliferation of the subjected neurites into the
appropriate delivery channel past the decision point.

Research extending the work with diffusible growth factors conducted for this
project would involve adjustment of these three contributors followed by multiple
experimental runs with the parameters adjusted. The revised protocol should be
applied to multiple potential growth factors, including those in the netrin family, based
on the literature. As mentioned in the paragraph describing the contribution of
experimental duration, the use of transgenic spiral ganglion that express fluorescent
molecules will greatly aid in the optimization of the device. Successful determination
the optimal concentrations of various growth factors for guiding spiral ganglion
neurite growth would then be applied to in vivo studies. Modeling and empirical
determination of a concentration gradient in vivo presents many difficulties.
Additional experimental considerations include initiation of adult spiral ganglion
outgrowth and delivery of growth factors from a modified cochlear implant. The in
vivo studies ultimately would conclude in experimentation and psycho-acoustic
assessment with animal (and eventually human) users of the cochlear implant. These
studies would compare the spectral resolution perceived by users of the cochlear
implant with and without the use of diffusible growth factors to assist in spiral ganglion neurite guidance to the implanted electrodes.
Metal Electrode Cell Culture Plate

Chapter IV describes a series of experimental protocols for the assessment of how electric fields affect the growth of spiral ganglion cells. A microfabricated cell culture plate has been created to present electric fields to developing spiral ganglion neurites. The plate is designed to allow for easy application of the desired electric stimulation (voltage or current) via a patterned computer board type interface that connects to a clamping female circuit board slot. The implemented plate consists of pairs of electrodes that stimulate three to four cell culture wells at a time in parallel. Biological screens have evaluated the effects of applying constant voltage potentials as well as various frequencies of a 10 volts peak-to-peak sine wave to spiral ganglion explants. Preliminary experimental results suggest that the survival of spiral ganglion explants is inversely proportional to the magnitude of applied voltage, and the frequency of sine wave oscillation, although these results are seriously questioned. Results have not been obtained that correlate electric fields with the orientation of neurite growth. This is due to faults in the design, fabrication, and experimental protocols associated with the current implementation of the device.

The metal electrode cell culture plate is constructed in two parts; the microfabrication of patterned titanium and gold electrodes on a glass substrate, and the molding of a set of cubic centimeter cell culture wells using PDMS and aluminum blocks. A 50mm x 75mm glass slide (from Dow Corning) is used as the substrate for the microfabrication of patterned electrodes. Evaporation of 200 angstroms of a titanium adhesion layer is followed by 2000 angstroms of the gold electrode surface.
Standard photolithographic patterning and chemical etching are then used to create the metal electrodes. The PDMS wells are allowed to reversibly bond to the glass slide with patterned electrodes then a silicon sealant is used to hold the two together throughout the experiment.

Testing of the functionality of the fabricated plate yielded poor results. The plate was designed for application of constant voltage in parallel across several wells at once. Any voltage greater than three volts led to degradation and destruction of the electrodes on the unit. This degradation is attributed to the oxidation-reduction of the titanium adhesion layer. Once the layer has gone into solution, the gold easily flakes off of the plate and the electrode becomes broken. When a constant current source was used to drive a single well at 0.1 microamps, rapid degradation of the adhesion layer led to the electrode breaking in less than ten seconds. The rapid degradation occurred as the current source exceeded five volts in attempt to drive the line. Instead of redesigning the device to handle a higher voltage differential, the biological assays were conducted with voltages held below the three-volt threshold of the device.

Preliminary biological assays were conducted in attempts to isolate a voltage and waveform that could potentially evoke a galvanotropic effect from spiral ganglion neurons. At the onset of the biological experiments literature pertaining to galvanotropism had not yet been reviewed. Therefore each week a new experiment with new variables was conducted to screen for possible winning combinations of voltage and waveform. The four documented experiments yielded results that suggest a relationship between the survival of spiral ganglion explants and the magnitude and
duration of a voltage applied across the cell culture well. These experimental results are considered not valid, as they are the results of isolated experiments (50% of which did not use controls) and because of the significant design flaws found in the unit used for stimulation. Based on the observation of the titanium adhesion layer degrading with the application of voltage across the well, it may be concluded that the observed dependencies are actually a result of the increased amount of titanium in the cell culture solution rather than a direct effect of the voltage itself.

Future research based on this work will acknowledge the flaws in design of the unit and design of the biological experimentation using the unit. The box coil method is suggested as the first technique to assess if the spiral ganglion exhibits galvanotropism. After indication that spiral ganglion neurites can be oriented to an electric field, a cell culture technique needs to be developed to bridge the gap between the non-invasive box coil method and stimulation in vivo with the cochlear implant itself. Microfabrication is not necessarily the recommended approach for developing such a technique. Possibly a better design would actually utilize a cochlear implant electrode array integrated into a cell culture plate. This would offer not only the assessment of how the liquid-electrode interface will affect the observed galvanotropic affect, but also how the electrode configuration specific to the cochlear implant can be used to stimulate different regions of an intact spiral ganglion in vitro.
APPENDIX A

MATERIAL AND METHODS:
FABRICATION OF MICROFLUIDIC UNIT I: micro channel component

Table Appendix.A.1: Materials and approximate quantity necessary to fabricate specialized components used in micro-channel component of the microfluidic unit

<table>
<thead>
<tr>
<th>Materials needed to create specialized components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5” diameter plastic beaker</td>
<td>1</td>
</tr>
<tr>
<td>1/2” wood cutting drill bit</td>
<td>1</td>
</tr>
<tr>
<td>High speed rotary drill</td>
<td>Dremell</td>
</tr>
<tr>
<td>Poly-Di-Methyl-Siloxane (PDMS) 10:1 polymer kit</td>
<td>100ml</td>
</tr>
<tr>
<td>6” diameter petri dish</td>
<td>1</td>
</tr>
</tbody>
</table>

