AN IMPROVED MODEL FOR NEURITE GUIDANCE VALIDATED
TO A NOVEL MICROPATTERNED IN VITRO SURROGATE

by

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A dissertation submitted to the
Graduate School-New Brunswick
Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences
University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements
For the degree of
Doctor of Philosophy
Graduate Program in Biomedical Engineering

Written under the direction of
Professor Troy Shinbrot

And approved by

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New Brunswick, New Jersey

MAY, 2011
Recent research on repair mechanisms in injured spinal cords focuses on the regeneration of neurites across the injury site. While integral to developing a therapy, regeneration does not ensure that neurites will reach their specific targets and develop functional recovery. Regenerating neurites are less likely to reach their targets given their potential for misguided exploration. To understand how the presence of numerous branches (e.g., spinal roots) along a spinal column may affect a neurite’s route, we (1) created a computational (in silico) model of neurite outgrowth which was applied to a pattern that resembles a spinal column with multiple roots, (2) engineered an in vitro model with identical patterns which was evaluated and compared to the in silico model, and (3) modified the patterns for both models to test how restricting neurite exploration into non-target roots affects neurite guidance to target roots.

The in silico model was constructed by analyzing dorsal root ganglion (DRG) neurite outgrowth on uniform laminin. We simulated a pattern that
resembles a 2-dimensional simplification of a spinal column with multiple roots. From this model, we found that the likelihood of neurites reaching a root diminishes exponentially with root distance from the neurite’s initiation.

For the in vitro model, neurites from E7 chick DRG explant cultures were grown on a micropattern, identical to the in silico model. In vitro and in silico neurite root preference were then compared, and were found to match significantly, thus validating the in silico model.

Finally, to understand the affect of non-target roots on guidance to target roots, we modified both models by removing non-target roots. We found that neurites were successfully guided to target roots when misguiding roots were removed, but only when targets are distant from the neurite’s origin in both models.

This research adds to the current understanding of neurite guidance in complex nervous systems and advances a predictive model for testing guidance strategies. Both of the in vitro and in silico models will enhance clinical or in vivo research, which focus on improving functional recovery following nervous system injury. These models will allow for a non-invasive, efficient, and cost effective way to test therapeutic guide strategies for treating nervous system injuries.
ACKNOWLEDGEMENTS

This work would not have been possible without the guidance and support of my advisors, family, and friends. I would like to thank the whole Biomedical Engineering department and Rutgers community who have helped me to keep my focus and make my time at Rutgers so delightful with the many activities, social gatherings, and day-to-day friendly conversations at the water cooler. Thank you to everyone at the KECK center and Biomedical Engineering building who are always helpful, have an open ear, and I will always call my friends.

I am also grateful to have committee members who have help with my professional development and allowed me to use their equipment to complete my research. Thank you to my advisor Dr. Troy Shinbrot for providing me with the necessary tools and opportunities to approach any scientific project objectively, for allowing me complete freedom in the lab so that I may build my confidence as a scientist, and teaching me how to narrow my focus while researching and writing. Thank you Dr. Helen Buettner for teaching me how to be a meticulous scientist, an objective writer, and guiding me toward pursuing my dream career. Thank you Dr. David Shreiber for also taking me under your tutelage and helping me to organize my thoughts about my research, reminding me to “know your data,” and to question everything.

I would also like to thank everyone at the W.M. KECK Center for Collaborative Neuroscience for helping me with acquiring time-lapse microscopy and fluorescent imaging late into the night. Thank you Dr. Noriko Goldsmith for making sure that the DECON microscope works and limiting the enormous
amount of user files that can slow the computer and cause frustrations for everyone using it. Thank you Chris Ricupero, Elina Tzatatzalos, and Dr. Aswani Valiveti for always being extremely helpful and supportive and of course letting me in the lab on the weekends to complete my work. I would also like to thank Dr. Bhaskar Mitra, and Dr. Jeffrey Zahn and his lab for their help while making silicon masters in the Rutgers Biomedical Engineering Clean room. Thank you Dr. Shawn Taylor for helping me improve my grammar and writing skills while writing my dissertation. I would also like to thank Medhi Doumi for his help in incorporating fasciculation into the computer simulation and Jonathan Chapellow for his help in the image processing of fluorescent images.

Finally, and most importantly I would like to thank my family for all their support, prayers, and wise words when it was difficult to choose my path and keep my focus while working on my PhD.
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1 INTRODUCTION

Potential therapies for central nervous system (CNS) injury have involved the promotion of axon extension across the non-restorative injury site in the adult CNS (Davies, Goucher, Doller, & Silver, 1999; Thuret, Moon, & Gage, 2006). Research has helped to further develop therapies through promoting regeneration and sprouting of both damaged and intact axons near the injury site (Schwegler, Schwab, & Kapfhammer, 1995; Bradbury & McMahon, 2006). Part of the success of these approaches hinges on the untested assumption that once regrowth of spinal axons is achieved, useable connections will be made and sensory or motor function will thereby be restored. Axons in the adult spinal column, however, have a very large number of paths to choose from, most of which are unsuitable for achieving functional recovery (e.g., efferent paths for afferent neurites or proximal spinal roots for motor pathways destined for distal targets). Therefore, a critical step to improve functional recovery following SCI is the development of strategies to increase the likelihood that neurites enter useable spinal roots (Harel & Strittmatter, 2006; Frank, 2006; Webber et al., 2008).

To understand the importance of neurite guidance, let us consider the case of a regenerating motor neurite in a patient suffering from paralysis. If a severed motor neurite, originating in the brain, is destined for the first lumbar spinal segment, then the regenerating neurite must avoid the first 20 cervical and thoracic spinal segments encountered. In addition, if the probability of taking the
correct path is $p$ at each decision point, i.e., spinal segment, then the probability of reaching the first lumbar root is $p^{20}$, or less than 1 in a million for the illustrative case when $p = \frac{1}{2}$. To direct new neurites to viable targets, future regenerative therapies will therefore require the incorporation of guidance strategies.

Motor and sensory spinal tracts have different origins and targets, which indicate that different guidance strategies may be needed to improve functional recovery and pathfinding during their regeneration. For example, in the descending corticospinal motor tract, neurons start in the cortex, enter the spinal cord, and exit into the periphery through the ventral root. Therefore, upper motor function relies on neurites exiting the spine at an upper spinal segment. If neurons grow past this upper spinal segment then functional upper motor recovery will not be restored. On the other hand, in the ascending spinothalamic sensory tract, neurons start in the peripheral nervous system (PNS) at a dorsal root, enter and synapse with neurons in the spinal cord, and form a path up the spinal cord to synapse with the thalamus. Thus lower sensory function relies on neurites traveling up the spine without exiting at any spinal segment. Therefore, if an axon intended to re-innervate a lower spinal segment exited an upper spinal segment before it reached the thalamus, then sensory recovery would not occur.

With these two examples, one can see that a single guidance strategy cannot serve the functional needs of the multiple different systems that are present in the spinal cord. If regenerating neurites follow spinal roots readily, they cannot serve distal sensory function; on the other hand, if they bypass spinal roots, they cannot provide proximal motor function. This very basic notion
underlines the importance of developing and testing therapeutically applicable guidance strategies. Developing a robust and experimentally validated simulation of neurite outgrowth as part of this work will allow for a non-invasive, efficient, and cost effective way to test therapeutic strategies for treating CNS injuries using guidance cues.

This dissertation is organized into five chapters. Chapter 2 covers the background of this research and discusses theories and factors that influence neurite migration. In addition, this chapter expands upon previous experimental techniques and computational models used to understand neuronal migration and guidance. Chapter 3 focuses on the development of an experimentally validated computational model based on \textit{in vitro} experiments. Chapter 4 compares neurite outgrowth patterns from an \textit{in vitro} and an \textit{in silico} model of neurites grown on a complex micropattern geometry, similar to a spinal column with multiple spinal roots. In addition, the hypothesis that neurite root preference decreases exponentially for roots that are distant from a neurite's initiation site when compared to nearby roots is addressed in this chapter. Chapter 5 focuses on comparing neurite outgrowth along a multiple root and a modified single root pattern. In addition, the hypothesis that restricting neurites from entering non-target roots increases the number of neurites that reach and enter a target root is examined in this chapter. Finally, the research findings are summarized and suggestions for future research directions are expanded on in the conclusion.
2 BACKGROUND

2.1 Anatomy

2.1.1 Neuron

A dorsal root ganglia (DRG) neuron is often divided conceptually into three parts: the cell body, the axon, and the growth cone, as shown in Figure 2.1. Dendrites are also a part of the anatomy of other neurons like hippocampal and cortical neurons.

![Figure 2.1. Image of neurite and components](image)

Contrast enhanced image of a growing E7 dissociated DRG neurite grown on uniform laminin (cell body, axon, and growth cone are labeled), taken with 40x phase contrast objective (black scale bar = 25 µm).

The anatomy of the pseudounipolar DRG, which do not produce the usual dendritic tree, is described below. The cell body contains organelles vital to growth and support of extending axons while the axon or nerve fiber and its growth cone serve to seek out viable targets to synapse with, and ultimately form connections for use in cell-to-cell communication. The growth cone is a dynamic structure at the tip of the axon that senses chemical and mechanical cues...
resulting in motility of neurites (Bamburg, 2003). During development, this structure is vital in sensing its environment so that axons or dendrites may synapse with appropriate targets (Caudy & Bentley, 1986; O’Connor, Duerr, & Bentley, 1990).

The growth cone is comprised of three domains: central, transient, and peripheral (Suter & Forscher, 2000), as shown in Figure 2.2.

![Figure 2.2 Image of growth cone regions and components](image)

**Figure 2.2 Image of growth cone regions and components**
Pictures are from (Suter & Forscher, 2000) of DIC and fluorescent images of a growth cone. (A) A high resolution DIC image of an *Aplysia* (Sea slug) growth cone on poly-L-lysine, (white scale bar = 10µm). The growth cone can be broken into two basic domains. As labeled in the micrograph: C – the dense organelle and microtubule rich central domain, T – transition zone, and P – the less dense actin rich peripheral domain. (B) The *Aplysia* growth cone in (A) is extracted and fixed with 1% Triton X-100. F-actin (red) is stained with rhodamine-phalloidin. Microtubules (green) are stained with a tubulin antibody. A white dashed line is added to mark the border between the central domain and the peripheral domain.

The central domain is an extension of the neurite process where the growth cone and axon join. Further out of the axon and into the growth cone is a transient zone that defines the central to peripheral domain boundary. Lastly, the peripheral domain is located beyond the transition zone and is the most dynamic portion of the growth cone. It consists of filopodia, long rod-like projections that
grow and retract from growth cone surface, and lamellipodia, web-like veils of cytoplasm that also spread and retreat often between filopodia. These structures sample the environment for favorable conditions and direct the body of the growth cone forward as the axon elongates and senses cues (Levitan & Kaczmarek, 2001).

2.1.2 Cytoskeleton

Neurites are composed internally of a heterogeneous network of filamentous structures known as the cytoskeleton. The major components of this network are microfilaments, microtubules, and neurofilaments (Levitan & Kaczmarek, 2001). The microtubules form the backbone of the axon, and act as both dynamic structural elements and tracks for organelle traffic – bringing materials from the cell body to the growing tip of the axon. Neurofilaments play a vital role in axon radial growth through filament assembly in vivo (setting the caliber of axon), which has been shown to allow for proper conduction velocity following maturation (Xu et al., 1996; Perrot, Lonchampt, Peterson, & Eyer, 2007). Microfilaments (polymerized actin) are vital to growth cone migration and response to environmental cues. Actin filaments act in concert with other molecular motors like myosin and membrane components for neuronal growth and secretion, without which the dynamic cytoarchitecture and diverse behavior of the growth cones would not exist (Levitan & Kaczmarek, 2001).

The filopodia of a growth cone allow for migration toward a preferred chemical substrate (e.g., attractive cue) by sensing the cue through receptors on the filopodia, attaching to the cue, and then contracting to create movement. The
neurite is enveloped in a membrane; the process is initiated by the addition of a vesicle to the filopodial leading edge followed by actin polymerization at the lead of the microfilament. Depolymerization at the base of the microfilament then leads to a force generated by the retrograde flow of actin and movement of the growth cone toward the attractive cue (Kandel, Schwartz, & Jessell, 2000).

2.2 Growth Cone Behavior

2.2.1 Axonal outgrowth

Growth cone outgrowth is associated with a continual cycle of polymerization and depolymerization of the contractile protein actin, previously mentioned. The resulting dynamic change in a cell’s internal architecture can be observed by examining the variation in flow and orientation of long filamentous actin termed F-actin (filamentous actin), and/or the decrease in sustained lamellar extension of growth cones migrating on uniform permissive substrates like laminin or poly-L-lysine (Danuser & Oldenbourg, 2000). Laminin in particular is an extracellular matrix (ECM) protein that is well documented in the literature to be an adhesive protein involved in many developmental events as a permissive cue in uniform concentrations and for guidance along gradients of increasing concentration (Kuhn, Schmidt, & Kater, 1995; Halfter, 1996; Bonner & O’Connor, 2001; Adams et al., 2005). Evidence for laminin’s role as a permissive substrate has been supported by past studies involving growth cones grown on uniform laminin at different concentrations. In particular, it was observed that while an increase in concentration promotes neurite outgrowth speed, directional persistence remained unaltered (Buettner & Pittman, 1991). In
addition, the observation that axons tend to follow relatively straight paths due to
the stiffness of axon microtubule bundles suggest how the different cytoskeletal
components may mediate different growth cone behaviors (Katz, George, &

Growth cone interactions with external cues have been studied
experimentally to identify the many different growth cone behaviors and
morphologies. A list of some external cues include: (1) different cell types
(Fallon, 1985; Caudy & Bentley, 1986), (2) chemical gradients (Gundersen &
Barrett, 1979; Zheng, Felder, Connor, & Poo, 1994), (3) contact cues (Clark,
Britland, & Connolly, 1993; Tai & Buettner, 1998; Withers, James, Kingman,
Craighead, & Banker, 2006; Song & Uhrich, 2007), and (4) extracellular matrix
density (Bonner & O’Connor, 2001; Snow, Smith, & Gurwell, 2002; Adams et al.,
2005). In this work, we are particularly interested in phenomena associated with
growth cone guidance and targeting. For example, growth cone turning by
filopodial dilation and veil extension or regression. To understand growth cone
guidance and targeting, we briefly review current literature on the topic.

2.2.2 Veil or lamellar extension or regression

Two mechanisms for growth cone steering are through veil extension
along a filopodium in contact with a local attractive cue (Figure 2.3A) or
regression by a repulsive cue. At its simplest, the net migration of the growth
cone can be viewed as progression toward the largest veil or lamellipodium,
where the lamellipodium becomes the leading edge of the growth cone. On the
other hand, if the filopodium contacts a repulsive cue, the veil retracts and the
growth cone turns away from the cue. In reality, growth cone migration is more complex and stochastic than this simple caricature indicates. For example, filopodia repeatedly extend and retract in response to cues in a way that is not uniform in space or steady in time (O’Connor et al., 1990). In addition, many cues can act as either attractors or repellers of a growth cone, depending on concentration or other factors (Zimmer, Kastner, Weth, & Bolz, 2007). Despite these complications, under fixed conditions and averaged over numerous filopodial extensions and retractions, the model described in Figure 2.3 portrays our best current understanding of growth cone response to simple cues.

![Figure 2.3 Growth cone behavior in the presence of attractive cue](image)

Pictures are from (Maskery & Shinbrot, 2005) caricatures of growth cone and axonal steering mechanisms. (A) Veil extension, in which the lamellipodial veil near an attractive cue is extended to support further growth in that direction. (B) Filopodial dilation, depicting the growth of filopodia in the direction of an attractive cue; typically filopodia that do not reach a supportive cue retract. The combined effect of (A) and (B) would be that the entire growth cone would migrate in the direction of an attractive cue (or away from a repulsive one). Commonly the trailing axon straightens to form a more gently curving apparent path than the original growth cone trajectory.

### 2.2.3 Filopodial dilation

Another mechanism for growth cone steering toward a cue occurs when a filopodium comes into contact with a local strong attractive cue, as shown in Figure 2.3B. Once in contact with the cue, the filopodium (which will become the leading edge of the growth cone and ultimately the axon) dilates and extends
over the cue, while the non-contacting filopodia retract. Once the growth cone covers the attractive cue, it can either stop extending, or continue retracting and dilating filopodia. This choice is dependent on the concentration of a number of guidance molecules (Isbister, Mackenzie, To, & O'Connor, 2003).

2.3 Guidance Mechanisms

2.3.1 Chemical cues

As mentioned, growth cone migration is mediated by the recruitment and polymerization of two cytoskeletal components. At the leading edge of the growth cone, actin polymerization establishes the lamellipodial and filopodial structures that permits the cone to extend and explore its environment, while further back toward the central zone of the growth cone (Figure 2.2), microtubules invade along oriented actin bundles to form the trailing axonal branch (for review, see: (Dent & Gertler, 2003)). Four major classes of neuronal guidance molecules that have been identified because of their roles in reorganizing actin and tubulin dynamics are the semaphorins, the netrins, the slits, and the ephrins (Song & Poo, 2001). Extracellular matrix (ECM) proteins like chondroitin sulfate proteoglycans (CSPG), also act as inhibitory guidance cues in the presence of other ECM proteins like laminin or fibronectin (Snow & Letourneau, 1992). Apart from the neurotropic non-diffusible cues mentioned above, other cues termed neurotrophic factors like neural growth factor (NGF), which does not belong to any of the major classes, act to direct neurite outgrowth by a diffusible chemical gradient (Gundersen & Barrett, 1979). Studies have localized these proteins on substrates near migrating growth cones to examine
the cone’s response to placement, concentration, and other factors (Gallo, Lefcort, & Letourneau, 1997; Snow et al., 2002).

