CHOICE POINT MODELS OF NEURAL AXONAL GUIDANCE WITH SOLUBLE CUES

AN ABSTRACT

SUBMITTED ON THE TWELFTH DAY OF MAY 2015

TO THE DEPARTMENT OF BIOMEDICAL ENGINEERING

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE

SCHOOL OF SCIENCE AND ENGINEERING OF TULANE UNIVERSITY

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

GARY CABATINGAN CATIG

APPROVED: Michael J. Moore, Ph.D.

Donald P. Gaver, Ph.D.

W. Lee Murfee, Ph.D.
During development, neurites sample the surrounding environment for mechanical and chemical guidance cues in order to reach their desired targets. Due to the nervous system’s limited ability to regenerate after experiencing trauma, approaches from neurite development have been applied to create improved neural therapeutic and regenerative strategies. The use of guidance cues to reconnect the neural network could overcome limitations of current clinical treatments and ensure full function restoration and inhibit neuropathic pain development. This dissertation presents *in vitro* models that incorporate soluble gradients of chemical cues to influence neural growth at a choice point.

Axonal growth can be guided to a desired destination through the use of soluble guidance cues. These cues are usually presented as gradients in order to induce an attractive or repulsive response. In order to advance the application of our laboratory’s hydrogel choice point model, we needed to integrate soluble cues. An approach for incorporating these soluble cues was incorporating a circular reservoir patterned directly into the construct to serve as a protein source for biomolecule diffusion. The objective of Aim 1 was to determine how changes in source well position, growth restrictive border composition, and source well concentration affected the spatial and temporal concentration of gradients within the growth permissive area. Increasing the well distance caused a longer sustained release of proteins resulting in longer maintained gradients with higher concentrations. Increasing the restrictive border composition slowed the diffusion of the protein, producing gradients with lower concentrations and lasting for shorter durations. Reducing the initial well concentration lessened the overall
gradient concentrations. In addition, a 2-D computational diffusion model was developed and compared to experimental results. Through the use of the computational model, we identified configurations and experimental processes that keep cells exposed to physiologically relevant concentrations of a desired chemical signal. Our results show the ability to control soluble gradient profiles within our biphasic scaffold and establish methods that inform future experiments aimed at exposing cells to concentrations found in vivo.

During development, axons frequently encounter choice points that they navigate by sensing environmental cues in order to reach their final destination. The objective of Aim 2 was to demonstrate the feasibility of growth factor induced axon guidance using a computationally informed experimental choice point model. Additionally, a biased turning neurite growth model was created as a predictive tool for evaluating newly microfabricated geometries. Our in vitro studies showed that the presence of a soluble gradient of nerve growth factor produced a more chemoattractive guidance ratio compared to conditions with no gradient. Corresponding computational simulations modified to match the choice point geometry, generated similar guidance ratio values for the gradient and no gradient conditions. Our results demonstrate an axonal guidance assay influenced by diffusible gradients at a choice point and supports the use of our computational growth model for the assessment of axonal growth patterns within newly fabricated geometries.

An issue with experimentally administering axonal guidance cues in Aim 2 was the need for multiple fillings. The use of exogenous cells that consistently secrete these cues could serve as a possible solution. The objective of Aim 3 was to demonstrate the
ability to direct axon guidance using exogenous cells. In our dual phased hydrogel system, keratinocytes and dermal fibroblasts were used as they are involved in cutaneous innervation. We developed viable co-cultures of neural and dermal cells in our choice point model. Secretions from keratinocytes alone produced a more chemoattractive response compared to the control. The combination of keratinocytes and fibroblasts induced a further measured chemoattractive response. Our results show that exogenous cells can direct neurite growth and can be used as a cue source for future axonal guidance experiments.

The results establish the utility of our system for observing axonal growth under the influence of soluble guidance cues. Obtaining a better understanding of the neurite-environmental interaction can lead to the development of improved neural therapeutic and regenerative strategies.
CHOICE POINT MODELS OF NEURAL AXONAL GUIDANCE WITH SOLUBLE CUES

A DISSERTATION
SUBMITTED ON THE TWELTH DAY OF MAY 2015
TO THE DEPARTMENT OF BIOMEDICAL ENGINEERING
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE
SCHOOL OF SCIENCE AND ENGINEERING OF TULANE UNIVERSITY
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
BY
GARY CABATINGAN CATIG

APPROVED:
Michael J. Moore, Ph.D.
Donald P. Gaver, Ph.D.
W. Lee Murfee, Ph.D.
ACKNOWLEDGEMENTS

The work discussed within this dissertation would not be possible without the guidance and support of Dr. Michael Moore. I would like to thank my lab colleagues, especially Lowry Curley, Elaine Horn-Ranney, Parastoo Khoshakhlagh, Joseph Majdi, Renee Huval, and Ashwin Sivakumar, who all provided valuable support and assistance during my tenure at Tulane. I would like to also express my gratitude to Dr. Don Gaver, Dr. Lee Murfee, Dr. Ron Anderson, Dr. Damir Khismatullin, John Sullivan, Cindy Stewart, Lori McGinley, Megan Ohar, and Anne Nguyen. I thank my parents, Mar and Vicky, and my brother Gilbert, for being a steady influence despite the distance. Lastly, I thank my wife, Rebecca for all she has done for me throughout this whole time.
# TABLE OF CONTENTS

## CHAPTER 1: INTRODUCTION

1

## CHAPTER 2: BACKGROUND

5

### 2.1 The Nervous System

5

#### 2.1.1 The central nervous system (CNS)

6

#### 2.1.2 The peripheral nervous system (PNS)

7

### 2.2 Axonal Pathfinding

8

#### 2.2.1 Physical cues

9

#### 2.2.2 Immobilized guidance cues

9

#### 2.2.3 Soluble guidance cues

13

### 2.3 Micropatterned in vitro Models

15

#### 2.3.1 Hydrogels in in vitro models

15

#### 2.3.2 Physical cues in in vitro models

18

#### 2.3.3 Immobilized cues in in vitro models

18

#### 2.3.4 Soluble cues in in vitro models

20

### 2.4 Computational Models

22

### 2.5 Motivation for Study

24

#### 2.5.1 Objective

24

#### 2.5.2 Design criteria

24

### 2.6 References

26

## CHAPTER 3: FORMATION AND CHARACTERIZATION OF SOLUBLE GUIDANCE CUE GRADIENTS IN DUAL HYDROGEL CONSTRUCTS FOR NEURAL GROWTH MODELS

36

### 3.1 Abstract

36

### 3.2 Introduction

37

### 3.3 Materials and Methods

40

#### 3.3.1 Dual hydrogel formation

40

#### 3.3.2 Protein diffusion experiments

40

#### 3.3.3 Curve fitting

47

#### 3.3.4 Fluorescence recovery after photobleaching (FRAP)

47

#### 3.3.5 Computational diffusion experiments

51

### 3.4 Results

52

#### 3.4.1 Protein diffusion

52

#### 3.4.2 FRAP results

59

#### 3.4.3 Computational analysis

61

### 3.5 Discussion

64

### 3.6 References

71
<table>
<thead>
<tr>
<th>CHAPTER 4: EXPERIMENTAL AND COMPUTATIONAL MODELS OF NEURITE EXTENSION AT A CHOICE POINT IN RESPONSE TO CONTROLLED DIFFUSIVE GRADIENTS</th>
<th>73</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Abstract ..........................................................................................................................73</td>
<td></td>
</tr>
<tr>
<td>4.2 Introduction ..................................................................................................................74</td>
<td></td>
</tr>
<tr>
<td>4.3 Materials and Methods .................................................. ...........................................78</td>
<td></td>
</tr>
<tr>
<td>4.3.1 Dual hydrogel formation .......................................................... ......................................78</td>
<td></td>
</tr>
<tr>
<td>4.3.2 Experimental diffusion profiles .......................................................... ...............................78</td>
<td></td>
</tr>
<tr>
<td>4.3.3 Computational diffusion profiles ....................................................... .............................81</td>
<td></td>
</tr>
<tr>
<td>4.3.4 Computational neurite growth .......................................................... .....................................82</td>
<td></td>
</tr>
<tr>
<td>4.3.5 Experimental neurite growth .......................................................... .......................................84</td>
<td></td>
</tr>
<tr>
<td>4.4 Results ..............................................................................................................................86</td>
<td></td>
</tr>
<tr>
<td>4.4.1 Diffusion experiments .......................................................... ..............................................86</td>
<td></td>
</tr>
<tr>
<td>4.4.2 Neurite growth experiments .......................................................... .......................................94</td>
<td></td>
</tr>
<tr>
<td>4.5 Discussion ..........................................................................................................................97</td>
<td></td>
</tr>
<tr>
<td>4.6 References ........................................................................................................................103</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 5: PERIPHERAL NERVE RESPONSE TO GUIDANCE CUE SECRETIONS FROM DERMAL FIBROBLASTS AND KERATINOCYTES IN A CHOICE POINT MODEL</th>
<th>107</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Abstract ..........................................................................................................................107</td>
<td></td>
</tr>
<tr>
<td>5.2 Introduction ..................................................................................................................108</td>
<td></td>
</tr>
<tr>
<td>5.3 Materials and Methods .................................................. ...........................................110</td>
<td></td>
</tr>
<tr>
<td>5.3.1 Co-culture media .......................................................... ...............................................110</td>
<td></td>
</tr>
<tr>
<td>5.3.2 Cell viability assay .......................................................... .................................................113</td>
<td></td>
</tr>
<tr>
<td>5.3.3 Dual hydrogel formation .......................................................... ..........................................114</td>
<td></td>
</tr>
<tr>
<td>5.3.4 Experimental neurite growth .......................................................... .....................................114</td>
<td></td>
</tr>
<tr>
<td>5.3.5 Secreted protein quantification .......................................................... ....................................118</td>
<td></td>
</tr>
<tr>
<td>5.4 Results ..............................................................................................................................118</td>
<td></td>
</tr>
<tr>
<td>5.4.1 Cell viability .......................................................... .........................................................121</td>
<td></td>
</tr>
<tr>
<td>5.4.2 Neurite growth experiment .......................................................... ......................................124</td>
<td></td>
</tr>
<tr>
<td>5.4.3 Protein quantification .......................................................... ..............................................130</td>
<td></td>
</tr>
<tr>
<td>5.5 Discussion ..........................................................................................................................126</td>
<td></td>
</tr>
<tr>
<td>5.6 References ........................................................................................................................130</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 6: CONCLUSION AND FUTURE STUDIES</th>
<th>133</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Abstract ..........................................................................................................................133</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. Bifurcated model curve fit parameters..................................................54

Table 2. Diffusion coefficient summary of fluorescein-casein in hydrogels...........60

Table 3. Straight channel model computational and experimental curve fit parameters .............................................................................................................63

Table 4. Media development for co-culture model..............................................112
CHAPTER 1: INTRODUCTION

The nervous system has a limited ability to regenerate after experiencing trauma. In the central nervous system the formation of the glial scar and the release of chemorepulsive cues at the injured site prevent network reformation. In the peripheral nervous system, misguided growth is the main cause preventing full sensory restoration. Therefore, there is a need to better understand the factors that influence axonal pathfinding. During development, neurites sample the surrounding environment for mechanical and chemical guidance cues in order to reach their desired targets. Due to the nervous system’s deficient healing response to trauma, approaches from neurite development have been applied to create improved neural therapeutic and regenerative strategies. The use of guidance cues to reconnect the neural network could overcome limitations of current clinical treatments and ensure full function restoration and inhibit neuropathic pain development. We hope to obtain better understanding of the neurite-environmental interaction by observing the effect of soluble guidance cues on neurite growth. Reformation network

Our objective is to examine and manipulate neuronal responses to diffusible guidance cues within a 3-D in vitro model. We pursued different methods to incorporate long term soluble gradients in a dual phased hydrogel system and evaluated the chemotactic effects on neural growth. By developing in vitro models with controllable and quantifiable soluble gradients of guidance cues, we hope to obtain more insight in axonal growth mechanics for regenerative strategies.
Aim 1: Establish defined diffusible gradients of proteins within specific regions of a biphasic tissue scaffold.

We adapted a dual phased hydrogel system to incorporate soluble protein gradients in designated cell permissive regions. A circular reservoir was patterned directly into the construct to serve as a protein source for biomolecule diffusion. Although diffusion occurred throughout the construct, soluble gradients were formed in the desired regions and quantified. A degree of control over gradient duration and profile was demonstrated by varying restrictive hydrogel composition percentage and the position of the well. Potential long term gradient formation of could be achieved by refilling the reservoir after a certain time duration. A computational diffusion model was developed in parallel to create an economical and high throughput alternative method to generate and characterize gradient profiles. These studies were a proof-of-concept model to demonstrate how soluble chemical cues could be presented and quantified.

Aim 2: Develop integrated models of neurite growth, which leverage macro-scale experimental studies against growth cone guidance in silico models.

We applied techniques used to create soluble gradients from Aim 1 into in vitro and in silico models of neurite guidance. A bifurcating culture area was used to mimic a choice point for neuronal cells and a well was placed in close proximity of one of the bifurcating channels to serve as a protein source of nerve growth factor. Long term gradients were established and maintained by refilling the well every two hours. A computational diffusion model, which was validated using experimentally derived concentration profiles, informed a neurite biased turning growth model utilizing the same
geometry as the experimental choice point. Neurite growth simulations were then compared to a corresponding *in vitro* study. Results indicated that when a soluble gradient was introduced within the choice point configuration, the biased turning model predicted experimental behavior closely. Both simulated and *in vitro* neurite growth studies displayed a significant chemoattractive response toward the bifurcating channel containing a nerve growth factor gradient. The integrated model of neurite growth described will allow comparison of experimental studies against growth cone guidance computational models applied to axon pathfinding at choice points.

**Aim 3: Develop quantitative evaluation of neurite growth at a choice point influenced by guidance cue secretions from dermal cells.**

The use of the co-culture was to understand how skin cells influence axonal development. Limitations from Aim 2 motivated the pursuit of incorporating co-cultures of guidance cue secreting cells into a choice point model. The cells served as a less labor intensive alternative of maintaining long term gradients compared to well refilling, and provided the opportunity to examine the effects of multiple cues as these cells produced more than a single guidance cue. Keratinoctyes and dermal fibroblasts were used as the secreting cells as both have been known to contribute to cutaneous innervations. The skin cells’ viability in our system was first determined. Neurite guidance studies at a choice point were then performed using the co-culture to observe the chemoattractive response the cell secretions induced in axonal growth. Also the release of nerve growth factor and glial cell-line derived growth factor, two guidance cues the cells are known to
secrete, was quantified. Through this study, our co-culture guidance platform proved to be a sufficient tool to study interactions between free neural endings and skin cells.

The findings in this dissertation provide a foundation for creating a 3D microenvironment to culture neural tissue under the influence of soluble gradients of guidance cues. Discovering how these cues direct axonal growth can lead to strategies to reform disrupted neuronal connections. The work presented here is significant as it was the first to form integrated models of neurite growth under the influence of a chemoattractive gradient at a choice point. Also, a novel co-culture guidance assay was developed to observe the axonal response to fibroblasts and keratinocytes simultaneously.
CHAPTER 2: BACKGROUND

2.1 The Nervous System

The nervous system is responsible for the coordination of all bodily functions and enables the body to react to shifting environmental conditions. The two main components of the nervous system, the central nervous system (CNS) and the peripheral nervous system (PNS), are comprised of masses of nerve cells called neurons and neuroglial cells that provide insulation, nutrients, and physical support for neurons. A typical neuron possesses a rounded area identified as the cell body and two types of protrusions that extend from the body, dendrites and axons. Each neuron usually has a single axon, which sends information in the form of nerve impulses. In contrast, a neuron can possess multiple dendritic extensions that receive electrochemical messages.

The two main responsibilities of the nervous system are related to sensory and motor functions. Located at the ends of peripheral neurons are sensory receptors which can detect a myriad of internal and external elements\(^1\). These elements can range from light and auditory intensities to body temperature and oxygen levels. The receptors convert environmental information to nerve impulses which are transmitted from peripheral nerves to the CNS where they are processed. Due to integrative function, an individual makes decisions and uses motor actions to act on these decisions. The motor functions of the nervous system use peripheral neurons, which carry impulses from the CNS to effectors. Effectors are outside the nervous system and include muscles that contract and glands that secrete when stimulated by nerve impulses. The motor functions in the PNS are either consciously controlled or involuntary.
2.1.1 The central nervous system (CNS)

The CNS consists of the brain and spinal cord and serves as the body’s control center. It is responsible for processing incoming signals and relaying commands to other parts of the body. Within the CNS, there are significantly more neuroglial cells than neurons\(^1\). Neuroglial cells, unlike neurons, have the capability to divide and consist of four main types of cell: microglial cells, oligodendrocytes, astrocytes, and ependymal cells. Microglial cells are found throughout the CNS and provide neuronal support. These cells are also responsible for phagocytizing bacterial cells and cellular debris. Oligodendrocytes are aligned along nerve fibers and provide an insulating layer of myelin around axons in the brain and spinal cord called the myelin sheath. Astrocytes and are commonly located between neurons and blood vessels. They provide structural support and regulate nutrient and ion concentrations. They also form scar tissue that fill up empty spaces proceeding CNS injury. Ependymal cells form an epithelia-like membrane that covers specialized brain parts and forms the inner linings that enclose spaces within the brain and spinal cord.