Table Appendix.A.2: Materials and approximate quantity necessary to fabricate microchannel component of the microfluidic unit

<table>
<thead>
<tr>
<th>Material and Consumables for Fabrication</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4” Si wafer-factory clean</td>
<td>1</td>
</tr>
<tr>
<td>Acetone</td>
<td>400ml</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>400ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>400ml</td>
</tr>
<tr>
<td>Microchem SU-8 10 photoresist</td>
<td>5ml</td>
</tr>
<tr>
<td>Microchem SU-8 100 photoresist</td>
<td>6ml</td>
</tr>
<tr>
<td>Microchem SU-8 developer</td>
<td>600ml</td>
</tr>
<tr>
<td>Poly-Di-Methyl-Siloxane (PDMS) polymer 10:1 kit</td>
<td>125ml : 12.5grams</td>
</tr>
<tr>
<td>6” x 6” x 1/8” piece of aluminum</td>
<td>1</td>
</tr>
<tr>
<td>5 minute epoxy</td>
<td>5 ml</td>
</tr>
<tr>
<td>5” diameter x 3/4” tall cylindrical plastic ring</td>
<td>Specialized component</td>
</tr>
<tr>
<td>PDMS ring holder</td>
<td>Specialized component</td>
</tr>
<tr>
<td>1/2” x 1/2” x 1” aluminum blocks</td>
<td>4</td>
</tr>
<tr>
<td>1/4” x 1/4” x 1” aluminum blocks</td>
<td>16</td>
</tr>
</tbody>
</table>
Table Appendix.A.3: Tool and manufacturers used to fabricate microchannel component of the microfluidic unit

<table>
<thead>
<tr>
<th>Equipment for Fabrication</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auto-cad software</td>
<td>Canvas 6.0</td>
</tr>
<tr>
<td>High resolution (&gt;1400dpi) transparency printer</td>
<td>Linotronic 330</td>
</tr>
<tr>
<td>Mask aligner</td>
<td>HTG</td>
</tr>
<tr>
<td>Sonciator</td>
<td></td>
</tr>
<tr>
<td>Digital hot plate</td>
<td>Coleman-Palmer</td>
</tr>
<tr>
<td>Evaporation chamber</td>
<td></td>
</tr>
<tr>
<td>Power drill</td>
<td>Makita</td>
</tr>
<tr>
<td>Digital scale</td>
<td></td>
</tr>
<tr>
<td>Mixing drill bit</td>
<td>Specialized Component</td>
</tr>
<tr>
<td>Scalpel with fresh blade</td>
<td></td>
</tr>
<tr>
<td>Glass pipette</td>
<td></td>
</tr>
<tr>
<td>(4) 5” diameter Pyrex glassware</td>
<td></td>
</tr>
<tr>
<td>300ml plastic Graduated beaker</td>
<td></td>
</tr>
<tr>
<td>20ml plastic graduated beaker</td>
<td></td>
</tr>
</tbody>
</table>

Table Appendix.A.4: Tools used for testing microchannel unit

<table>
<thead>
<tr>
<th>Cleaning and Testing</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissection Microscope</td>
<td></td>
</tr>
<tr>
<td>Inverted Microscope</td>
<td></td>
</tr>
<tr>
<td>Fine surgical tweezers</td>
<td></td>
</tr>
<tr>
<td>DMEM media</td>
<td></td>
</tr>
<tr>
<td>3cc cut syringe</td>
<td>Specialized Component</td>
</tr>
</tbody>
</table>
OVERVIEW OF METHOD:  
FABRICATION OF MICROFLUIDIC UNIT I: microchannel component

Day 0  
Specialized component creation  
Create Mixing drill bit  
Create Plastic Ring  
Create PDMS ring holder

Mask Creation  
Design Masks with auto-cad tool  
Print with high-resolution printer onto transparencies and cut out individual masks

Day 1  
Clean wafer  
Clean with acetone  
Clean with isopropyl alcohol  
Evaporate solvents at 230°C for 1 hour

50 micron channel layer  
Level and Pre-heat hotplate to 40°C  
Apply 5ml SU-8 10 photoresist  
Spin at 600RPM; 5 seconds acceleration, 15 seconds full speed  
Pre-bake; 40°C to 50°C, 50°C to 90°C 20 minutes, 90°C 20 minutes, cool  
Expose for 250 seconds (parameter set to Paul Yu’s HTG in EBU1 1106)  
Post exposure bake; 50°C to 90°C 10 minutes, 90°C 10 minutes, cool

500 micron well layer  
Level and Pre-heat hotplate to 40°C  
Apply 6ml SU-8 100 photoresist  
Spin at 600RPM; 10 seconds acceleration, 30 seconds full speed  
Pre-bake; 40°C to 50°C, 50°C to 95°C 1 hour, 95°C 1 hour 30 minutes, cool  
Align and expose for 700 seconds (parameter set to Paul Yu’s HTG in EBU1 1106)  
Post exposure bake; 50°C to 90°C 10 minutes, 90°C 20 minutes, cool

Development of SU-8 photoresist  
Agitate 15 minutes in developer  
Transfer to clean developer and agitate 13 minutes  
Transfer to clean developer and agitate 2 minutes  
Transfer to isopropyl alcohol and agitate 30 seconds  
Blow-dry with N2
Hardening Bake and Solvent Evaporation

Hardening Bake; 30°C to 120°C 1 hour, 120°C 1 hour, cool slowly
Place in evaporator under vacuum for at least 36 hours

Day 3

Prepare wafer for molding
Clean 6” x 6” aluminum sheet and set wafer onto center with 5 minutes epoxy