2.3.2 Contact cues (micropatterns)

One of the earliest studies to examine outgrowth in an engineered microenvironment employed the use of patterning ECM proteins at the micron scale (protein micropattern) to examine preferential outgrowth and attachment of sensory neurons (Letourneau, 1975b; Letourneau, 1975a). As the technology of the semiconductors began to boom in the 80’s scientists started to explore how to use micromachining to micropattern proteins. One method examined was the use of photolithography to create these micropatterns and align neurons in a stripe pattern (Kleinfeld, Kahler, & Hockberger, 1988). This study was one of the first to coat non-permissive substrates and restrict cellular secreted proteins from adhering to the non-patterned regions (Corey & Feldman, 2003). Since then, various techniques including photolithography, agar, plasma treatment, microfluidics, and micro-contact stamp printing have been developed to create different patterns and gradients (von Philipsborn et al., 2006; Vahidi, Park, Kim, & Jeon, 2008). In particular, two methods known as microscale initiated patterning (µPIP) (Figure 2.4A) and micromoulding in capillaries (MIMIC) (Figure 2.4B) have gained acceptance because they offer the ability to create uniform patterns that are easily reproducible using only a plasma generator and a polydimethylsiloxane patterned (PDMS) stamp (Kim, Xia, & Whitesides, 1996; Langowski & Uhrich, 2005).
Figure 2.4 Micropatterning Techniques
Two different patterning techniques from (Kim et al., 1996; Langowski & Uhrich, 2005) that show how to create a patterned geometry. (A) Illustration of how to create micropatterns by applying a method called µPIP. (B) Illustration of how to create micropatterns by applying a method called MIMIC.

The advent of photolithography and soft lithography processes have allowed scientists to examine the growth cone dynamics and neurite outgrowth along different reproducible geometries with micron precision boundaries and compare experiments to computational models (Kleinfeld et al., 1988; Kim et al., 1996; Xia & Whitesides, 1998; Withers et al., 2006). Micropatterned extracellular proteins have allowed scientists to examine and quantify the effects of contact cues on neurite orientation, outgrowth, and morphology (Hammarback, McCarthy, Palm, Furcht, & Letourneau, 1988; Clark et al., 1993; Tai & Buettner, 1998; Song & Uhrich, 2007). This precision is important not only to control cellular migration on a micron scale, but also to imitate the environment cells detect in computationally models (Buettner, Pittman, & Ivins, 1994).
2.4 Spinal Cord Injuries and Therapies

As previously mentioned, growth cones are located at the tip of axons and are the highly dynamic motile element that allow neurons to search and reach their synaptic target during development (Sperry, 1963; O’Connor et al., 1990; Mueller, 1999). All growth cones consist of the same cytoskeletal components, but it is the growth cone’s ability to sense chemical or contact cues through their specific receptors while migrating during development, that guide it to a unique target. Researchers have scrutinized and classified sensory and motor axon tract formation and pathfinding in the spinal cord during development (Landmesser, 1978; Honig, 1982; Wang & Scott, 1999). If injury occurs in the adult central nervous system (CNS), a neuron may not be able to restore connection with its target organ along these tracts due to inhibitory chemical factors (Nogo), demyelination, apoptosis, and/or physical barriers (cysts, glial scaring, or cavities) developed post-injury. Post-injury effects result in restricted neurite outgrowth, growth cone collapse, and degeneration of the spinal tract, which hinders regeneration of neurites and results in sustained partial or total loss of sensory or motor function (Snow, Steindler, & Silver, 1990; Jones, McDaniel, & Popovich, 2005; Horky, Galimi, Gage, & Horner, 2006; Silver & Miller, 2004).

Many animal studies have shown that by exposing neurons to growth promoting neuroprotective factors or cellular transplantation axons may grow across the injury site on the circumferential white matter that is spared (Xu, Chen, Guenard, Kleitman, & Bunge, 1997; Davies et al., 1999; Thuret et al.,
2006; Silver & Miller, 2004). While these approaches provide hope for future SCI therapies, the limited number of axons that cross the injury site prevent complete recovery. Researchers have also tried to improve outgrowth through the use of chemicals that neutralize the inhibitory affects of myelin-associated glycoproteins (MAG, ex. Nogo-A) or components of the glial scar (ex. chondroitin sulfate proteoglycan, CSPG) near the injury site. Partial functional recovery has been reported in some of these experiments, possibly due to the removal of the inhibitory effects from chemicals released post-injury and regeneration of lesioned axons. Another opinion, however, is that synaptic plasticity and axonal sprouting underlies the functional improvements stimulated by these experimental treatments (Bradbury & McMahon, 2006). If this is true then this could be a cause of concern since research has shown that axonal sprouting may result in neuropathic pain and autonomic dysreflexia by inappropriate rewiring (McClellan, 1999; Bradbury & McMahon, 2006). Experiments employing “treadmill training” as a therapy have also been used to increase corticospinal tract (CST) connectivity locally at the dysfunctional spinal segment, and shown increased functional recovery through rewiring of the central pattern generator (CPG) (Calancie et al., 1994; Thomas & Gorassini, 2005). Many SCI researchers agree that a treatment combining the therapies described above will improve growth across the injury site and synaptic rewiring, but if neurites fail to reach suitable targets, full functional recovery will inevitably be restricted (Calancie et al., 1994; Thomas & Gorassini, 2005). To establish functional recovery, we propose that neurites will need to be guided to suitable targets, else
they will tend to form aberrant paths and may establish unusable connections (McClellan, 1999; Harel & Strittmatter, 2006).

2.5 Computational Model

2.5.1 Stochastic and deterministic processes

Computational modeling of growth cone migration is vital for understanding mechanisms of motility, how environmental cues (haptotactic or chemotactic gradients) affect guidance, and the patterns of emerging properties in simulated biological environments. In the past, researchers have used quantified parameters from experimental time-lapse video microscopy data to accurately model a neurite’s trajectory (Buettner & Pittman, 1991; Buettner et al., 1994; Wang, Liu, Diefenbach, & Jay, 2003). The growth cone lamellipodial centroid, measured at short uniform time intervals, is an example of a parameter that has been used to model the stochastic and deterministic nature of the growth cone’s trajectory. Researchers have also used a continuum model by taking into account the concentration of tubulin at the terminal tip and the resources of a growth cone’s primary subunits to simulate neurite elongation successfully (McLean, Ooyen, & Graham, 2004). In addition, the role of myosin 1c and uniform laminin concentrations on growth cone persistence time, the average amount of time before a significant change in the direction of cell movement occurs, and root mean squared (RMS) speed has been examined using a persistence random walk model (McLean et al., 2004). Probability functions and statistical properties may also be fit to experimental data to
examine possible mechanisms contributing to environmental cues (McLean et al., 2004).

Neurite guidance and pathfinding to targets have also been studied in the presence of contact cues and guidepost permissive regions to imitate the native environment during development by modeling the filopodial dynamics and lamellipodial centroid migration (Buettner et al., 1994; Buettner, 1996). Guidance in the presence of inhibitory or attractive cues have also been analyzed using other parameters like growth cone search angle and angular direction of growth (Maskery, Buettner, & Shinbrot, 2004). A key conclusion from modeling outgrowth in the presence of guidance cues is that there are three distinct phases that describe whether a growth cone will turn, continue in the same direction, or is currently in a transition period (Maskery et al., 2004). Others researchers have modeled a growth cone’s signal transduction network to reveal important mechanisms behind the different growth cone behavior, but it is still difficult to recreate the growth cone migration completely using this model (Aletti & Causin, 2008).

Data gathered from fractionating the axonal growth process provided evidence that elongation of axons can be successfully modeled as a two-dimensional stochastic walk and indicated neurite outgrowth has a bias (drift) in the forward direction (Aletti & Causin, 2008). Autocorrelation analysis from centroid data taken every 30 seconds also indicated that this growth cone motion exhibits at least two different patterns of outgrowth at this time-scale (Odde & Buettner, 1995). The first pattern is a random walk with a drift
where $x_i$ and $y_j$ are the $x$ and $y$ positions of the growth cone centroid and $i$th interval and $\mu(.)$ is the constant drift components and $a_{(\cdot,i)}$ is the random movements modeled as a Gaussian distribution (Aletti & Causin, 2008). The second pattern exhibits an autoregressive behavior (first order stochastic differential equation)

\begin{align}
  x_i &= \rho_x x_{i-1} + a_{x,i} \\
  y_i &= \rho_y y_{i-1} + a_{y,i}
\end{align}

(2.2)

where $\rho(\cdot)$ is a constant representing a fraction of the next iterations movement and the rest of the variables are the same as the random walk with drift model (Aletti & Causin, 2008). Despite the complexity of the mechanisms involved in cytoskeletal dynamics, a realistic phenomenological replica of experimental axonal growth has previously been simulated based on the autoregressive and persistent random walk models (Maskery et al., 2004; Maskery & Shinbrot, 2005).

In the present work, we calibrate this model by setting parameters to agree with *in vitro* neurite outgrowth statistics and applying this calibrated simulation to outgrowth on a patterned substrate containing numerous roots intended to mimic roots in the spinal column. This model improves upon previous *in silico* models of neurite outgrowth, by including the effect of the
geometry of the nervous systems, which our findings show is an important factor that has not been examined previously. In addition, we compare this simulation side-by-side with experiments using chick DRG explants in a protein micropattern of identical geometry to further validate the computational model. Previous work on promoting functional recovery following nervous system injury focuses primarily on regrowth of neurites in the post-injury environment that inhibits neurite regrowth. Neurite regrowth, however, will not result in functional recovery unless the neurites reach their appropriate targets. Our improved predictive model provides a tool for exploring questions related to neurite guidance, and in developing guidance strategies to promote functional recovery after nervous system injury. One such strategy, that promotes neurite guidance by restricting neurites from taking aberrant paths, is demonstrated as part of this research.
3 COMPUTATIONAL MODEL OF NEURITE GUIDANCE ON A PATTERN

3.1 Chapter 3 Summary

This chapter focuses on the construction of an *in silico* simulation, which models growth cone wandering within an apical branching geometry that resembles a spinal column with multiple spinal roots. The goals of the simulation include: (1) to establish the likelihood that a neurite will reach a desired root and (2) to create a predictive model for future examination of guidance strategies.

The current simulation is based on previous models that replicate embryonic chick and rat dorsal root ganglia (DRG) outgrowth, as validated using time-lapse microscopy (Burt, ) and experimental parameters of neurite outgrowth (Buettner, 1994). The current study modifies the previous model that now includes channel geometrical factors (e.g. root widths, angles, and distances) and growth cone parameters evaluated from neurite outgrowth on uniform substrates (e.g., growth cone search angle (δ), average outgrowth ($r_d$), and outgrowth uncertainty ($r_s$)).

3.2 Background

Nervous system injury therapies will benefit from mathematical and computational assessment of how successful neurites reach a desired target. Simulations allow for a non-invasive, efficient, and cost effective way to test therapeutic strategies for treating CNS injuries. Computational models of neurite
migration, developed from in vitro experiments, have previously been used to understand mechanisms of motility, neurite polarity, and how environmental cues (haptotactic or chemotactic gradients) affect guidance (Katz et al., 1984; Buettner & Pittman, 1991; Buettner et al., 1994; Wang et al., 2003; McLean et al., 2004; Forciniti, Schmidt, & Zaman, 2009; Mortimer et al., 2009). Simulations have also been used to assess the effects of growth cone cytoskeletal dynamics on neurite targeting (Buettner, 1996). Work in our laboratory has demonstrated that growth cone migration can be effectively simulated using an integrated random walk (Maskery et al., 2004; Maskery & Shinbrot, 2005). In the present work, we calibrate this model, setting parameters to agree with neurite outgrowth statistics in experiments of chick dorsal root ganglion (DRG) neurons grown on a uniform laminin substrate. We then apply this calibrated simulation to outgrowth on a patterned substrate containing numerous roots, intended to mimic roots in the spinal column. We compare the simulation side-by-side with experiments using chick DRG explants in the identical geometry in the subsequent chapter. We emphasize that the simulations are calibrated on a uniform substrate, while the comparisons between simulations and experiment described in later chapters are performed on patterned substrates. We use these comparisons to quantitatively evaluate the hypothesis that the probability of reaching subsequent roots decreases exponentially as $p^n$ ($p$ is a fixed probability and $n$ is the number of roots from the originating explant). This theoretical expectation is not a forgone conclusion, because real neurites fasciculate (i.e., a neurite growing along...
existing neurites), cling to boundaries, wander in a pulsatile fashion, and otherwise exhibit behaviors that might be inconsistent with the expectation.

### 3.3 Methods and Materials

#### 3.3.1 Explant culture and time-lapse microscopy

Neurite outgrowth parameters in the simulation were acquired by analyzing independent time-lapse microscopy images of embryonic day seven (E7) dorsal root ganglia (DRG) explants grown on uniform laminin coated MatTek 14mm glass-bottomed petri dishes. Dishes were coated with laminin (25µg/ml) and incubated for 4 hours at 37°C in 5% CO\(_2\) and then washed three times with a F12/DMEM 1:1 mixture. DRGs were dissected from the lumbar region of specific pathogen free chicken embryos (Charles Rivers) in cold calcium and magnesium free (CMF) Hank’s Balanced Salt Solution (HBSS) under a dissection microscope. A single whole DRG was then plated on the laminin-coated glass in 600µl of serum free media (SFM) for eight hours followed by the addition of 1.4ml of SFM. SFM is composed of 1:1 (v/v) DMEM/Ham’s F-12, 2 mM L-glutamine, 50 U/ml, penicillin/streptomycin, 10 mg/ml glucose, 1X MEM vitamins, 20 µg/ml N3, 1X ITS+1 premix (100 µg/ml insulin, 55 µg/ml transferrin, 50 ng/ml selenium), 0.1% bovine serum albumin (BSA), 50 ng/ml neural growth factor (NGF) (R&D Systems, 556-NG), and 100 mM ascorbic acid. N3 consists of 5 ml N2 supplement (Invitrogen, 17502048), 250 µl 1X ITS+1 premix, and 10 µl of 2 mg/ml corticosterone. All reagents were purchased from Sigma unless stated otherwise. The DRG was grown in SFM overnight before taking time-lapse images. Images were captured every 30 seconds for 1 hour with a 40x objective.
(5.8 pixels/micron) using an onstage DECON incubator (Rutgers Collaborative KECK center) that maintained the temperature at 37°C with 5% CO₂.

3.3.2 Dissociated DRG culture

Migration of dissociated DRGs was also examined. Following dissection of DRGs (~15), cells are pelleted by centrifugation at 12,000 rpms for 2.5 minutes and the excess HBSS was aspirated off. To aid in dissociation, 750 µL of 1x trypsin-EDTA was added to the pellet and the solution was incubated in a 37°C water bath for 30 minutes. 750 µL of F12/DMEM 1:1 (v/v) mixture was added to dilute the trypsin-EDTA. The dissociated cells were then pelleted again by centrifugation at 12,000 rpms for 2.5 minutes and solution was aspirated off. 500µL of serum free media (SFM) was added and dissociated neurons were resuspended by triturating 10-15 times to break up any remaining cell-cell interactions. To remove the debris and deactivate remaining trypsin, the cell suspension was centrifuged at 200 rpm for 7 minutes in a 3.5% BSA gradient (w/v) in DMEM. The excess 3.5% BSA solution was then aspirated off and the cells were resuspended in 500 µL SFM. After the dissociated neurons were counted with a hemocytometer, the cells were seeded onto the MatTek glass bottom petri dishes at a low cell density of 2.5x10³ cells/well to minimize cell-cell interactions. Dissociated neurons were then cultured for 1 hr at 37°C at 5% CO₂ in 100 µL of media followed by the addition of 2 ml of media and incubation overnight before imaging.
3.3.3 Analysis of growth cone motility from time-lapse images

Time-lapse image sequences from DRG neurons (Figure 3.1) were analyzed to evaluate the rate of neurite outgrowth and the growth cone search angle. These data were used to build a realistic computational model. Growth cone centroid and base were manually tracked using ImageJ 1.41 (NIH) (Abramoff, Magalhaes, & Ram, 2004).

Figure 3.1 Time-lapse sequence and selection
Images were taken from time-lapse microscopy (30-second intervals) of a growth cone outgrowth on a featureless laminin substrate. By outlining the lamellipodium region (gray outline), the growth cone centroid (white circle) was estimated. The base of the growth cone (black circle) and axon angle (black line) was also measured. (white scale bar = 10 µm)

As illustrated in Figure 3.2A, the growth cone centroid (xc,yc) was calculated by outlining the lamellipodium and excluding filopodia (Buettner, 1994). The base of the growth cone (xb,yb) was selected as the point where the axon broadens to larger than the average width of an axon (~1 µm, from average measurements of dissociated neurites). The angle from the growth cone centroid to growth cone base with respect to the x-axis of each successive movement was calculated to find the growth cone orientation, Φi, defined in Figure 3.2B. The change in orientation was then used to calculate the search angle, δi. The rate of centroid movement was converted from Cartesian to polar coordinates (ri, θi) to calculate the respective outgrowth and current direction shown in Figure 3.2C. Uncertainty
of outgrowth was also calculated as the change in outgrowth. Neurite outgrowth, uncertainty of outgrowth, and search angle measurements were fit to probability distributions using MATLAB’s Statistical Toolbox. All data were found to fit significantly to their respective distributions after performing a two-sample Kolmogorov-Smirnov test \( p > 0.05 \).

![Figure 3.2 Analysis of time-lapse microscopy](image)

**A** An image from time-lapse microscopy with an outline around the lamellipodium region of the growth cone (gray lines within dashed box), the estimated centroid (white circle), and the base of the growth cone (black circle). **B** An enlarged view of the growth cone’s next position (30-second interval) illustrates how its orientation, \( \Phi_i \), was calculated by estimating the angle between the growth cone centroid and the growth cone base. The search angle \( \delta_i = \Phi_{i+1} - \Phi_i \) was calculated by finding the change in growth cone orientation. **C** A further enlargement of growth cone centroids at subsequent time increments indicates how the outgrowth, \( r_i \), and current direction, \( \theta_i \), of the growth cone centroid was measured from location \( (x_c, y_c) \) at time \( i \), to location \( (x_{c_{i+1}}, y_{c_{i+1}}) \) at time \( i+1 \). The axon is highlighted with a black line in (A) and (B). (white scale bar = 10 µm)

### 3.3.4 Computational simulation

The computational model of neurite outgrowth is based on a 2D random walk, where each progressive iteration, \( i \), is dependent on the previous one. Neurite outgrowth is adjusted based on the three parameters: average outgrowth, \( r_d \), outgrowth uncertainty, \( r_s \), and current direction, \( \theta_i \), of a neurite’s tip described in the previous section and shown in Figure 3.3A. The distance parameter, \( r_i \), is the sum of the average outgrowth, \( r_d \), and the outgrowth uncertainty, \( r_s \), which are respectively gamma and Gaussian distributed. The
maximum uncertainty of outgrowth is \( r_s \). The current direction is modified by the growth cone’s search angle, \( \delta_i \), to the left and right of its current angle, \( \theta_i \), as illustrated in Figure 3.3A.