During CNS injury, the network of nerve fibers is often disrupted and severed resulting in damage to the surrounding tissues and loss of function. At the beginning of the healing process, the distal ends of the disconnected axons form dystrophic growth cones that sample the damaged glial environment\(^2\). As time progresses, inflammatory cells and reactive astrocytes are recruited to the damaged site to form a glial scar. During scar formation, there is an increased release of chondroitin sulphate proteoglycans, which limits regeneration due to their chemorepulsive properties. In addition, the presence of myelin associated inhibitors from undamaged oligodendrocytes and myelin debris can
further limit axonal regrowth \(^{3-5}\). As a result, the combined effect of molecular inhibitors and scar formation make it difficult for complete axon repair.

### 2.1.2 The peripheral nervous system (PNS)

The PNS consists of nerves that branch out of the CNS and connects it to other parts of the body. The PNS is divided into the somatic and autonomic nervous systems, which control conscious and unconscious activities respectively. The somatic nervous system consists of the nerve fibers from the spine and skull that connect the skin and skeletal muscles to the CNS\(^1\). The autonomic nervous system connects the internal organs within the body cavity to the CNS. The Schwann cells are the primary neuroglial cells found in the PNS and are critical in the formation of the myelin sheath that wrap around axons.

In contrast to CNS injury, PNS regeneration after injury can result in some restorative sensory function, although the outcome after repair can vary. Following PNS injury, axonal sprouting will occur at the first node of Ranvier proximal to the injury\(^6\). Macrophages are recruited to the distal stump region to phagocytize myelin and axon debris during Wallerian degeneration. Also during this stage, Schwann cells begin to proliferate and align inside the basal lamina forming Büngner bands. Regenerating axons, which are directed by multiple guidance molecules, extend and navigate across the lesion gap allowing the axons to enter into the basal lamina tubes and rows of Schwann cells. The Schwann cells will migrate along the regenerating axons and gradually sort them until a single Schwann cell remyelinates a single axon. The regenerating axons preferentially grow in contact with the old basal lamina and the Schwann cell membrane, although the old basal lamina is broken down and replaced by a new basal lamina created.
by the Schwann cells. Eventually, the Schwann cells form a thin myelin layer surrounding the axon. Finally, axon sorting and target finding occurs to reconnect the network and restore function. Reinnervation of the PNS is commonly inadequate because misdirected axons, hyperinnervation, and polyneuronal innervation prevent complete restoration.

The wound healing response of both the CNS and PNS presents challenges in reforming synaptic connections. Utilizing axonal pathfinding can be a key approach in developing neural therapeutic strategies. Identifying which environmental factors influence axonal growth and how they influence guidance can help direct axons to their appropriate destination.

2.2 Axonal Pathfinding

Most of what is known about axonal pathfinding has been obtained from the developmental biology of neurons. While maturing, axons regularly encounter choice points where the collaborative effort of various environmental factors helps guide them to their desired targets. Physical cues such as the mechanical properties of the extracellular matrix and adjacent tissue can affect neuron growth. For example, studies have shown neurite growth is adversely affected by the stiffness of its surroundings. The stiffer surroundings prevent neurite penetration into an area\textsuperscript{7-9}. Moreover, chemical signals highly influence neurite growth. These chemical signals may be presented as either freely diffusible or contact mediated guidance cues and can initiate attractive or repulsive responses. Positive guidance cues can vary from a simply permissive substrate, in an otherwise nonpermissive region, to actual chemoattractive signals. Negative guidance
cues span from repulsive cues to robust inhibitory signals that can serve as barriers to neuron growth\textsuperscript{10,11}.

2.2.1 Physical cues

The mechanical properties surrounding neural tissue can play a significant role in axonal behavior including mechanobiology, motility, extension, and stem cell differentiation\textsuperscript{12-15}. Neurons are able to detect and react to various physical external stimuli such as stretching, pressure, compression, and touch. Previous studies have observed the neuronal cell response to mechanical properties. For example, a study utilizing polyacrylamide gels showed that lower substrate flexibility improved cell adhesion to glyoxyl agarose and encouraged neurite branching\textsuperscript{16}. Similarly, softer substrates increased branching by as much as three fold compared to stiffer gels\textsuperscript{8}. Stiffness also influenced axonal growth as there was an inverse relationship found between neurite extension and substrate stiffness\textsuperscript{7}.

Other physical cues that may affect cellular function include the porosity and pore size of the substrate. Porosity is important to cells with regards to cell affinity and viability as it influences cell binding, movement, intercellular signaling, and molecular transport\textsuperscript{17}. The model pore network should limit dead space, unconnected pores, and tortuosity to ensure proper mass transport of media and nutrients to cells encapsulated within\textsuperscript{18}.

2.2.2 Immobilized guidance cues

Extracellular Matrix Molecules

Factors present in the extracellular matrix (ECM) play important roles in nervous system developmental processes including cell migration, growth cone guidance, neurite
extension, and synapse formation. The ECM is the predominant component of nervous system tissue and is comprised of collagen and noncollagenous glycoproteins such as laminin, fibronectin (FN), and tenasin (TN), chondroitin sulfate proteoglycans (CSPGs), and heparin sulfate proteoglycans (HSPGs)\(^\text{19,20}\). The main surface receptors for ECM molecules are integrins, which consist of noncovalently linked α and β subunits arranged to form the heterodimetric protein. Integrins can transmit chemical and mechanical changes within the growth cone. The binding of integrins in neuronal cells can result in cell migration, axonal extension, growth cone guidance, cell anchorage, and differentiation\(^\text{21,22}\).

Laminin (LN) possesses chemoattractive properties as it has been shown to stimulate neurite guidance and extension in the PNS and CNS\(^\text{23,24}\). There are different varieties of LN that are present throughout the nervous system. LN-9 and LN-11 are found in the perineurial region of the nerve fiber while LN-2 is within the endoneurial basal lamina\(^\text{25}\). LN-1 is the most commonly used LN for neuronal studies as it is the most commercially available. Previous studies have shown the ability of LN-1 to enhance adhesion and outgrowth of CNS and PNS associated neurons\(^\text{26}\). Other types of LNs could be potentially useful in nerve guidance experiments, but are currently difficult to isolate and procure significant quantities.

Fibronectin, another ECM molecule, is commonly found in the bloodstream, connective tissue and basement membrane. FN is produced by multiple cell types including fibroblasts, epithelial cells and chondrocytes\(^\text{27}\). It is a vital component in wound healing and development, but its purpose with regards to neural cells is to support neurite extension and migration\(^\text{19,20,28}\). Its chemoattractive properties and ability to
interact with integrins make it an advantageous factor to incorporate into neural studies.

Tenascins (TN), CSPGs, and HSPGs are ECM molecules with contact mediated chemorepulsive properties. TN-C and TN-R are the members of the TN family that display repulsive cues, but each of the domains initiates differing responses in neurite guidance, migration, and extension. It has been shown in retinal ganglion cells that the presence of LN and FN were able to overcome the inhibitory effects of TN-R on neurite outgrowth. In contrast, CSPGs and HSPGs impede olfactory axon outgrowth, and their repulsive effects restrain these axons to a precise path during development. CSPGs have also been found within the glial scar after CNS injury where they prevent axonal reinnervation. Although both CSPGs and HSPGs have inhibitory effects, their mechanisms of action differ. Experimental results utilizing hippocampal neurons revealed CSPG stabilized neurons via integrin activation while HSPG destabilized the same cells by growth cone collapse.

**EPHRINS**

Ephrins are a class of immobilized proteins that are bound to membranes. They bind to ephrin receptors (Eph) and are predominantly repulsive, though they have the ability to be attractive. The ephrin ligands are classified into two groups based on their extracellular domain sequence. The first group, A-ephrins, are glycosylphosphatidylinosital membrane anchored, while the other group, B-ephrins, are transmembrane proteins. In vertebrates, there are nine EphA receptors that bind to six A-ephrin ligands (ephrinA1-ephrinA6) and six EphB receptors that bind to three B-ephrin ligands (ephrinB1-ephrinB3). Although they favor binding to their specific ephrin
protein, EphA4 and EphB2 may be activated by either type of ephrin protein \cite{38,39}. A previous study has demonstrated that ephrinA2-EphA3 axonal binding causes axonal detachment \cite{36}. The work of Marquardt et al showed when axons express either ligand or receptor, ephrinA to EphA binding caused growth cone repulsion while the reverse signaling of EphA to ephrinA resulted in growth cone attraction in the CNS \cite{40}.

**SEMAPHORINS**

Semaphorin biomolecules are divided into soluble and insoluble proteins. They are known for their growth cone collapsing properties \cite{41}, but have recently been found to attract extending axons \cite{42}. They are a large class of proteins made up of nearly 500 amino acids and are further classified into eight subclasses based on different characteristics such as membrane anchorage or secretion \cite{43}. The two primary receptors that mediate semaphorin activity are plexin and neuropilin \cite{44-46}. The majority of semaphorins directly bind to plexin via a single protein domain, though class 3 semaphorins interact differently. Sema3A initially binds to neuropilin with the newly formed complex activating plexin, which causes growth cone collapse \cite{45,46}. Class 3 semaphorins along with their receptors are involved in the development of multiple parts of the nervous system including cranial and spinal nerves, the hippocampus, the cortex, and the olfactory system \cite{47}.

Semaphorins can be soluble or membrane bound proteins that may cause long or short range chemorepulsive responses. In a sciatic injury model, five class 3 semaphorins experience an upregulation in expression along with their respective receptors \cite{48}. Depending on the location in the nervous system, class 3 semaphorins may be attractive or repulsive. During spinal cord injury, class 3 semaphorins aided in the inhibitory
effects of glial scar formation. Similarly, Sema3A and Sema3C initiated growth cone collapse resulting in repelled sensory neurons in both dorsal root ganglia and sympathetic chain ganglia. In cortical neurons, Sema3A exhibited a similar repulsive effect, however Sema3C exhibited an attractive effect.

1.2.3 Soluble guidance cues

**NEUROTROPHINS**

Neurotrophins are secreted cytokine proteins that are involved in cell survival, differentiation, proliferation, and plasticity. The four major neurotrophins are brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). P75 neurotrophin (p75NTRs) and tropomyosin receptor kinases (Trks) are the receptors most involved with neurotrophins. NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 forms complexes with TrkC. P75NTR attaches with high affinity to NGF but also has a low affinity for the other neurotrophins. The neurotrophins have chemoattractive properties and are known to play a role in the wound healing process. Controlled delivery of NGF has increased peripheral nerve regeneration and nerve fiber thickness and aided the regeneration of myelinated and unmyelinated axons. On the other hand, NT-3 stimulated repair of only myelinated axons. During spinal cord injury, NT-4/5 induced axonal regrowth, but did not result in any functional recovery. Similarly, the presence of BDNF has been shown to increase neurite extension after spinal cord injury.

**NETRINS**

Netrins are guidance molecules predominantly found on the vertebrate floor plate. They have been known to exhibit a bimodal nature depending on the activated receptor
It has been shown to attract commissural axons to the midline during development of the spinal cord. The chemoattractive response is associated with the immunoglobulin family transmembrane protein receptors deleted in colorectal cancer and neogenin. Binding of these receptors leads to axon guidance and cell adhesion. Conversely, the uncoordinated-5 receptors are linked to the chemorepulsive response. Though primarily associated with spinal cord development, netrin is involved in development of additional CNS areas including the retina, cerebellum, substantia nigra, and corpus striatum.

**SLIT**

Slits are secreted proteins that assist in axonal guidance through their chemorepulsive properties. There are currently three types of slits (slit1-3) that bind to four different receptors, all in the transmembrane robo family (robo-1-4). During development, slits play vital roles including guiding commissural axon at the midline, enabling spatiotemporal patterning and neurite projections in the brain, and leading axonal extensions in the optic system. In addition, the various slits play vital roles in nerve regeneration and maintenance. Following CNS injury, there is an upregulation of slit-1 in the cerebellum, slit-3 in the spinal cord, and slit-1 and slit-3 by microglial cells. Specifically as a response to brain injury in the hippocampus, slit-2 expression is increased. It is believed that slits help in regenerative failure through their repulsive affects. Alternatively during PNS injury, slit-1 expression increased, slit-2 expression decreased, and slit-3 expression was unaffected. It is suggested that slit-1 aids in nerve elongation while slit-2 remodels dendritic branching around the soma.

**GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR**
Glial cell line-derived neurotrophic factor (GDNF) is a small protein which has promising potential for neural therapeutic strategies. It has been shown to interact with GDNF family receptor alpha-1 and 2\textsuperscript{86,87}. This neurotrophic factor has beneficial regenerative effects, including the increased survivability of dopaminergic neurons afflicted with Parkinson’s disease\textsuperscript{88,89}. Furthermore, it has been shown to increase differentiation, fiber outgrowth and dopamine release in neurons\textsuperscript{90,91}. Also gradients of GDNF have induced and directed axon growth of neurons into the striatum in vivo using its chemoattractive properties\textsuperscript{92,93}.

2.3 Micropatterned \textit{in vitro} Models

One approach to observing neural growth behavior is through in vitro model systems. Lab on a chip technologies have great potential in creating high throughput platforms that can recreate the nervous system environment. Through such systems, individual variables can be isolated and their role in neurite guidance studied. Other benefits of \textit{in vitro} models include reduced costs and easier reproducibility compared to \textit{in vivo} models.

2.3.1 Hydrogels in \textit{in vitro} models

Significant research has focused on creating biomimetic microenvironments for neural cell culture. Hydrogels have been commonly used in micropatterned lab on a chip technologies due to their beneficial properties. Their water-swollen cross-linked networks allow hydrogels to imitate both the mechanical properties and architecture of the ECM where cells can grow in three dimensions\textsuperscript{65,94}. Furthermore, the stiffness of hydrogels can be finely tuned to observe its influence on neurite outgrowth\textsuperscript{7,8}. Various
guidance cues can be also incorporated within the hydrogel at known concentrations either as immobilized \(^{95,96}\) or freely diffusible proteins \(^{97,98}\).

**POLYETHYLENE GLYCOL**

Polyethylene glycol (PEG) is a hydrogel that has been commonly used as a biomaterial for cell culture. It has high biocompatibility and lacks immunogenicity \(^{99,100}\). It also has the ability to resist protein adsorption and does not support cell attachment due to the hydrated surface \(^{101,102}\). The beneficial properties are a result of PEG chains high mobility related to water-binding ability and conformational flexibility. There are various methods to synthesize PEG hydrogels, with the most common through photoinitiated chain polymerization \(^{103}\). In this process, long polymethacrylate chains are crosslinked with PEG. The pros of this process are rapid and robust gel formation, but the drawbacks include non-uniform degradation and heterogeneous nanoscale structure. The PEG chain length determines the molecular mesh size while the methacrylate concentration influences the molecular mesh size and the crosslinking density. Another approach to synthesizing PEG is through step growth polymerization, where a reaction occurs between comonomer solutions containing complementary reactive groups \(^{104}\). The resulting hydrogel has a more homogenous network structure compared to photoinitation, which can lead to superior mechanics. In this case, the PEG chain and concentration dictate the mesh size while the PEG crosslinker length helps determine the crosslink density.

**AGAROSE**

Agarose, a polysaccharide based hydrogel from seaweed, has also been utilized in tissue engineering applications \(^{96,105}\). It dissolves at temperatures above 70°C and sets at
temperatures below 45°C. When cooled, agarose chains form hydrogen bonds and a hydrophobic relationship that helps create double helices. It is assumed the generation of these double helices and their aggregation creates the formation of a solid agarose hydrogel 106.

**PURAMATRIX**

Puramatrix is an L-amino acid based hydrogel that is highly soluble in pure water and has the tendency to form an unusually stable β-sheet structure. It has many beneficial properties that make it a favorable hydrogel for cellular studies. First it forms a network of nanofibers similar to the native extracellular matrix 107. This simulates an in vivo environment for cell growth, migration, and differentiation. Second, it can be broken down into natural L-amino acids, which can then potentially be used by the surrounding tissue or excreted in urine. In addition, it is synthetic and free of chemical and biological contaminants usually present in animal derived biomaterials. Moreover, it is stable and resistant to digestion to enzymes. Finally it is immunologically inert and possesses an insoluble macroscopic membrane.

Puramatrix spontaneously assembles to form stable macroscopic matrices while in the presence of monovalent cations. During this process, oligopeptides form intermolecular hydrogen bonds in conventional β sheets on the peptide backbones 108. At the same time, side chains of positively and negatively charged residues form intermolecular ionic bonds and methyl groups of alanine form hydrophobic β sheet interactions. As a result, a peptide of alternating hydrophilic and hydrophobic amino acid residues adopts a β sheet structure; one polar surface with charged ionic side chains and one non-polar surface with alanines.
2.3.2 Physical cues in in vitro models

Lab on a chip technologies that evaluated the effects of stiffness on cells have been fabricated using photopolymerization of hydrogels, or the crosslinking of hydrogels through light exposure. Through this process, temporal and spatial control of the polymerization and fabrication of intricate geometries can be achieved \(^{109,110}\). This process also allows the formation of physical gradients during the polymerization stage through varying the light exposure using a grey scale or sliding scale photomask. For example, a polyacrylamide gel was formed using the grey scale with elastic modulus ranging from ~2.5 to ~11 kPa over a length of 18 mm \(^{111}\). Similarly, the use of a sliding scale resulted in stiffness gradients in hydrogels composed of hyaluronic acid \(^{112}\) and PEG \(^{109}\).