Prepare aluminum block, plastic ring, and PDMS ring holder for molding
Sonicate in acetone 5 minutes
Sonicate in isopropyl alcohol 5 minutes
Air dry in fume hood

Prepare PDMS for molding
Mix 125ml PDMS with 12.5 grams curing agent for 50 minutes using power drill
Vacuum mix until all bubbles are gone (approximately 45 minutes)

Mold the PDMS
Place plastic ring, PDMS ring holder, and aluminum blocks on wafer and aluminum sheet
Pour mixed PDMS onto wafer
Heat; 20°C to 75°C 1 hour, 75°C 24 hours, cool

Day 4

Separate Individual Units
Remove plastic ring, PDMS ring holder, and aluminum blocks
Cut molded PDMS into four individual units sized to 60mm petri dish

Clean and Test Individual Units
Remove excess PDMS and SU-8
Test Fluidic Continuity
DETAILED METHOD:
FABRICATION OF MICROFLUIDIC UNIT I: microchannel component

Specialized component creation: Mixing drill bit
1. Mixing drill bit is created by modifying 1/2” paddle shaped wood cutting drill bit
2. Use rotary drill with carbon fiber attachment to remove sharp points from the bottom of the bit, and to create triangular grooves in the sides of the paddle
3. Use rotary drill with stone grinding attachment to smooth and round all edges

Specialized component creation: Plastic ring
4. Plastic ring is created by cutting a 3/4” thick piece of the plastic beaker using the carbon fiber attachment on the rotary drill
5. After the ring is cut, sides are made flat by depressing the ring on hot plate at 100°C for 5 to 10 seconds then transferring to a piece of aluminum foil on a flat table top to cool quickly

Specialized component creation: PDMS ring holder
6. PDMS ring holder is created by placing the plastic ring inside the 6” petri dish and molding PDMS in the outer ring of space between the two
7. Mix 100ml of PDMS with 10 grams of curing agent for 30 minutes using electric drill
8. Place beaker of mixed PDMS in vacuum chamber under vacuum until all bubbles are gone (approximately 45 minutes)
9. Place flat side of plastic ring down inside the Petri dish
10. Pour PDMS into outer space between the petri dish side walls and the plastic ring
11. Carefully balance Pyrex glass beaker on plastic ring and place approximately 100 gram mass inside the beaker to secure the plastic ring to the petri dish
12. Allow 24 hours to cure
13. Slice the PDMS ring holder and plastic ring in one location to allow for removal without tearing

Mask Creation
1. Lay out 50 micron and 500 micron masks used to create the device. The 50 micron mask defines the channels. The 500 micron mask defines the interface between the channels and the wells (see figure 89: detailed mask view), in addition to barriers which will hold the aluminum block in place on the wafer during molding
2. Print the mask design to file for a linotronic330 printer
3. Send the file to a printing facility (UCSD pre-press services) for printing a film positive of the file
4. Receive the transparencies, and use scissors to cut out the two masks individually
Clean wafer
1. Remove visible dirt and grime with acetone and clean room paper
2. Sonicate in acetone for 10 minutes
3. Transfer to Pyrex container with methanol quickly
4. Sonicate in methanol for 10 minutes
5. Blow dry with N2
6. Heat on hotplate in fume hood to 230°C for 1 hour and cool to room temperature

50 micron channel layer
1. Adjust hotplate position in clean room fume hood until center of plate is completely level
2. Pre-heat hotplate to 40°C
3. Warm up HTG mask aligner by turning on arc lamp
4. Apply 5ml SU-8 10 photoresist, use clean glass pipette to spread over entire wafer
5. Spin at 600RPM with 5 seconds acceleration and 15 seconds full speed
6. Place wafer on hotplate at 40°C
7. Carefully prevent bubbles by spreading photoresist over forming holes with pipette
8. Heat to 50°C as slow as necessary while preventing bubbles in photoresist
9. Equilibrate photoresist (not many bubbles forming) approximately 2 minutes at 50°C
10. Heat from 50°C to 95°C for 20 minutes (120°C/min) preventing bubbles until 65°C
11. Hold at 90°C for 20 minutes and let cool to room temperature
12. Place wafer on mask aligner, align microchannel mask to Si wafer notch
13. Expose for 250 seconds (parameter set to Paul Yu’s HTG in EBUI 1106)
14. Post exposure bake 50°C to 90°C for 10 minutes (240°C/minute)
15. Hold at 90°C for 10 minutes and cool to room temperature

500 micron well layer
1. Pre-heat leveled hotplate to 40°C
2. Apply 6ml SU-8 100 photoresist, use clean glass pipette to spread over entire wafer
3. Spin at 600RPM with 10 seconds acceleration and 30 seconds full speed
4. Place wafer on hotplate at 40°C
5. Carefully prevent bubbles by spreading photoresist over forming holes with pipette
6. Heat to 50°C as slow as necessary while preventing bubbles in photoresist
7. Equilibrate photoresist (not many bubbles forming) approximately 2 minutes at 50°C
8. Heat from 50°C to 95°C for 1 hour (45°C/min) preventing bubbles until 65°C
9. Hold at 95°C for 1 hour 30 minutes and let cool to room temperature
10. Place wafer on mask aligner, align 500 micron well mask to microchannels on wafer
11. Expose for 700 seconds (parameter set to Paul Yu’s HTG in EBUI 1106)
12. Post exposure bake 50°C to 90°C for 10 minutes (240°C/minute)
13. Hold at 90°C for 20 minutes and cool to room temperature

*Development of SU-8 photoresist*
1. Prepare 3 Pyrex containers with 200ml SU-8 developer and 1 with 200ml isopropyl alcohol
2. Place wafer in developer and agitate gently for 15 minutes
3. Move wafer to clean developer and agitate gently until all visible photoresist not part of the pattern is gone (approximately 13 minutes)
4. Move wafer to clean developer and agitate gently for 2 minutes
5. Move wafer to isopropyl alcohol and agitate gently for 30 seconds
6. Blow dry wafer carefully with N2 gun, be sure to blow from several angels to remove solvent