Figure 3.3 Schematic of parameters that affect simulated neurite growth
(A) Growth of a neurite’s tip, defining the growth cone centroid’s position, is calculated using a persistent random walk model in the simulation. The future location, \((x_{i+1}, y_{i+1})\), depends on the prior trajectory defined by \((r_i, \theta_i)\), the mean distance traveled, \( r_d \), the maximum variation in distance traveled, \( r_s \), and the growth cone search angle, \( \delta_i \). (B) When a non-permissive edge (gray rectangle) is encountered (gray square), the neurite first reverts back to its previous position \((x_i, y_i)\) and direction \((\theta_i)\). Then a new neurite tip location \((x_{i+1}, y_{i+1})\) is recalculated after selecting a new search angle, \( \delta_{adj} \), at random from a Gaussian distribution. The previous steps are then repeated until the subsequent neurite tip position avoids the non-permissive region (black circle).

The experimentally fit distributions from time-lapse data were incorporated into the simulation, and values were randomly generated during the simulation using MATLAB’s Statistical Toolbox. In this way, the simulated growth cones were engineered to produce identical statistics to those seen in vitro on a featureless
substrate. Polar coordinates \((r_i, \theta_i)\) are converted to Cartesian coordinates before adding the next growth to the current position in the equations below:

\[
x_{i+1} = x_i + r_{i+1} \cos(\theta_{i+1}) \\
y_{i+1} = y_i + r_{i+1} \sin(\theta_{i+1})
\] (3.1) (3.2)

When a neurite encounters a non-permissive boundary (shown in Figure 3.3B), the neurite reverts back to its previous position \((x_i, y_i)\) and direction \((\theta_i)\). A new direction is then recalculated from summing the new search angle \((\delta_{adj})\), which is randomly selected from a Gaussian distribution, and previous direction \((\theta_i)\). If the new neurite tip position evaluated encounters the edge again then these two steps are repeated until the boundary is avoided. Once an angle is selected that allows the neurite tip to avoid the edge, the neurite tip continues migrating based on the previously described persistent random walk. The simulated change in direction of the neurite tip at the non-permissive edge is based on previous experimental observations examining growth cone turning behavior, which has been described as the growth cone reorienting along the substrate edge and then growing along or away from the edge (Tanaka & Kirschner, 1995).

The simulation was initiated by placing a number, \(n\), \((n = 100\) in the simulations for each case studied) of neurites at random positions along the base of the pattern with neurite current angles parallel to the primary channel’s edge.
Figure 3.4 Simulated neurite outgrowth on a multiple root pattern
Simulation of neurites (yellow) and growth cones (red) on a multiple root pattern designed to mimic the spinal column and roots. The start, defining where the DRG explant neurites would enter the primary channel for companion *in vitro* studies, is labeled blue. Neurites continued to grow straight at 45° after entering a root, as observed from experimental samples. (white scale bar = 200 µm)

Each neurite grows for a different number of iterations, simulating the time-dependent neurite entrance into the micropattern environment. When a simulated neurite enters a root, it is not permitted to turn backwards, and may only continue moving forward. This concurs with observations from time-lapse videos using micropattern stripes 100 µm wide or smaller. Simulations of growth cone outgrowth were run with 100 neurites (1 iteration = 30 seconds and 1 unit = 174 µm), producing results such as those shown in Figure 3.4. Red and blue circles represent the respective neurite end points and origins. Each simulation of 100 neurites is replicated 20 times. The simulation ran for an equivalent
number of iterations that matched 2.5 days, the average time from when neurites entered the primary channel until neurites reached $b = 8$ in vitro.

### 3.3.5 Calculating neurite preference for different root entry widths

Computational neurite preference measurements were developed to examine how root entry widths affect neurite guidance. Root entry widths greater than (111.6 µm), less than (37.2 and 74.4 µm), and the same as the primary channel width (100 µm) were selected. Neurite root preference, $PF_b$, and the probability that the neurite will choose a divergent trajectory into a root, $PR_b$, were calculated. $PF_b$ was calculated as the fraction of neurites that entered a root, $Nr_b$, relative to the total number of neurites that entered all roots, $\sum Nr_b$. $PR_b$ was calculated as $Nr_b (x_{i+1} > 0.588$ units and $b > y_{i+1} > b-0.422)$ divided by the number of neurites that entered, $Nr_b$, and avoided the root, $Nl_b$, i.e. took a straight trajectory ($Nl_b = Nl_{b+1} + Nr_{b+1}$ or $Nl_b = 100 - \sum Nr_b$). Simulated $PF_b$ was fit to exponential curves, $PF_b = \alpha e^{\beta b}$, using MATLAB’s Curve Fitting Toolbox, where $b$ is the root number, and coefficients $\alpha$ and $\beta$ are constants fit to data points using the least squares method.

### 3.4 Results

#### 3.4.1 Growth cone outgrowth, search angle, and outgrowth uncertainty fit to distributions

Data from average growth cone centroid outgrowth distance ($r_d$) fit significantly to a gamma distribution (Figure 3.5A), $\Gamma(\kappa, \theta)$, where $\kappa = 6.8$ µm and
\( \theta = 0.38 \, \mu\text{m} \), and the uncertainty of outgrowth (\( \Delta r \) or \( r_s \)) fit significantly to a normal distribution (Figure 3.5C), with mean 0 and \( \sigma = 0.32 \, \mu\text{m} \).

**Figure 3.5 Distributions fit to growth cone outgrowth and search angle**

Plot of the growth cone centroid (A) outgrowth rate, (B) search angle, and (C) outgrowth uncertainty probability histograms, which are fit respectively to gamma, normal, and normal probability density functions (pdf). Fit distributions for growth cones 1, 2, and 3 are respectively shown as different black lines.

Since events of neurite extension, average persistent outgrowth distance, are a combination of random events (actin polymerization (Betz, Koch, Lim, & Kas, 2009) and microtubule assembly (Odde, Tanaka, Hawkins, & Buettner, 1996)) and gamma distributions generally arises in connection with compound random events there is an appropriate theoretical basis for the use of the distribution (Buettner & Pittman, 1991). The addition of a stochastic variable, uncertainty of outgrowth distance, to average outgrowth distance accounts for other fluctuations
in the outgrowth process like dynamic instability of microtubule assembly (Mitchison & Kirschner, 1984). In addition, search angle (δ) data fit significantly to a normal distribution (Figure 3.5B), with mean 0° and σ = 12°. The distribution matched expected behavior since neurite outgrowth migrates predominately in forward trajectory, presumably due to the stiffness in the microtubules (Katz et al., 1984; Katz, 1985; Buettner, 1994). A two-sample Kolmogorov-Smirnov test, comparing experimental data to a hypothesized continuous distribution, indicates that there is a significant fit for all of these distributions (significance level of 0.05) (Table 3.1, Table 3.2, and Table 3.3).

### Table 3.1 Kolmogorov-Smirnov (K-S) test of growth cone outgrowth

Two parameters (scale and shape) of the gamma distribution fit to growth cone outgrowth data using maximum likelihood estimation for three different DRG samples.

<table>
<thead>
<tr>
<th>Growth Cone Outgrowth (Gamma distribution)</th>
<th>a (scale), μm</th>
<th>b (shape), μm</th>
<th>K-S test, null hypothesis: sample fits a gamma distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Cone #1</td>
<td>3.50±0.43</td>
<td>0.15±0.019</td>
<td>p = 0.53</td>
</tr>
<tr>
<td>Growth Cone #2</td>
<td>3.24±0.40</td>
<td>0.22±0.030</td>
<td>p = 0.60</td>
</tr>
<tr>
<td>Growth Cone #3</td>
<td>3.41±0.42</td>
<td>0.20±0.026</td>
<td>p = 0.89</td>
</tr>
</tbody>
</table>

### Table 3.2 Kolmogorov-Smirnov (K-S) test of growth cone search angle

Mean, μ, and standard deviations, σ, of normal distributions fit to growth cone search angle data using maximum likelihood estimation for three different DRG samples.

<table>
<thead>
<tr>
<th>Growth Cone Search Angle (Normal distribution)</th>
<th>μ (mean), °</th>
<th>σ (standard deviation), μm</th>
<th>K-S test, null hypothesis: sample fits to a normal distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Cone #1</td>
<td>-0.51±0.53°</td>
<td>5.7°±0.37°</td>
<td>p = 0.92</td>
</tr>
<tr>
<td>Growth Cone #2</td>
<td>0.0045±1.2°</td>
<td>12.7°±0.83°</td>
<td>p = 0.62</td>
</tr>
<tr>
<td>Growth Cone #3</td>
<td>0.043±1.5°</td>
<td>16.4°±1.1°</td>
<td>p = 0.30</td>
</tr>
</tbody>
</table>

### Table 3.3 Kolmogorov-Smirnov (K-S) test of growth cone outgrowth uncertainty

Mean, μ, and standard deviations, σ, of normal distributions fit to growth cone outgrowth uncertainty using maximum likelihood estimation for three different DRG samples.

<table>
<thead>
<tr>
<th>Growth Cone Outgrowth Uncertainty (Normal distribution)</th>
<th>μ (mean), μm</th>
<th>σ (standard deviation), μm</th>
<th>K-S test, null hypothesis: sample fits to a normally distributed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Cone #1</td>
<td>-0.0048±0.036</td>
<td>0.40±0.026</td>
<td>p = 0.71</td>
</tr>
<tr>
<td>Growth Cone #2</td>
<td>0.0040±0.051</td>
<td>0.55±0.036</td>
<td>p = 0.78</td>
</tr>
<tr>
<td>Growth Cone #3</td>
<td>-0.0097±0.50</td>
<td>0.50±0.032</td>
<td>p = 0.87</td>
</tr>
</tbody>
</table>
3.4.2 Analysis of growth cone motility and persistence time

To confirm the correspondence between experiments and simulations, mean-squared displacements (MSD) of the two data sets were compared. MSD of the growth cone centroid was calculated for both experiments and simulations on a uniform substrate to examine persistence time, \( P_v \), and random motility, \( \mu \), which describes growth cone motility and tortuosity (Buettner, 1994; Wang et al., 2003). These two parameters were estimated by fitting a theoretical persistent random walk model to MSD measured from experiments (Figure 3.6A) and simulations (Figure 3.6B) using nonlinear regression \((r^2 > 0.98)\). Average random motility and persistence time were compared between the \textit{in vitro} experiments \((n = 3\) replicates\) and \textit{in silico} simulations \((n = 100\) replicates\) of neurite outgrowth for 1 hour on a uniform substrate. Both parameters for experiments and simulations matched within a 95% confidence interval.

Figure 3.6 Persistent random walk model fit to mean-squared displacement
Theoretical persistent random walk model fit to MSD measurements from (A) \textit{in vitro} experiments \((n = 3\) replicates\) and (B) simulations \((n = 100\) replicates\) grown on a uniform permissive substrate. Average random motility, \(<\mu>\), and persistent time, \(<P_v>\), was compared between experiments and simulations to examine outgrowth tortuosity. Error bars in (A) are standard errors of the mean (SEM).
3.4.3 Neurite root entry width affects neurite root and trajectory preference

Neurite root preference along the multiple root pattern in the simulation is affected by root entry width. Neurites prefer the first root more than distant roots (b = 3 through 6) when root entry width is greater than the primary channel width, as shown in Figure 3.7A. Neurites also prefer to enter roots more than continuing up the primary channel as root entry width is increased (Figure 3.7B).

![Figure 3.7 Neurite preferences for roots with different entry widths](image)

Neurite preference as a function of root entry width was examined. (A) Neurite root preference ($PF_b$) is shown for root widths greater than (111.6 μm), equal to (100 μm), or smaller than (74.4 and 37.2 μm) the primary channel (100 μm) in the simulation. When root width is decreased the exponential decay of $PF_b$ decreases for increasing $b$ and $PF_b$ significantly decreases (paired one-tailed t-test, $p < 0.05$) for smaller root widths in $b = 1$. $PF_b$, however, is not significantly different for all root widths in $b = 2$. $PF_b$ differed significantly when comparing the same root widths for $b = 1$ and 2 (unpaired two-tailed t-test, $p < 0.05$) and $b = 2$ and 3 (paired two-tailed t-test, $p < 0.05$). (B) Probability of a neurite choosing a divergent trajectory ($PR_b$) decreases as root width decreases. $PR_b$ did not significantly differ along all root numbers when root widths are smaller than the primary channel (74.4 μm and 37.2 μm). Error bars are SEM.

3.5 Discussion

We have developed a simulation of growing neurites that matches neurite outgrowth from explants on a featureless substrate. This model uses outgrowth parameters from explant neurites, since explants will be used in the in vitro multiple root pattern. To achieve the goal of developing an experimentally
validated model, we incorporated experimentally-fit outgrowth parameters into the model.

3.5.1 Compare dissociated and explant neurite outgrowth

To compare the difference in neurite behavior between dissociated and explant neurites, we tracked the growth cone’s centroid migration under the same in vitro conditions (Figure 3.8A). Fitting MSD in vitro data to the theoretical persistent random walk model allowed us to estimate persistence time, RMS speed, and random motility coefficients for dissociated (Figure 3.8B & C) and explant neurons (Figure 3.8D-F).

This analysis indicates that root mean squared speed of dissociated neurites is significantly faster (unpaired one-sample t-test, $p < 0.05$) than explant neurons, while persistence time remains relatively constant (Table 3.4). DRGs will be used to model outgrowth dynamics and neurite root preference in all experiments, because the cell body (i.e., origin) of a dissociated neurite moves.

**Table 3.4 Comparison of dissociated and explant neurite outgrowth behavior**

Dissociated and explant neurite’s RMS speed and persistence time fit to theoretical persistent random walk model. Average RMS speed differed significantly (unpaired two-sample t-test assuming, $p < 0.05$) between dissociated and explant neurite but persistence time was not significantly different.

<table>
<thead>
<tr>
<th>In vitro neurites</th>
<th>Root Mean Squared Speed ($S$, $\mu$m/min)</th>
<th>Persistence Time ($P_v$, min)</th>
<th>Random motility ($\mu$, $\mu$m$^2$/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissociated (N = 2)</td>
<td>1.1±0.09</td>
<td>78±5.2</td>
<td>46±4.2</td>
</tr>
<tr>
<td>Explant (N = 3)</td>
<td>0.57±0.04</td>
<td>75±11</td>
<td>12±0.29</td>
</tr>
</tbody>
</table>
Figure 3.8 Growth cone centroid analysis of trajectory and mean squared displacement

Growth Cone centroid trajectory for dissociated (gray) and explant (black) neurites. Regression fit of the persistent random walk model (gray circles) and MSD (black line) for the dissociated (B & C) and explant (D-F) neurites. Insets plots show residuals from curve fits, which further confirms that the persistent random walk model is a good fit to MSD data. Error bars are SEM.

3.5.2 Pattern geometry selection and root entry width’s influence on neurite preference

To examine how root entry width influences neurite preference, we ran the same neurite simulations after changing the entry width of roots. We found that
neurite root preference, $PF_b$, exponentially decreases for roots farther from the neurite’s initiation point, as shown in Figure 3.7A. In addition as root width was decreased, $PF_b$ exponentially decreased less for increasing $b$. Because fewer neurites entered the first roots encountered when root width was decreased, more neurites reached subsequent roots, resulting in an increase in $PF_b$ for these roots. Conversely, increasing the root entry width allowed more neurites to enter $b = 1$ and less overall neurites to search or enter the successive roots, resulting in a smaller $PF_b$ for $b$’s $> 1$.

Another interesting finding was that $PF_b$ did not significantly differ for any root entry width at $b = 2$. In addition, we found that if the root entry width was greater than or equal to the primary channel width, then the probability of a neurite taking a divergent trajectory was significantly different (paired two-tailed t-test, $p < 0.05$) for all root numbers, shown in Figure 3.7B. The reasons of these results are unclear, but it may be due to neurite outgrowth uncertainty in the simulation.

It is unknown if root entry width plays an important role in guidance strategies for neurites that project from the spinal cord through the intervertebral foramina space or vise versa during development. If a mechanism of neurite guidance based on the intervertebral foramina space did exist then we would expect descending tract motor neurons to encounter larger intervertebral foramina from cervical to lumbar spinal segments. Even though intervertebral foramina area does increase down the spinal column (Cunningham, 1918) (e.g., from spinal segments L1 to L5 (Modi, Suh, Song, & Yang, 2008)) it is difficult to
discern whether intervertebral foramina space may influence neurite guidance since developmental studies have shown that spinal rootlets and vertebrate (i.e., intervertebral foramina space) develop in parallel (Hamburger & Hamilton, 1992; Shapiro, 1992). How this factor may play a role in guidance is an interesting unanswered developmental question.

Root entry width for future simulations was selected to match the anatomy of a chicken’s nervous system. Previous work on mapping the neural projections in a developing chicken hindlimb, shown in Figure 3.9 (Honig, Frase, & Camilli, 1998a; Wang & Scott, 1999), allowed us to determine the root entry and primary channel widths.