Porosity gradients have also been formed for tissue engineering applications. One method involved spinning a two-material system of collagen and glycosaminoglycan to create a tubular network with radial gradients in pore size and porosity \(^{113}\). Tripathi et al fabricated an agarose/gelatin hydrogel where the agarose was self-gelated at low temperatures while the gelatin was simultaneously cross-linked by glutaraldehyde. The resulting hydrogel contained pore size varying from 45 μm to 260 μm \(^{114}\). In a separate study, Dubrueil et al cryogenically cross-linked gelatin scaffolds with embedded parallel channels. This produced cone like porous structures with a pore size range from 30 μm to 330 μm \(^{115}\).

2.3.3 Immobilized cues in in vitro models

Immobilized cues have been known to affect neuronal cells during development. Differing strategies and approaches have been established in order to observe contact
mediated cues on neuron behavior. One such assay is the stripe assay, in which alternating stripes of cell membrane fractions are created on an adherent substrate using a silicone matrix. The silicone matrix is composed of parallel microchannels, which serve as a mask exposing certain areas of the surface. One cue is circulated within the microchannels to bond with the exposed surface and then removed. The silicone matrix is then removed and a second cue is deposited on the surface, which only attaches to regions without the first cue. Cells grown on the surface are now exposed to two substrates and preference for either cue can be detected after a few days in culture. This assay has been used to understand guidance in various systems including the thalamocortical system, the olfactory system, and the hippocampus. A drawback of the assay is it only reflects a preference between two cues, and does not indicate an attractive or repulsive response. In addition, the homogenous presentation of cues and the abrupt transition from one strip to the next may not be representative of in vivo environments.

Another type of assay for the study of substrate-bound cues includes a discontinuous gradient. One method to form these discontinuous gradients involves using a microdispenser for ink jet printing. The spaces between prints can be achieved by altering the number of drops per point. Using this method, Turcu et al micropatterned laminin onto a surface to support neuronal growth. Though this process is relatively quick and simple, the disadvantages include low spatial resolution and the inability to robustly print on non-polymeric surfaces. Discontinuous gradients may also be formed by microcontact printing. Here, a patterned stamp is used to print onto a substrate using physical contact. Varying sized dots and lines with desired spacing can be easily
produced creating long stable patterns\textsuperscript{123}. The main drawback of this method is that it does not permit the printing of multiple types of cues or varying concentrations onto the surface.

A third assay involves a continuous gradient of substrate-bound cues. One of the first methods for forming a continuous immobilized gradient incorporated a guidance factor solution into a drop of Hank’s solution. The proteins diffused radially and adsorbed onto the surface of a substrate\textsuperscript{124}. Although a gradient was formed, there was limited control of the gradient properties and lack of long term binding of the protein. Later, microfluidic gradient mixers were utilized to improve spatial control and reproducibility of the gradient, however these methods still relied on adsorption for attachment\textsuperscript{125,126}. Photoimmobilization has proven to be a promising technique to construct high-resolution substrate bound cues. Light-sensitive hydrogels provided spatial control of the protein placement within them. Thiol-based chemistries have been utilized extensively due to their ability to entrap proteins inside the network when exposed to ultraviolet (UV) light\textsuperscript{127,128}. Photolabile nitrobenzyl based caging moieties functionalized into hydrogels can covalently bond to proteins after UV exposure\textsuperscript{96,129}. Much like in the formation of stiffness gradients in hydrogels, a grey scale or sliding mask can be used to form immobilized gradients.

\textit{2.3.4 Soluble cues in in vitro models}

There are numerous methods to introduce soluble guidance cues into in vitro models. The most common and simplest method is the diffusion source-sink method where diffusion occurs between two solutions\textsuperscript{130,131}. One solution, containing the desired protein, serves as the source. The second solution, which contains either no desired
protein or a lower concentration of the desired protein than the source, serves as the sink. The solutions are placed on opposite ends of a channel and the source solution is allowed to diffuse towards the sink creating a gradient. Though creating gradients through this method is easy, the reliance solely on diffusion can be time consuming in order to use in cell studies.

A second method involves thorough mixing of the two solutions to establish a gradient. A tree-like gradient generator begins with the placement of two solutions, similar to the source-sink method at the start of the microdevice. Flow is introduced into the device causing mixing of the solutions and serial dilution at each successive step, or branch, of the tree. This is a relatively rapid process compared to the source-sink as gradients hundreds of microns long are formed in a minute $^{132,133}$. The gradient shape can be influenced by the microchannel network design. A significant drawback to this method is the gradient is only stable during fluid flow. Once flow is turned off, diffusion occurs bringing the concentration to equilibrium. A second strategy involves the use of a dynamic mixing apparatus. Multiple syringe pumps are used simultaneously to pump different solutions at controllable flow rates into the mixer. The mixed solution is then deposited into a mold to be stabilized and used later $^{134,135}$. Varying gradients with controllable shapes can be produced using the dynamic mixer by changing the ratio of solutions mixed.

Printing has also been used to incorporate soluble gradients within hydrogels. Rosoff et al printed lines of increasing concentrations of guidance molecules on top of a collagen gel $^{97}$. After 3 hours, a smooth gradient was formed by diffusion of the molecules into the space in between each line. Using this method, the outgrowth
behavior of dorsal root ganglion (DRG) neurons was observed under high sensitivity of NGF gradients. Though very fine control of the gradient shape was achieved by this method, the printer required to produce the gradients is very costly.

Co-culture assays have also been used to study the effects of guidance cues. These co-cultures involve an explanted tissue containing developing neurons cultured with cells that secrete a desired guidance cue. The secreting cells can either secrete the desired guidance cue naturally, or be transfected to cause the secretion. Since the cells are continually secreting, they can serve as a long term source of guidance cues. Some disadvantages of a co-culture assay include the difficulty to experimentally determine the shape of the formed gradient and the ability to isolate the chemotactic effect of a single guidance cue as cells are secreting more than a single component into their environment.

2.4 Computational Models

Computational models are useful tools that can be used to acquire greater understanding of neurite growth patterns. The use of these models can help optimize studies establishing baseline parameters for testing. In addition, the validity of the computational approach can be confirmed by with matching in vitro results. Previous work by Ming et al. showed that axonal growth behavior could be modeled similar to the chemotactic response of bacteria where the neurite detected a gradient and moved in a zig-zag pattern toward the source. Other models have shown neurite paths to travel a smoother path when influenced by a gradient. In these models the direction an axon moves is based on its level of polarization. A neurite with little polarization is more inclined to follow a random path, while a highly polarized neurite would follow a more
A neurites polarity increases as more guidance cue is detected leading to a less variable path as it approaches the source.

One *in silico* model used to determine axonal chemotactic growth patterns is a biased turning model (BTM)\(^{143}\). In the BTM, a signal to noise ratio (SNR) is calculated to determine the direction an axon will move. The SNR is calculated using the fractional change in concentration across the growth cone (µ), the growth cones current direction (θ) and the gradient direction (θ\(_{NGF}\)). Using equation 1, an SNR can be calculated where γ is a dimensionless value which represents the background growth factor concentration.

\[
\text{SNR} \propto |\mu| \sqrt{\frac{\gamma}{(1+\gamma)^3}} \sin(\theta - \theta_{NGF})
\]  

At each time step, the axon grows a set distance in its current direction. Afterwards, a random number is obtained from a Gaussian distribution with a mean equal to the SNR and a variance of 1. If the random number obtained is positive, the axon turns to the right a designated angle and turns to the left the designated angle if the number is negative.

Another computational model, from the same group, used growth-rate modulation (GRM) to determine neurite response to a gradient\(^{144}\). Similar to the BTM, the GRM calculated an SNR, but in this case, the value of µ was the fractional concentration change along the length of the entire growth cone. The SNR for the GRM was also calculated using the equation 2.

\[
\text{SNR} \propto |\mu| \sqrt{\frac{\gamma}{(1+\gamma)^3}} \cos(\theta - \theta_{NGF})
\]

The GRM also differed from the BTM because at each time step the neurite would turn a set angle either to the left or right in an impartial manner, but would grow a varied distance, L, based on the SNR. L was calculated using equation 3 where \(N(\text{SNR},1)\) is a
random number generated from the Gaussian distribution with a mean of the SNR and a variance of 1, and $k$ is an experimentally derived constant.

$$L = G(1 + k \times N(SNR,1))$$  \hspace{1cm} (3)

### 2.5 Motivation for Study

#### 2.5.1 Objective

The overarching goal of this study is to observe the axonal response to chemoattractive soluble guidance cues at a choice point using computational and experimental models. Our work improves upon current studies through the use of a choice point model, which constrains axonal growth to a certain point where they must choose a path from two options based on the presented guidance cues. Many present experimental studies observe the effect of guidance cues in an unrestrained environment as opposed to a choice point. For those studies that use a choice point, none utilize an integrated computational approach as a predictive tool for axonal growth. In addition, although there have been studies exploring the effect of multiple guidance cues, no study has employed a co-culture of three different cell types. Eventually the knowledge obtained from the choice point model can be applied towards wound healing therapies.

#### 2.5.2 Design Criteria

To fulfill our objective, we must design models that include the following criteria: i) constrain neurite growth into a choice point geometry; ii) introduce and maintain soluble gradients within physiologically relevant ranges; iii) develop an integrated computational model that simulates diffusion of soluble cues and axonal growth response to the cues. Previously in this chapter, we have described a multitude of factors that influence neurite growth and methods to integrate these factors into *in vitro* models. The
following chapters investigate how diffusible cues may be incorporated into a single nerve growth substrate, and how the presence of the diffusible cues affects axon divergence at a choice point. In Chapter 3, the source-sink method is used to introduce soluble proteins in the construct and techniques are developed to quantify and control the formed concentration gradients. In Chapter 4, the techniques developed in Chapter 3 are used to form soluble gradients of NGF to direct axon growth at a choice point. A corresponding integrated neurite growth model is used to predict axonal growth behavior. In Chapter 5, the co-culture method is used to form soluble gradients in a choice point axonal growth model.
2.6 References


Jain, A., Kim, Y. T., McKeon, R. J. & Bellamkonda, R. V. In situ gelling hydrogels for conformal repair of spinal cord defects, and local delivery of BDNF


74 Brose, K. *et al.* Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* 96, 795-806 (1999).

75 Li, H. S. *et al.* Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. *Cell* 96, 807-818 (1999).


CHAPTER 3: FORMATION AND CHARACTERIZATION OF
SOLUBLE GUIDANCE CUE GRADIENTS IN DUAL HYDROGEL
CONSTRUCTS FOR NEURAL GROWTH MODELS

Curley JL et al. Biofabrication 2014; 6: 035026)

Diffusible guidance cues can direct neurite growth in the process of chemotaxis. This chapter covers the proof of concept introducing quantifiable soluble factors as gradients within a dual hydrogel system. This work is in support of **Aim 1**.

**Aim 1: Establish defined diffusible gradients of proteins within specific regions of a biphasic tissue scaffold.**

3.1 Abstract

Chemotaxis is the movement of cells in response to detecting environmental soluble gradients, which may initiate attractive or repulsive response. Developing *in vitro models* that incorporate diffusible gradients is important to achieve better understanding of cell guidance, particularly in the case of the nervous system. The present study was designed to introduce and maintain soluble biomolecular gradients within specific regions of a dual phased hydrogel construct experimentally. In addition, we developed a computational model that will inform us of the optimal experimental design that will allow us to produce and sustain guidance cues. A circular well was patterned into the construct to serve as a reservoir that allowed biomolecule diffusion into the cell permissive region. Steeper profiles and longer duration of soluble gradients
could be achieved by increasing the distance of the well and lowering composition percentage of the cell restrictive hydrogel. Precise knowledge and control of the gradient profile is necessary to expose neurites to optimal and physiologically relevant concentrations of a guidance cue. If the concentration is too low, the receptors at the growth cone are unable to encounter enough guidance cues to promote turning. On the other hand if the concentration is too high, receptors are too saturated and overwhelmed to detect any noticeable gradient. Strategic placement of the well also exposed desired regions of the construct to a soluble gradient while other areas were unexposed. Use of the computational model could provide an inexpensive and high throughput alternative method to generate concentration profiles compared to experimental studies.

3.2 Introduction

Cells regularly respond to various factors within their surroundings. These factors, which include contact mediated cues, freely soluble cues and mechanical properties, can influence their growth, differentiation, and migration during cellular maturation. Lab on a chip technologies serve as useful tools in studying in vitro cell growth and behavior. A biomimetic milieu can be recreated with fine control of the microenvironmental conditions cells are exposed to. Through the use of such tools, a better understanding of the cell-environment interaction can be achieved. We adapted a previously developed micropatterned biphasic hydrogel system to incorporate diffusible protein gradients in specific regions of the construct\(^1\). In addition, a computational model was also created to simulate diffusion and replicate experimentally produced concentration profiles. In doing so, we hope to create a cellular environment that presents gradients of soluble cues within physiologically relevant concentrations.
A main component of the cellular microenvironment is the extracellular matrix (ECM). The primary function of the ECM is to provide cells with mechanical support. The mechanical properties cells experience may induce structural changes of the cytoskeleton which can cause a cellular response known as mechanotransduction. Additionally, mechanical property gradients can connect mismatched tissues including bone-cartilage interfaces and dentino-enamel junctions. A second function of the ECM is to expose cells to spatiotemporally regulated biochemical signals. Molecular gradients of these signals can play an important role in biological functions including chemotaxis, morphogenesis, and wound healing.

Recently, hydrogel based microfabricated chip technologies have been proven useful in investigating cellular responses to the microenvironment. Hydrogels are useful materials utilized in chip technologies because their water-swollen cross-linked networks can closely emulate both the mechanical properties and architecture of the ECM. In addition, the hydrogels can be easily manipulated to create fine control over environmental factors. For example, studies have shown that stiffness can affect cellular growth, cytoskeletal organization, differentiation, cell signaling, and process extension. Additionally, hydrogels can be used to expose cells to a variety of chemical signals by two methods. First, these proteins can be immobilized within the hydrogel network. Previous work observed the effects of immobilized cues on cell attachment, alignment, migration, and neurite extension. Moreover, the chemical signals can be present as freely soluble factors which are used to observe processes such as chemotaxis and angiogenesis.
When observing the effect of soluble chemical gradients on cell behavior, it is important to take note of the concentration ranges the cells are exposed to. If the concentration is too low, a cellular response may not be induced. In contrast, if the concentration is too high, cellular receptors may be saturated and too overwhelmed to induce an appropriate response. The concentration profiles of soluble chemicals can be experimentally determined, but can be time consuming each time a new material or geometry is introduced. Computational diffusion models can be a more efficient and cost effective method to determine these profiles compared to in vitro models. Through the use of the computational model, we will identify configurations and experimental processes that keep cells exposed to physiologically relevant concentrations of a desired chemical signal. This is described in §3.4.2.

Previously, we developed a cell culture model capable of constraining neurite growth in specific geometries. This hydrogel construct was composed of an outer cell restrictive border which contained growth within a specific cell permissive region. We adapted this biphasic system to incorporate a circular well, which served as a protein source to introduce a freely soluble protein gradient within designated parts of the cell permissive region. Through our study we hope to accomplish two goals:

1) Introduce soluble gradients within our construct and observe the effect that well placement, restrictive border hydrogel composition, and well solution concentration had on the gradient profile.
2) Use both experimental and computational models to determine the correct configuration that exposed neural tissue to physiologically relevant concentrations for a neurite growth assay.

3.3 Materials and Methods

3.3.1 Dual hydrogel formation

The dual constructs were produced as previously described\(^1\),\(^6\). First, a solution was prepared by dissolving polyethylene glycol (PEG) into phosphate buffer solution (PBS). Next, a six well polyester membrane Transwell\(^\circledR\) permeable support (Corning, Corning, NY) was treated with Rain-X (SOPUS Products, Houston, TX) to reduce meniscus formation. Afterwards, a digital micro-mirror device (DMD) serving as a dynamic photomask (Texas Instruments, Dallas, TX) and an ultraviolet (UV) light source (EXFO, Quebec, Canada) were used to irradiate the PEG solution inside the tissue culture insert for 55 seconds. The UV exposure crosslinked the PEG directly onto the insert forming the cell restrictive border with a bifurcated void. Agarose was dissolved in PBS at 1% w/v by heating and sonicating for 30 minutes. To form the cell permissive region, approximately 1 μL of the agarose solution was added into the bifurcated void and inserts were chilled at 4 °C for 1 hour to allow for gelation.

3.3.2 Protein Diffusion Experiments

In this section, we describe two models:

a) a bifurcated model that demonstrates the ability to introduce soluble gradients into the construct and methods to control the gradient.
b) a straight channel model that uses computational and experimental approaches to determine experimental processes to present physiologically relevant concentrations of a guidance cue.