*Hardening Bake and Solvent Evaporation*
1. Heat wafer on hotplate from 30°C to 120°C for 1 hour (90°C/minute)
2. Hold at 120°C for 1 hour and let cool to room temperature
3. Place in evaporator under vacuum for at least 36 hours

*Prepare wafer for molding*
1. Clean 6” x 6” aluminum sheet with acetone and isopropyl alcohol using clean room paper
2. Mix 5ml of 5 minute epoxy on the center of the aluminum sheet for 60 seconds
3. Carefully press wafer onto center of aluminum sheet so that the epoxy leaks from the edges of the wafer

*Prepare aluminum blocks and for molding*
1. Remove visible dirt and grime from aluminum blocks, plastic containment ring, and outer PDMS ring holder with acetone and clean room paper
2. Sonicate in acetone for 5 minutes, stirring occasionally
3. Transfer to Pyrex container with isopropyl alcohol quickly
4. Sonicate in isopropyl alcohol for 5 minutes, stirring occasionally
5. Air dry all components in the clean room fume hood on clean room paper

*Prepare PDMS for molding*
1. Fully charge batteries to hand held electric drill
2. Clean the mixing drill bit with acetone and isopropyl alcohol using clean room paper
3. Clean the two plastic graduated beakers (300ml and 20ml) with acetone and isopropyl alcohol using clean room paper, blow dry with N2 gun
4. Pour 125ml PDMS into 300ml plastic graduated beaker
5. Measure 12.5 grams of PDMS curing agent into 20ml plastic graduated beaker
6. Pour 1/3 of the curing agent into the PDMS beaker
7. Mix with electric drill on high for 15 minutes, be sure to mix entire beakers contents, but be careful to not rub the side of the beaker very frequently as it will shred
8. Pour 1/3 of the curing agent into the PDMS beaker and mix for 15 minutes
9. Pour last 1/3 of the curing agent into the PDMS beaker and mix for 20 minutes
10. Place beaker in vacuum chamber and apply vacuum until ALL bubbles are gone (approximately 45 minutes), breaking the vacuum occasionally to expedite the neurite

**Mold the PDMS**
1. Place the plastic ring and the PDMS ring holder around the Si wafer on the aluminum sheet adjusting as necessary to create the best seal possible
2. Carefully place the aluminum blocks onto the wafer in the slots created with the 500 micron layer of photoresist
3. Slowly pour all 125ml of the mixed PDMS onto the wafer
4. If a small amount of bubbles formed around the lips of the aluminum block during pouring, pop with a clean glass pipette
5. If a large amount of bubbles formed during pouring of the PDMS, place entire piece in vacuum chamber and apply vacuum as long a necessary to remove all bubbles
6. After bubbles are removed, place entire piece on leveled hotplate in the fume hood
7. Heat from 20C to 75C for 1 hour (55C/minute)
8. Hold at 75C for 24 hours and cool to room temperature

**Separate Individual Units**
1. Carefully remove the PDMS ring holder and the plastic ring
2. Slowly peal off the molded PDMS layer from the Si wafer, once a part of the mold has come off of the wafer, do not let it touch the wafer again
3. Transfer the mold to a clean piece of aluminum foil
4. Gently remove the aluminum blocks with gloves on by pulling the block in one direction while pulling the PDMS in the other direction. Separating the PDMS from each side of the block in this manner, than gently wiggle the block out of the PDMS mold
5. With all aluminum block removed, cut the four units apart using a scalpel and fresh blade. Firmly place an aluminum ruler across the region to be cut, and slide the scalpel across the ruler.

6. The individual units are stored in 60mm petri dish containers. Cut edges and corners of the units as needed to allow the unit to fit with 2mm space on all sides in the dish.

**Clean and Test Individual Units**

1. Remove unit from petri dish and observe channel-side-up under dissection microscope. Remove excess PDMS and SU-8 with fine dissection tweezers by gently tearing.

2. Carefully remove PDMS in front of microchannel well, and at microchannel – well interface if necessary.

3. Gently run tweezers along length of channels to be sure all SU-8 is removed.

4. Clean by vigorously spraying with high pressure 70% ethanol from squirt bottle.

5. Dry channel-side-up on clean chem.-wipe or paper towel.

6. Observe again under dissection microscope and inverted microscope, clean as necessary.

7. When it looks clean and free of excess PDMS, adhere unit to clean petri dish.

8. Fill center well with colored fluid, such as DMEM.

9. Remove plunger from 3cc syringe, and cleanly cut off the top (the side where the needle goes).

10. Replace the plunger in the opposite direction.

11. Use the cut 3cc syringe to create a vacuum in each of the 4 fluidic chambers; firmly depress the open side of the syringe on the well with the plunger fully depressed, then raise the plunger and hold for 10 seconds. The vacuum should be such that while the plunger is raised, the entire unit can be picked up by the syringe.

12. If all four wells have fluid or foamy bubbles inside, the unit is ready to be rinsed with ethanol and sterilized for use.

13. If any of the wells do not have any fluid or foamy bubbles, observe the unit under the inverted microscope and attempt to determine what is inhibiting flow. Correct with tweezers and scalpel if possible, otherwise discard unit.
APPENDIX B.