![Figure 3.9 Patterns of sensory and motor neuron projections into the peripheral and central nervous system of a chicken embryo](image)

Figures taken from Ref. (Honig et al., 1998a) and (Wang & Scott, 1999) illustrate the pattern of sensory and motor neurite organization along the spinal column and roots. (A) Whole-mount view of the crural plexus and major nerve trunks in the anterior thigh of an embryo; anterior is at the top. DRG in the embryo was injected with Dil at Stage 31 (E7); Dil was photoconverted to a brown reaction product. Sart, sartorius nerve; Femo, femorotibialis nerve; Addu, adductor nerve; CFL, cutaneous femoralis lateralis nerve; CFM, cutaneous femoralis medialis nerve, LS, lumbrosacral. (B) Double immunofluorescence indicates how sensory axons, yellow, and motoneuron axons, red, are organized along the spinal root and spinal column. The image is a cross section from the right side of a stage 29 embryo. This embryo was sectioned transverse to the long axis of the left thigh. Spinal nerves LS1, LS2 and LS3 are shown arising from their respective DRGs, coursing ventrally and distally, and then meeting and projecting posteriorly, toward the tail. In each spinal nerve, sensory axons are grouped into several bundles that are interspersed with motoneuron axons. (black scale bar = 200 μm and white scale bar = 100 μm).
Through comparison of dorsal rootlet and spinal column widths from Figure 3.9A, we found that the spinal column (primary channel) is approximately twice as large as the rootlets in the lumbrosacral segments. Therefore, we chose a root entry width of 74.4 µm, (root width, 50 µm) and a primary channel width of 100 µm (i.e., primary channel is twice as large as the root width) to simulate the anatomy of a chicken spinal roots and column. Even though root width may vary depending on the root or position along the spinal column, as shown in Figure 3.9B, our simulation can be easily modified to match the boundary conditions of the neural system in question. By using the initial and boundary conditions developed in this model, future work will examine how to increase the number of neurites that reach and enter a specific target.
4 ENGINEERED IN VITRO MODEL TO EXAMINE NEURITE TARGET PREFERENCE

4.1 Chapter 4 Summary

The main focus of this chapter is on the development and analysis of an *in vitro* model of neurite outgrowth on a complex micropattern. In this model neurites from E7 dorsal root ganglia (DRG) explants were grown on a laminin micropattern designed to mimic branching in a spinal column, identical to the *in silico* model described in chapter 3. The micropattern design allows for neurites to enter any of the alternate pathways (“roots”), and is organized so that neurite root preference can be evaluated. Neurite root preference decreased exponentially for roots farther from the neurite’s initiation point, which agrees with results from the *in silico* model. These results help to establish a predictive *in silico* model for testing and comparing how restricting neurites from entering undesired roots affects guidance, which will be evaluated in chapter 5.

4.2 Background

Potential therapies for spinal cord injuries (SCIs) have involved the promotion of axon extension across the non-restorative injury site (Davies et al., 1999; Bradbury & McMahon, 2006; Thuret et al., 2006). The success of these approaches depends in part on the untested assumption that once regrowth of neurites is achieved useable dendritic and axonal connectivity will be made, thus restoring function. Axons in the adult spinal column, however, have a very large
number of paths to choose from, most of which are unsuitable for achieving functional recovery, e.g., efferent paths for afferent neurites or proximal spinal roots for motor pathways destined for distal targets. Therefore, a critical step to improve functional recovery is to increase the likelihood of neurites entering useable spinal roots by developing effective neurite guidance strategies (Harel & Strittmatter, 2006; Frank, 2006; Webber et al., 2008).

During development, a neuron’s growth cone, dynamic motile element located at the tip of the axon, searches for its synaptic targets (Sperry, 1963; O’Connor et al., 1990; Mueller, 1999). The growth cone is directed to viable targets by its ability to sense chemical and contact guidance cues (Tessier-Lavigne & Goodman, 1996; Chilton, 2006). While some guidance cues exist after development, their distributions differ, especially following an injury (Koeberle & Bahr, 2004; Harel & Strittmatter, 2006). These changes in cue distribution modify the environmental signals and affect the guidance of any regenerating neurites. In addition, following SCI, multiple factors prevent neurite regeneration, including: the release of inhibitory factors (Nogo), axon demyelination, apoptosis, and physical barriers (cysts, glial scaring, or cavities) that may be developed post-injury. Consequent restricted neurite outgrowth, growth cone collapse, and axonal degeneration further hinders regeneration of neurites resulting in sustained partial or total loss of sensory or motor function (Snow et al., 1990; Jones et al., 2005; Silver & Miller, 2004).

Animal studies have shown that axons are able to grow past an injury site along uninjured or protected myelinated motor and sensory routes through the
The use of cellular transplantation and/or neurite exposure to growth promoting neuroprotective factors (Xu et al., 1997; Davies et al., 1999; Thuret et al., 2006; Silver & Miller, 2004). Increased outgrowth past the injury site is also achieved by neutralizing factors that cause inhibitory effects such as myelin-associated glycoproteins (e.g., Nogo-A) or components of the glial scar (e.g., chondroitin sulfate proteoglycan, CSPG) near the injury site (Bradbury & McMahon, 2006). Many SCI researchers agree that a therapy combining these strategies described above will be necessary to successfully promote growth past the injury site and synaptic rewiring; however, if neurites fail to reach suitable targets full functional recovery will be restricted (McClellan, 1999; Harel & Strittmatter, 2006; Thuret et al., 2006). To establish functional recovery, we propose that in addition to these therapies, neurites will need to be guided to suitable targets; otherwise, neurites tend to form aberrant paths and may not produce usable connections (McClellan, 1999; Harel & Strittmatter, 2006; Thuret et al., 2006). In support of this proposal, researchers have recently shown that a combination of developmental chemotropic guidance and strategies to promote regeneration past the injury can successfully help guide regenerating neurites to synapse with their appropriate targets in the brainstem (Alto et al., 2009).

We highlight the importance of neurite guidance by examining the probability that a regenerating axon reaches its desired target. Consider the case of a regenerated motor neurite originating in the brain and destined for the first lumbar spinal segment. To reach the desired root, the regenerating neurite must avoid the first 20 cervical and thoracic spinal segments encountered. If the
probability of taking the correct path is $p$ at each decision point, then the probability of reaching the lumbar roots is $p^{20}$, or less than 1 in a million for the illustrative case when $p = \frac{1}{2}$. To direct new neurites to viable targets, future regenerative therapies will therefore require the incorporation of guidance strategies.

These therapies will benefit from mathematical and computational assessment of how successful neurites are in reaching a desired target. In particular, simulations allow for a non-invasive, efficient, and cost effective way to test therapeutic strategies for treating CNS injuries. Some previous computational models of neurite migration, developed from in vitro experiments, have helped us to understand mechanisms of motility, neurite polarity, and how environmental cues (e.g., haptotactic or chemotactic gradients) affect guidance (Katz et al., 1984; Buettner & Pittman, 1991; Buettner et al., 1994; Wang et al., 2003; McLean et al., 2004; Forciniti et al., 2009; Mortimer et al., 2009). In addition, neurite targeting has been examined from computational models based on growth cone cytoskeletal and filopodial dynamics (Buettner, 1996). Work in our laboratory has demonstrated that growth cone migration can be effectively simulated using an integrated random walk (Maskery et al., 2004; Maskery & Shinbrot, 2005).

In the present work, we calibrate this latter model, setting parameters to agree with neurite outgrowth statistics in experiments of chick dorsal root ganglion (DRG) neurons grown on a uniform laminin substrate. This simulation, calibrated on a featureless substrate, is then applied to a patterned substrate
containing numerous side channels intended to crudely mimic roots in the spinal column. We compare the simulation side-by-side with *in vitro* experiments using chick DRG explants in identical laminin micropattern geometry. We emphasize that the simulations are calibrated on a uniform substrate, while the comparisons between simulations and experiments are performed on patterned substrates. We therefore use the final simulation to quantitatively evaluate the hypothesis that the probability of neurites reaching subsequent side channels, or roots, decreases exponentially (i.e., as $p^n$, where $p$ is a fixed probability and $n$ is the number of roots from the originating explant). This theoretical expectation is not a forgone experimental conclusion, because real neurites fasciculate, cling to boundaries, wander in a pulsatile fashion, and otherwise exhibit behaviors that might be inconsistent with the expectation.

In this chapter, we: (1) confirm that neurites grown *in vitro* obey the probabilistic rules outlined above, (2) evaluate the actual probability, $p$, exhibited in a branching pattern with multiple sequential roots, and (3) produce an experimentally validated *in silico* simulation to facilitate evaluations of future guidance strategies. This work will further the field of neurite guidance by showing that neurites preference for distant targets decreases exponentially in complex geometries. We hope that by applying this rule when developing therapies, future guidance strategies will increase the number of neurites that reach their targets, thus improving functional recovery.
4.3 Methods and Materials

4.3.1 Master creation

The multiple root micropatterned geometry that we use for the *in vitro* work is shown in Figure 4.1.

![Figure 4.1](image)

*Figure 4.1 DRG neurites grown on a spinal column resembling micropattern*

(A) *In vitro* growth of DRG neurons (neurofilament, yellow) on a micropattern (laminin, green) designed to emulate roots branching off of the spinal column. Blue, dapi, stain of nuclei is indicated by red arrow. (B) Enlarged view of gray box visible in upper right of (A). (white scale bars = 500 µm and 200 µm respectively in A and B)

The pattern was designed in AutoCAD (LT 2002, San Rafael, CA) and made into a dark chrome mask (Microfabrication Laboratory, University of California at Berkeley). The mask was used to make photoresist trenches (70 µm) on a silicon wafer master using photolithography. To create the master, a silicon wafer was first cleaned and pre-baked at 300°F for two hours. 2025 SU-8 negative photoresist was spin coated and soft baked with a temperature ramp from 65°C to 95°C for two minutes. The mask was aligned to the master using
an EV Group 620 Mask Aligner and exposed to UV light for 18 seconds. Following exposure, the silicon wafer was baked at 65°C for three minutes and ramped up to 95°C for seven minutes. The wafer was immersed in developer for 10 minutes to remove unexposed photoresist and cleaned with isopropanol. Following creation of the master, an elastomer base and curing agent (SYLGARD elastomer kit (Dow Corning, Midland, MI)) were mixed in a 10:1 ratio (w/w), poured over the master and cured at 65°C overnight. The resulting polydimethylsiloxane (PDMS) was peeled off the master manually and cut into segments to make PDMS stamps. Before placing the stamps onto glass coverslips for protein coating, the PDMS was cleaned by sterile autoclaving, sonicating in 70% ethanol for 30 minutes, and UV treating.

4.3.2 Protein patterning

PDMS stamps were first placed on a 22 mm cover glass (Fisher Scientific). The underside of the cover glass was marked at the center of the pattern with a marker to guide where to place the DRG (Figure 4.1A) when plating. The assembly was treated with oxygen plasma with the stamps in place on the coverslip, in a PX-250 plasma generator with an environment of 60% O₂ and 40% N₂ for 60 seconds at 670 mtorr and 100 Watts. The MicroMolding in Capillaries (MIMIC) process (Kim et al., 1996) was used to coat the coverslip with laminin (25 µg/ml). Following 4 hours of incubation at 37°C in 5% CO₂ the stamp was removed and the cover glass was washed three times with a F12/DMEM 1:1 mixture. 0.5% bovine serum albumin (BSA) was then added for 30 minutes at room temperature (Thompson & Buettner, 2001). BSA helped to restrict neurites
from growing off of the pattern. In addition, BSA does not bind to the laminin protein and when BSA adsorbs onto a surface in the presence of laminin it does not interfere with the permissive laminin pattern (Esch, Lemmon, & Banker, 1999; Dertinger, Jiang, Li, Murthy, & Whitesides, 2002). The laminin pattern was confirmed by indirect immunostaining (Figure 4.1B, green overlay) and replicated the design of 100 µm wide primary channel with 50 µm wide roots at 45° from the primary channel.

4.3.3 Explant culture

DRGs were dissected from the lumbar region of embryonic day seven (E7) specific pathogen free chickens (Charles Rivers) in cold calcium and magnesium free (CMF) Hank’s balanced salt solution (HBSS) under a dissection microscope. A single whole DRG was then plated on the laminin-patterned glass in 600 µl of serum free media (SFM) for eight hours followed by the addition of 1.4 ml of SFM. SFM is composed of 1:1 v/v DMEM/Ham’s F-12, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin, 10 mg/ml glucose, 1X MEM vitamins, 20 µg/ml N3, 1X ITS+1 premix (100 µg/ml insulin, 55 µg/ml transferrin, 50 ng/ml selenium), 0.1% BSA, 50 ng/ml neural growth factor (NGF) (R&D Systems, 556-NG), and 100 mM ascorbic acid. N3 consists of 5 ml N2 supplement (Invitrogen, 17502048), 250 µl 1X ITS+1 premix, and 10 µl of 2 mg/ml corticosterone. All reagents were purchased from Sigma unless stated otherwise.

4.3.4 Immunostaining

DRGs grown on the micropatterned laminin substrate for 3-4 days were fixed with 4% paraformaldehyde for 15 minutes and then washed with CMF PBS.
Samples were blocked with 10% normal goat serum (Sigma, G9023) in immunobuffer (1% BSA, 0.5% Triton-X100, and CMF PBS) for 1 hour. Laminin and neurofilament were labeled using indirect immunostaining. Samples were incubated in primary rabbit anti-laminin (1:100, Sigma, L9393) and primary mouse anti-neurofilament (1:1000, 68 kDa, and 1:5000, 200 kDa, Sigma) for 1 hour and then washed before adding secondary FITC goat anti-rabbit (1:100, Sigma, F0382) and Alexa 546 goat anti-mouse (1:250, Invitrogen, A11018) for 45 minutes. In some cases, Alexa 647 conjugated phalloidin (1:100, Invitrogen, A22287) was added for 20 minutes to examine growth cone density. Nuclear stain DAPI (100 ng/ml, Sigma, D9542) was added for 20 minutes before mounting coverslips onto slides using inkfusing Fluoro-Gel (Electron Microscopy Science, 17985-10).

4.3.5 Microscopy and image processing

Fluorescence and difference interference contrast (DIC) images were acquired using a monochrome Zeiss AxioCam, CCD camera mounted onto an inverted Zeiss 200M deconvolution inverted microscope (Rutgers Center for Collaborative Neuroscience) with a 20x objective (Plan-apochromat). Fluorescence images were captured at a constant exposure time of 300 – 500 ms with a scale of 0.3263 µm/pixel. Neurofilament grayscale images were used to calculate neurite preference for each root from neurofilament pixel area.

All original grayscale neurofilament and DIC images were converted into binary images (values of 0 and 1) using a segmentation algorithm programmed in MATLAB (MathWorks Inc., Natick, MA) before calculating areas. MATLAB’s
Image Processing Toolbox was used for all image processing. Original neurofilament fluorescent images contained background fluorescence that concealed neurite information and caused incongruous segmentation. Background fluorescence was partially removed by subtracting the background of the image, found by morphologically dilating and then eroding the original grayscale image (MATLAB’s imopen function). Dilation assigns the maximum pixel value to all pixels within the local structure element (neighborhood), while erosion assigns the minimum pixel value to all pixels within the neighborhood (Gonzalez & Woods, 2002; Costantino et al., 2008). To avoid including background in neurofilament calculations, a disk-shaped structure element with a 100-pixel radius (> 30 µm) was selected for dilation and erosion. The background-reduced image was then filtered using a local Laplacian and nonlinear range filter to create two new images that respectively isolated fine neurofilaments and neurofilament edges.

Neurofilament segmentation was accomplished by partitioning the grayscale pixel values of all three processed images into two separable clusters (background and neurofilament) using MATLAB’s kmeans function. K-means clustering calculates two cluster centroids, chosen initially to be 0 or 1, by minimizing the sum of squared Euclidian distances between pixel values and cluster centroids. Algorithmically, first each pixel is assigned to the cluster centroid with the smallest distance between the pixel value and cluster centroid. Then after all pixels are assigned to a cluster centroid, the pixel values within each cluster are averaged to recalculate each centroid. These two steps, pixels
reassignment to cluster centroids and centroid position recalculation, are repeated until the centroids’ positions remain unchanged. The resulting binary image of neurofilament is shown in Figure 4.2.

![Figure 4.2 Binary neurofilament image and measurements](image)

**Figure 4.2 Binary neurofilament image and measurements**

Binary image of neurofilament stain overlaid on top of laminin pattern boundaries (gray line border). Total area within each root, $T_{A_b}$, is measured by manually selecting regions of neurofilament within a root number, $b$, (dotted white box in $b = 3$, $T_{A_3}$). Neurofilament areas within a fixed region of the root and primary channel were also measured respectively to the right and left of the root bifurcation point (dashed white boxes in $b = 1$, enlarged view of boxes labeled $AR_1$ and $AL_1$). Comparison of areas is used to examine neurite trajectory and root preference at each bifurcation point. (white scale bar = 200 µm)

To validate whether neurofilament stain is an accurate metric for neurite area, we compared the areas from image processed DIC images to neurofilament images. Background from the DIC images was first subtracted from the original image using the same morphological operation, imopen, described for neurofilament images. The new image was then filtered separately by a local entropy (9 x 9 neighborhood) and a standard deviation (5 x 5 neighborhood) filter after observing pixel value randomness and variance along the axons (Hamahashi, Onami, & Kitano, 2005). These two images along with
the background reduced DIC image were segmented into two pixels clusters, ‘cells’ and ‘background’, using the same k-means clustering method described earlier. Laminin stain was also segmented to isolate fluorescence using k-means clustering to visualize the pattern’s edges while selecting regions of interest.

4.3.6 Calculating neurite preference and fasciculation

The binary neurofilament images produced were used to calculate the probability of root entry by any neurite, \( PE_b \), for each root, where \( b \) defines the order of roots (i.e., root numbers) from the entrance into the primary channel. \( PE_b \) was calculated by dividing the frequency, \( M_b \), of any continuous neurofilament observed to enter 20 microns past a root entry by the total number of samples measured (\( N = 20 \) replicates).

Neurite preference for a particular root, \( PT_b \), was calculated by dividing the total neurofilament area, \( TA_b \), in a root, \( b \), by the total neurofilament area within all roots, \( \sum TA_b \), for both neurofilament and DIC binary images. \( TA_b \) was measured by counting the number of white pixels within an outlined region from the \( b \)-th root entry to the farthest-reaching neurite tip within the side borders of the root (dotted white box in \( b = 3 \) of Figure 4.2). Side borders were determined by overlaying the laminin binary image onto the neurofilament and DIC images. Roots with neurites over 50 microns away from the root entry and with no neurites observed in them were not measured.