\textit{a. Bifurcated Model}

At the time of development, the most commonly used geometry for our constructs included a bifurcated region where neural cells grew. A circle was placed near the point of bifurcation on the photomask in order to produce a construct and a circular well with a radius of 500 µm simultaneously (figure 1). In this configuration, the well was placed close to the bifurcation point as this was the only area in our construct that could accommodate its size. The present design was only used to prove the ability of establishing soluble gradients within our construct. A modified version of the current setup was used in the following chapter to produce a choice point model. The use of this model would be more relevant to axonal guidance studies. Two variables of our system were examined to observe their effects on the gradient profile. The distance between the well and point of bifurcation, as indicated by the length $L_4$ in figure 1, was set at 115 µm for the close configuration or 545 µm for far configuration. The other variable analyzed was the outer PEG composition, which was set at either 10\% w/v or 20\% w/v.
Figure 1: Dimensions of the bifurcated model: $R_1 = 572.5 \, \mu m$; $R_2 = 500 \, \mu m$; $L_0 = 1625 \, \mu m$; $L_1 = 895 \, \mu m$; $L_2 = 395 \, \mu m$; $L_3 = 625 \, \mu m$; $L_4 = 115 \, \mu m$ (close) or 545 \, \mu m (far); $\theta = 90^\circ$. 
The circular well was filled with 0.4 μL of 140 nM fluorescein-conjugated bovine serum albumin (BSA) (Molecular Probes, Eugene, OR) in PBS and fluorescent images were taken at the time points 0, 15, 30, and 60 minutes to monitor the BSA diffusion throughout the dual hydrogel construct. Using ImageJ software (National Institutes of Health, Bethesda, MD), the intensity profile for each image was measured using a straight, 270 pixel long line drawn across the length of the agarose gel. Pixels were binned in groups of ten pixels per bin to give twenty seven points of average relative intensity. The relative intensity was averaged over three experiments (n=3) obtaining a mean relative intensity and standard deviation per point. A calibration curve was then acquired from images of known concentrations of fluorescein-BSA contained in wells of similar thickness to the dual hydrogel constructs. Using the calibration curve, the relative intensity was converted to concentration of nM. A representative calibration curve is shown in figure 2. In the equation of the curve, y is the concentration in nM and x is the intensity.
Figure 2: Calibration curve used to convert intensity from fluorescent images to concentration.

\[ y = 1.22 \times 10^{-2} x + 0.626 \]

\[ R^2 = 0.998 \]
An alternative experiment using the same bifurcated model was used to explore the feasibility of sustaining a long term gradient of fluorescein-BSA within physiological ranges. Three tests were performed using the 10% close configuration, but the concentration of the fluorescein-BSA in PBS was reduced from 140 nM to 70 nM (n=3). The well was refilled after one hour resulting in a total time of 2 hours of monitored diffusion. Similar methods as described above were used to characterize the gradients, but the relative fluorescence was measured using a 300 pixel line, binned into 30 points of average relative intensity, and then converted into concentration (nM).

b) Straight Channel Model

Diffusion experiments as described above were performed to isolate a gradient in a specified region of a construct using a straight channel model. This alternative configuration was comprised of a single channel with wells on opposite ends (figure 3a). The PEG used was 10% w/v but instead of using fluorescein-BSA as the diffusing protein, fluorescent conjugated nerve growth factor (NGF) at a concentration of 40 nM was placed in one of the wells while the other remained empty (figure 3b). Two experimental diffusion profiles were obtained, the first of which was in the region closest to the filled well and the other in the region on the opposite end near the empty well. Diffusion was observed for 180 minutes and three tests were performed (n=3). If neural cells were initially seeded in the center circular region, this geometry can be used to observe the effects of NGF, a known chemoattractant, on neurites. Both neurite growth and extension can be potentially observed in this model.
Figure 3: Straight channel configuration. (a) Dimensions of the straight channel model: $R_1 = 625 \, \mu m$; $R_2 = 598 \, \mu m$; $L_0 = 3375 \, \mu m$; $L_1 = 982 \, \mu m$; $L_2 = 250 \, \mu m$. (b) Micropatterned configuration for the single channel diffusion experiments with one well left empty and the other filled with fluorescent conjugated nerve growth factor (NGF) $^{17}$. 
3.3.3 Curve Fitting

In order to characterize the gradients formed experimentally, the averaged concentration profiles at each time point were fitted to an exponential curve using SigmaPlot software (Systat Software, Inc., San Jose, CA) with the following equation:

\[ C = C_0 e^{\frac{x}{x_0}} \]  

In the equation \( C \) is the concentration (nM) and \( x \) is the position (µm). To characterize the diffusion profiles, two parameters were used. The first parameter, represented by \( C_0 \), was the absolute magnitude of the concentration in units of nM. The other parameter represented by \( x_0 \), was the decay length and had units of µm. The \( R^2 \) value was also obtained to compare how closely the experimental data fit the exponential equation.

3.3.4 Fluorescence Recovery After Photobleaching (FRAP)

Bulk hydrogels of PEG and agarose were prepared as previously described in §3.3.1 and loaded with fluorescent-casein in PBS at a concentration of 3800 nM. Casein was previously used to simulate the diffusion of NGF because the molecular weights of both proteins are similar \(^{18} \). FRAP experiments were then performed on the loaded hydrogels using a Zeiss LSM 510 Meta microscope (Zeiss, Oberkocken, Germany). A circular region with a radius of 26 µm was bleached for a total time of 1.31 seconds. Fluorescent images were taken before photobleaching and immediately after every 0.26 seconds for approximately 14 seconds. A method by Brandl et al. was used to calculate the diffusion coefficients of NGF in both hydrogel \(^{19} \). To determine the experimental recovery curve from the fluorescent images, the mean fluorescence intensities within the bleached region, \( I_{frap}(t) \), and a reference region \( I_{ref}(t) \) were calculated at each time point using ImageJ software. In the next step, \( I_{frap}(t) \) was normalized to the intensity before
bleaching, \( I_{\text{frac}(\text{pre})} \), and corrected for any bleaching effects that may have occurred during image taking using equation (2).

\[
f(t) = \frac{I_{\text{ref}(\text{pre})}}{I_{\text{ref}(t)}} \times \frac{I_{\text{frac}(t)}}{I_{\text{frac}(\text{pre})}}
\]

In the above equation \( f(t) \) is the normalized fluorescence intensity inside the bleached region and \( I_{\text{ref}(\text{pre})} \) is the fluorescence intensity inside the reference region before bleaching. The \( f(t) \) value was further normalized to the full scale using equation (3).

\[
F(t) = \frac{f(t) - f(0)}{f(\text{pre}) - f(0)}
\]

Here, \( f(0) \) is the normalized fluorescence intensity immediately after bleaching and \( f(\text{pre}) \) is the normalized fluorescence intensity prior to bleaching. A least squares fit of the expression in equation (4) was performed on the experimental recovery curve using Matlab (MathWorks, Natick, MA) to determine the characteristic diffusion time \( \tau_D \), which has units of seconds, and the mobile fraction \( k \), which is unitless.

\[
F(t) = k \times e^{-\frac{\tau_D}{2t}} [I_0(\frac{\tau_D}{2t}) + I_1(\frac{\tau_D}{2t})]
\]

\( I_0 \) and \( I_1 \) are the modified Bessel functions of the first kind of zero and first order. In order to see how close the experimental data fit the expression, the acquired resnorm value from Matlab was subtracted from 1. Once the \( \tau_D \) value was obtained, the diffusion coefficient (\( D \)) can be calculated using equation (5) where \( w \) is the radius of the bleached region.

\[
D = \frac{w^2}{\tau_D}
\]

Three tests were performed per group (\( n=3 \)). The experimentally determined diffusion coefficients were used in the computational diffusion model described in the next section.
A brief summary of the FRAP process and how the intensities are obtained can be found in figure 4.
Figure 4: Images of the photobleaching process and how the intensities of the bleached and referenced site are determined. Scale bars = 25 µm.
3.3.5 Computational Diffusion Experiments

A 2D computational model was constructed in MATLAB to simulate the diffusion of nerve growth factor (NGF) throughout a construct. The model possessed the same dimensions as the single channel configuration (figure 3) and consisted of two distinct domains, a rectangular region, which represented the entire construct, and a single channel, representing diffusion through PEG and agarose respectively.

The interface between the two distinct domains was modeled as a narrow region having a continuous transition between the properties of each domain, representing a region where the agarose and PEG hydrogels were interwoven. A well was placed at either end of the channel to serve as protein sources. A fixed concentration of 40 nM of NGF was placed in one of the wells and allowed to diffuse over three hours. The unsteady diffusion equation was solved using a finite difference method where $D_n$ is the diffusivity in the different hydrogels, $C$ is the concentration, and $F$ is a source function.

$$\frac{\partial C}{\partial t} = D_n \nabla^2 C + F(x,y,t)$$  (6)

The difference method used was an alternating direction implicit method where a Heaviside step function determined the diffusivity at different regions in the domain, thus drawing the two distinct boundaries and their transitional region. The outer boundary conditions of the PEG domain were $C = 0$ representing the low concentration of the culture media surrounding the hydrogel domain. At the boundary between the agarose ($n = 1$) and PEG ($n = 2$) domains, the flux is assumed to be continuous. Since this is a 2D
model, we neglect transport to the media beneath the porous membrane, which will be discussed in the limitations. At different time points, the computational profile was observed with a length of 1.5 mm with point 0 being closest to the NGF well.

3.4 Results

3.4.1 Protein Diffusion

a) Bifurcated Model

We explored the ability of delivering a transient concentration gradient of soluble biomolecules within the cell permissive region of the bifurcated model (figure 1). In figure 5a, a fluorescent image is shown depicting the presence of fluorescein-BSA within the construct at time 30 min. Three separate configurations were tested with fluorescein-BSA in the well: 1) 115 μm from the void in 10% w/v PEG (10% close), 2) 545 μm from the void in 10% w/v PEG (10% far), and 3) 545 μm from the void in 20% w/v (20% far) (figure 5b-d). In all three cases, diffusion occurred quickly enough that an appreciable gradient was detected as soon as images were taken near time 0 min (figure 5). As the fluorescein-BSA diffused through the gels, a gradient of fluorescence was perceived within the agarose gel before tapering off. In the 10% close group, a slight gradient with a maximum concentration near 10nM was formed at t ~ 0. The fluorescein-BSA concentration in agarose peaked at 30 minutes after filling with a maximum concentration near 35 nM. The gradient at this time encompassed a larger length than the previous time point. Later, the profile began to lessen with a maximum concentration close to 20 nM. When the well was placed further from the agarose (10% far), the maximum protein concentration in agarose still occurred at time 30 min but at a maximum concentration 38 nM. Not only did the profile reach a higher maximum concentration, but the
concentration profile diminished more gradually compared to the 10% close scheme as the maximum concentration at time 60 min was slightly below 30 nM. Thus, by changing the distance between the protein well and the PEG void, the concentration profile showed a more gradual diffusion of fluorescein-BSA through the agarose gel, and the concentration gradient was maintained for a longer period of time. For the 20% far scheme, the maximum concentration achieved at time 30 min was lower than the 10% far scheme with a maximum concentration near 32 nM. After 1 hour, the profile decreased with a maximum concentration around 23 nM. For the higher percentage PEG, the diffusion rate for the fluorescein-BSA was slower resulting in less pronounced gradients. The curve fit parameters determined for the three experimental groups at each time point follow similar trends (table 1). For all three groups, the absolute concentration ($C_0$) peaks and the decay length ($x_0$) is at its lowest at $t = 30$ min.
Table 1: Curve fit parameters from the averaged gradients for each configuration at different time points

<table>
<thead>
<tr>
<th>Configuration</th>
<th>t (min)</th>
<th>C₀ (nM)</th>
<th>x₀ (µm)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% PEG 115 µm</td>
<td>~0</td>
<td>10.3</td>
<td>1241.9</td>
<td>0.837</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>32.0</td>
<td>776.4</td>
<td>0.974</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>17.8</td>
<td>1641.8</td>
<td>0.947</td>
</tr>
<tr>
<td>10% PEG 545 µm</td>
<td>~0</td>
<td>10.2</td>
<td>1905.5</td>
<td>0.706</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>34.6</td>
<td>820.3</td>
<td>0.964</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>25.1</td>
<td>1431.8</td>
<td>0.96</td>
</tr>
<tr>
<td>20% PEG 545 µm</td>
<td>~0</td>
<td>13.8</td>
<td>1209.4</td>
<td>0.796</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>27.2</td>
<td>839.6</td>
<td>0.876</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>18.1</td>
<td>1387.9</td>
<td>0.906</td>
</tr>
</tbody>
</table>
The 10% close configuration was used to establish long term gradients by refilling the well after 1 hour. In these trials the concentration of fluorescent-BSA was reduced from 140 nM to 70 nM. From the 3D plot, we were able to maintain gradients with the maximum concentration not exceeding 20 nM (figure 6a). The profiles exhibited similar trends as before with the maximum concentration occurring at 30 minutes before reducing over time. After refilling the well after 1 hour, we recreated similar profiles in the second hour as in the first. Though the magnitude of the gradient at time 30 min was less than at time 90 min, the gradients have similar steepness (figure 6a). Sixty minutes after refilling (time 120 min), the gradient profile resembled the profile at time 60 min. In addition, values of the curve fitting parameters of $C_0$ and $x_0$ were reproduced after refilling the well. Though not a direct match of absolute magnitude at each time point, they do follow similar trends between fillings, with peaks in magnitude shortly after each time the well is filled and reaching the lowest values after 60 minutes (figure 6b). A closer link between fillings can be observed from the changes in decay length over time. After each filling, the shortest decay length occurs close to $t \sim 0$ and increases over time reaching its largest value after 60 minutes (figure 6c). It should be noted that in the range of $0 \leq t \leq 15$ min after the second filling, the data results are less detailed than after the first filling. Therefore, the curve fit information in this range may not be directly comparable.
Figure 6: Diffusion of soluble fluorescein-BSA through dual hydrogel construct with sustained gradient. The well was filled with fluorescein-BSA at 0 min and refilled after 60 min. (a) 3D plot of mean concentration of fluorescein-BSA after time 0, 30, 60, 90, and 120 min relative to position in construct. (b) Absolute magnitude of the concentration at the starting position over time. (c) Graph of the decay length over time. Error bars represent standard deviation.
b) Straight Channel Model

In the single channel configuration we were able to introduce a gradient at locations close to the NGF reservoir (figure 3b). In figure 7a, x = 0 represents the point closest to the filled well. At time 15 min, a steep gradient is established with a maximum concentration approximately 3.6 nM. After 180 minutes, the gradient is no longer present and the profile is at a near constant concentration of 0.6 nM. During the same time span, no gradient is formed on the opposite end of the channel nearest the empty well (figure 7b). In the figure, x = 0 represents the point closest to the empty well. In this region, the concentration profiles exhibit a more subtle gradient and do not change over time. At times 15 min and 180 min the gradients had concentration ranges from 0.1 to 0.9 nM.
Figure 7: Diffusion profiles in the single channel construct. (a) Experimental NGF concentration profile demonstrating concentration gradient in near channel upon establishment. (b) NGF concentration profile in far channel was minimal and in the opposite direction. Concentration values were determined along the center of the channel with position 0.0 mm corresponding to the end of the channel nearest the soluble NGF reservoir (a) or empty well (b)^17
3.4.2 FRAP Results

The diffusion coefficient of fluorescent-casein was determined in both hydrogels performing a least squares curve fit of the experimental data to equation (4). A representative image of the curve fit in agarose is shown in figure 8. These diffusivity values were then used in the computational model simulating NGF throughout our construct. The coefficients of diffusion for casein in 10% PEG and agarose were calculated to be $32.2 \pm 6.8 \mu m^2/sec$ and $107.4 \pm 1.0 \mu m^2/sec$ respectively. Table 2 is a summary of the diffusion coefficients of casein in each hydrogel and how their experimental recovery curve matched the least squares curve fit.
Figure 8: Representative experimental fluorescence recovery curve in agarose is shown by the * symbols. The solid line is the least squares curve fit to equation (4).

Table 2: Diffusion coefficient summary of fluorescent-casein in hydrogels

<table>
<thead>
<tr>
<th></th>
<th>10% PEG</th>
<th>Agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusivity</td>
<td>32.2 ± 6.8</td>
<td>107.4 ± 1.0</td>
</tr>
<tr>
<td>(µm²/sec)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - resnorm</td>
<td>0.958 ± 0.008</td>
<td>0.965 ± 0.009</td>
</tr>
</tbody>
</table>
3.4.3 Computational Analysis

We wanted to make a computational model to mimic the experimental diffusion of proteins within our dual phased construct. In the future, the in silico model will be used as a tool to identify configurations and experimental processes that keep cells exposed to physiologically relevant concentrations of a neurite guidance cue.

Computationally generated concentration profiles were obtained using the straight channel geometry (figure 3b). At time 15 min, the simulated profile has a maximum concentration slightly greater than 3 nM and a steeper slope compared to the later time points. At time 60 min the maximum concentration decreased to less than 2 nM and the profile regressed to a more subtle gradient. At time 180 min, the profile has a near constant concentration of 0.65 nM.