MATERIALS AND METHODS:
FABRICATION OF MICROFLUIDIC UNIT II: fluidic delivery component

Table D.1: Materials Necessary for Fabrication of Fluidic Delivery Plates

<table>
<thead>
<tr>
<th>Materials</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 1/2” x 2 1/2” x 1/8” piece of bicarbonate Plexiglas</td>
<td>2</td>
</tr>
<tr>
<td>20 gauge plastic tubing</td>
<td>(4) 2’ pieces</td>
</tr>
<tr>
<td>20 gauge syringe needles</td>
<td>4</td>
</tr>
<tr>
<td>Syringe locking two way stoppers (one side male/one side female)</td>
<td>4</td>
</tr>
<tr>
<td>1/8” x 1” bolt and matching nuts</td>
<td>4</td>
</tr>
<tr>
<td>Drill press</td>
<td>1</td>
</tr>
<tr>
<td>Drill bits 1/16”, 3/16”, 1/4”, 1/3”</td>
<td>1</td>
</tr>
<tr>
<td>5 minute epoxy</td>
<td>10 ml</td>
</tr>
<tr>
<td>Paper copy of 500 micron layer mask</td>
<td>1</td>
</tr>
<tr>
<td>Lid of 60mm petri dish</td>
<td>1</td>
</tr>
</tbody>
</table>
DETAILED METHOD:
FABRICATION OF COMPRESSION AND FLUIDIC DELIVERY PLATES

Drilling the ports into the Plexiglas
1. Peal paper protective layer from both sides of one piece of Plexiglas
2. Use paper print of mask to locate and mark with pencil center of fluid chamber wells (labeled A in figure 103), and center of cell culture well (labeled B in figure 103)
3. Use 1/16” drill bit on fluid chamber marks on one piece
4. Use 1/3” drill bit on cell culture well on one piece, widen the well as indicated in the diagram
5. With other piece of Plexiglas, mark four corners of the plate (labeled C in figure 103) such that the petri dish lid is within the bounds of the marks
6. Drill 1/8” deep taps in Plexiglas (half the thickness of the Plexiglas) using the 3/32” drill bit in the four corners marked above
7. Hold both pieces together tightly, placing the tapped surface facing up
8. Load 1/16” drill bit and lower bit with power off aligning the bit with the center of one of the taps
9. Slowly drill through the center of the tap while holding the two pieces of Plexiglas together
10. Place the bolt through the new hole, and tighten the nut
11. Repeat step 7 through 10 for the remaining three taps
12. With two pieces bolted together in alignment, label the plate with a marker on two sides such that the marking spans both plates and can be used to identify orientation

Affixing the tubes and connectors to the Plexiglas
1. Feed the four pieces of tubing through the four fluidic chamber ports leaving an excess of 1cm on the side of the Plexiglas that faces towards the bottom piece
2. Mix 5ml of 5 minutes epoxy for 1 minute
3. Apply epoxy to tubing-Plexiglas interface on both sides of the Plexiglas, gently push and pull tubing 3mm in either direction to apply epoxy throughout
4. After epoxy sets (5 minutes), slide syringe needle >1cm into the 4 ends of the tubes. A pair of pliers may be used to assist in pulling the tubing over the needle
5. Screw on 2-way stoppers to the four needle ends
6. Test that all tubing allows flow using a syringe filled with colored fluid, if flow is inhibited cut down epoxy end of tubing until flow is normal

Labeling tubes and plate
1. Tape the tubes in three locations as indicated in figure 88 to reduce tangling
2. Label the top plate with a marker indicating side A and B, and growth factor + and – (see diagram)
3. Label the tape with “+” and “-“ to indicate which line is which, especially at the top of the tubing where the syringe will attach
APPENDIX C

MATERIALS AND METHODS:
APPLICATION OF MICROFLUIDIC UNIT: cell culture and staining

Table Appendix.C.1: All Materials Necessary for Experiment

<table>
<thead>
<tr>
<th>Materials</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Microchannel component of microfluidic unit</td>
<td></td>
</tr>
<tr>
<td>Fluidic delivery component of microfluidic unit</td>
<td></td>
</tr>
<tr>
<td>Lid or base of 1000µl pipette tip box</td>
<td></td>
</tr>
<tr>
<td>IO Gas sterilization bag</td>
<td></td>
</tr>
<tr>
<td>(2) 60cc syringes</td>
<td></td>
</tr>
<tr>
<td>(2) 10cc syringes</td>
<td></td>
</tr>
<tr>
<td>(4) 3cc syringes</td>
<td></td>
</tr>
<tr>
<td>High Precision digital Syringe pump</td>
<td></td>
</tr>
<tr>
<td>1000µl, 200µl, 20µl Micropipette</td>
<td></td>
</tr>
<tr>
<td>Phosphate Buffered Solution (PBS)</td>
<td></td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s Media (DMEM)</td>
<td></td>
</tr>
<tr>
<td>(3) Sterilized 60mm petri dish</td>
<td></td>
</tr>
<tr>
<td>Poly-L-Lysine</td>
<td></td>
</tr>
<tr>
<td>Human Laminin</td>
<td></td>
</tr>
<tr>
<td>Fetal Calf Serum</td>
<td></td>
</tr>
<tr>
<td>Hepes Buffer</td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td></td>
</tr>
<tr>
<td>NT3</td>
<td></td>
</tr>
<tr>
<td>70% Ethanol</td>
<td></td>
</tr>
<tr>
<td>High Temperature Instant Sterilization device</td>
<td></td>
</tr>
<tr>
<td>Gross Dissection Scissors large</td>
<td></td>
</tr>
<tr>
<td>Gross Dissection Scissors small</td>
<td></td>
</tr>
<tr>
<td>(2) Fine dissection tweezers</td>
<td></td>
</tr>
<tr>
<td>Scalpel with blade</td>
<td></td>
</tr>
<tr>
<td>Sterile swab</td>
<td></td>
</tr>
<tr>
<td>Inverted Microscope</td>
<td></td>
</tr>
<tr>
<td>½ fine dissection tweezers</td>
<td></td>
</tr>
<tr>
<td>4% paraform aldehyde</td>
<td></td>
</tr>
<tr>
<td>Media I: Serum Based (1.25ml total)</td>
<td>Concentration</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>DMEM</td>
<td>To 1.25 ml Total</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>10%</td>
</tr>
<tr>
<td>Hepes Buffer</td>
<td>25 mM</td>
</tr>
<tr>
<td>Penicillin</td>
<td>300 U/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Media II: Growth Factor +/- (10 ml total)</th>
<th>Concentration</th>
<th>Volume Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>To 10 ml Total</td>
<td>9.55 ml</td>
</tr>
<tr>
<td>Hepes Buffer</td>
<td>25 mM</td>
<td>250 µl</td>
</tr>
<tr>
<td>Glucose</td>
<td>6 mg/ml</td>
<td>100 µl</td>
</tr>
<tr>
<td>Penicillin</td>
<td>300 U/ml</td>
<td>30 µl</td>
</tr>
<tr>
<td>N2</td>
<td>30 µl/ml</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