Since the \( PT_b \) measurement increased the bias of neurite root preference for neurites that traveled farther up the root (shown in Figure 4.2), we developed a new bulk area measurement to reduce the biasing. The new neurite root
preference measurement, $PF_b$, is based on neurofilament area a fixed distance into the $b$-th root, $AR_b$, and is calculated by dividing $AR_b$ by the total fixed neurofilament area within all roots, $\sum AR_b$. The fixed area within $AR_b$ is 400 pixels along the length of the root, starting from the root entry, and 400 pixels wide ($1.6e5$ pixels = $1.7 \text{ cm}^2$), as shown in Figure 4.2’s dashed white box labeled $AR_1$ to the right of the primary channel.

A neurite’s probability to enter a root, $PR_b$, (i.e., divergent trajectory) instead of continuing to grow along the primary channel, $PL_b$, (i.e., straight trajectory) was also examined. $PR_b$ was calculated by comparing $AR_b$ to the neurofilament area within a fixed area in the primary channel, $AL_b$. $AL_b$ is the number of neurofilament pixels within a 400 by 400 pixel square region of the primary channel bordering the $AR_b$ region. All regions selected are equal in area (dashed white boxes labeled $AR_1$ and $AL_1$ for $b = 1$ in Figure 4.2). $AR_b$ and $AL_b$ were used to calculate $PR_b$ for each $b$ in the equation below:

$$PR_b = AR_b / (AR_b + AL_b), \quad (4.1)$$

$AR_b$ and $AL_b$ selected regions are equal in area. $PR_b$ is analogous to $p$ mentioned in the introduction for $b = 1$ only.

The number of neurite bundles formed from neurites that have fasciculated was also determined using a 400 pixels long profile plot parallel to the primary channel at the root entry. Because neurite bundles are larger than a single axon, each continuous white pixel was classified as a bundle of axons if
larger than a single axon. The frequency of bundles, $B_b$, at $b$ for all samples was then counted and divided by the total number of samples ($N = 20$ replicates) to estimate the average number of neurite bundles, $F_b$.

4.3.7 Compare neurite preferences calculated from different neurite area measurements

Using fluorescent microscopy, we observed that neurites could overlap. These neurites may have been neglected when measuring bulk neurite area, 2D area, to calculate neurite root preference, $PF_b$, as described in the previous section. $PF_b$ was recalculated with a new area measurement that includes the overlapping neurites. This new area, z-stack area, was measured from the cross-section of neurites (orthogonal to the xy-plane) along the root entry of a z-stack image. Z-stacks were acquired using a 63x oil emersion objective (0.3 µm/pixel) on an Olympus IX2-DSU spinning-disk confocal microscope. Images were imported into ImageJ using the LOCI plugin. A straight line was drawn perpendicular to the neurites within the fluorescently labeled root using the straight-line tool. The cross-section image of the z-stack was reconstructed using the reslice tool and then processed into a binary image using k-means clustering. A contrast enhanced and background removed image, original image with the morphologically dilated and eroded image subtracted, was used to create the binary image. Objects in the image that were considered to be non-neurite were removed using the morphological operation imopen, with a disk-shaped structure element (diameter ≤ 1 µm (Bergers et al., 2002)). The probability of a neurite choosing a divergent trajectory, $PR_b$, was also
recalculated as described in the previous section using z-stack area measurements of neurofilament at root entries, $AR_b$, and after the roots in the primary channel, $AL_b$. Then $PF_b$ and $PR_b$ were calculated and compared for both area measurements: 2D bulk and z-stack.

### 4.3.8 Computational simulation

The computational model of neurite outgrowth is based on a 2D random walk, where each progressive iteration, $i$, is dependent on the previous one. Neurite outgrowth is adjusted based on the three parameters: average outgrowth, $r_d$, outgrowth uncertainty, $r_s$, and current direction, $\theta_i$, of a neurite’s tip described in the previous section and shown in Figure 3.3A. The distance parameter, $r_i$, is the sum of the average outgrowth, $r_d$, and the outgrowth uncertainty, $r_s$, which are respectively gamma and Gaussian distributed. The maximum uncertainty of outgrowth is $r_s$. The current direction is modified by the growth cone’s search angle, $\delta_i$, to the left and right of its current angle, $\theta_i$, as illustrated in Figure 3.3A. The experimentally fit distributions from time-lapse data were incorporated into the simulation, and values were randomly generated during the simulation using MATLAB’s Statistical Toolbox. In this way, the simulated growth cones were engineered to produce identical statistics to those seen in vitro on a featureless substrate. Polar coordinates ($r_i, \theta_i$) are converted to Cartesian coordinates before adding the next growth to the current position as shown in equations 3.2 and 3.3.

When a neurite encounters a non-permissive boundary (shown in Figure 3.3B), the neurite reverts back to its previous position ($x_i, y_i$) and direction ($\theta_i$). A
new direction is then recalculated from summing the new search angle ($\delta_{\text{adj}}$), which is randomly selected from a Gaussian distribution, and previous direction ($\theta_i$). If the new neurite tip position evaluated encounters the edge again then these two steps are repeated until the boundary is avoided. Once an angle is selected that allows the neurite tip to avoid the edge, the neurite tip continues migrating based on the previously described persistent random walk. The simulated change in direction of the neurite tip at the non-permissive edge is based on previous experimental observations examining growth cone turning behavior, which has been described as the growth cone reorienting along the substrate edge and then growing along or away from the edge (Tanaka & Kirschner, 1995).

The simulation was initiated by placing a number, n, (n = 100 in the simulations for each case studied) of neurites at random positions along the base of the pattern with neurite current angles parallel to the primary channel’s edge. Each neurite grows for a different number of iterations, simulating the time-dependent neurite entrance into the micropattern environment. When a simulated neurite enters a root, it is not permitted to turn backwards, and may only continue moving forward. This concurs with observations from time-lapse videos using micropattern stripes 100 µm wide or smaller. Simulations of growth cone outgrowth were run with 100 neurites (1 iteration = 30 seconds and 1 unit = 174 µm), producing results such as those shown in Figure 3.4. Red and blue circles represent the respective neurite end points and origins. Each simulation of 100 neurites is replicated 20 times. The simulation ran for an equivalent
number of iterations that matched 2.5 days, the average time from when neurites entered the primary channel until neurites reached $b = 8$ in vitro.

### 4.3.9 Calculating in silico neurite preference

Neurite preference measurements from experimental and simulated data were compared to assess the accuracy of the simulation. Evaluation of neurite preferences for the simulation was calculated and modified to match the four different experimental neurite preference assessments: $PE_b$, $PT_b$, $PF_b$, and $PR_b$. Both $PE_b$ and $PT_b$ measurements were calculated in identical ways for experimental and simulated data. Similar to experimental samples, $PE_b$ for the simulation could be observed from the output. $PE_b$ was calculated by dividing the frequency, $M_{i+1}$, of root entry by any neurite ($x_{i+1} > 0.588$ units and $b > y_{i+1} > b - 0.422$) in all samples (N = 20 replicates) by the total number of samples. In addition, $PT_b$ for the simulation was evaluated similarly as experimentally measured $PT_b$ because the total neurite area in each root, $TA_b$, could be estimated from the total distance a neurite traveled in a root. $TA_b$ for the simulation’s $PT_b$ measurement was calculated by summing the distance traveled by all neurites within $b$ from the root entry ($x_{i+1} > 0.588$ units) until the end of the simulation. Because individual neurites could be identified in the simulation we did not need to use indirect estimates of neurites, as done in experiments.

Therefore, $PF_b$ was calculated in the simulation as the fraction of neurites that entered a root, $N_{r_b}$, relative to the total number of neurites that entered all roots, $\sum N_{r_b}$. $PR_b$ was calculated in the simulation as $N_{r_b} (x_{i+1} > 0.588$ units and $b > y_{i+1}$
> b-0.422) divided by the number of neurites that entered, \( N_{rb} \), and avoided the root, \( N_{lb} \), i.e., took a straight trajectory (\( N_{lb} = N_{lb+1} + N_{rb+1} \) or \( N_{lb} = 100 - \sum N_{rb} \)).

### 4.3.10 Compare simulation to experimental data using curve fitting

Experimental and simulated \( PT_b \) and \( PF_b \) were fit to exponential curves, \( y = ae^{b} \), using MATLAB’s Curve Fitting Toolbox, where \( y \) is either \( PT_b \) or \( PF_b \), \( b \) is the root number, and coefficients \( a \) and \( b \) are constants fit to data points using the least squares method. Both exponential curve fits were also compared between the experiment and simulation to check if the exponential coefficients matched within a 95% confidence interval. Experimental and simulated probabilities of neurites entering a root at a decision point, \( PR_b \), were fit to a line, \( PR_b = \xi b + \lambda \), where coefficients \( \xi \) and \( \lambda \) are constants fit to data points using least squares. \( PR_b \) was fit to a line to examine if the same linear trend observed from experimental data was captured in simulations.

### 4.4 Results

#### 4.4.1 Probability that a root is entered by one or more neurites (\( PE_b \))

\( PE_b \) was evaluated to examine if neurites enter a particular root more often than others. Root entry by neurites was observed more frequently for nearby than distant roots in both in vitro and in silico models (Figure 4.3).
Figure 4.3 Probability of root entry by one or more neurites (PE_b)
Neurite entry into a root was determined if any neurofilament was detected in the root. Experimental and simulated data were both averaged for 20 replicates. Error bars are standard errors of the mean (SEM).

The average probability of experimental root entry of neurites into b, PE_b, decreased linearly (PE_b = -0.12b + 1.1, for b = 1 through 6 inclusive, r^2 = 0.97, N = 20). For simulations, PE_b decreased less rapidly with increasing b (PE_b = -0.020b + 1.0, b = 1 through 6 inclusive, r^2 = 0.84, N = 20), as shown in Figure 4.3. On average, at least one neurite was observed to enter each of the first three roots in the simulation for every sample (M_b = 20 for all 20 replicates, when b = 1 through 3 inclusive).

4.4.2 Neurite root preference (PT_b & PF_b)

Neurite root preference was measured to examine if it exponentially decreased for increasing b and if the simulation matched the experimental data. Neurite root preference was observed to decrease exponentially with increasing root number according to y = ae^{bb}, in the first eight roots for both experiments and simulations. As shown in the inset plot to Figure 4.4, neurite root preference, PT_b, which is based on neurofilament area, decreases exponentially as a function of increasing root number.
Figure 4.4 Neurite root preference (PT$_b$ & PF$_b$)
Percent of neurofilament area occupied within a fixed distance into the first eight roots is shown for both experimental (black squares) and simulation (gray triangles) data. Data are fit to exponentials indicated: $r^2$ values for experimental and simulation data are 0.955 and 0.996 respectively. Experimental PF$_b$ was significantly larger for the labeled $b$ when compared to the next root number (unpaired one-tailed t-test, $p < 0.05$, N = 20). Similar results were found using a multiple comparison test with Tukey’s least significant difference procedure along a 95% confidence interval. Percent of total neurofilament area, PT$_b$, in the first eight roots is shown in the inset. Error bars are SEM.

Neurofilament stain was found to be a valid indicator for neurite location by comparing $PT_b$ calculated from DIC and neurofilament images for all $b$ (N = 13), shown in Figure 4.5.

Figure 4.5 Comparison of DIC and Neurofilament PT$_b$
Percent of DIC and neurofilament area occupied within each of the first eight roots, PT$_b$, is shown for experimental data. Data are fit to exponentials indicated: respective $r^2$ values for DIC (black circles) and neurofilament (NF, gray triangles) data are 0.955 and 0.996. Residuals for each fit are plot in the inset. Error bars are SEM

Calculation of PF$_b$ also confirmed that the exponentially decreasing trend is based on root number and is not an artifact of neurites extending farther along
nearby than distant roots. Both experimental \((r^2 = 0.955)\) and simulated \((r^2 = 0.996)\) data fit to exponential curves, as shown in Figure 4.4. In addition, the coefficients, \(\alpha\) and \(\beta\), for both exponential curves overlapped within 95% confidence intervals of each other and both \(PF_b\) data were not significantly different for all \(b\) \((N = 20)\). Significantly larger experimental \(PF_b\) was found for \(b = 1\) and \(3\) when compared to there next respective root number (unpaired one-tailed t-test, \(p < 0.05\), \(N = 20\)), as shown in Figure 4.4.

4.4.3 Percent of neurites that choose a divergent trajectory (\(PR_b\))

\(PR_b\) was evaluated to examine if it was constant for all roots and if the simulation matched the experimental \(PR_b\). The fraction of neurites that will choose a divergent trajectory, \(PR_b\), remained relatively constant in simulations (average \(PR_b\) for all roots 0.18±0.01) but decreased for increasing \(b\) in the experiments. As shown in Figure 4.6, experimental \(PR_4\) differed significantly to the previous three roots (unpaired two-tailed t-test, \(p < 0.05\), \(N = 20\)). In addition, experimental \(PR_5\) differed significantly between \(b = 1\) or \(3\) (unpaired two-tailed t-test, \(p < 0.05\), \(N = 20\)), as shown in Figure 4.6. Experiment and simulation average \(PR_b\) data did not differ significantly for \(b = 5\) and \(6\) (unpaired two-tailed t-test, \(p > 0.90\), \(N = 20\)). Both experiments and simulations suffered from the same increased uncertainty caused by small sample sizes (i.e., that few neurites reach distant roots) far from the explant. To limit this variability, roots 7 and 8 were excluded from the analysis.
Figure 4.6 Probability of neurites choosing a divergent trajectory (PR\_b)
Probability of neurites choosing a divergent trajectory for the first six roots showed a linear trend for experimental and simulated data. Experimental (black squares) and simulation (gray triangles) data fit within the predicted 95% confidence intervals of their respective linear curves. Experimental PR\_b was significantly larger for the labeled b when compared to b = 4 (unpaired two-tailed t-test, \( \ast \) p < 0.05, N = 20). In addition, experimental PR\_b differed significantly for the labeled b when compared to b = 5 (unpaired two-tailed t-test, \(*\ast\) p < 0.05, N = 20). Similar results were found using a multiple comparison test with Tukey’s least significant difference procedure along a 95% confidence interval. Error bars are SEM.

4.4.4 Comparison of data from 2D bulk and z-stack area

Two different neurofilament area measurements, 2D bulk and z-stack, were compared for PF\_b and PR\_b to validate whether the 2D bulk area accurately captured the experimental neurite preference along the multiple root pattern. Neurite root preference was observed to decrease exponentially with increasing b for both 2D bulk, PF\_b\_2D, and z-stack, PF\_b\_Zstack, area measurements (N = 5), as shown in Figure 4.7A. Both area measurements fit significantly to exponential curves (PF\_b\_2D, \( r^2 = 0.95 \), and PF\_b\_Zstack, \( r^2 = 0.94 \)) and the coefficients, \( \alpha \) and \( \beta \), for both exponential curves overlapped within 95% confidence intervals of each other. PF\_b for b = 4 and 6, however, differed significantly when comparing different area measurements (paired two-tailed t-test, \( p < 0.05 \), N = 5), as shown in Figure 4.7A. On the other hand, PR\_b was not significantly different when comparing different area measurements for all b (N = 5), as shown in Figure 4.7B.
Figure 4.7 Compare neurite preference for z-stack area to 2D bulk area measurements

$PF_b$ and $PR_b$ were compared along all root numbers for two different area measurements: 2D bulk and z-stack. (A) Data fit to exponential curves indicate respective $r^2$ values for 2D bulk (black squares) and z-stack (gray triangles) $PF_b$ to be 0.95 and 0.94. Different area measurements for $PF_b$ differed significantly for $b = 4$ or 6 (paired two-tailed t-test, $p < 0.05$, $N = 5$). (B) $PR_b$ for the first six roots showed a similar linear decreasing trend for 2D and z-stack area data. 2D and z-stack $PR_b$ data were not significantly different for all $b$. Error bars are SEM.

### 4.4.5 Neurite fasciculation

Neurite bundle caliber at the root entry was measured to quantify if there was a trend of decreasing fasciculation (i.e., smaller axon widths) for increasing $b$ in vitro. Fasciculation was observed within the first roots encountered along the primary channel from time-lapse data (data not shown), indicating that neurites grew over each other. In addition, neurofilament binary images showed that neurites had 2-3 times larger calibers for roots closer than farther from the entry of the primary channel. The maximum width of these bundles was observed to decrease in roots farther up the pattern, as shown in Figure 4.8 inset.
Fasciculation was established by measuring the width of neurofilament stained axons at the entry of all roots. The maximum bundle width (inset) and average number of bundles (main plot) both decreased with distance from the explant. Maximum bundle widths (inset) were significantly larger for the labeled $b$ when compared to the next root number (unpaired one-tailed t-test, $*p < 0.05$, $N = 20$). Similar results were found for a multiple comparison test using Tukey’s least significant difference procedure along a 95% confidence interval. Error bars are SEM.

For example, maximum bundle width was significantly larger for $b = 1$ and 3 when compared to the next root number (unpaired one-tailed t-test, $p < 0.05$, $N = 20$), as shown in Figure 4.8 inset. In addition, as shown in the main plot of Figure 4.8, the average number of neurite bundles, $F_b$, at the root entry remained at one or more until the fifth root ($b < 5$), where no bundles (average number of neurite bundles $\leq 1$) were observed ($N = 20$ replicates).

### 4.5 Discussion

#### 4.5.1 Bulk neurite area measurement to examine neurite root preference

We examined neurite root preference on a micropattern with multiple roots by estimating the number of neurites in each root from a bulk measurement of neurofilament area. This measurement allowed us to avoid the intrinsic difficulties of counting overlapping neurites. Neurofilament area was selected as an appropriate marker for mature neurites and used to estimate neurite root
preference at a fixed time point (3-4 days). In addition, the neurofilament stain allowed us to estimate the number of fasciculated neurites (Honig, Petersen, Rutishauser, & Camilli, 1998b; Snow, Smith, Cunningham, McFarlin, & Goshorn, 2003), and distinguish between single or bundled neurites at the root entry.