The experimental profiles exhibit similar trends to those generated by the computational model. In order to determine how closely the computational and experimental profiles corresponded, the % difference was calculated using the following equation:

\[
\text{% difference} = \frac{\int_{a}^{b} |f(x) - g(x)| dx}{\int_{a}^{b} |f(x)| dx}
\]  

(7)

In equation (7), f(x) is the exponential equation of the best fit curve of the simulated profile and g(x) is the exponential equation of the best fit curve of the experimentally generated profile. At time 15 min, the experimental maximum concentration attained approximately 3.6 nM. This profile corresponded closely with the computational model, as the calculated percent difference was 18.9%. At time 60 min, the experimental profile had a reduced steepness compared to time 15 min and had a maximum concentration of approximately 2 nM. When compared to the simulated profile, the percent difference
was 15.9%. After 180 minutes, the experimental profile no longer maintained a gradient and has a near uniform concentration near 0.65 nM. A percent difference of 10.2% was found between the two profiles at time 180 min.

The curve fit parameters for both the computational and experimental concentration profiles exhibit comparable behavior (table 2). There is an increase in absolute concentration between 15 and 30 minutes. At $t = 30$ min, the absolute concentration has reached its peak and begins to decline at each time point afterwards. Conversely, the decay length decreases initially between 15 and 30 minutes. After $t = 30$ min, the decay length begins to increase over time. At the latter time points, $r^2$ values begin to dip below 80% indicating at these times the profiles may no longer exhibit exponential behavior.
Figure 9: Computational and experimental concentration profiles of soluble NGF diffusion through agarose regions, demonstrating the initial gradient formation and behavior through time 17.

Table 3: Curve fit parameters of the computational concentration profiles and the averaged experimental concentration profiles at different time points.

<table>
<thead>
<tr>
<th>t (min)</th>
<th>SIMULATION</th>
<th>EXPERIMENTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C₀ (nM)</td>
<td>x₀ (µm)</td>
</tr>
<tr>
<td>15</td>
<td>3.43</td>
<td>521.4</td>
</tr>
<tr>
<td>30</td>
<td>3.66</td>
<td>832.6</td>
</tr>
<tr>
<td>45</td>
<td>3.17</td>
<td>1145.9</td>
</tr>
<tr>
<td>60</td>
<td>2.61</td>
<td>1509.7</td>
</tr>
<tr>
<td>75</td>
<td>2.13</td>
<td>1957.3</td>
</tr>
<tr>
<td>90</td>
<td>1.74</td>
<td>2531.0</td>
</tr>
<tr>
<td>105</td>
<td>1.44</td>
<td>3291.6</td>
</tr>
<tr>
<td>120</td>
<td>1.2</td>
<td>4340.3</td>
</tr>
<tr>
<td>135</td>
<td>1.02</td>
<td>5858.2</td>
</tr>
<tr>
<td>150</td>
<td>0.87</td>
<td>8216.9</td>
</tr>
<tr>
<td>165</td>
<td>0.76</td>
<td>12312.2</td>
</tr>
<tr>
<td>180</td>
<td>0.66</td>
<td>20973.2</td>
</tr>
</tbody>
</table>
3.5 Discussion

Biomolecular gradients are important to cellular behavior and are involved in chemotaxis, mechanotransduction, and morphogenesis. To better understand how gradients influence cells, precise knowledge is needed of both the range and the steepness of the concentrations the cells are exposed to. We provided a method to introduce soluble gradients within the cell permissive regions of our dual hydrogel construct and characterized the concentration profiles at various times. A reservoir was strategically placed to allow proteins to diffuse into the system and gradients were maintained. Using the bifurcated model (figure 1), we varied the parameters of well distance, outer PEG composition, and protein concentration. By altering these parameters, we exhibited a degree of control over the soluble gradients. By increasing the distance between the protein well and PEG void, a more sustained release of the fluorescein-BSA into the agarose was achieved. Because of this, the gradient was maintained longer and reached a higher maximum concentration in the 10%/545μm compared to the 10%/115μm (figure 5). This relationship can also be observed in the curve fit parameters (table 1). The absolute magnitude of the concentration ($C_0$) is higher at time 30 min and 60 min in the 10% PEG 540 μm group compared to the 10% PEG 110 μm group. In contrast, increasing the PEG composition had an opposite effect on the gradient. For the 20% PEG/545 μm, the maximum concentration reached was lower at time 30 min and 60 min compared to 10% PEG/545 μm. Similar behavior is found in the curve fit parameters with $C_0$ being less in the 20% PEG compared to the 10% PEG at both 30 minutes and 60 minutes after filling. The fluorescein-BSA had a reduced diffusion rate in the 20% PEG resulting in the reduced concentrations. The magnitude of the gradient was also
controlled by the initial protein concentration in the well. In the refilling experiment, when the protein concentration was reduced from 140 nM to 70 nM, the resulting gradient ranged from 0 to 15 nM. In these studies, our diffusing protein was BSA. BSA can serve as an analog for the known guidance cue netrin because they possess similar molecular weights ($M_n \sim 70$ kDa). Bhattacharjee et al. showed a gradient of soluble netrin varying from 0-2.8 nM across a 500 µm space induced axon turning in cortical neurons $^{23}$. A separate study by Kothapli et al. illustrated how a soluble gradients in the range of 0 nM to 14.2 nM across a 1200 µm length induced the maximum positive turning effect in DRG neurites $^{24}$. By reducing the concentration of the protein solution in the well, we created gradients that were more physiologically relevant to those able to influence cells *in vitro*.

Our initial tests show a gradient was maintained for 1 hour, however the time scale for gradient studies for cells is typically a few days. In order to overcome this limitation, the well was refilled to retain the gradients for longer periods. From figure 6a we replicated similar profiles after refilling the well after 1 hour. The concentration profiles exhibit similar shapes with higher concentrations found after the second filling. This could be due to the additive effect of the new fluorescein-BSA combining with the remnant BSA from the initial filling. Moreover, the absolute concentration and decay length follow similar trends after each filling (figure 6b and 6c). Following the second filling of the well, the steepness profiles over time are more linear than the initial filling (figure 6c). This could be due to a burst release of the protein leading to the gradient steepness observed between time 0 min and 10 min, along with some level of fluorescein-BSA saturation in the PEG, and to a lesser degree within the agarose. The higher decay
length after refilling imply burst release may only be a factor for the initial filling and not for ensuing refills. Although a current set up refilled the well after only 1 hour, creating gradients over longer periods could easily be accomplished by changing the construct geometry and parameters we examined in §3.2.2. Alternatively, the well could be filled with loaded microspheres or cells that secrete the desired biomolecule to provide a steadier source of soluble cues.

After showing the ability to create gradients and control their profiles within the cell permissive region of our construct, our next step was to isolate the gradients in a specified area (figure 5). A different geometry was used to test this consisting of a single channel and a well placed at either end (figure 3). One of the wells was filled with fluorescently labeled NGF with the hope of establishing a gradient in the half of the void closest to the well while leaving the further half unaffected. In the region closest to the well, we observed a gradient at time 15 min that tapers off and resorts to almost uniform concentration at time 180 min. In the region on the opposite end however, the concentration profile seems unchanged during the same span. This selective placement of a gradient can be helpful for cell guidance models where migration or growth is induced into one area over another.

Using projection lithography, any number of geometries can be fabricated for our construct. To experimentally determine the gradients formed for each potentially new geometry would be time consuming and require lots of resources. Therefore it would be helpful to have a quick, reliable, and cost effective method to generate the concentration profiles of any geometry. A computational model would be a handy tool to generate these profiles and could greatly reduce the costs of materials compared to experiments.
We developed a computational model that simulated diffusion with our dual hydrogel system and tested it using the single channel configuration (figure 3). Visually, the profiles generated by the \textit{in silico} model show that the gradient formation and behavior over time closely resemble that of the experimentally derived curves (figure 9). Analysis of the curve fit parameters support the above statement as $C_0$ and $x_0$ exhibit similar behavior and have comparable values (table 3). Between 15 and 30 minutes, $C_0$ increases, but continually decreases after 30 minutes as time progresses. Alternatively, the decay length becomes larger at each time point after filling. When comparing the profiles between the computational and experimental there is close correspondence at each time point. At the time points of 15 min, 60 min, and 180 min, the percent difference between the simulated and actual curves were 18.9\%, 15.9\% and 10.2\% respectively. Though the computational and experimental profiles are a close fit, they are not a direct match. This could be due to the computational model not factoring loss of protein through the bottom of the permeable membrane. The computational diffusion model can be easily adapted to fit any geometry and only the diffusion coefficients of the desired protein in the desired hydrogels are needed to perform simulations. These diffusion coefficients can be found using the FRAP process in §3.3.4.

Not only can the computational model reduce the number of experimental trials thus preserving resources, but simulations can also help optimize experiments before they are performed. For future cellular studies we would like to study the chemoattractive properties of NGF within the straight channel geometry. We were able to introduce NGF gradients in the channel closest to the well within the range of 0 - 5 nM. In this range, preferential neurite outgrowth has been induced towards the high end of the gradient \textsuperscript{18}. 
Using the computational model, the frequency the well needs to be filled with the desired guidance cue can be obtained. From figure 9, we see the concentration profile leveled off near a constant concentration of 0.65 nM after 180 minutes. We also noticed that the decay length experienced the highest jump amongst total length of our study between $165 \leq t \leq 180$ min. These results lead to filling the well every 3 hours for axonal guidance experiments.

One of the main drawbacks of our approach to forming gradients is the short length they were maintained. While we established gradients that were sustained a few hours, other methods such as protein immobilization can result in stable long term gradients that last several days to indefinitely. We can overcome this limitation by simply refilling the well at specific time points as our data indicated the ability to recreate concentration profiles after refilling. In addition our method provides a more accurate representation of biomolecule interaction compared to immobilized cues. For example, bound molecules may not have their appropriate binding site exposed for receptor attachment and therefore may not initiate the desired response. Moreover, most methods of binding proteins involve exposure to variable amounts of light utilizing either a sliding or gradient grayscale mask. As the typical process relies on the same chemistry to crosslink the polymer and to conjugate the ligands to the network, the gradient tethering can alter the crosslink density of the hydrogel and change mechanical properties of the exposed region. As a result it is difficult to assess whether the cell behavior is solely caused by the bound chemical gradient. On the other hand, our gradients consist of freely soluble proteins with active binding sites readily available and do not require additional UV exposure to form leaving the mechanical properties unadulterated.
Although any cell type can be incorporated within our dual phased hydrogel construct, our focus is to observe neural cell behavior under the influence of soluble factors. In the first part of our study, we used fluorescein-BSA as an analog for the chemoattractive cue netrin as they share similar molecular weights ($M_n \sim 70$ kDa). The ranges of the gradient achieved and maintained using fluorescein-BSA were within the physiological range to induce axon turning in cortical neurons $^{23}$ and to induce the maximum turning effect in DRG neurites $^{24}$. A smaller chemoattractive biomolecule, NGF ($M_n \sim 26$ kDa), was as easily incorporated into our system to form gradients in the range to affect neural growth. A study by Rosoff et al. showed that a gradient with an average absolute concentration on the order of 1.0 nM resulted in a more chemoattractive guidance ratio$^{18}$. Using our microfabrication process, we hope to develop choice point geometries that expose axons to soluble guidance cues to direct growth into a desired region.

We demonstrated a versatile biphasic system that can be easily altered to any geometry and incorporate any size soluble cue. By placing a protein source well in a strategic location, a gradient can be induced in a specific region of our construct, leaving other areas unexposed. Through modifying the composition of the outer restrictive border, the distance of the well, and the concentration placed within the well, some degree of control over the gradient magnitude and steepness was achieved to ensure cells encounter physiologically relevant levels. Also a corresponding diffusion model was developed to simulate biomolecule diffusion in order to create a quick and cost effective method to characterize the gradients. Both the in vitro and in silico models can be useful
tools in recreating microenvironments where cellular behavior is observed when influenced by a freely soluble biomolecule gradient.
3.6 References


During development, axons regularly encounter choice points where a multitude of environmental factors influence growth and direct them to their appropriate targets. This chapter covers the development of integrated models, which apply experimental and computational approaches to observe axonal response to soluble cues at a choice point, in support of Aim 2.

Aim 2: Develop integrated models of neurite growth, which leverage macro-scale experimental studies against growth cone guidance in silico models.

4.1 Abstract

Axons are guided toward desired targets through a series of choice points that they navigate by sensing cues in the cellular environment. A better understanding of how microenvironmental factors influence neurite growth during development can inform strategies to address nerve injury. Therefore, there is a need for biomimetic models to systematically investigate the influence of guidance cues at such choice points. We ran an adapted in silico biased turning axon growth model under the influence of nerve growth factor and compared the results to corresponding in vitro experiments. We examined if growth simulations were predictive of neurite population behavior at a choice point. We used a biphasic micropatterned hydrogel system consisting of an outer
cell restrictive mold that enclosed a bifurcated cell permissive region and placed a well near a bifurcating end to allow proteins to diffuse and form a gradient. Experimental diffusion profiles in these constructs were used to validate a diffusion computational model that utilized experimentally measured diffusion coefficients in hydrogels. The computational diffusion model was then used to establish defined soluble gradients within the permissive region of the hydrogels and maintain the profiles in physiological ranges for an extended period of time. Computational diffusion profiles informed the neurite growth model, which was compared with neurite growth experiments in the bifurcating hydrogel constructs. Results indicated that when applied to the constrained choice point geometry, the biased turning model predicted experimental behavior closely. Results for both simulated and *in vitro* neurite growth studies showed a significant chemoattractive response toward the bifurcated end containing an NGF gradient compared to the control, though some neurites were found in the end with no NGF gradient. The integrated model of neurite growth we describe will allow comparison of experimental studies against growth cone guidance computational models applied to axon pathfinding at choice points. As a variety of different geometries can be created through our microfabrication method, the integrated *in silico* model could provide an efficient and cost effective method to observe the growth behavior in these newly produced configurations and a means to develop experimental processes that expose axons to physiologically relevant levels of a desired guidance factor.

**4.2. Introduction**

During development, axons navigate the complex environment of the human body in order to properly wire the nervous system. The growth cone, the mobile portions of
axons found at their tip, steer and elongate axons in response to a multitude of environmental factors during development and regeneration. For instance neurite outgrowth can be affected by the mechanical properties of their environment, where stiffer surroundings prevent neurite penetration into an area\textsuperscript{1,2}. Moreover, molecular gradients play an important role in guiding neurites where growth cones detect chemotactic and haptotactic signals. These signals elicit attractive and repulsive responses to direct axons to their appropriate targets\textsuperscript{3-6}. Axonal pathfinding is thought to be reducible to a series of “choice points” at which axons navigate via the interplay of guidance cues\textsuperscript{7}. Prominent and extensively studied examples of choice points include the spinal cord midline and the optic chiasm, where the combinations of attractive and repulsive receptor-ligand interactions dictate whether an axon can traverse to the contralateral side or will remain ipsilateral\textsuperscript{5,8,9}. We created a bifurcated tissue construct to mimic the choice points that axons regularly encounter and introduced a soluble gradient of nerve growth factor (NGF) in one of the channels. We investigated computationally whether an NGF gradient can account for neurite divergence at a choice point and if the simulated growth patterns were predictive of experimental neurite population behavior. In doing so, we hope to obtain better understanding of the neurite-environment interaction, which can then be used to develop neural therapeutic and regenerative strategies.

Significant research has focused on creating a biomimetic microenvironment for neural cell culture. Hydrogels have been proven to be advantageous materials for tissue engineering. Their water-swollen cross-linked networks allow them to imitate both the mechanical properties and architecture of the extracellular matrix where cells can grow in
three dimensions\textsuperscript{10,11}. Cells cultured within 3D environments have exhibited dissimilar behavior compared to 2D environments such as morphology\textsuperscript{12} and gene expression\textsuperscript{13}. Neuronal cells in particular displayed more biomimetic electrophysiological properties including resting membrane potentials, calcium dynamics, voltage-gated ion channel functionality, and action potential propagation when cultured in 3D\textsuperscript{14-17}. Furthermore, various guidance cues can be incorporated within the hydrogel either as immobilized\textsuperscript{18-20} or freely diffusible proteins\textsuperscript{21,22}. One particular protein, NGF, is a soluble cytokine from the neurotrophin family that has been extensively studied\textsuperscript{23-25}. It has the ability to promote neuronal survival and provides nourishment for neurons\textsuperscript{26}. When used as treatment for peripheral nerve injuries, NGF enhanced nerve regeneration and inhibited neuronal changes caused by damage\textsuperscript{26,27}. More importantly, when present as a gradient, NGF serves as a neuronal guidance cue\textsuperscript{21,28}.

Computational models are useful tools that can be used to acquire greater understanding of neurite growth patterns. The use of these models can help optimize experimental studies by establishing baseline parameters for testing. In addition, the mechanisms proposed for neurite guidance can be validated with comparisons to complementary \textit{in vitro} work. A number of \textit{in silico} simulations have been used to predict growth in response to various environmental factors. For example, Forciniti et al developed a model to observe neuronal response to topographical physical cues and immobilized chemical cues\textsuperscript{29}. On the other hand, Mortimer et al developed a biased turning model (BTM) where soluble NGF gradients helped determine the angle that neurites turned\textsuperscript{30}. A separate model employed growth rate modulation (GRM) where gradients helped establish the length neurites grew at each time point\textsuperscript{31}. Although these
studies provide general insight on axonal growth behavior under environmental influence, none of them address navigation at choice points that are normally encountered by growing axons.