*Mix 10ml, shake and divide:*

| NT-3                                      | 2.5 µl (25ng/ml) | 0 µl        |

Table Appendix.C.2: Cell Culture Media Constituents
OVERVIEW OF METHOD:
CELL CULTURE AND STAINING WITH THE MICROFLUIDIC UNIT

Day 0
Specialized component creation
Create ½ fine tweezers

Cleaning and Sterilization of Microfluidic Unit
Clean fluidic delivery component
Clean microchannel component
IO gas sterilize unit

Coat Cell Culture Plates
Poly-L-Lysine in DMEM
Human Laminin in PBS

Day 1
Preparation of Cell Culture Media
Serum based Media
Growth Factor +/- Media
Equilibrate in Incubator
Load in Syringes

Preparation of Microfluidic Unit
Load Microchannels
Fill fluidic wells
Compress Structure

Extraction of Spiral Ganglion Explants
Gross Dissection – cochlea extraction
Fine Dissection – spiral ganglion extraction

Loading of Microfluidic Unit
Cutting Spiral Ganglion
Transfer and Position in Microfluidic Unit

Day 2 (18 hours later)
Change Media
Exchange Serum Based Media with Growth Factor +
Start Fluidic Pump
Connect Syringe Pump
Open Valves
Start Pumping at .25 μl / min

**Day 3 and 4**
*Observe explants*

**Day 5 (72 hours after pumping starts)**
*Stop Pump*

*Fix Explants*
4% Paraformaldehyde
Wash twice

*Primary antibody Immunostain*
Membrane Lyse
Block
Primary Antibody

**Day 5 or 6**
*Secondary antibody Immunostain*
Wash
Block
Secondary Antibody
Wash
View
DETAIL METHOD:
CELL CULTURE AND STAINING WITH THE MICROFLUIDIC UNIT

Specialized component creation: Create ½ fine tweezers
1. Break a pair of fine surgical tweezers into two halves. Pliers, a Dremmel drill with carbon fiber attachment, or any other means is appropriate.
2. Test with complete Microfluidic unit assembled; tweezer half may be manipulated into the channel region of the unit while observing with an inverted microscope.

Cleaning and Sterilization of Microfluidic Unit
1. Load (1) 60cc syringe with 70% ethanol, other 60cc syringe will be kept empty.
2. Clean outside of fluidic delivery component with warm water and soap.
3. Clean tubes of fluidic delivery component by injecting with 70% ethanol.
4. Flush tubes of fluidic delivery component by injecting with air.
5. Leave valves open at the end of the fluidic lines.
6. Clean microchannel component by vigorously spraying with 70% ethanol on channel side.
7. Dry channel side up on clean Chem-wipes.
8. Check that channels are clean; adhere microchannel component to clean 60mm petri dish and observe with inverted microscope, clean again as necessary.
9. Place microfluidic unit in clean lid or base of 1000µl pipette tip box, including top and bottom of the microfluidic component, the microchannel component in a clean petri dish, and the four associated nuts and bolts.
10. Place microfluidic unit and lid into a sterilization bag, seal and label for IO gas sterilization.
11. Deliver the sealed bag to a gas sterilization facility for pick up the following day.

Coat Cell Culture Plates
1. Prepare 20µl of Poly-L-Lysine (PLL) in 3.8ml of DMEM to a final concentration of.
2. Prepare 30µl of Human Laminin in 3ml PBS to a final concentration of 10µg/ml.
3. In cell culture hood, pour PLL solution onto a sterile 60mm petri dish lid.
4. Swirl the lid so that the entire surface is covered by the solution.
5. Place lid in cell culture incubator at 37°C, 5% CO2, and 95% humidity for 1 hour.
6. Wash with PBS; vacuum or pour off contents of lid, fill with PBS, and vacuum or pour off PBS.
7. Pour Laminin solution onto the lid and swirl to cover entire surface.
8. Place lid in refrigerator overnight at 4°C.
**Preparation of Cell Culture Media**

1. Gather various components of the media as outlined in the table above; PBS is stored at 4C, all others are stored at –80C or –20C
2. All following steps are carried out in cell culture hood unless otherwise stated, clean gloves are used in all steps
3. Add all constituents of Media I: Serum Based (see table 109) to sterile screw cap container to make 1.25 ml per plate, shake well to mix
4. Add constituents of Media II: Growth Factor +/- except for NT-3 (see table 109) to sterile screw cap container to make 10 ml per plate, shake well to mix
5. Transfer 5ml of mixed Media II: Growth Factor +/- to fresh sterile screw cap container, label new container “+” and old container “-”
6. Add 25ng/ml NT-3 to Media II container marked “+”, shake well
7. Label (2) 100mm sterile petri dishes “+” and “-“ on both the lid and the base
8. Pour the Media II from each of the two screw cap containers into the labeled petri dishes
9. Place the petri dishes in the incubator to be used for tissue culture at 37C with 5% CO2 and 95% humidity with the lids off
10. Allow 10 minutes for gas equilibration of the media
11. If the incubator is not close to the cell culture hood, prepare a “clean” area by the incubator by spraying the area with 70% ethanol, and laying down a sheet of sterile gauze
12. In the clean area (or hood) prepare (4) 3cc syringes and (2) 5cc syringes; label (2) 1cc syringes with a “+” and (1) 5cc syringe with a “+”, affix a 10 gauge needle to both 5cc syringes
13. Remove one petri dish from the hood at a time, and fill the 2 appropriate 3cc syringes with 1.2cc of media, and the 5cc syringe with the remaining media
14. Tap and expel excess air from the syringes, replace the needle cap on the 5cc syringe, then place them back into the incubator
15. Repeat steps 13 and 14 for other petri dish and syringes

**Preparation of Microfluidic Unit**

1. All following steps occur in a clean cell culture hood, with clean gloves at all times
2. Prepare coated plate; discard laminin coating from the petri dish lid, rinse with PBS, and discard PBS. Allow the plates to air dry in the culture hood (crystal formation is okay)
3. Bring the 5cc syringes to the cell culture hood, removing the covered needles for step 4 and replacing the covered needles directly afterward
4. Fill fluidic delivery component lines with Media II; attach appropriate syringe (“+” syringe to “+” fluidic line) to each line and fill the line, closing the valve when full
5. Place microchannel component on completely dry petri dish lid, apply some pressure to initiate adhesion
6. Micropipette 500 µl of Media I into the center well, and 200 µl into the surrounding wells
7. Prepare sterile cut-syringe; remove plunger from 3cc syringe, cut with sterile scissors or scalpel blade at the top of the syringe (needle side), and replace plunger in opposite direction
8. Depress cut-syringe on microfluidic unit so that it completely surrounds the opening of a fluidic well and pull plunger so that the maximum vacuum is created
9. Hold for 5 seconds, making sure that vacuum seal stays continuous, and that the fluid levels in the adjoining wells does not get too low, if the fluid levels become too low, immediately stop suction and re-fill the appropriate wells to prevent bubble formation
10. Check that media appear in the fluidic well; use the inverted microscope to see that there is not a significant amount of bubbles in close proximity to the microchannel entrance. If a lot of bubbles are found, or if the fluid has not reached the well repeat steps 8 and 9
11. Repeat steps 8 through 10 for all (4) fluidic wells
12. Place (4) bolts in proper direction in the bottom fluidic delivery plate and set plate on clean surface of the cell culture hood with bolts facing up
13. Place the petri dish lid and microchannel component assembly onto the bottom fluidic delivery component plate
14. Lightly slide top plate of the fluidic delivery component onto bolts with proper orientation, check that the tubing coming from the bottom of the plate fits into the fluidic wells of the microchannel component
15. Fill the fluidic wells with proper media II before clamping: depressing one side of the top plate slide appropriate syringe needle to the top of fluidic well and fill to the top, repeat this for all (4) fluidic wells maintaining pressure on the plates to keep the wells from leaking
16. When all four wells are filled to the top, depress the entire plate and screw on all (4) nuts
17. Proper tightness is achieved when excess fluid has been excluded from space between top plate and top of the microchannel component
18. Assembled microfluidic unit is placed into sterile 1000ml pipette tip lid
19. Additional 500ml of Media I: Serum Based is added to center well
20. Unit is transferred to cell culture incubator until surgeries are complete

**Extraction of Spiral Ganglion Explants**
1. Area surrounding dissection microscope is prepared by spraying with 70% ethanol, a sterile swab is laid down next to the microscope
2. (2) 60mm petri dishes are filled approximately half full with PBS
3. Gross dissection scissors (large and small) and tweezers, fine dissection tweezers (2) are sterilized in high heat and placed on sterile swab
4. Fresh scalpel blade #15 is affixed to the scalpel handle
5. Gross Dissection – cochlea extraction
6. Three or four day old rat pup is held over a 60mm petri dish top while the head is cut off using the large gross dissection scissors. The body is saved for proper disposal
7. Holding the snout with the gross dissection tweezers, the fine dissection scissors are used to separate the jaw from the skull and to loosen the skin from the scalp
8. The skull is cut in half down the medial line on both the top and the bottom of the head, carefully to avoid damaging the cochlea
9. The snout is cut from the back of the skull, and is disposed with the body
10. The two halves of the brain casing are gently separated, and the tweezers may be used to carefully scoop out the brain from either half
11. View the following under low magnification on the dissection microscope, with the light sources above the work area and facing down. Use fine dissection tweezers for all following steps
12. With the inside skull exposed on both halves, bone is gently picked away to expose the cochlea
13. Intact cochlea are transferred to the PBS filled 60mm petri dish for fine dissection
14. Fine Dissection – spiral ganglion extraction
15. Increase magnification, move light sources towards the table top facing the sides of the dish
16. Remove soft tissue from protective bone cover
17. While holding base of cochlea, gently unravel the three layers of the tissue
18. The spiral ganglion is identified by its fur like texture. Very gently separate this structure from the others. Be especially careful not to touch the tissue with the tweezers too much, as every touch kills neurons
19. Once separated, transfer the ganglion to the second petri dish full of PBS. Pick up the spiral ganglion by capturing it in a bubble of solution that is carefully maintained between the tips of the tweezers to avoid touching and drying out the tissue
20. Gently push the ganglion to the bottom of the plate, stretching it out across the bottom as much as possible without damaging the tissue