Two alternative approaches, time-lapse microscopy and staining of the growth cones, were also attempted to quantify neurite root preference. Both approaches, however, yielded unreliable results. For example, time-lapse microscopy of explants allowed us to see neurites as they entered a root. But due to overlapping neurite trajectories, we found that some growth cones traveled up a pioneer axon or bundle of axons undetected. This was indicated by temporary expansions in the bundle width coinciding with filopodial projections. Additional investigations were attempted using dissociated cultures in place of intact explants, to reduce the density of neurites and permit accurate counting. But time-lapse images revealed constantly moving cell bodies that interfered with our ability to detect where a given growth cone originated, and hence how many roots a given neuron passed. Another approach was to count the number of growth cones within a root stained with phalloidin, which identifies F-actin. These stains, however, prevented us from discerning single neurite tips since growth cones fasciculate and also grow on top of each other.

4.5.2 Comparison of in vitro and in silico neurite root preference

We compared simulated and experimental neurite root preference, by normalizing the total neurofilament area within each root relative to that of all roots. Both simulated and experimental data fit to an exponential curve. This
was expected for the simulation because each root entry is independent and neurite motion is stochastic. In experiments, however, the same conclusion was unpredictable since neurite trajectories are often modified by other previous neurites’ outgrowth through neurite-neurite interactions (Wiencken-Barger, Mavity-Hudson, Bartsch, Schachner, & Casagrande, 2004). Nevertheless, our data indicates that these neurite-neurite interactions (Hentschel & van Ooyen, 1999) have negligible affect on the exponentially decreasing neurite root preference measurement, as shown in Figure 4.4 and Figure 4.7A.

While the fraction of total neurofilament area helped to validate similarities and differences between the simulation and experimental data, the measurement also assessed the distance neurites traveled into each root. Therefore, a new experimental measurement of neurite root preference was calculated that removed the distance biasing by measuring neurofilament area within a fixed distance into each root (Figure 4.2 inset). Since this experimental measurement approximates neurite root preference, it was compared to the fraction of neurites that enter each root relative to the total number of neurites that enter all roots in the simulation. The simulation’s neurite root preference was recalculated in exactly the same way as experimentally measured neurite root preference, to examine if the proportion of neurite number is correlated to proportion of neurite area. We found that both measurements of neurite root preference, based on neurite area and neurite number, were not significantly different and fit to exponential curves ($r^2 > 0.98$) shown in Figure 4.9. In addition, parameters from exponential fit curves overlapped within 95% confidence intervals of each other,
demonstrating that the normalized area is significantly correlated to proportions of neurite number.

Figure 4.9 Compare simulation and experimental measurements
Two different neurite root preference measurements were compared in the simulation. Measurements were based on neurite area 130.5 µm up each root (gray triangle) while the other is based on neurite number (black squares). Data from both measurements fit to similar exponential curves ($r^2 > 0.98$) within a 95% confidence interval and were not significantly different for all root numbers. Error bars are SEM.

4.5.3 Comparison of *in vitro* and *in silico* neurite trajectory preference

Given the strong correlation between the experimental neurofilament area and the simulated number of neurites entering a root, the simulation’s neurite trajectory preference was calculated using the fraction of neurites that choose a divergent over a straight trajectory. These data allow us to determine the likelihood that neurites reach and enter different roots by combining the calculated probabilities for a neurite to avoid ($PL_b$) or enter ($PR_b$) a root ($b$). For example, to ensure that a neurite enters $b = 2$ (a nearby root) the neurite must avoid the first root ($PL_1$) and then enter the second root ($PR_2$). This possibility carries the probability $p = (0.74)(0.22) = 0.16$ based on *in vitro* data. On the other hand, for a neurite to enter $b = 6$ (a more distant root) the neurite must avoid the first five roots ($PL_b$ for $b = 1$ through 5 inclusively) and then enter the
sixth root ($PR_b$), which has combined probability $p = 0.06$ based on *in vitro* data. These examples show how the likelihood of a neurite entering a distant root can be significantly less than the likelihood of entering a nearby root.

In addition, this new measurement allows us to examine the differences between simulations and experiments by examining the hypothesis that $PR_b$ does not differ for any root and independent of root distance from the explant. We showed in Figure 4.6 that simulated probabilities of neurites entering roots were, as expected, independent of distance from the explant, $b$, while experimental probabilities decreased for increasing $b$. This finding indicates a discrepancy between simulations and experiments, which may be associated with experimental sources of variability.

To limit experimental variability, multiple experiments were repeated and only those experimental replicates whose furthest neurite reached roots 6 through 9 were used in our analysis (26 out of 36). In addition, some experiments exhibited neurites that were raised out of the focal plane of the microscope, presumably due to neurites lifting off of the substrate. These replicates were discarded to avoid inaccurate calculations of neurofilament area (5 out of 36). Anomalous samples in which neurofilament left the primary channel or the roots were likewise omitted to further reduced variability in the samples (7 out of 36).

Simple statistical fluctuations may account for the minor differences that persist between simulations and experiments. In addition, systematic variations due to experimental effects such as fasciculation that were not included in the
simulation may explain this discrepancy. *In vitro* neurites were observed to fasciculate in the primary channel, which might inhibit neurites from diverging to explore roots (Bentley & Keshishian, 1982). Fasciculation can also increase the rigidity of the axon bundle (Rutishauser & Edelman, 1980), which may be due to the stiffness of microtubules (Dent & Gertler, 2003), and indirectly decrease the likelihood for a neurite to bend or wander into a root. Whether this would produce a bias favoring exploration along the primary channel instead of a root is uncertain. Future simulations that incorporate fasciculation will improve our understanding of how neurite fasciculation affects guidance in these complex geometries.

4.5.4 Fasciculation decreases probability for neurites to reach distal side roots

To determine where fasciculation is prevalent *in vitro*, the width of axon bundles at each entry point was measured. Since bundle width diminishes with distance from the explant, it follows that more neurites fasciculate - presumably because more neurites are present – nearer to the explant than farther from it. To determine whether a root contained single neurites, we measured the number of connected pixels 1 micron or less in width. We found that single neurites occupy roots 5 through 8 on average after 3-4 days of incubation, as shown in Figure 4.8. In addition, significant changes in $PF_b$ and maximum bundle width at $b = 1$ and 3 indicate that other unknown phenomena may be occurring between these and subsequent neighboring roots.
Since our in vitro cultures have a heterogeneous cell population (neurons, Schwann cells, and satellite cells (Bernardini, de Stefano, Tata, Biagioni, & Augusti-Tocco, 1998)), bundling in culture may also be a result of Schwann cells wrapping around multiple neurites (Corfas, Velardez, Ko, Ratner, & Peles, 2004). Specifically, non-neuronal cells tend to migrate and proliferate along existing axonal tracts in the PNS and modify the pattern of fasciculation based on the rate of non-neuronal cell migration along the axons. Following staining with both phalloidin (a marker for growth cones and non-neuronal cytoskeleton, F-actin) and nuclear stain DAPI, we observed non-neuronal cells along the axons mainly at the entrance of the primary channel and proximal roots. This further demonstrates that other factors may be influencing guidance and/or reorganization of neurites in the early roots.

4.5.5 Future Studies

In future studies, we plan on modifying the pattern or adding factors that affect neurite fasciculation and guidance to develop neurite guidance strategies in complex micropattern geometries. Since fasciculation, as well as defasciculation, is important in developmental pathfinding (Zipser, Morell, & Bajt, 1989; Fambrough & Goodman, 1996; Wiencken-Barger et al., 2004) understanding it will benefit the development of future neuronal repair strategies. In addition, the influence of non-neuronal mitotic cell proliferation in our culture may have affected the levels of bundling. This can be reduced by using an anti-mitotic agent like Mitomycin C for Schwann cells to examine if these cells can affect guidance at the roots in vitro (Enver & Hall, 1994; Kleitman, Wood, &
Bunge, 1998). In future work, fasciculation’s influence will also be minimized by adding factors known to influence fasciculation. Therefore, we plan on repeating these experiments with soluble defasciculating agents such as anti-G4/L1, PSA mimotope cyclic peptide (p1 or p2), or colominic acid (polysialic acid, PSA, analogue) (Honig & Rutishauser, 1996; Honig et al., 1998b; Torregrossa et al., 2004) and hope this will shed light on how fasciculation influences guidance in multiple root micropatterns.

Recent success using chemotropic guidance to guide regenerating supraspinal neurites, following SCI, to find their appropriate nearby target in the spinal cord has provided support for the use of guidance cues in SCI therapies (Alto et al., 2009). These therapies will benefit greatly from the development of in vitro models that examine the influence that guidance cue concentration has on neurite guidance at nearby decision points (Wittig, Ryan, & Asbeck, 2005; Ryan, Wittig, Evans, Dazert, & Mullen, 2006). The different roles that both chemical and contact cues play in the relative guidance of neurites to distant or nearby targets, however, are still unknown. Because of the multiple different systems and complex organization that are present in the spinal cord, a single guidance strategy cannot serve the functional needs of a regenerating spinal cord. For example, if regenerating neurites follow roots readily they cannot reach distant targets; on the other hand, if they bypass the first roots encountered then they cannot enter nearby roots. Therefore, we must develop and test therapeutically applicable guidance strategies using multiple guidance cues. Currently, we are working on placing non-permissive cues to improve neurite guidance to target
roots at different distances along the pattern. Therapies aimed at guiding regenerating peripheral neurons to a specific target following an injury, i.e., preferential motor reinnervation (Brushart, 1988; Madison, Archibald, & Brushart, 1996), can also benefit from using similar computational simulations to optimize guidance strategies through assessment of targeting success rates in complex geometries.
5 COMPARISON OF IN SILICO AND IN VITRO NEURITE TARGET ROOT PREFERENCE

5.1 Chapter 5 Summary

This chapter focuses on comparing simulation results with experimental data concerning neurites grown on multiple and single root patterns. Some important questions we address in this study include: (1) whether more neurites enter a target root when restricted from entering non-target roots and (2) whether simulated neurite outgrowth patterns match in vitro neurite outgrowth on a modified pattern.

We hypothesize that the number of neurites that enter a target root increases when neurites are restricted from entering non-target roots. Comparing neurite outgrowth patterns between multiple (control) and single (experimental) root micropatterns allow us to determine whether the removal of non-targets roots affects guidance to a target root.

5.2 Introduction

5.2.1 Complex geometry of nervous system

As the nervous system develops, neurites reach their appropriate targets and form complex connections through guidance strategies. Guidance is accomplished through a combination of attractive, repulsive, non-permissive, and permissive cues which ensure that neurites reach their targets and make proper neural connections. Following an injury to an adult central nervous system,
these connections are interrupted, which results in a loss of sensory and/or motor function. In this case, functional recovery requires that regenerating neurites must first reach their target before they can reform their previous connections. But an absence of guidance cues and presence of complex branching patterns in the adult nervous system result in an extremely low probability for neurites to reach their target as described in the introduction to this dissertation.

Branching patterns along peripheral and central nervous systems present many possible routes for a regenerating neurite. One such example, in the peripheral nervous system (PNS), is the heavily branched spinal nerves from either the brachial, lumbar, or lumbrosacral plexuses (Yan & Hitomi, 2004; Matejcik, 2010). These plexuses organize motor nerve fibers so that they innervate the correct target muscles. Another example, in the central nervous system (CNS), is the ascending sensory tract within the spinal column. Neurites in this tract must travel long distances up the spinal column to their targets in the brainstem; however, there are many dorsal rootlets that neurites may mistakenly follow (Fraher, 1999; Tashiro et al., 2001). To understand how guidance cues influence neurite targeting in these complex geometries, researchers have employed in vitro models of neurite outgrowth.

5.2.2 In vitro and in silico models to examine guidance questions

In vitro models have been used to examine how soluble or substrate bound cues affect guidance. Using microfluidics and surface chemistry, gradients have been created or presented as a combination of different cues to isolate a neurite’s response to various microenvironments. Wittig and Ryan
(Wittig et al., 2005; Ryan et al., 2006) used microfluidics to examine neurite growth preference along an alternate-choice microfluidic platform in the presence of neurotrophin-3 (NT-3), an attractive soluble cue. Guidance cues have also been bound to a substrate to examine neurite pathfinding (Dertinger et al., 2002; Li, Liu, & Hoffman-Kim, 2008; Millet, Stewart, Nuzzo, & Gillette, 2010). One method using substrate bound cues to study neurite guidance is a stripe assay, which assesses a neurite’s affinity for different substrates (Evans et al., 2007; Shi, Nedelec, Wichterle, & Kam, 2010). A combination of substrate and diffusible microfluidic gradients of guidance cues (Joanne Wang et al., 2008) have also been used to understand how neurites are guided to specific targets when presented with multiple cues.

*In vitro* models have also been used to address specific questions about the developing nervous system. For example, Ti1 growth cones have been shown to integrate attractive and repulsive cues while reorienting themselves at specific cells (i.e., guidepost cells) in the limb of a developing grasshopper (Bentley & Keshishian, 1982; Isbister & O’Connor, 2000). To mimic the topology and substrate affinity for guidepost cells observed *in vivo*, researchers have used micropatterns to examine the developmental question of how guidepost cues influence axonal pathfinding (Hammarback & Letourneau, 1986; Kuhn et al., 1995). Computational models based on these *in vitro* models and *in vivo* neurite pathfinding events have also improved our understanding of how neurite guidance is regulated by its environment (e.g., guidepost cells and substrate affinity) (Buettner et al., 1994; Buettner, 1996).
5.2.3 Substrate permissivity during development influences neurite guidance

Tissue permissivity (i.e., neurite adhesive strength to an extracellular matrix from a non-instructive cues (Lemmon, Burden, Payne, Elmslie, & Hlavin, 1992)) is a neurite guidance strategy that plays a distinct role in neurite pathfinding. We note that this strategy is observed in many different areas during development. For example, non-permissive cues in the ventral metencephalon and boundary of the mesencephalon guide vestibular afferent neurites emanating from cranial nerve VIII to their targets in the cerebellar primordium (rhombic lip) (Tashiro et al., 2001). In addition, dorsal migration of medial ganglionic eminence (MEA) cells, which develop into interneurons in the neocortex, are restricted from migrating away from the neocortex by ventromedially surrounded non-permissive tissue (Wichterle, Alvarez-Dolado, Erskine, & Alvarez-Buylla, 2003). In both of the examples above, non-permissive cues act by restricting neurites from taking aberrant paths toward non-target regions in the central nervous system.

In this study, we examine how a guidance strategy that restricts neurites from entering non-target roots by removing the non-target roots from the pattern influences neurite guidance a specific root. We also show that this guidance strategy is more beneficial for distant than nearby target roots and that a predictive computational model has been built which matches in vitro data.
5.3 Methods and Materials

5.3.1 Combined single and multiple root micropattern

For the *in vitro* work, a new pattern (Figure 5.1) was used that combines the previously described comb-like micropattern with multiple roots (control pattern) along side a y-micropattern with only one root (experimental pattern). The experimental pattern was designed to examine the likelihood of neurites to reach and enter a target root (single root) when restricted from entering all other non-target roots (i.e., roots removed from the pattern). The target root on the experimental pattern is the single root. Similarly, the target root on the control pattern is same distance up the primary channel as the experimental pattern’s target root (mirrored across the dashed gray line in Figure 5.1). By comparing neurite area within the target roots of both patterns, we were able to determine how neurite area is modified in the target root when all non-target roots are removed.

Three variations in the pattern were created by changing the target root’s location on the experimental pattern (2nd root shown in Figure 5.1A, 5th root shown in Figure 5.1B, and 6th root shown in Figure 5.1C). The purpose of using different patterns was to examine the affect of removing non-target roots on neurite area within near (2nd root) and distant (5th or 6th root) target roots.
Figure 5.1 Combined multiple and single root micropattern
A new pattern incorporating the multiple root pattern (labeled “Control”) along side a y-pattern with one root (labeled “Exp”). Three different target roots were selected to examine how neurite area is modified when neurites are restricted from entering non-target roots for a (A) nearby (i.e., target root number 2 in the control pattern) or distant (i.e., target root numbers (B) 5 and (C) 6 in the control pattern) target root. Dashed gray lines separate the two patterns shown above. (white scale bar = 400 µm)

Roots 5 and 6 were selected as distant target roots to ensure that a significant neurite area was measured in the target root of the control pattern. This is necessary for making a comparison between target roots of both patterns, because neurite area decreases exponentially with root distance from the explant for multiple root patterns. The second root was used as a nearby target root, instead of the first root, since neurite area within the first root was expected to not change when non-target roots were removed from the pattern. On the other hand, it was unknown whether neurite area within the second root would change when non-target roots were removed.

The pattern was designed in AutoCAD (LT 2002, San Rafael, CA) and made into a dark chrome mask (Microfabrication Laboratory, University of
California at Berkeley). The mask was used to make photoresist trenches (70 µm) on a silicon wafer master using photolithography. Following creation of the master, an elastomer base and curing agent (SYLGARD elastomer kit (Dow Corning, Midland, MI)) were mixed in a 10:1 ratio (w/w), poured over the master, and cured at 65°C overnight. The resulting polydimethylsiloxane (PDMS) pattern was then cleaned and used as a stamp.

5.3.2 Protein patterning

PDMS stamps were first placed on a 22 mm cover glass (Fisher Scientific), whose underside was marked at the center of the pattern with a marker to guide the placement of DRGs during plating. The assembly was treated with oxygen plasma with the stamp in place on the coverslip in a PX-250 plasma generator with an environment of 60% O₂ and 40% N₂ for 60 seconds at 670 mtorr and 100 Watts. Subsequently, the MicroMolding in Capillaries (MIMIC) process (Kim et al., 1996) was used to coat the activated coverslip with laminin (25 µg/ml). Following 4 hours of incubation at 37°C in 5% CO₂, the stamp was removed and the cover glass was washed three times with a 1:1 mixture of F12/DMEM. The coverslip was then placed in 0.5% bovine serum albumin (BSA) at room temperature for 30 minutes (Thompson & Buettner, 2001). BSA was used to restrict neurites from growing off of the pattern. BSA does not bind to the laminin protein and when BSA adsorbs onto a surface in the presence of laminin it does not interfere with the permissive laminin pattern (Esch et al., 1999; Dertinger et al., 2002). The laminin pattern was confirmed by indirect
immunostaining and mimicked the original pattern of a 100 μm wide primary channel and 50 μm wide side roots at 45° from the primary channel.