We have created both computational and experimental models to investigate the effect of soluble gradients on axonal growth at a choice point. Previously, we developed a cell culture model capable of maintaining robust neurite growth in a 3D environment while constraining neurites in specific geometries. This dual hydrogel construct was composed of an outer restrictive border which contained growing neurites within a specific permissive region. We adapted this biphasic system into a choice point model under the chemoattractive influence of soluble NGF gradients. Neurites were grown to a bifurcating point where the presence of a gradient influenced growth into one channel over the other. We sought to develop in vitro and in silico models in biomimetic environments to allow better insight of how guidance cues influence navigation of a choice point. We demonstrate an integrated model which leverages macro-scale experimental studies against growth cone guidance computational models. The results suggest that models of growth cone guidance may predict emergent behavior such as population neurite guidance at a choice point, and that a soluble gradient of a single factor is not likely sufficient to direct all neurites toward one path. Such use of in vitro and in silico models in tandem may be a powerful way to investigate complex phenomena with respect to neurite guidance. This approach may be useful for the study of neural pattern formation during development, or the routing of regenerating axons toward proper targets in neural repair.
4.3 Materials and Methods

4.3.1 Dual hydrogel formation

The dual hydrogel constructs were produced as previously described\textsuperscript{19,32}. An outer cell restrictive layer of 10% w/v polyethylene glycol (PEG) dissolved in phosphate buffer solution (PBS) was crosslinked directly onto collagen-coated PTFE Transwell® Permeable supports (Corning, Corning, NY) using a digital micro-mirror device (DMD) as a dynamic photomask (Texas Instruments, Dallas, TX) and an ultraviolet (UV) light source (EXFO, Quebec, Canada). An inner cell permissive region was created by adding Puramatrix (BD Biosciences, Bedford, MA) into micro patterned voids in the PEG hydrogels. The Puramatrix was diluted to 0.15% w/v in deionized water and supplemented with 1 µg/mL soluble laminin (Invitrogen, Carlsbad, CA).

4.3.2 Experimental diffusion profiles

To study the diffusive profiles formed within the cell permissive region, a 1200 µm diameter circular well, which served as a protein source, was placed 424 µm from one of the bifurcating channels (figure 10a). The well was filled with 0.45 µL of 20 nM fluorescein-conjugated casein (Invitrogen, Carlsbad, CA) in PBS. Casein was previously used to simulate the diffusion of nerve growth factor (NGF) since they share similar molecular weights\textsuperscript{21}. Fluorescent images were taken at various time points over a two hour period to monitor fluorescein-casein diffusion into the Puramatrix gel. Using ImageJ software (National Institutes of Health, Bethesda, MD), the intensity profile for each fluorescent image was measured using a 100 pixel straight line in the channel closest to the well. Pixels were binned in groups of five pixels per bin to give 20 points of average relative intensity. The relative intensity was averaged over three experiments.
to give a mean relative intensity per point. The mean relative intensity was used to determine the concentration of fluorescein-casein in the samples by interpolating the data from a calibration curve generated from known concentrations contained in PEG reservoirs.
Figure 10: Diffusion set up with bifurcating void for cell growth and a circular well placed distal to one of the bifurcating ends. (a) Dimensions of bifurcated construct: $R_1 = 800 \, \mu\text{m}$; $R_2 = 600 \, \mu\text{m}$; $L_0 = 940 \, \mu\text{m}$; $L_1 = 1000 \, \mu\text{m}$; $L_2 = 510 \, \mu\text{m}$; $L_3 = 840 \, \mu\text{m}$; $L_4 = 424 \, \mu\text{m}$; $\theta = 90^\circ$. (b) Guidance Ratio (GR) calculated using data from the top channel closest to the well (i) and the bottom channel furthest from the well (ii). Scale bar = 500 $\mu\text{m}$.
To sustain a gradient of fluorescein-casein within physiological range, the well was refilled two and four hours from the start of the experiment. These time points were determined from the diffusion computational model described later. The concentration profiles at each time point fit the following exponential equation:

\[ C = C_0 e^{-Bx} \]  

In the equation \( y \) is concentration, \( x \) is position, \( C_0 \) is related to absolute concentration, and \( B \) is the decay parameter with units of \( \text{mm}^{-1} \). The decay parameter was obtained from the best-fit exponential curves at each time point and can be used as a measure of the gradient slope.

**4.3.3 Computational diffusion profiles**

A 2D computational diffusion model was constructed in MATLAB (MathWorks, Natick, MA), which possessed the same dimensions as the *in vitro* dual hydrogel system and consisted of two distinct domains, a rectangular region and bifurcating void region, representing diffusion through PEG and Puramatrix respectively. The diffusion coefficients of casein in both hydrogels were determined with fluorescence recovery after photobleaching (FRAP) and accompanying calculations used previously \(^{33}\). The procedure is described in §3.3.4. The diffusion coefficient for Puramatrix was 569.6 \( \mu \text{m}^2/\text{s} \) and 332.9 \( \mu \text{m}^2/\text{s} \) for PEG. The interface between the two distinct domains was modeled as a narrow region having a continuous transition between the properties of each domain, representing a region where the Puramatrix and PEG hydrogels were interwoven. The diffusion equation was solved computationally as described in §3.3.5.
4.3.4 Computational neurite growth

An agent based model was used where the concentration was discretized on an Eulerian finite-difference grid and the neurite paths moved through the domain in a Lagrangian manner by sampling local concentration. One-way coupling was used to interpret concentration and move the neurite with a biased random walk. To do so, the neurite growth was modeled using a biased turning model (BTM) similar to one described by Mortimer et al.\textsuperscript{30} Initially 300 neurites were randomly placed on the surface of a circle with a radius of 100 μm in the non-bifurcated end of the growth permissive region. This represents each axon’s initial starting point on the surface of a DRG explant. At each time step, the NGF concentration was sampled 10 μm to the left and 10 μm to the right of each axon’s current position. This concentration difference between the left and right was calculated as representative of the concentration difference across the growth cone (μ). The BTM modeled a signal to noise ratio (SNR) surrounding the growth cone and used a Bayesian approach to develop a formula, which simulates how the neurons respond to the detected gradient. In the model, the SNR was dependent on μ, the growth factor background concentration (γ), and an empirically set scaling factor (k). This scaling factor provided a parameter inherent in the computational model that could be adjusted to follow experiments more closely. In equation (3), the SNR and γ are dimensionless, k is in \( \frac{mL}{mg} \), and μ is in \( \frac{mg}{mL} \).

\[
\text{SNR} = k \cdot \mu \left( \frac{\gamma}{\sqrt{(1 + \gamma)^3}} \right)
\]  

(2)
Based on previous simulations that exhibited the chemoattractive response of neurites to a static gradient of NGF, $\gamma = 0.001$ and $k = 5 \frac{ml}{mg}$. Since $k$ and $\gamma$ were unchanged, their value in the SNR could be represented with the constant $K$,

$$K = k \frac{\gamma}{\sqrt{(1+\gamma)^3}}$$

(3)

The only value changing was $\mu$, which was dependent on the current concentration values. Therefore equation (3) could be reduced to:

$$SNR = K \times \mu$$

(4)

For each neurite, a random number was generated from a normal distribution with a mean of zero and a variance of 1. The random number was then added to the SNR and if that sum was positive, the neurite turned to the left at an angle of $\delta_0$. If the sum was negative, then it turned to the right at an angle of $\delta_0$. In our model, $\delta_0 = 6^\circ$. After determining the direction, the neurite would then grow a set distance of 4 $\mu$m at each time step.

Neurites started at the nonbifurcating end of the construct and grew to the choice point under constant 20 ng/mL NGF concentration for 2 days. Once at the choice point, two growth conditions were observed. The first condition, the control, involved constant 10 ng/mL NGF concentration throughout the construct. The other condition involved constant 10 ng/mL NGF concentration throughout the construct with 20 nM of NGF added to the reservoir every 2 hours for 2 days. To determine the simulated effect of NGF on neurite growth at the choice point, the position of each neurite was monitored.

A guidance ratio (GR) was used as a simple way to quantify the neurite response using data in either the gradient channel (i) or the non-gradient channel (ii) (figure 10b). In this case, separate counts were made when a neurite occupied an area in the top
channel closest to the well, channel i (T), or in the bottom channel furthest from the well, channel ii, (B) at each time step. The GR was then calculated by taking the difference between T and B normalized by the total counts.

\[
GR = \frac{(T - B)}{(T + B)}
\]

Using this GR, a positive number indicated a chemoattractive response and a negative value indicated a chemorepulsive one. Each condition was simulated 16 times, and a two-tailed student’s t-test of equal variance with a 5% confidence interval was used to establish significance.

4.3.5 Experimental neurite growth

All procedures involving vertebrate animals were approved by the Institutional Animal Care and Use Committee. Dual hydrogel constructs containing live dorsal root ganglion explants were prepared as previously described. Collagen-coated PTFE cell culture inserts were soaked overnight in adhesion medium (Neurobasal medium supplemented with L-glutamine, NGF, 10% fetal bovine serum (FBS) and penicillin/streptomycin (pen/strep), (Gibco-Invitrogen, Carlsbad, CA). Four dorsal root ganglia (DRG) isolated from embryonic day 15 Long-Evans rat pups (Charles River, Wilmington, MA) were placed on the surface of each cell culture insert membrane and maintained in serum-free growth medium (Neurobasal medium supplemented with B27, L-glutamine, NGF and pen/strep) at 37°C, 5% CO₂ for four hours.

After four hours, the medium was replaced with 500 µL growth medium containing 10% PEG/0.5 % Irgacure 2959 (Ciba Specialty Chemicals, Basel, Switzerland). The projected photomasks were aligned around the adhered DRG using a visible light source and inverted microscope, and the PEG constructs of the dual hydrogel
system effectively confined the DRG after UV irradiation for 55 seconds. Inserts were washed with PBS containing pen/strep to remove uncrosslinked PEG solution and to prevent contamination. Puramatrix was introduced via micropipette into the bifurcating void in the PEG construct, inducing gelation of Puramatrix. The DRG explants in the dual hydrogel systems were cultured in an incubator for four days with medium changes every 48 hours to allow for neurites to grow to the choice point. After four days, the medium was replaced with low NGF medium (10 ng/mL). At this time, one group of DRGs was exposed to a soluble NGF gradient placed in a reservoir distal to one end of the bifurcating channel. The gradient was established by adding 0.45 μL of 20 nM NGF to the well every 2 hours for 2 days to allow the neurites to reach the end of either bifurcating channel. A group of explants maintained in low NGF conditions with no gradient served as a control.

Neurite outgrowth in the dual hydrogel systems was evaluated using standard immunohistochemical techniques. After fixing the DRG in 4 % paraformaldehyde for 2 hours, neurites were tagged with mouse monoclonal [2G10] to neuron-specific beta III tubulin primary antibody, followed by fluorescent tagging with Cy3.5 conjugated goat-anti-mouse secondary antibody (AbCam, Cambridge, MA). The staining and tagging steps were carried out in PBS containing 0.1 % saponin and 0.2 % bovine serum albumin (BSA) for 1 hour, followed by three 10 minute washes in PBS with 0.1 % saponin. Fluorescent images were acquired with a Nikon AZ100 stereo zoom microscope (Nikon, Melville, NY) for conventional fluorescence. Confocal imaging of β III tubulin stained neurites in both channels was performed throughout the depth of the constructs using a Zeiss LSM 510 Meta microscope (Zeiss, Oberkocken, Germany) and were processed
with Image J software. Maximum intensity Z-stacks were obtained from the confocal images of each channel. In order to quantify neurite growth, automatic thresholding (mean) was used, followed by pixel volume analysis for each channel. A guidance ratio (GR) evaluating the fluorescent pixels in channel i and channel ii (figure 10) was used to determine the effect soluble NGF had on growth. It was observed from previous GR studies using the dual phased system that the apparent universal presence of large number of neurites at the PEG/Puramatrix interface produced a large amount of error as it tended to normalize results across conditions\textsuperscript{34}. Therefore the region of interest used for the GR calculations included only the bulk Puramatrix and excluded the border of every channel, as depicted in figure 10. A student’s t-test of equal variance was used to establish significant difference between conditions. Samples with neurite growth that did not reach the choice point were discarded.

4.4. Results

4.4.1 Diffusion experiments

In the laboratory experiments, fluorescent casein, which served as a model of soluble NGF, was introduced into the dual hydrogel system via a reservoir placed near one of the bifurcating channels. As time increased, casein diffused throughout the construct (figure 11). Initially, there was high fluorescent intensity concentrated within the well (time 1 min). At time 30 min, 60 min, and 120 min the casein diffused radially from the well and as absolute intensity was reduced, the area containing fluorescence became larger at each successive time point.
Figure 11: 20 nM fluorescein-conjugated casein diffused throughout the PEG/Puramatrix construct over a time of 120 minutes. Scale bars = 500 µm.
Casein concentrations were measured along both bifurcating channels at different time points as described in §4.3.2. The position closest to the bifurcating point was designated as 0 μm and the position closest to the reservoir well was designated as 750 μm (figure 12). A transient concentration gradient formed in the bifurcating channel closest to the well, or the top channel, and diffusion occurred quickly enough that an appreciable gradient was apparent close to time 1 min (figure 12a). The minimum concentration at 1 min was approximately 0.4 nM and the maximum was close to 1.1 nM resulting in a steep slope. As the fluorescein-casein diffused through the gels, a gradient was observed before eventually resolving to uniform concentration. At time 30 min the gradient profile reached its highest absolute concentration with a range between 0.8 to 1.6 nM in the close channel. After 2 hours (time 120 min), the gradient began losing shape and had a relatively stable concentration around 0.5 nM. In comparison, the channel furthest from the well, or the bottom channel, did not experience a gradient and maintained relative constant concentration close to 0 nM at the same time intervals.
Figure 12: Experimental diffusion profiles over two hours in the bifurcating channel closest to the well (TOP) and furthest from the well (BOTTOM).
The gradient could be sustained within the same concentration ranges upon refilling the well with additional protein solution (figure 13a-c). In the experiment, the well was filled initially at time 0 min (1st filling) and refilled at 120 min (2nd filling) and 240 min (3rd filling). At 30 minutes after each filling, the gradient reached its highest concentration value establishing a steep gradient. As time progressed, the profile steepness and maximum concentration decreased at each time point until 120 minutes after each filling, where the profiles were flatter with a near constant concentration. Moreover, comparable decay parameter values could be obtained after each filling (Figure 13d). At the start of the first and second filling, the decay parameters were similar with values near 1.0 μm\(^{-1}\) and 0.8 μm\(^{-1}\) at the first two time points (figure 13d). However in the latter half, the decay parameter decreased with values closer to 0.6 μm\(^{-1}\) for the first filling and values below 0.6 μm\(^{-1}\) after the second filling. The decay parameter after the third filling, although exhibiting similar trends, is much lower compared to the first two fillings. The steepness began near 0.7 μm\(^{-1}\) and ended close to 0 μm\(^{-1}\).
Figure 13: Results from refilling the well every two hours. The presented concentration profiles were measured in the top bifurcating channel closest to the well. (a) Concentration profiles initial filling (1st Filling). (b) Concentration profiles after refilling the well after 120 minutes of initial filling (2nd Filling). (c) Concentration profiles after refilling the well after 240 minutes of initial filling (3rd Filling). (d) Decay parameter with respect to time after each filling.
An in silico model was developed to simulate diffusion of biomolecules in the hydrogels. Initially, 20 nM of biomolecule originated inside the well and allowed to diffuse for 120 minutes. We successfully created a computational model of diffusion through the two domains and monitored the profiles at different times throughout the construct. Figure 14a is the computer generated diffusion profile after 30 minutes. The ranges of concentrations varied between 0 nM and 3 nM. When comparing the NGF profiles in the center of the channel closest to the well, similar values were observed between the computational and experimental studies (figure 14b). At time 30 min, the gradient in the computational model fell in the range of approximately $1.0 \text{ nm} < C < 2.0 \text{ nM}$. The experimental model possessed similar values in the range between $1.0 \text{ nM} < C < 2.3 \text{ nM}$. At time 60 min, both the computational and experimental model possessed concentration profiles between $1.0 \text{ nM} < C < 1.3 \text{ nM}$. At time 120 min, a constant profile close to $0.5 \text{ nM}$ was observed computationally. Likewise, the experimental profile exhibited a similar constant concentration near $0.5 \text{ nM}$. Although not a direct match at the different time points, the two profiles correspond very closely.
Figure 14: Computational diffusion results. (a) Diffusion profile of entire construct at time 30 minutes. (b) Comparison between computational and experimental diffusion profiles in channel closest to the well. Position 0 μm is close to the point of bifurcation and 750 μm is at the end of the channel.
4.4.2 Neurite growth experiments

The diffusion profiles from the simulations were then used to inform a computational neurite growth model which was based on an established BTM model by Mortimer et al. that we adapted for constrained geometries. Neurite growth in a bifurcating choice point was simulated in the presence of an NGF gradient in the top channel (gradient) or under uniform NGF concentration (no gradient). Representative images of the growth patterns show unbiased growth for the neurites grown in uniform concentration and preferential growth towards the channel with a gradient (figure 15). In the absence of an NGF gradient, the simulation suggested neurites would exhibit no preference for either channel, as quantified in figure 15a with a neurite growth guidance ratio of 0.0118 ± 0.0200 (n=16). Conversely, when grown in the presence of an NGF gradient in the top channel, the simulation resulted in a preference for that channel with a chemoattractive guidance ratio of 0.1560 ± 0.0417 (n=16). A student t-test showed a significant difference (p< 0.05) suggesting the NGF gradient would be sufficient to influence neurite guidance toward one channel but not to attract all neurites to that channel.

In vitro studies mimicking the same conditions used in simulations were performed to compare to the computational model. Like the computational model, the experimental studies confirmed that a gradient of NGF was sufficient to induce a significant preference for one channel at a bifurcating choice point (figure 15b), while some neurites still entered the channel with no NGF gradient. In the absence of a gradient, there was a slight preference for the top channel, as indicated by a guidance ratio of 0.0395 ± 0.0216 (n=16). With the NGF gradient present in the top channel, however, there was a statistically significant (p<0.05) preference for the channel with the
NGF gradient with a chemoattractive guidance ratio of $0.1622 \pm 0.0577$ (n=14). These results show that the computational model predicted experimental behavior with a high degree of accuracy. As in the simulations, some neurites grew into the bottom channel, indicating that the gradient was not sufficient to attract all neurites to a single channel.
Figure 15: (a) Computational neurite growth results. Simulated neurite growth patterns with and without an NGF gradient and corresponding calculated guidance ratio. (b) Experimental neurite growth results. Experimental neurite growth patterns with and without an NGF gradient and corresponding calculated guidance ratio. A significant difference (p < 0.05) was detected between the gradient and no gradient conditions for both computational and experimental results. Scale bar = 500 μm.
4.5. Discussion

To our knowledge, this is the first report of a system of integrated experimental and computational models intended to mimic axon pathfinding at a choice point. In our present study, we examined the guidance properties of an NGF gradient for its ability to direct neurites into a desired channel. A previously described, biphasic culture system was modified and incorporated into an integrated computational model. This model used experimentally determined NGF diffusion profiles to inform a previously well established neurite guidance biased turning model (BTM) that was adapted for a constrained choice point geometry. The simulations demonstrated an NGF induced chemoattractive response as neurites exhibited biased growth towards the gradient channel. Corresponding experimental work revealed that the in silico model predicted the choice point behavior remarkably accurately (figure 15). Although a chemoattractive response was observed, absolute control was not achieved as neurites also extended into the channel without the NGF gradient. These observations suggest that a single guidance cue alone may not be sufficient to guide all axons toward one path at a choice point and that multiple factors may need to be present to achieve this.

Experimentally, we demonstrated the establishment and maintenance of a soluble gradient within a desired region of a dual hydrogel system. In a two hour span, the concentration in the bottom channel remained constant and close to 0 nM (figure 12). During the same two hours, the gradient within the top channel, which had concentrations between 0.1 nM and 1.5 nm, remained within physiological conditions 21,35,36 (figure 12). At concentrations below this level, the receptors at the growth cone are unable to encounter enough NGF to promote turning. At concentrations above this level, receptors
are too saturated and overwhelmed to detect any noticeable gradient. Although a clear
gradient can be preserved for 2 hours, it takes several days for significant neurite growth.
In order to overcome this limitation, we refilled the reservoir every two hours. Similar
concentration profiles were replicated (figure 13 a-c and comparable decay parameter
trends were recreated (figure 13d) with each refilling.

These diffusion profiles were reproduced computationally in a similar dual phased
environment. When compared to the acquired experimental data, the computational
model generated similar concentration profiles (figure 14b). At time 30 min, both
gradients closely correlated with slightly higher experimental concentrations found in the
first 0.2 mm closest to the well. A closer correspondence can be found in the latter two
time points. At time 60 min, both gradients displayed a slight gradient with a maximum
concentration value near 1.2 nM and a minimum value 1 nM. At time 120 min, each
profile regressed to a flat profile with a constant concentration of 0.5 nM throughout.
Due to the similar concentration profiles between the experimental and computational
models, we feel confident that our in silico model accurately simulates the diffusion of
NGF within our construct.

We created an integrated neurite growth model influenced by generated NGF
profiles in a dual phased construct. There have been earlier attempts to model NGF
influence on neurite guidance. One model developed by Mortimer et al. used Growth-
Rate Modulation (GRM) where neurites turned a random angle, but the neurite growth
was dependent on the gradient. Mortimer et al also developed a BTM, where the
gradient affected the neurite turning angle. In our study, we adapted the BTM to fit our
biphasic system. One of the differences between the two BTMs is the calculation of the
SNR, which is used to determine the axon turning angle. In the Mortimer model, the SNR requires knowing the direction the axon is traveling, the direction of the gradient, and how far apart those two directions are. In our model, the SNR relies solely on the concentration difference across the growth cone at its current location. This was done to keep the variables based solely on what a growth cone could sense and not need external information. Additionally in both Mortimer models, neurites grew freely in a single domain where as our system consisted of distinct cell permissive and restrictive regions. The use of the dual phased domain allowed us to create a choice point for the neurites to encounter. A model by Krottje and Van Ooyen incorporated four circular obstacles representing blood vessels within their neurite growth region, but these obstacles did not serve as boundaries. The neurites were able to circumvent the obstructions by interactions with the gradient, but could have still grown within the circular zones.

Although our system of integrated models employed a bifurcating choice point, the design can be easily altered to accommodate any number of configurations. The Dynamic DMD photomask can generate any desired geometry while any diffusible cue can be utilized in our construct. Once the guidance cue diffusion coefficient is measured by FRAP, the value can be substituted into the computational diffusion and neurite growth programs. Also we have previously shown some degree of control over the gradient profile. By changing the PEG composition and the distance of the well, the gradient steepness and duration can be altered. Through the computational model, we can predict an optimized configuration that can keep neurites exposed to physiological concentrations as well as the frequency of refilling the well to sustain these concentrations.
Our growth simulations, like prior work on which it is based,\textsuperscript{30,31}, exhibited chemoattractive patterns when influenced by an NGF gradient (figure 15a). Building upon those previous reports, our system involved regions of constrained growth, which required the neurites to traverse a 45° angle. Though this angle may seem large, growth cones have been shown to make sharp turns up to 90°\textsuperscript{38,39}. The combined use of the patterned construct and guidance cue gradient allowed greater control of the angle the neurites navigated, in a manner similar to guidance at natural choice points. The neurite growth model may be further adapted to match changes in experimental configurations.

The agent based neurite growth model served as a valuable tool to predict neurite population behavior under the influence of NGF. In both the experimental and simulated studies with no gradient, the neurites exhibited negligible preference for either channel with GR values close to zero. The GRs for the \textit{in silico} and \textit{in vitro} trials were \(-0.0118 \pm 0.0200\) and \(0.0395 \pm 0.0216\) respectively. Similarly, the results of the experimental and simulated studies with a gradient shared similar values with a GR of \(0.1560 \pm 0.0417\) for the computational and a GR of \(0.1622 \pm 0.0577\) for the experimental. After performing the statistical analysis, both gradient groups were found to be significantly more chemoattractive than the control groups. From these results, the inclusion of soluble NGF gradients can account for neurite divergence at a choice point. However, because neurites were also present in the non gradient channel, we conclude that NGF alone cannot account for absolute guidance. Most likely, multiple cues working in tandem, as experienced \textit{in vivo}, are needed for precise control. This integrated \textit{in vitro} -\textit{in silico} model approach will thus be helpful for the study of other cues responsible for choice
point navigation, including neural patterning during development, or axon routing during regeneration after neural injury.

Throughout the course of our study, we did encounter some limitations. For example, although comparable decay parameter trends could be recreated after refilling the well, halfway through the second filling and after the third filling, the decay parameter was decreased (figure 13d). This could be attributed to prolonged exposure beneath epifluorescence light dehydrating the construct while images were obtained. The extended light exposure could have caused premature degradation of both hydrogels leading to loss of integrity and quicker diffusion speeds. Dehydration should not be encountered during in vitro cellular studies since the constructs would not be exposed to light. In addition, although we can sustain the gradient long term, the process can be arduous as it requires refilling the well every two hours. In order to alleviate some of the stress of maintaining the gradient, later iterations of the choice point model could incorporate a few strategies. For example, loaded microspheres could be placed in the well to sustain release for longer periods extending the length of time needed between refilling. Another approach could involve creating a co-culture by seeding cells in the well that may secrete guidance cues for a more permanent solution. Finally, we were unable to isolate the NGF gradient completely since it was also present in growth media. NGF could not be removed in the media because it is needed for survival and growth promotion \(^{40,41}\). Therefore future studies could involve studying the roles of other soluble guidance factors that do not also serve as survival factors to determine the degree of their influence. For example, Neurotrophin-3 could be used for a different chemoattraction study \(^{42}\) or Semaphorin-3A could be used for chemorepulsion \(^{43,44}\). To create a more
biomimetic environment, multiple cues could also be used to incorporate attractive and repulsive cues\textsuperscript{34,45} or the study the synergistic effects of two cues\textsuperscript{46}.

In conclusion, we observed neurite response to soluble NGF gradients within a choice point neurite growth model. Soluble concentration gradients of NGF were introduced within our biphasic micropatterned construct and successfully modeled computationally. The validated simulated concentration profiles informed an integrated neurite growth simulation which was then compared to macro-scale experimental observations. When applied to this constrained choice point geometry, the neurite growth simulation predicted experimental behavior with striking accuracy. Both the \textit{in silico} and \textit{in vitro} neurite growth models exhibited significantly more chemoattractive guidance ratios with a gradient compared to the control. Though our present work served well as an initial study, further work is necessary to create cell culture microenvironments and corresponding computational models that incorporate multiple guidance factors in order to gain a more precise understanding of neurite growth behavior at choice points. Fortunately the versatility of our constructs allows a variety of geometries and guidance cues to be examined for future work.
4.6 References


CHAPTER 5: PERIPHERAL NERVE RESPONSE TO GUIDANCE CUE SECRETIONS FROM DERMAL FIBROBLASTS AND KERATINOCYTES IN A CHOICE POINT MODEL

Observing axonal guided growth due to interactions with skin cell secretions may provide insight during cutaneous innervations. This chapter develops a co-culture environment that incorporates dorsal root ganglia, dermal fibroblasts, and keratinocytes to examine axonal response to skin cell secretions. This work is in support of **Aim 3**.

**Aim 3: Develop quantitative evaluation of neurite growth at a choice point influenced by guidance cue secretions from dermal cells.**

**5.1 Abstract**

Lack of suitable *in vitro* techniques that sufficiently mimics cutaneous innervation has hindered progress in understanding interactions between nerve fibers and the skin. Precise spatial control is needed to ensure limited contact between neuronal cell bodies and skin cells, but ensure adequate exposure of sensory nerve endings to skin cell secretions. We developed a dual phased hydrogel system that isolated peripheral neurons from dermal fibroblast and keratinocyte skin cells, but allowed nerve ending exposure to guidance cues through diffusion. We proved skin cell viability within our construct and observed the chemoattractive response the cell secretions induced in axonal growth. The release of the known guidance cues nerve growth factor and glial cell-line-derived growth factor was quantified from these cells. Use of this model could provide insight on how skin cells influence patterning of cutaneous nerves and development of sensory receptors.
5.2 Introduction

Limitations from Aim 2 motivated the pursuit of incorporating co-cultures of guidance cue secreting cells into our choice point model. The cells served as a less labor intensive alternative of maintaining long term gradients compared to well refilling, and provided the opportunity to examine the effects of multiple cues as most cells produced more than a single guidance cue. The dermis was a good source of cells that naturally secrete neurite guidance cues as keratinocytes and dermal fibroblasts have both been known to contribute to cutaneous innervation.\(^1,2\)

The skin is densely innervated by the peripheral nervous system (PNS). Free periphery nerve endings are found in close proximity to and regularly interact with skin cells to provide the structural basis for sensory function. Skin cells have been shown to produce a variety of axonal guidance factors including both attractive\(^3,4\) and repulsive cues\(^5\). The opposing effects of these factors can allow precise control of cutaneous innervation. Keratinocytes, the main cellular component of the epidermis, have been shown to secrete the attractive guidance cue glial cell-line-derived neurotrophic factor (GDNF) and to a lesser extent nerve growth factor (NGF).\(^1\) In contrast, dermal fibroblasts, the main cellular component of the dermis, have been shown to secrete more NGF than GDNF.\(^1\) Very little work has been done exploring the mechanisms regulating cutaneous innervation due to a lack of in vitro techniques that suitably mimic the in vivo process. In this study we propose a dual phased microfabricated hydrogel construct as an appropriate biomimetic environment to observe neurite response to skin cell secretions.

Substantial research has been directed at creating biomimetic microenvironments for cell culture. Hydrogels have been shown to be promising materials to be used in
tissue engineering because of their beneficial properties. For example, their water-swollen cross-linked networks allow them to imitate both the mechanical properties and architecture of the extracellular matrix \(^6,7\). This provides a 3D environment for cells to grow in. Additionally, many \textit{in vitro} studies utilizing hydrogels have taken a variety of approaches to incorporate chemical cues to observe chemotactic and haptotactic cellular response to these factors. For example, a number of studies have immobilized proteins within the hydrogel network \(^8,9\). Similarly, soluble guidance cues have been incorporated into hydrogels using a variety of methods including printing \(^10\), source-sink diffusion \(^11,12\), and tree-like gradient generators \(^13,14\). Co-culture assays are an alternative method to induce soluble gradients as they can serve as a long term source. This method involves explanted neural tissue cultured with cells that secrete a desired guidance cue. The secreting cells can either secrete the desired guidance cue naturally \(^1,2\) or be transfected to cause secretion \(^15,16\).

NGF is a soluble cytokine from the neurotrophin family that has been extensively studied \(^17-19\). It has the ability to promote neuronal survival and provides nourishment for neurons \(^20\). In addition, when used as a treatment for peripheral nerve injuries, NGF enhanced regeneration and inhibited neuronal changes caused by damage \(^20,21\). More importantly, when present as a gradient, NGF served as a neuronal guidance cue \(^10,22\). Another potential guidance cue is GDNF. It has beneficial regenerative effects such as increased survivability in dopaminergic neurons afflicted with Parkinson’s disease \(^23,24\). Furthermore, it has been shown to increase differentiation, fiber outgrowth, and dopamine release in neurons. Also similar to NGF, gradients of GDNF induced and directed axon growth of neurons into the striatum in vivo \(^25,26\).
Here we have developed an assay to investigate the effects of skin cell secretions on cutaneous innervation. Previously, we developed a cell culture model that supported robust neurite growth in a 3D environment while confining the growth within specific geometries. This dual hydrogel construct consisted of an outer restrictive border which limited growth within a specific permissive region. We modified this biphasic system into a choice point geometry that could present soluble gradients to specified regions of the construct. Neurites grew to a bifurcating point where the exposure to a gradient would induce preferential growth into one channel over the other. We first sought to ensure the skin cell viability within our system and to verify and quantify the secretion of the guidance cues GDNF and NGF under these conditions. Neurite guidance studies were then performed at a choice point to observe the attractive response of each skin cell type individually and in tandem. We demonstrate an in vitro model that can provide insight into cutaneous innervation by observing peripheral nerve response to guidance cues produced by keratinocytes and dermal fibroblasts.

5.3. Materials and Methods

5.3.1 Co-culture media

In order to create the co-culture media, we first looked at the media used for each cell type that was used. The primary DRG neurons were cultured in neurobasal medium supplemented with B27, GlutaMax, NGF, and penicillin/streptomycin (Pen/Strep) (Life Technologies, Carlsbad, CA). Adult human epidermal keratinocytes (HEKa) were grown in EpiLife media supplemented with Pen/Strep and S-7 supplement (Life Technologies, Carlsbad, CA). Although no B-27 supplement was added, there was some present in the EpiLife media. For the adult human dermal fibroblasts (HDFa), the media used was
Media 106 supplemented with Pen/Strep and LSGS supplement (Life Technologies, Carlsbad, CA). Table 4 summarizes the media used for the different cell types and the final one used for co-cultures. After looking at previous studies\textsuperscript{2,27,28} and using table 4, the final co-culture media chosen was EpiLife supplemented with GlutaMax, NGF, Pen/Strep, B-27, and S-7. EpiLife was chosen as earlier work showed it could sustain both DRGs and HEK\textsubscript{2,27,28}. Our media differed from prior work as it contained NGF, because since our model placed the NGF source far away from the DRG cell bodies, we wanted to ensure the neural cells had enough NGF to survive. The LSGS supplement was not added to the media because HDF\textsubscript{a} are a robust cell type that can be maintained in most types of media.
Table 4: Media used for each cell type with the final media utilized for the co-cultures

<table>
<thead>
<tr>
<th>Item</th>
<th>DGR</th>
<th>Keratinocytes</th>
<th>Dermal Fibroblasts</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurobasal Media</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EpiLife Media</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media 106</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>GlutaMax</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>B-27</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-7</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSGS</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>
5.3.2 Cell viability assay

The cell viability of both keratinocytes and dermal fibroblasts in a newly developed co-culture media was tested using HEKa and HDFa (Life Technologies, Carlsbad, CA). The purpose of this test was to ensure our co-culture media was capable of sustaining both types of skin cells. The co-culture media developed was a modified version of one used previously for co-cultures of HEKa and rat dorsal root ganglion (DRG)\textsuperscript{2,27,28}. Our media consisted of EpiLife® medium with 60 μM calcium supplemented with penicillin streptomycin (pen/strep) (1% v/v), supplement S7 (1% v/v), B27 (2% v/v), glutaMAX (0.02%), and 20 ng/mL of nerve growth factor (NGF) (Life Technologies, Carlsbad, CA). A 10% poly (ethylene glycol) (PEG) construct was created as previously described\textsuperscript{29,30} with a circular void in the center of the construct with a diameter of 1.6 mm. The construct was polymerized directly onto collagen-coated PTFE Transwell® permeable supports (Corning, Corning, NY) using a dynamic photomask (DMD) (Texas Instruments, Dallas, TX) and an ultraviolet (UV) light source (EXFO, Quebec, Canada). The PEG was irradiated for 36 seconds and washed afterwards with phosphate buffer solution (PBS) containing pen/strep to remove uncrosslinked PEG and prevent contamination. Either cell type was seeded into the circular void by pipetting 0.45 μL of cell suspension with a concentration of 500,000 cells / mL into the circular void. Cells were maintained in the co-culture media for two days in an incubator (37°C, 5% CO\textsubscript{2}). After two days, a LIVE/DEAD® viability/cytotoxicity kit (Life Technologies, Carlsbad, CA) was used to analyze HEKa and HDFa viability in the co-culture. Fluorescent images were taken and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) to count the number of cells stained alive and dead. To quantify the percent
viability, the number of living cells was divided by the total number of living and dead cells. Three tests per cell type were performed (n = 3).

5.3.3 Dual hydrogel formation

The method used to produce the dual hydrogel constructs is similar to the process described in section 2.1 used to make the PEG constructs. An outer cell restrictive layer of 10% w/v PEG dissolved in phosphate buffer solution was crosslinked directly onto collagen coated permeable supports using the DMD and a UV light source. Instead of a circular void, a bifurcated void was patterned into the construct with a well placed 424 μm away from one of the bifurcating channels. Afterwards, an inner cell permissive region was created by adding Puramatrix (BD Biosciences, Bedford, MA) into the micro patterned bifurcating void in the PEG hydrogel. The Puramatrix was diluted to 0.15% w/v in deionized water and supplemented with 1 μg/mL soluble laminin (Life Technologies, Carlsbad, CA).

5.3.4 Experimental neurite growth

All procedures involving vertebrate animals were approved by the Institutional Animal Care and Use Committee. Dual hydrogel constructs containing live DRG explants were prepared as previously described. The dual phased constructs (described in Section 2.3) used in these studies were stored in Neurobasal medium supplemented with B27 (2%v/v), glutaMAX™ (1% v/v), pen/strep (1% v/v) and NGF (20 ng/mL) overnight in an incubator (37°C, 5% CO₂). DRGs were first isolated from embryonic day 15 Long Evans rat embryos (Charles River, Wilmington, MA). A single DRG explants was then placed on top of the circular end of the cell permissive void and
gently pressed into the Puramatrix gel. Afterwards, the culture was incubated at 37°C for 2 days in the Neurobasal medium.

After 2 days, the Neurobasal medium was removed and replaced with the co-culture media. At this time, either the HEKa, HDFa, or a combination of the two cell types were seeded into the well by pipetting 0.45 µL of cell suspension with a concentration of 500,000 cells / mL (Figure 16). The cell constructs were incubated for two weeks with media changes every 48 hours to allow the neurites to grow to the end of either bifurcating channel. Constructs with DRGs only and no cells in the well were used as a control. A total of four groups, keratinocytes (K), dermal fibroblasts (DF), keratinocytes and dermal fibroblasts (K + DF), and the control, with at least 7 samples for each were tested (n ≥ 7).
Figure 16: Geometry of neurite guidance assay with bifurcating void for cell growth and circular well, where skin cells seeded, placed distal to one of the bifurcating ends. Guidance Ratio calculated using data from the top channel closest to the well (i) and the bottom channel furthest from the well (ii). Scale bar = 500 µm.
Neurite outgrowth in the dual hydrogel systems was evaluated using standard immunohistochemical techniques. The cells were first fixed in 4% formaldehyde at 37°C for 2 hours. Afterwards, the neurites were tagged with mouse monoclonal (2G10) to neuron-specific beta III tubulin primary antibody followed by fluorescent tagging with Cy3.5 conjugated goat-anti-mouse secondary antibody (AbCam, Cambridge, MA). Both the staining and the tagging steps were performed in PBS containing 0.1% saponin and 0.2% bovine serum albumin overnight in a refrigerator followed by three 15 minute washes in PBS with 0.1% saponin. Confocal imaging of β III tubulin stained neurites in both channels was performed throughout the depth of the constructs using a Nikon A1 confocal laser microscope system (Nikon, Melville, NY) and was processed with ImageJ software. Maximum intensity Z-stacks were obtained from the confocal images of each channel. In order to quantify neurite growth, automatic thresholding (mean) was used, followed by pixel volume analysis for each channel. A guidance ratio (GR) evaluating the fluorescent pixels in channel I (T) and channel ii (B) (figure 1) was used to determine the effect the secretions from each cell type had on neurite growth. The GR was calculated using the same method in §4.3.5. It was previously observed that neurites tend to accumulate at the PEG/Puramatrix interface, which can lead to large amount of error 32. Therefore the region of interest used for the GR calculations included solely the bulk Puramatrix and excluded the border of every channel as depicted in figure 1. An analysis of variance and a Tukey test was used to establish significant difference between the groups. Samples with neurite growth that did not reach the choice point were discarded.
5.3.5 Secreted protein quantification

Both HEKa and HDFa were cultured separately on 6 well plates in a volume of 1 mL co-culture media. Every 48 hours, media was changed until confluency was achieved. After confluency, media was collected for analysis and replaced every 48 hours for a total of 6 days. The protein quantification of the HEKa and HDFa supernatants at each time point were measured using enzyme-linked immunosorbent assay (ELISA). The supernatant of each cell type was diluted twofold and tested for NGFβ and GNDF using a beta NGF human ELISA and GDNF human ELISA kit respectively (Abcam, Cambridge, MA). A Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA) was used to read optical density, which was then converted to concentration using a calibration curve. Three tests were performed for each time point and each protein type (n = 3).

5.4. Results

5.4.1 Cell viability

The results of the cell viability assay demonstrate the effectiveness of the new co-culture media to sustain the two types of skin cells. The fluorescent images found in Figures 2a-b are the merged images of the live stain (green) and dead stain (red). The merged images of the HDFa display healthy spread morphology on the collagen coated surface (Figure 17a) with cells conforming to a flat triangular or polygonal shape. In addition the HDFa have migrated throughout the growing area as they occupy a substantial portion of the circular void. Similarly, the HEKa, though not as numerous as the HDFa, have adhered and spread onto the coated surface (Figure 17b). Furthermore, the formation of contact mediated networks can be observed between the neighboring cells. The calculated cell viability of both cell types in the co-culture media are high with
91.7 ± 1.7 % of HDFa and 86.2 ± 3.9 % of HEKa surviving (Figure 17c). It should also be noted that as in the case of DRGs, the PEG served as a cell restrictive layer for both skin cell types as all growth was constrained to the circular void.
Figure 17: Merged live/dead fluorescent image of (a) fibroblasts and (b) keratinocytes. (c) Cell viability of the two cell types. Scale bar = 250 µm.
5.4.2 Neurite Growth Experiment

HDFa and HEKa were introduced to a choice point model to observe their influence on neurite growth. Cells were placed in a well located near one of the bifurcating channels (figure 16). Three different configurations were tested: DF which contained only HDFa, K which contained only HEKa, DF + K which had both HDFa and HEKa, and a control. Representative confocal images for each group are shown in Figures 4a-d. In the orientation of the images, the well containing cells was closest to the top channel. For the control, there does not seem to be any biased growth into either channel as the neurites seem to be equally distributed (Figure 18a). This is further verified by the low chemoattractive GR value of 0.0219 ± 0.0652 (n = 8). The GR values increase between the DF, K, and DF + K groups respectively. A slight chemoattractive response is observed in the DF group (Figure 18b). A corresponding GR value of 0.0787 ± 0.0667 (n=7) was calculated for this group indicating a slim chemoattractive response. The presence of HEKa had a substantial attractive response as both the K and DF + K groups had double digit GR values. A more distinguishable chemoattractive response is observed in the K group compared to the previous two groups (Figure 18c). A higher GR was calculated for this group with a value of 0.1803 ± 0.0602 (n = 8). The largest chemoattractive response is observed in the combined group of DF + K group with a calculated GR of 0.3131 ± 0.1138(Figure 18d). A graph summary of the GR results for each group is presented in Figure 18e. Using an analysis of variance and a Tukey test with a significance level of (p<0.05), the DF + K group was determined to be significantly greater than the other three groups. The K group also induced a significant response as it was found to be significantly larger than the control. HDFa seemed to have
no effect on neurite guidance as no significant difference found between the DF group and either the control or the K group.
Figure 18: Representative confocal images of neurite growth patterns for (a) control group, (b) DF group with dermal fibroblasts (c), K group of keratinocytes, and (d) DF + K group with both dermal fibroblasts and keratinocytes. (e) Summary graph depicting calculated guidance ratios for each group. * indicates a significant difference (p<0.05). Scale bar = 500 µm
5.4.3 Protein Quantification

Samples of culture supernatants of each cell type was collected every two days for six days and analyzed using an ELISA. The results show that both HDFa and HEKa secreted NGF during the designated time span. HDFa secreted 12.00 ± 5.93 pg/mL of NGF on day 2, 18.47 ± 5.75 pg/mL on day 4, and 31.46 ± 0.17 pg/mL on day 6 (Figure 19a). During the same time points, HDKa secreted 5.02 ± 1.36 pg/mL, 9.70 ± 0.25 pg/mL, and 14.14 ± 0.72 pg/mL (Figure 19a). The only time point where there was a significant difference in secretion was at day 6 (p<0.05). In contrast, HEKa secreted more GDNF than HDFa at each time point. HEKa secreted 23.87 ± 6.08 pg/mL, 45.00 ± 4.17 pg/mL, and 68.12 ± 5.59 pg/mL on days 2, 4 and 6 respectively (Figure 19b). HDFA released 4.44 ± 1.59 pg/mL, 8.05 ± 2.48 pg/mL, and 14.22 ± 2.85 pg/mL on the same days (Figure 19b). A student t-test found significant differences between the amounts of GDNF secreted at each time point (p<0.05).
Figure 19: (a) Concentration of NGF released by both cell types. (b) Concentration of GDNF released by both cell types. * indicates a significant difference (p<0.05).
5.5. Discussion

In our present study, we found our current model was capable of maintaining the skin cell types of HDFa and HEKa. Both cell types had high viability within our system with a value of $91.7 \pm 1.7\%$ for HDFa and $86.2 \pm 3.9\%$ for HEKa. Although the initial number of cells seeded into the construct was the same, after the cells were stained for viability, there seemed to be more HDFa than HEKa. This is because the time for HDFa to reach confluency (~4 days), is almost half the time for HEKa to reach confluency (~7 – 8 days). It is also noteworthy that the restrictive nature of the PEG limited growth within the circular growing area. This can keep the skin cells and DRGs isolated from each other as cell migratory activity has been proven problematic in analyzing axonal guidance. In addition, as our micropatterning process can easily produce constructs of any geometry, the cell source well can be placed anywhere within the construct. This provides improved placement of the secreting cells with respect to the DRGs. Previous co-culture studies, that isolated DRGs from HEKa, relied on commercially available chamber systems with predetermined dimensions. The inability to alter these dimensions severely limited the spatial control.

The set-up for our in vitro model provides a microenvironmental environment that closely resembles phenomena that occurs in vivo. As mentioned earlier in this section, the skin cells are isolated from the neuronal cell bodies and the secreted guidance cues diffuse through the hydrogel and react only with the neurite extensions. Other studies have used co-cultures in close contact with each other, but these models are not representative of cutaneous innervation. In the body, DRGs extend long axons to the periphery where the skin is only in contact with the nerve endings. In addition, to our
knowledge, this study is the only one that incorporates homogenous populations of cells from distinct layers of the skin simultaneously as shown in the DF + K group. There has been previous work that looked at the cells independently or used skin biopsies of specific layers of skin. The studies with skin biopsies contained melanocytes, Langerhans cells, Merkel cells, and inflammatory cells along with keratinocytes in the epidermis while macrophages and adipocytes were also present in the dermis.

From our results, the groups containing keratinocytes induced a greater chemoattractive response in neurites than the group containing fibroblasts only or the control. The combined secretions of HEKa and HDFa generated the largest chemoattractive response and the response was significantly larger than all the other groups (Figure 4e). The K group also induced a significantly higher attractive response compared to the control. Both these results could indicate the importance of HEKa during cutaneous innervation. As keratinocytes are the main cell type of the epidermis, and dermal fibroblasts are the main cell type of the dermis, this mixed cell group could be representative of the interfacial region between the two layers of skin. Observations by Hsieh et al. showed that periphery free nerves spent significant time growing along the epidermis/dermis border during innervation.

After observing the chemoattractive response the secretions from the HEKa induced, we quantified the release of guidance cues from both cell types. Although these cells secrete numerous factors, we focused on NGF and GDNF because these proteins are the only ones to have been previously quantified. Earlier work utilized antibodies in culture media to identify the presence of guidance factors secreted by cells, but quantifying the amount released provides a better idea of the concentration of proteins.
that the neurites are exposed to. Our results are consistent with those of Roggenkamp et al. as we found HEKa produced more GDNF than NGF and that HDFa produced more NGF than GDNF, however in our work, a significant difference was observed after 6 days of protein production.

In the neurite growth experiments, we observed the groups with HEKa produced influential attractive growth towards the channel with the well. Additionally, protein quantification analysis showed that at each time step, the HEKa released significantly more GDNF than the HDFa. Although not the sole factor, these findings suggest GDNF could be a key guidance factor for nerve development and growth within the skin.

The DF group did not induce a significantly different guidance response but these results do not exclude NGF from a prominent role in periphery nerve guidance. Past work has shown that atopic keratinocytes, which experience an increased production of NGF, attracted proprioceptive neurites. The lack of NGF response could be explained by the inherent NGF found in the growth media. The concentration of NGF in the media is significantly larger than the concentration cells produce, making it more difficult to detect an NGF gradient within the construct. Moreover, there was a disconnect of the time span between the neurite growth experiment, which was two weeks, and the protein quantification, which was six days. We only monitored the release of guidance cues for half the time the guidance experiment was run. The protein release may not follow the same behavior after six days.

Though our co-culture system has potential application for cutaneous innervation studies, there are some limitations. For example, the cells used as the guidance cue source secrete multiple factors, some of which that are still being identified. It is difficult
to isolate a response to a single factor. To overcome this shortcoming, instead of using secreting cells, a solution of a single factor can be placed in the well and allowed to diffuse through the construct as demonstrated in Aim 2. This can ensure the neurites only interact with one factor. Another limitation is that the shape and concentrations of the gradient formed in our construct cannot be characterized. However, a validated computational model can be used as a predictive tool for the gradient formation\(^{36}\).

In conclusion, we demonstrated a cell co-culture that can be used to study the development of cutaneous innervations. This system accurately represents \textit{in vivo} occurrences by limiting neural/skin interactions, only involving nerve endings and the proteins secreted from dermal fibroblasts and keratinocytes. Compared to conventional methods, the micropatterning process allowed precise spatial control of the location of the skin cells. By using our system, we showed the chemoattractive abilities of HEK\(_a\) alone and in combination with HDF\(_a\).
5.6 References


CHAPTER 6: CONCLUSION AND FUTURE STUDIES

In these studies we explored methods of forming, quantifying, and controlling soluble gradients within a biphasic hydrogel system. We then applied these methods to incorporate gradients of guidance cues into in vitro studies to observe the chemotactic response on axonal growth. Our first iteration employed a refillable well to establish and maintain long term gradients and used a choice point to test the chemotactic response of nerve growth factor. An integrated computational model was developed as a predictive tool for experimental growth studies. The second iteration utilized the same choice point, but relied on secreting cells as the guidance cue source. Secreting cells served as a better alternative to refillable wells as the gradients they formed were less labor intensive and provided multiple guidance cues. There were limitations associated with methods of

There were limitations associated with both methods of establishing soluble gradients. Well refilling was labor intensive and time consuming as it required fillings every two hours. For the secreting cells method, it was difficult to distinguish the individual effects each guidance cue induced or know all the guidance cues present. The use of loaded microspheres into the reservoir could serve as a nice median between these two methods as they can sustain a longer release of factors requiring less maintenance. Also microspheres can be loaded with solely known factors so individual chemotactic effects can be isolated.

In addition, all the in vitro axon guidance studies mentioned here introduced guidance factors into a single channel of the choice point. Geometric changes to the construct could be easily made to introduce a second well near the other bifurcating
channel for a separate guidance cue. Through such configurations, a competitive aspect could be observed between guidance factors. Further studies incorporating the keratinocytes and dermal fibroblasts could also be performed using this two well system. Each cell type has been known to attract a specific type of neurite so this configuration could be implemented into an axonal segregation assay.
Gary Catig is a kid born and raised in Valencia, California. He obtained his Bachelor of Science in Materials Science and Engineering from the Johns Hopkins University and his Master of Science in Biomaterials Science from New York University. He continued his pursuit of higher education at Tulane University during the fall of 2009. He has worked in various fields throughout his career from microprocessors to bone grafts, and from toothbrushes to his current field of neural microengineering.