Loading of Microfluidic Unit
1. Use the scalpel with blade #15 under high magnification to cut the complete spiral ganglion into (4) equivalent sized pieces, with ideal surgical extraction from one rat, (8) pieces should be obtained total
2. Take out the microfluidic unit from the incubator
3. Transfer (3) or (4) pieces of spiral ganglion from the petri dish to the center well of the microfluidic unit by carefully capturing it in a bubble of PBS between the tips of the tweezers
4. Push floating pieces under the media repetitively until they all sink to the bottom of the well
5. Gently push two explants approximately 1-2 millimeters away from the two microfluidic trapezoids
6. Spray the inverted microscope with 70% ethanol and heat sterilize the specialized ½ fine tweezers tool
7. Transfer the microfluidic unit to the cleaned inverse microscope to view the microchannel structure with high resolution
8. Carefully use the ½ tweezers to clear out any air bubble captured directly in the trapezoid
9. Use the ½ tweezers to gently push the explant into the trapezoid, as close to the channel outlet as possible. The explant will easily adhere to the tweezer, use quick but controlled pushing to avoid this as much as possible
10. After both explants are pushed in place, check once more with the inverted microscope that they are still within the trapezoid, as they are carried out with small movements of the fluidic tubing

Day 2 (18 hours later)

Change Media
1. Under the dissection microscope, gently withdraw the media from the center well using a P1000 micropipette, leaving a small meniscus around the bottom of the well
2. Replace the media with 500 microliters of 50% Growth Factor +, 50% Growth Factor - media

Start Fluidic Pump
1. The syringe pump should be sprayed with 70% ethanol including the top, bottom and power cord then placed in the 37°C incubator
2. Set the pump to 500 microliters per minute and place the 3cc syringes prepared during Preparation of Cell Culture Media
3. Start the pump and run until all four syringes have droplets forming at the mouth
4. Connect the microfluidic tubes to the syringes with the valves off
5. With all syringes connected, turn on the valves
6. Set the syringe pump to 0.25 µl / min
7. Start the pump and close the incubator door

Day 3 and 4

Observe explants
1. Move inverted microscope next to the incubator
2. Spray microscope with 70% ethanol to sterilize and put on a fresh pair of gloves
3. Carefully adjust syringe pump to allow the microfluidic unit to reach the microscope
4. Remove the unit from the incubator and observe explants with the microscope
5. Observe that fluid level has increase from the pumping action
Day 5 (72 hours after pumping starts)

Stop Pump
1. Turn off the syringe pump
2. Close the four syringe valves
3. Disconnect the microfluidic units from the syringes

Fix Explants
1. Wash with PBS:
   a) With the dissection microscope, use a P1000 micropipette to gently remove
      media from the center well, leaving a small meniscus around the edges
   b) Fill the well with 800 µl of PBS
   c) Use P1000 to remove the PBS from the well
2. Fill the well with 800 µl of 4% Paraformaldehyde
3. Channel suction filling:
   a) Connect two fluidic tubes that deliver to the same explant to a fully depressed
      3cc syringe with a ‘y’ connector
   b) Load the 3cc syringe into a ‘withdrawing’ syringe pump
   c) Withdraw the syringe at .15ml/min at the 5cc setting for 3 minutes. If the fluid
      level in the center well becomes low immediately refill!
   d) Close both valves simultaneously
   e) Detach the fluidic tubes from the 3cc syringe ‘y’ connector
4. Repeat Channel suction filling with other explant on the unit
5. Wait 25 minutes after filling both sides of the unit with 4% paraformaldehyde
6. Wash with PBS twice

Primary antibody Immunostain
1. Fill the well with 800 µl of Triton
2. Channel suction filling: Side A followed by Side B
3. Wait 10 minutes after filling both sides of the unit with Triton
4. Wash with PBS
5. Fill the well with 800 µl of 1% Donkey Serum
6. Channel suction filling: Side A followed by Side B
7. Wait 25 minutes after filling both sides of the unit with 1% Donkey Serum
8. Mix the primary antibody; 1 µl Rabbit anti-Neurofilament in 500 µl 1% Donkey Serum (final concentration = )
9. Use the P1000 micropipette to remove the Donkey Serum from the center well,
    leaving a meniscus around the bottom of the well
10. Fill the well with 500 µl of the primary antibody solution
11. Channel suction filling: Side A followed by Side B
12. Place the entire unit, covered with aluminum foil to reduce evaporation, in the 4C refrigerator overnight
Day 6

Secondary antibody Immunostain

1. Use the P1000 micropipette to remove the Primary Antibody from the center well, leaving a meniscus around the bottom of the well
2. Wash with PBS
3. Fill the well with 800 µl of Triton
4. Channel suction filling: Side A followed by Side B
5. Wait 10 minutes after filling both sides of the unit with Triton
6. Wash with PBS
7. Fill the well with 800 µl of 1% Donkey Serum
8. Channel suction filling: Side A followed by Side B
9. Wait 25 minutes after filling both sides of the unit with 1% Donkey Serum
10. Mix the secondary antibody in the dark; 2.5 µl Donkey anti-Rabbit purified antibody in 500 µl 1% Donkey Serum (final concentration = )
11. Use the P1000 micropipette to remove the Donkey Serum from the center well, leaving a meniscus around the bottom of the well
12. Fill the well with 500 microliters of the secondary antibody solution
13. Channel suction filling: Side A followed by Side B
14. Place the entire unit, covered with aluminum foil to reduce evaporation and keep out light, on a countertop at room temperature
15. Wait 2 ½ hours
16. Wash with PBS
17. Fill the well with 800 µl Triton
18. Channel suction filling: Side A followed by Side B
19. Wait 10 minutes after filling both side of the unit with Triton
20. Wash with PBS
21. Fill the well with 800 µl PBS
22. View unit under fluorescent microscope
23. To greatly increase optical clarity, remove the top and bottom plates CAREFULLY labeling on the petri dish which channel was filled with what type of growth factor media
Chapter REFERENCES