5.3.3 Explant culture

Dorsal root ganglia (DRGs) were dissected from the lumbar region of embryonic day seven (E7) specific pathogen free chickens (Charles Rivers) were placed in cold calcium and magnesium free (CMF) Hank’s Balanced Salt Solution (HBSS) under a dissection microscope. Each DRG was then plated on the laminin-patterned glass in 600 μl of serum free media (SFM) for eight hours followed by the addition of 1.4 ml of SFM. The SFM is composed of 1:1 (v/v) solution of DMEM/Ham’s F-12, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin, 10 mg/ml glucose, 1X MEM vitamins, 20 μg/ml N3, 1X ITS+1 premix (100 μg/ml insulin, 55 μg/ml transferrin, 50 ng/ml selenium), 0.1% BSA, 50 ng/ml neural growth factor (NGF) (R&D Systems, 556-NG), and 100 mM ascorbic acid. N3 consists of 5 ml N2 supplement (Invitrogen, 17502048), 250 μl 1X ITS+1 premix, and 10 μl of 2 mg/ml corticosterone. All reagents were purchased from Sigma, unless stated otherwise. Explants were incubated for 3-4 days in vitro.

5.3.4 Immunostaining

DRGs cultured on the micropatterned laminin substrate were fixed with 4% paraformaldehyde for 15 minutes and then washed with CMF PBS. Samples were blocked with 10% normal goat serum (Sigma, G9023) in immunobuffer (1% BSA, 0.5% Triton-X100, and CMF PBS) for 1 hour. Laminin and neurofilament were labeled using indirect immunostaining. Samples were incubated in primary
rabbit anti-laminin (1:100, Sigma, L9393) and primary mouse anti-neurofilament
(1:1000, 68 kDa, and 1:5000, 200 kDa, Sigma) for 1 hour and then washed
before adding secondary FITC conjugated-goat anti-rabbit (1:100, Sigma, F0382)
and Alexa 546 (1:250, Invitrogen, A11018) for 45 minutes. Nuclear stain DAPI
(100 ng/ml, Sigma, D9542) was added for 20 minutes before mounting coverslips
onto slides using inkfusing Fluoro-Gel (Electron Microscopy Science, 17985-10).

5.3.5 Microscopy and image processing

Fluorescence and difference interference contrast (DIC) images were
acquired using a monochrome Zeiss AxioCam CCD camera mounted onto an
inverted Zeiss 200M deconvolution inverted microscope (Rutgers Collaborative
KECK center) with a 20x objective (Plan-apochromat). Fluorescence images
were captured at a constant exposure time of 300 – 500 ms with a scale of
0.3263 µm/pixel. Neurofilament grayscale images were used to calculate neurite
area for each root from neurofilament pixel area.

All original grayscale neurofilament were converted into binary images
(values of 0 and 1) using a segmentation algorithm programmed in MATLAB
(MathWorks Inc., Natick, MA) before calculating areas. MATLAB’s Image
Processing Toolbox was used for all image processing. Original neurofilament
fluorescent images contained background fluorescence that concealed neurite
information and caused incongruous segmentation. Background fluorescence
was partially removed by subtracting the background of the image, found by
morphologically dilating and then eroding the original image (MATLAB’s imopen
function), from the original grayscale-image. Dilation assigns the maximum pixel
value to all pixels within the local structure element (neighborhood), while erosion assigns the minimum pixel value to all pixels within the neighborhood (Gonzalez & Woods, 2002; Costantino et al., 2008). To avoid including background in neurofilament calculations, a disk-shaped structure element with a 100-pixel radius (> 30 µm) was selected for dilation and erosion. The background-reduced image was then filtered using a local Laplacian and nonlinear range filter to create two new images that respectively isolated fine neurofilaments and neurofilament edges.

Neurofilament segmentation was accomplished by partitioning the grayscale pixel values of all three processed images into two separable clusters (background and neurofilament) using MATLAB’s kmeans function. K-means clustering calculates two cluster centroids, chosen initially to be 0 or 1, by minimizing the sum of squared Euclidian distances between pixel values and cluster centroids. Algorithmically, each pixel is first assigned to the cluster centroid with the smallest distance between the pixel value and cluster centroid. Then after all pixels are assigned to a cluster centroid, the pixel values within each cluster are averaged to recalculate each centroid. These two steps, pixel reassignment to cluster centroids and centroid position recalculation, are repeated until the centroids’ positions remain unchanged. This results in a binary image of neurofilament.
5.3.6 Analysis of neurite root and trajectory preference using neurofilament area

Total neurofilament area along a pattern, $TAP_{Tb}$, was measured from the binary image. This measurement was calculated by counting the number of white pixels within a selected polygon region that enclosed the neurofilament area from the entry of the primary channel to edges of where the neurites extended along the patterns. Comparison of total neurofilament area within the control and experimental patterns allowed us to examine the presence of a bias for neurite area along either pattern for different target root numbers, $Tb$.

In addition, total neurofilament area, $TA_{Tb}$, within a target root, $Tb$, and neurofilament area a fixed distance (400 pixels) into target roots, $AR_{Tb}$, was measured as shown in Figure 5.2A. $TA_{Tb}$ was measured by counting the number of white pixels within an outlined region from the $Tb$-th root’s entry to the farthest-reaching neurite tip within the side borders of both single and multiple root patterns (dotted blue boxes in Figure 5.2A). Side borders were determined by overlaying the laminin binary image onto the neurofilament images as shown in Figure 5.2A. The fixed area within $AR_{Tb}$ is 400 pixels along the length the root, starting from the root entry, and 400 pixels wide (1.6e5 pixels = 1.7 cm$^2$ neighboring the primary channel) shown as dashed yellow boxes within the roots in Figure 5.2A. Probability of a neurite entering the target root, $PET_{Tb}$, was calculated by dividing the total neurite area within a target root, $TA_{Tb}$, by the total area along the pattern, $TAP_{Tb}$ (experimental or control).
Area measurements of neurofilament for both patterns

Binary images of neurofilament stain (white) overlaid on top of laminin pattern (gray background) for control and experimental patterns with $T_b = 5$. Neurites on both patterns originated from the same explant. (A) Total area, $TA_{T_b}$, within the target root of the control and experimental patterns is measured by manually selecting regions of neurofilament (dotted blue boxes). In addition, $TA_{T_b}$ for $T_b = 1$ of the control pattern was measured. Neurofilament areas within a fixed region of the root, $AR_{T_b}$, and primary channel, $AL_{T_b}$, were also measured at the root bifurcation point (dashed yellow boxes). Comparison of areas was used to examine neurite trajectory and root preference for both patterns. (B) Total neurite area within and above the target root, $TWA_{T_b}$, including non-target roots was also measured (dashed yellow polygon) and used to calculate a second neurite trajectory preference. (white scale bar = 400 µm)

A neurite’s probability of choosing a divergent trajectory, $PR_{T_b}$, was calculated by comparing $AR_{T_b}$, to neurofilament area within a fixed area in the primary channel, $AL_{T_b}$. $AL_{T_b}$ is the number of neurofilament pixels within a 400 by 400 pixel square region of the primary channel (shown in Figure 5.2A as the dashed yellow boxes bordering the $AR_{T_b}$ region). $AR_{T_b}$ and $AL_{T_b}$ were then used to calculate $PR_{T_b}$ for each $T_b$ in the equation below:

$$PR_{T_b} = \frac{AR_{T_b}}{AR_{T_b} + AL_{T_b}}, \quad \text{(5.1)}$$

$AR_{T_b}$ and $AL_{T_b}$ selected regions are equal in area. In addition, a second measurement of a neurite’s probability of choosing a divergent trajectory, $TPR_{T_b}$,
was calculated by dividing the total neurite area within the target root, $T_{TA_{Tb}}$, (dotted blue boxes in Figure 5.2B) over the total neurite area within and above the target root, $T_{W{A_{Tb}}}$, including non-target roots (area within dashed yellow polygon in Figure 5.2B).

By comparing these measurements, we examined whether more neurites reached and entered a target root when neurites were presented with less paths (i.e., non-target roots removed). Neurofilament area in the first root of the control pattern was also calculated using the same neurite area measurements. The neurofilament area measurements from the first root of the control pattern were compared to the target root of the experimental pattern because both roots are the first roots encountered along their respective patterns. $PET_{Tb}$, $PR_{Tb}$, and $TPR_{Tb}$ were evaluated for target roots of each pattern and the first root number, $b$, encountered in the control pattern ($b = 1$). Only samples whose neurites were restricted to the laminin pattern and grew past the target root on both patterns were analyzed.

5.3.7 Modified computational simulation of neurite outgrowth along the experimental and control pattern

The model of neurite outgrowth is based on a 2D random walk where each progressive iteration, $i$, is dependent on the previous one. Neurite outgrowth is adjusted based on three parameters: average outgrowth, $r_d$, outgrowth uncertainty, $r_s$, and current direction, $\theta$, of a neurite’s tip shown in Figure 3.3A. The distance parameter, $r_i$, is the sum of the average outgrowth, $r_d$, and the outgrowth uncertainty, which is Gaussian distributed with $r_s$ being the
maximum uncertainty of outgrowth. The current direction is modified by the search angle, $\delta_i$, of the growth cone to the left and right of its current angle as illustrated in Figure 3.3A. Polar coordinates $(r_i, \theta_i)$ are converted to Cartesian coordinates before adding the next growth to the current position as shown in equations 3.2 and 3.3.

The simulation is initiated by placing a number, $n$, ($n = 100$ in the simulations described here) of neurites at random positions along the base of a branched pattern identical in geometry to the experimental laminin pattern, and with initial neurite current angles parallel to the primary channel's edge. Each neurite grows for a different number of iterations, simulating the time-dependent neurite entrance into the micropattern environment. When a neurite encounters a non-permissive boundary (shown in Figure 3.3B), the neurite reverts back to its previous position $(x_i, y_i)$ and direction ($\theta_i$). A new direction is then recalculated from summing a search angle, which is randomly selected from a Gaussian distribution, and the previous direction ($\theta_i$). If the new neurite tip position evaluated encounters the edge again then these two steps are repeated until the boundary is avoided. Once an angle is selected that allows the neurite tip to avoid the edge the neurite tip continues migrating based on the previously described persistent random walk. The simulated change in direction of the neurite tip at the substrate edge is based on previous experimental observations examining growth cone turning behavior, which has been described as the growth cone reorienting along the substrate edge and then growing along or away from the edge (Tanaka & Kirschner, 1995). When a simulated neurite
enters a root, it is not permitted to turn backwards, and may only continue moving forward. Simulations of growth cone outgrowth were run for 100 neurites (1 iteration = 30 seconds and 1 unit = 174 µm). Each simulation is replicated 20 times. The simulation was run till neurites reached the average root number reached in vitro \((b = 9)\). Simulated neurites were run on both the multiple and single root patterns.

Unrepeated random numbers from a random number generator, i.e., unseeded random numbers, were used for all variables in the simulation \((r_s, r_d, \text{ and } \delta_i)\). Results from the simulation with unseeded random numbers were compared to simulations with identical random numbers between samples \((N = 20)\), i.e., seeded random numbers, for all patterns to examine if the random numbers used in the simulation affects neurite outgrowth patterns. Because the random numbers are identical between control and experimental patterns, neurite outgrowth differences along the patterns were only based on the differences between the patterns.

### 5.3.8 Calculating computational neurite preference

Computational neurite preference measurements were developed to compare the simulation to in vitro results. The patterns replicated the in vitro design of a 100 µm wide primary channel and side roots at 45° from the primary channel with root widths of 50 µm (entry width of 74.4 µm). A neurite’s probability of entering a target root, \(PET_{Tb}\), and the probability of choosing a divergent trajectory into a target root, \(PR_{Tb}\) or \(TPR_{Tb}\), were calculated. \(PET_{Tb}\) was calculated as the fraction of neurites that entered a root, \(Nr_{Tb}\), relative to the
total number of neurites in the simulation (n = 100). $PR_{Tb}$ and $TPR_{Tb}$ were calculated the same way, $N_{r_{b}} (x_{i+1} > 0.588 \text{ units and } b > y_{i+1} > b-0.422)$ divided by the number of neurites that entered, $N_{r_{b}}$, and avoided the root, $N_{l_{b}}$, i.e., took a straight trajectory ($N_{l_{b}} = N_{l_{b+1}} + N_{r_{b+1}}$ or $N_{l_{b}} = 100 - \sum N_{r_{b}}$). The model’s multiple root pattern was modified to test how neurite preference changed when the original non-permissive boundary of multiple roots was adjusted to include only a single root that resembled the experimental pattern. Three different target roots ($Tb = 2, 5, \text{ and } 6$) were selected to match the geometry used in vitro. The number of neurites within each target root for the control and experimental patterns were then compared.

5.3.9 Comparison of in silico and in vitro models

In silico $PET_{Tb}$, measured from the number of neurites within a target root, was compared to in vitro $PET_{Tb}$, which was measured based on the total neurofilament area within a target root. $PET_{Tb}$ for $Tb = 2, 5, \text{ and } 6$ were compared for the control and experimental patterns. In addition, control pattern $PET_{Tb}$ for the first root number ($b = 1$) was compared between in silico and in vitro data. $TPR_{Tb}$ was compared between in vitro and in silico data for experimental patterns with target roots 2, 5, and 6. The simulation matched in vitro data if $PET_{Tb}$ and $TPR_{Tb}$ were not significantly different for nearby and distant target roots (unpaired two-tailed t-test, $p > 0.05$).
5.4 Results

5.4.1 Comparison of total neurite area along the *in vitro* experimental and the control patterns \((TAP_{Tb})\)

Total neurofilament area, \(TAP_{Tb}\), along control and experimental patterns were compared to examine if neurites extended equally along both patterns. Average total neurite area for the control pattern was not significantly different to the experimental pattern for all target root number patterns shown in Figure 5.3.

![Figure 5.3 Total neurite area along different target root number patterns (TAP_{Tb})](image)

Average neurite area along the control pattern (white bar) was not significantly different to experimental patterns (gray bar) for target root numbers 2 \((N = 6)\), 5 \((N = 10)\), and 6 \((N = 8)\). Error bars are standard errors of the mean (SEM).

5.4.2 Comparison of neurite area in target root *in vitro* \((TA_{Tb} \text{ or } AR_{Tb})\)

Average total neurite area in a target root, \(TA_{Tb}\), significantly increased (paired one-tailed t-test, \(*p < 0.05\)) for distant target roots \((Tb = 5 \text{ and } 6)\) when neurites were restricted from entering non-target roots (comparison of target roots on control and experimental patterns), shown in Figure 5.4A. \(TA_{Tb}\) for a nearby target root \((Tb = 2)\), however, was did not differ significantly for control and experimental patterns (paired two-tailed t-test, \(p = 0.62\)). These results
indicated that the number of non-target roots encountered by neurites before a target root and the target root distance from the base of the pattern affects $TA_{Tb}$.

$TA_{Tb}$ for the target root of the experimental pattern and $TA_{Tb}$ for the first root number of the control pattern ($b = 1$) were also compared since both are the first roots that neurites encounter along their respective patterns. These values differed significantly (unpaired two-tailed $t$-test, $p < 0.05$), which further indicated that $TA_{Tb}$ is affected by a root’s distance from the base of the pattern. $TA_{Tb}$ along $b = 1$ for all control patterns were not significantly different.

$TA_{Tb}$ biased area measurements for roots whose neurites extended farther along the length of the root (i.e., nearby roots). Therefore, another area measurement using neurite area a fixed distance along the length of the root, $AR_{Tb}$, was examined to compare neurite area within target roots of different patterns. $AR_{Tb}$ significantly increased (paired one-tailed $t$-test, $p < 0.05$) for

Figure 5.4 Compare neurite area in target roots ($TA_{Tb}$ & $AR_{Tb}$)
Neurite area within the target roots and first roots encountered ($b = 1$) along the experimental and control patterns were compared for the three different target root numbers ($Tb = 2$, 5, and 6). (A) $TA_{Tb}$ was significantly larger (paired one-tailed $t$-test, *$p < 0.05$) for distant target roots along experimental (dark gray bar) rather than control patterns (light gray bar). Total neurite area along a nearby target root was not significantly different when comparing experimental and control patterns. (B) Similar results were found for $AR_{Tb}$ measurements along distant and nearby target roots of experiment and control patterns. Error bars are SEM.
distant target roots \((b = 5 \text{ and } 6)\) when neurites were restricted from entering non-target roots in the experimental pattern, which is shown in Figure 5.4B.

5.4.3 Comparison of the probability of a neurite choosing a divergent trajectory into a target root \textit{in vitro} \((PR_{Tb} \text{ or } TPR_{Tb})\)

\(PR_{Tb}\), measured from fixed area, differed significantly (unpaired two-tailed \(t\)-test, \(\ast p < 0.05\)) for \(Tb = 5\) when compared to \(PR_{Tb}\) for other experimental pattern target roots (dark gray bars), shown in Figure 5.5. \(PR_{Tb}\), however, for all experimental pattern target roots should not be significantly different, especially in the case of \(Tb = 5 \text{ and } 6\), which are adjacent roots. To reexamine this unexpected result, a new measurement \(TPR_{Tb}\), which accounts for the total area within and above the target root, was calculated, shown in Figure 5.5A.

\[
\text{TPR}_{Tb} \text{ for all target roots of the experimental pattern (dark gray bars) were not significantly different to } b = 1 \text{ (white bars) and the target roots (light gray}}
\]
bars) of the control patterns. In addition, $TPR_{Tb}$ for the different target root numbers of the experimental pattern (dark gray bars) and $b = 1$ for all control patterns (white bars) were not significantly different. A similar overall trend of decreasing $TPR_{Tb}$ was observed for increasing root numbers in the control pattern (light gray bars) which was previously observed.

5.4.4 Comparison of neurite preference in target root in silico

$PET_{Tb}$ for unseeded and seeded simulations were not significantly different for all control and experimental target roots. $PET_{Tb}$ increased significantly (unpaired one-tailed t-test, $p < 0.05$) for experimental distant target roots ($Tb = 5$ and 6) when compared to control distant target roots for seeded and unseeded simulations, shown in Figure 5.6A.

![Figure 5.6 Comparison of seeded and unseeded random numbers in silico](image)

(A) Probability of neurites entering a target roots, $Tb$, ($Tb = 2, 5, \text{ and } 6$) for unseeded or seeded simulations of neurites on control or experimental patterns. Probability of a neurite entering a target root for seeded and unseeded simulations were not significantly different for all patterns; however, experimental distant target root numbers ($Tb = 5$ and 6) differed significantly (unpaired two-tailed t-test, $p < 0.05$) from control distant target root numbers. (B) The ratio of neurites in a target root on a control versus experimental pattern for seeded (white) or unseeded (gray) simulations is shown. Ratio of neurites in the control to experimental target root for seeded and unseeded simulations were not significantly different. Error bars are SEM.
The fraction of neurites that entered the target roots of the control pattern to the experimental pattern for both unseeded and seeded random numbers were not significantly different for all \( Tb \), shown in Figure 5.6B. Since the unseeded random numbers are more relevant to the \textit{in vitro} experiments we selected these to compare against the \textit{in vitro} data.

5.4.5 Comparison of \textit{in vitro} and \textit{in silico} data (\( PET_{Tb} \) and \( TPR_{Tb} \))

\textit{In silico} and \textit{in vitro} data (\( PET_{Tb} \) and \( TPR_{Tb} \)) were compared to examine if simulated neurite outgrowth patterns matched \textit{in vitro} experiments. \textit{In silico} and \textit{in vitro} \( PET_{Tb} \) were not significantly different when respective patterns were compared. \textit{In vitro} \( PET_{Tb} \), however, was significantly different (unpaired two-tailed t-test, \#p < 0.05) to \textit{in silico} \( PET_{Tb} \) for distant target roots of the control patterns (\( Tb = 5 \) and 6). Restricting neurites from entering non-target roots (experimental pattern) significantly increased \( PET_{Tb} \) (unpaired one-tailed t-test, \*p < 0.05) for both \textit{in silico} (white bars) and \textit{in vitro} (gray bars) distant target roots (\( Tb = 5 \) and 6), shown in Figure 5.7A. \( TPR_{Tb} \) for experimental target roots were not significantly different for \textit{in silico} (white bars) or \textit{in vitro} (gray bars) data when comparing \( Tb \) (unpaired two-tailed t-test, \( p \geq 0.84 \)). The \textit{in vitro} \( TPR_{Tb} \), however, was significantly greater than \textit{in silico} \( TPR_{Tb} \) (unpaired one-tailed t-test, \#p < 0.05), as shown in Figure 5.7B.
Figure 5.7 Compare in silico and in vitro PET$_{Tb}$ and TPR$_{Tb}$ for target roots

(A) Restricting neurites from entering non-target roots (experimental patterns) significantly increased (unpaired one-tailed t-test, *p < 0.05) the probability of neurites entering distant target roots ($Tb = 5$ and 6) for both in silico and in vitro data. PET$_{Tb}$, however, was significantly different for distant target roots along the control pattern when comparing in silico and in vitro data (unpaired two-tailed t-test, #p < 0.05). (B) Even though TPR$_{Tb}$ is constant for in silico or in vitro data, in vitro TPR$_{Tb}$ was significantly larger than TPR$_{Tb}$ calculated from the simulation (unpaired one-tailed t-test, #p < 0.05). Error bars are SEM.

5.5 Discussion

5.5.1 Neurite area searched before and after target root

Neurite area in distant target root numbers ($Tb = 5$ and 6) increased when neurites were restricted from entering non-target roots. Neurites along the control patterns, however, were also observed to enter roots more distant than the target roots, which is a part of the area searched by neurites. To examine how restricting neurite entry into non-target roots affects neurite search area, we compared the difference in percent of total neurite area along the pattern beyond the target roots for each pattern shown in Figure 5.8A (percent of total neurite area on pattern within dashed yellow polygons). We found that the percent difference in neurite area searched either after (white bars) or before (gray bars) the target roots were not significantly different when comparing the different target root numbers ($Tb$), as shown in Figure 5.8B.
Figure 5.8 Percent difference in neurite area searched after and before target roots (experimental-control)

(A) The neurite area searched after (yellow dashed polygons) or before (blue dashed polygons) the target root is shown above for the control and experimental patterns (Tb = 5). Calculating the difference in the percent of total neurite area occupied within yellow or blue polygons illustrated for experimental and control patterns evaluated the percent difference in area searched. (B) Percent differences in area searched before or after were not significantly different for all target root numbers. Neurite search area after and before distant target roots (Tb = 5 and 6), however, had respectively significantly larger and smaller percent difference (paired one-tailed t-test, *p < 0.05) than a nearby target root (Tb = 2). Error bars are SEM.

The mean difference in neurite area after the target root, however, was significantly greater than a standard normal distribution (paired one-tailed t-test, *p < 0.05) only for distant target root numbers (Tb = 5 and 6). The mean difference in neurite area before the target root shown in Figure 5.8A (percent of total neurite area on pattern within dashed blue polygons) was significantly less than a standard normal distribution (paired one-tailed t-test, *p < 0.05) only for distant target root numbers (Tb = 5 and 6). The low sample number (N = 6) may account for the high variability in percent difference in area searched for a nearby target root (Tb = 2).
5.5.2 Reasons for the differences between *in silico* and *in vitro* results

The *in silico* and *in vitro* results did not match for particular target root numbers of certain patterns. Three reasons that may account for the differences in the simulation include: (1) too many neurites reach the distant roots along the pattern, (2) multiple neurite turning behaviors are not taken into account at the non-permissive edge, and (3) fasciculation is not taken into account in the simulation. Possible explanations for why the *in silico* data does not entirely match the *in vitro* data are described below.

First, to examine whether the number of neurites that reached distant target roots ($Tb = 5$ or $6$) in the simulation modifies $PET_{Tb}$ we compared 20 replicates of 100 neurites grown for different iterations on the control pattern. 100 neurites were either grown for a constant (7800 iterations) or a uniformly distributed random number of iterations (1 to 7800 iterations). We found no significant difference for *in silico* $TPR_{Tb}$ after comparing recalculated $TPR_{Tb}$ using constant or random uniformly distributed iterations. Neurite probability of entering a target root, however, did significantly increase (unpaired one-tailed t-test, $p < 0.05$) when neurites were grown for a constant number of iterations along the control and experimental patterns for distant target roots. The increase in neurite number is potentially caused by more neurites reaching the distant target roots, which would not have been possible with a smaller number of iterations. Since the *in vitro* $PET_{Tb}$ data is significantly less than the *in silico*
PET_{Tb} for distant roots, future simulations will be fit to in vitro data by reducing the number of neurites that reach the distant roots.

Second, we examined how neurite turning behavior at the pattern’s non-permissive boundary, (i.e., edge) may affect the TPR_{Tb}. Studies of neurites growing on non-permissive or less permissive boundaries indicate that neurites have many behaviors at these boundaries (Gomez & Letourneau, 1994; Tanaka & Kirschner, 1995). In our simulation, neurites that encountered the edge randomly selected a new angle until they avoided the edge; however, neurites sometimes regress, stall, or die back before regrowing along or away from the edge of the pattern. If neurites are allowed to grow along the edge of the pattern, instead of selecting a random new angle to grow within a small range, neurites are more likely to enter a root, which may increase the probability of a neurite taking a divergent trajectory into a root. The model was modified so a neurite that encounters an edge dies back to its previous positions and then changes its current angle to 90° and re-enters the program until another edge is encountered again. Even with this modification the in silico TPR_{Tb} was not significantly different to TPR_{Tb} for the original simulation.

Third and final, we examined how fasciculation, i.e., a neurite growing along existing neurites, affects neurite root preference by influencing neurites to either be guided into or away from a root. Fasciculation was observed in all in vitro experiments along the micropattern. Previous studies have shown from in vitro data that when neurites from an explant interact with existing axons at shallow angles (< 30°) they tend to fasciculate, while neurites that impinge on an
existing axon at more obtuse angles tend to cross over the existing axon (Roberts & Taylor, 1982; Kapfhammer & Raper, 1987). Using the angle of incidence between neurites crossing over to incorporate fasciculation into the program we developed a new function. This function allows neurites growing nearby (1 µm, approximate diameter of an axon) and at similar angles to follow the previous neurite’s trajectory.

To simulate fasciculation at each iteration, the angle θi of each neurite tip was compared with that of the neurite segments lying within a distance rc, a conform radius (Figure 5.9).

![Figure 5.9 Schematic of how fasciculation was incorporated into the program](image)

Growth of a neurite's tip, defining the growth cone centroid's position, is calculated using a persistent random walk (blue line) as previously described when there are no nearby neurites. When a growing neurite (red line) detects a nearby neurite within a conform radius (rc, green circle), the neurite may fasciculate with the nearby neurite by adjusting its current angle so that it follows parallel to the nearby neurite for at least one iteration. A neurite will fasciculate in the simulation if the difference between the growing neurite’s (black line) current angle, θi, and a nearby neurite’s (blue line) angles (θi and θi−1) are less than the conform angle, θc. To simulate fasciculation, the growing neurite’s next angle (θi+1) is set to the nearby neurite’s angle (θj).

We use the conform radius, rc, to accommodate distinct cases in which neurites fasciculate side-by-side within an axon-width’s distance (1 µm). If a neurite
segment is found within $r_c$ of any growing tip, the angle of attach to the tip of the segment is evaluated, and if the angle is less than or equal to $\theta_c$, then the tip adopts the direction of the nearby segment. Neurites that are outside of the angle of fasciculation travel without interruption. If there is more than one eligible neurite segment to choose from, the neurite tip picks the angle of the closest neurite. Thus we simulate fasciculation as the convergence of growth cone trajectories with existing neurites as illustrated in Figure 5.9.

We compared 20 replicates of 100 neurites grown on the single root pattern (experimental pattern) with fasciculation incorporated into the *in silico* model. $TPR_{Tb}$ increased as conform angle was increased ($0^\circ$, $30^\circ$, and $90^\circ$) for $Tb = 2$ and $6$, but not for $Tb = 5$, as shown in Figure 5.10.

![Figure 5.10 Comparing *in silico* and *in vitro* $TPR_{Tb}$ when fasciculation was incorporated into the *in silico* model](image)

The probability of a divergent trajectory is not significantly different for either *in silico* or *in vitro* data when comparing target root numbers. *In vitro* $TPR_{Tb}$ was not significantly different to *in silico* $TPR_{Tb}$ when conform angle was $90^\circ$ for $Tb = 2$ or $6$. *In vitro* $TPR_{Tb}$, however, was significantly different (unpaired two-tailed t-test, $*p < 0.05$) to *in silico* $TPR_{Tb}$ when conform angle was $90^\circ$ for $Tb = 5$. Error bars are SEM.

One reason for this unexpected result may be that the distance from the neurite's initiation influences neurite entry into a target root. A similar unexpected decrease for *in vitro* $PR_b$ was also observed for the experimental pattern of $Tb =$
5 (Figure 5.5A). Nevertheless, *in silico* and *in vitro* \( TPR_{Tb} \) was not significantly different for nearby \( (Tb = 2) \) and a distant target root \( (Tb = 6) \) when fasciculation was incorporated into the *in silico* model (only when \( \theta_c = 90^\circ \)). Even though fasciculation was observed to occur primarily in roots near the neurite initiation *in vitro*, fasciculation was also observed in distant roots. Further research on how fasciculation may influence a neurite from leaving the primary channel and entering a root may provide insight into another possible guidance strategy for promoting a neurite’s migration farther along the pattern. Future research should also focus on other factors affecting whether neurites following pioneering neurites into roots, such as non-neuronal – neurite interactions or neurite branching.

### 5.5.3 Clinical Implications

As we have shown, the likelihood of neurites reaching a distant target root is improved by using a strategy that restricts neurites from entering non-target roots (i.e., removal of non-target roots). Future therapies may employ a similar strategy to nervous system injuries in which non-target regions are avoided by regrowing neurites; not by removing these regions but by placing non-permissive agents (e.g., BSA) at key decision points along a regenerating neurite’s path. Similar strategies have been observed during brain development when vestibular axons migrate to the cerebellum (Tashiro et al., 2001). Future research will focus on placing other guidance cues such as attractive and repulsive cues along a micropattern with a complex geometry to examine other methods that promote neurite guidance to targets. In particular, it will be beneficial to see how other
cues influence nearby target guidance since we have developed a strategy to promote neurite guidance to a distant target.

Neurite guidance strategies developed *in vitro*, however, may not result in the same effect when applied to the *in vivo* system because of dissimilarities between the two systems. For example, many other factors not taken into account *in vitro* but present *in vivo*, such as physical obstacles and different extracellular matrix cues, may influence neurite guidance. In addition, the *in vivo* system is 3-dimensional whereas the *in vitro* system is 2-dimensional. Others, however, have shown that modified *in vitro* experiments may be used to examine how this extra dimension influences guidance (Li & Folch, 2005; Kofron, Fong, & Hoffman-Kim, 2009) and this will be an important factor to investigate in future research.
6 CONCLUSION AND FUTURE WORK

Based on the results described in the preceding chapters, three important conclusions can be drawn. First, without guidance the likelihood that a regenerated neurite will reach a desired target is effectively zero; hence guidance strategies are necessary. Second, although a single guidance strategy cannot serve the functional needs of the multiple different systems that are present in the spinal cord, a strategy that restricts neurites from entering non-target regions promotes neurite guidance to distant targets but not nearby targets. Third, neurite tips do not obey simple first order dynamics (Voyiadjis, Doumi, Curcio, & Shinbrot, 2011); thus necessitating the development of more complex computational models that are more accurate.

To examine the likelihood of a neurite reaching a desired target without cues, we developed an in vitro and an in silico model which provide a theoretical framework and an experimental platform to answer questions about guidance strategies in a simplified environment. We verified the in silico model by using an in vitro model with identical micropattern geometries as the in silico model. In particular, our simulation predicted that the likelihood of a neurite reaching a target exponentially diminishes with distance from its initiation point, which was later verified by experiments in the in vitro system. These results establish that our simulation makes accurate predictions with respect to in vitro neurite outgrowth behavior when a factor affecting neurite behavior is incorporated (e.g., complex geometry of permissive/non-permissive boundaries). More importantly
because the likelihood of a neurite reaching a target exponentially diminishes with distance from its initiation point, the likelihood of a neurite reaching a distant target is effectively zero. Therefore, guidance strategies are necessary to promote neurite guidance to these distant targets.

Using the *in silico* and *in vitro* models, we tested a simple guidance strategy to investigate if the likelihood of neurites reaching a target root may be modified. In this strategy, neurites were restricted from entering undesired roots by removing these roots from the micropattern. This results in a significant increase of neurites that reach and enter the distant target root but not when the target root is near the neurite’s initiation point. Results from this research support the use of similar strategies in nervous system injury therapies, in which neurites are restricted from entering non-target regions when a target is distant from the neurite’s initiation. As previously mentioned in chapter 5, we speculate that a similar strategy using non-permissive agents (e.g., BSA) placed at key decision points along a neurite’s path to its distant target may increase the number of neurites that reach its target. In addition, a strategy using a guidance cue that restricts neurite entry into non-target regions (e.g., repulsive cues) may also be advantageous for directing neurites to their distant target.

To support the hypothesis that guidance cues play distinct roles in pathfinding, we note that three (the ephrins, semaphorins, and slits) of the four known major protein guidance cues act predominately as inhibitory or repulsive cues during neurite outgrowth (Maskery & Shinbrot, 2005). Each of these families has many types of protein members; for example there are over 20
different kinds of semaphorins (Pasterkamp & Kolodkin, 2003). However, only one of the major protein guidance cues plays a role as an attractive cue (the netrins) and this has only two members. The far greater diversity and redundant number of repulsive cues suggests that they may play a preferred role in neurite guidance (de Wit & Verhaagen, 2003; Maskery & Shinbrot, 2005).

Finally, we reassessed the robustness of our predictive model by comparing it to an identical \textit{in vitro} experiment using the same modified single root pattern. We found that the \textit{in silico} and \textit{in vitro} models matched significantly for a distant and a nearby target root, but only when fasciculation was incorporated into the simulation. This finding indicates that neurite tips do not obey simple first order dynamics alone, and thus future simulations need to test and include more complex models in order to be accurate.

Future work may use the \textit{in vitro} model as a tool to enhance the \textit{in silico} model by assessing the impact of variables that are not taken into account in the \textit{in silico} model. This will be useful in the further development of more sophisticated guidance strategies. For example, neurites preferred divergent trajectories more \textit{in vitro} than \textit{in silico} in regions where neurites fasciculated more. Therefore the impact of \textit{in vitro} fasciculation on neurite guidance could be evaluated by comparing the two models, since the \textit{in silico} model did not originally incorporate fasciculation. In addition, fasciculation’s potential role in neuronal repair could also be evaluated by examining its influence on guidance \textit{in vitro}. 
Particular emphasis was also placed on limiting the number of important variables in the simulation that influence neurite outgrowth while still accurately depicting experimental outgrowth behavior and developing a simulation whose boundaries can be easily modified. These aspects of the simulation were stressed so that future work may examine different neurite behaviors and neural systems after modifying a small number of parameters in the model. The current simulation includes channel geometrical factors (e.g. root widths, angles, and distances) and growth cone parameters evaluated from neurite outgrowth on uniform substrates (e.g., growth cone search angle ($\delta$), average outgrowth ($r_d$), and outgrowth uncertainty ($r_s$)). An example of another nervous system that the simulation could easily be modified to match is the peripheral nervous system. Therapies aimed at guiding regenerating peripheral neurons to a specific target following an injury (e.g., preferential motor reinnervation) will also benefit from using this computational simulation to assess of how successful neurites are in reaching their targets in complex geometries.

In summary, we have developed a framework and a strategy for testing and using cues in neural repair. Further in vivo studies will need to be conducted to take this research into the realm of clinically relevant applications, and remains the subject of future direction for this research.
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ACKNOWLEDGMENT OF PREVIOUS PUBLICATIONS

Parts of this work have previously been published:
