

**COVER PAGE**

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## 1. ORIGINAL AIMS OF THE PROJECT

- 1) Computational modeling: The focus of this aim was to construct a computational model to simulate neurite outgrowth based on biological experiments. Simulated neurites were grown on a pattern that branches repeatedly, as occurs in the spinal cord with multiple spinal "roots". The simulation was then used to test the hypothesis that more neurites travel into roots near a neuron when attractive cues (i.e. a chemical agent that neurons are attracted to) are placed at the nearby root. We also hypothesized that more neurites reach and enter roots far from a neuron when repulsive cues (i.e. a chemical agent that neurons avoid) are placed at undesired roots before and after the distant target root. Results from this aim tell us how cue choice and placement can affect targeting.
- 2) *In vitro* experiments: The focus of this aim was to manufacture a microfabricated experimental equivalent to the geometry and cue arrangements of Aim 1. Permissive and repulsive cues were to be imprinted on a micropattern with multiple roots, on which embryonic chick sensory neurites would be grown. These results would provide us with both experimental validation (and indications for improvements) of the computational model of Aim 1, and permit us to quantitatively evaluate the hypothesized relevance of cue choice to neurite guidance.
- 3) Optimization: The focus of this aim was to use data obtained from Aims 1 & 2 to determine how both simulations and experiments respond to attractive vs. repulsive cues, and to predict optimal conditions to maximize the likelihood that neurites reach and enter a specific target root.

## 2. PROJECT SUCCESSES

Research on spinal cord injury (SCI) repair focuses on developing mechanisms to allow neurites to grow past an injury site. As motivation for this work, we observe that numerous divergent paths (i.e. spinal roots) are present along the spinal column, and hence guidance strategies must be devised to ensure that regrowing neurites avoid blind alleys, which for example may already be enervated, but instead reach viable targets. Therefore, we chose to examine how neurites grow along a pattern with many alternate pathways ("roots") along a branching protein micropattern (Fig. 1). Alongside these experiments, we developed a computational simulation with the same geometry as our experiments (Fig. 2), which was validated by comparison with independent biological experiments and used to test guidance strategies. We were successful in growing and analyzing experimental neurite outgrowth on the micropattern with multiple roots, optimizing our computational simulation to match experimental neurite outgrowth behavior, and evaluating how restricting neurite entry into non-target roots may increase the chances for neurites to reach and enter a distant target.

The first project success was creating the computational model (Fig. 2) based on observed neurite outgrowth behavior on an experimental micropattern (Fig. 1). By comparing the computational and experimental systems, we quantified neurite preference for different roots along the pattern, neurite decisions at the root entry, and what other factors most strongly appear to influence guidance. For example, by analyzing the neurite outgrowth patterns in each system we found (see Fig. 3) that a neurite's preference ( $PF_b$ ) for a particular root decreases exponentially with respect to the number of roots away a neurite's origin. Consequently, the likelihood of a neurite reaching a distant root can be vanishingly small for both the simulation and experiments. We also evaluated the probability that neurites will choose a divergent trajectory ( $PR_b$ ) into a root instead of continuing to grow straight along the primary channel. Initially, we assumed that  $PR_b$  would be constant for all roots, which was true for the simulations; however, experimentally there was a significant decrease in  $PR_b$  as neurites moved further along the pattern (Fig. 4). These results were described in Paper 2, below. This difference may be attributed to other factors such as neurites interacting and growing along one another, i.e., fasciculation or bundling, which was observed in experiments. Fasciculation was found primarily within the first roots encountered along the primary channel (Fig. 5) since the neurite's width in these roots was greater than the average width of one neurite (width > 1  $\mu\text{m}$ ). This finding was unexpected and indicates how factors such as fasciculation may have influenced guidance within these early roots, i.e., early decisions points along a neurites path. Fasciculation was studied and described in Paper 1, below.

The second project success was showing that our simulation matched our experimental data of neurites grown on a multiple root pattern. Our simulation is based on experimental neurite outgrowth data taken from time-lapse microscopy (Fig. 6) and analyzed using MATLAB's Statistical and Curve Fitting Toolboxes (Figs. 7 and 8). This result represents a starting point for future strategies to optimize the likelihood that neurites will reach appropriate targets in the regenerating nervous system, and provides a new computational tool to evaluate the feasibility and expected success of neurite guidance in complex geometries (Fig. 2).

The final project success was the evaluation of neurite outgrowth on a modified multiple root pattern to examine how non-permissive cues can influence neurite guidance to distant target roots ( $Tb$ ) along a complex micropattern. The new pattern combined the previous multiple root micropattern (control pattern) along side a y-pattern with only one root (experimental pattern) for both experiments and simulations. Three different micropattern designs were created to examine how the target root's distance from the primary channel entry and number of non-target roots before the target root affect neurite area for a target root near ( $2^{\text{nd}}$  root,  $Tb = 2$ , shown in Fig. 9A) and distant ( $5^{\text{th}}$  and  $6^{\text{th}}$  roots,  $Tb = 5$  and 6, respectively shown in Figs. 9B and 9C) from the primary channel entry. The target root on the experimental pattern is the single root. Similarly, the control pattern's target root is at the same location and distance up the primary

channel as the experimental pattern's target root (mirrored across the primary channel).

By comparing neurite area along the target roots of both patterns, we quantified the changes in neurite area searched within a target root when other roots were removed. Total neurite area or neurite area within a fixed distance of a target root was measured and is shown respectively as dotted blue or dashed yellow boxes in Fig. 10. We found that average total neurite area,  $TA_{Tb}$ , for distant target roots ( $Tb = 5$  and  $6$ ) significantly increased when neurite's were restricted from entering non-target roots in the experimental pattern (Fig. 11A).  $TA_{Tb}$  for a nearby target root ( $Tb = 2$ ), however, was not different when comparing the control and experimental patterns. The target root in the experimental pattern and  $b = 1$  in the control pattern were also compared since they are the first root that a neurite encounters along their respective patterns. These values differed significantly which indicates that neurite preference is affected by a root's distance from the base of the pattern as well as the number of non-target roots encountered before the target root. Similar results were found when neurite area was measured a fixed distance within the target roots,  $AR_b$  (Fig. 11B). These results have been collected in Paper 3, below.

In summary, we have successfully shown that because of the complexity of spinal connections, the *a priori* chance of a nerve axon reaching a specified target in the spinal column without guidance can be expected to exponentially decrease with distance to the target (Fig. 3). Thus distant targets will have vanishing likelihood of being reached, even by actively growing neurites, without an intentional guidance strategy. In addition, we have developed an integrated program of computational simulations (Fig. 2) combined with biological experiments (Fig. 1) to identify guidance strategies to significantly increase the chances that regrowing neurons will reach distant usable targets and thereby restore function.

### 3. PROJECT CHALLENGES

One project challenge was developing a metric for neurite entry into a root since we could not visualize single neurites during time-lapse microscopy. We decided to examine 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. 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). This measurement allowed us to avoid the intrinsic difficulties of counting overlapping neurites.

Two different approaches investigated to quantify neurite root preference included: (1) time-lapse microscopy and (2) staining of the neurite's growing tip, the growth cone. Both approaches, however, proved difficult to accurately quantify. Time-lapse microscopy of neurites allowed us to see neurites as they entered a root. Although tracking neurite tips is time consuming, it was hoped that this would provide definitive data for the numbers of neurites that enter different roots. In the event, we found that some growth cones traveled up along

other neurites undetected due to overlapping of the cone with pre-existing neurite arbors. This overlap was detected by noting neurite tips that disappear and reappear from view, often coinciding with temporary expansions in the bundle width. Additional investigations were attempted using isolated single neurites, to reduce the density of neurites and permit accurate counting. But time-lapse images revealed constantly moving cells that interfered with our ability to detect where a given growth cone originated, and hence how many roots a given neuron passed. Staining neurite tips, was also investigated and used to count the number of growth cones within a root, however single neurite tips often grow on top of each other, preventing us from distinguishing individual tips.

Another project challenge was the placement of guidance cues (as described in the aims) along the multiple root pattern. Two complications we encountered were reproducibly placing a guidance cue on the pattern without disrupting the protein micropattern underneath. We attempted to place the cues on the pattern using PDMS stencils, however, it was particularly difficult to align the stencil with the micropattern underneath. In addition, we tried placing a small drop onto the particular location using a microscope and syringe with small gauge but this sometimes disrupted the pattern underneath. The best method for continuation studies would be to outsource this patterning process using micropattern printing technology.

#### **4. IMPLICATIONS FOR FUTURE RESEARCH AND/OR CLINICAL TREATMENT**

We have examined neurite outgrowth patterns on micropatterns with multiple diverging roots, which mimic the spinal column's multiple roots. Both computational simulations and experiments were performed, indicating that the probability of neurites entering a root decreases exponentially with distance from a neurite's initiation along the multiple root pattern. This implies that future regenerative therapies are exceedingly unlikely to produce viable connections, especially to distant targets, even once central nervous system obstacles developed post-injury have been overcome. We argue, therefore, that new guidance strategies, especially the judicious application of non-permissive cues at unwanted nearby spinal roots, will be necessary to establish functional recovery whenever the neurite's target is distant from the origin of the neurite.

The simulation developed is a first prototype of a neurite guidance model. We have validated the simulation with comparison both to growth on featureless substrates on to growth on micropatterned branching substrates. To improve applicability of our simulation so as to inform future therapeutic approaches, further embellishments may be desirable. These include important effects such as fasciculation, branching, and responses to guidance cues, all of which can be included and experimentally validated in a straightforward manner. In addition, the use of non-permissive cues to guide neurites to distant targets demonstrates that these cues could improve the target area searched by neurites when placed at specific locations. Future research should clearly apply the guidance strategies developed in this study to animal studies *in vivo*. These studies would

**FIGURES FOR FINAL NARRATIVE:**

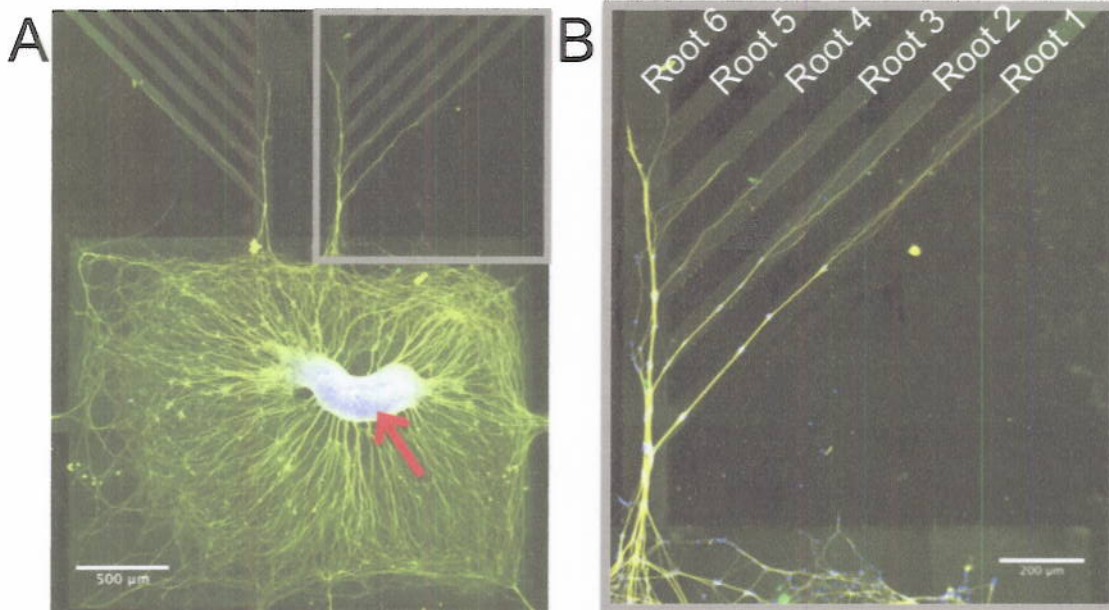


Fig. 1. (A) *In vitro* growth of DRG neurons (neurofilament, yellow) on a micropattern (laminin, green) designed to emulate roots branching off of the spinal column. The red arrow indicates where the DRG explant (DAPI, blue) was placed. (B) Enlarged view of gray box visible in upper right of (A).

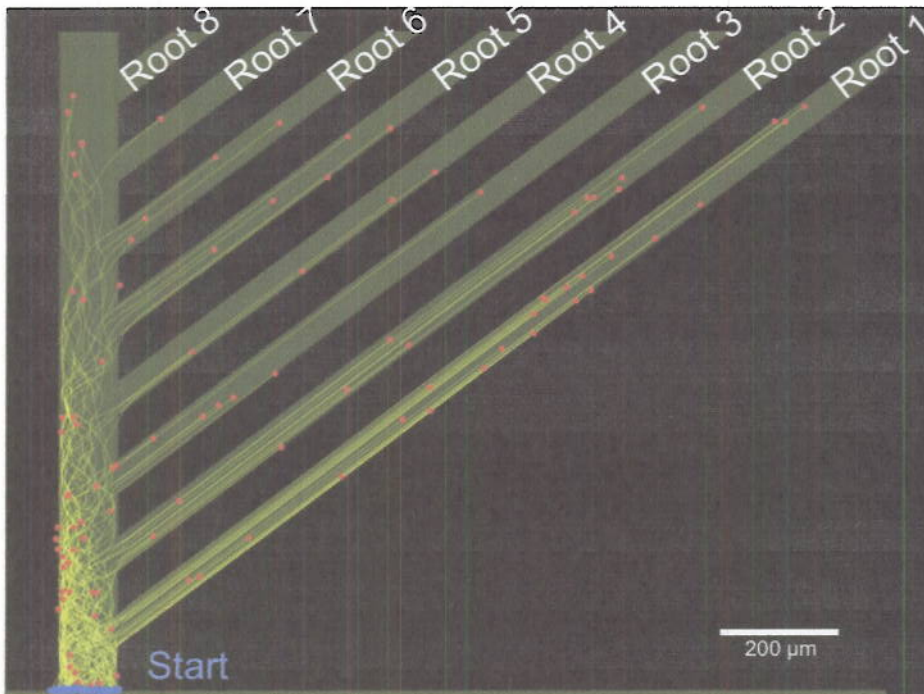


Fig. 2. Result from the simulation of neurite trajectories (yellow lines) and growth cone centroids (red circles) on a multiple root pattern designed to mimic the complex *in vitro* micropattern geometry. The start, defining where neurites enter the primary channel for companion *in vitro* studies, is labeled as blue circles. Neurites continued to grow straight at 45° after entering a root, as observed from experimental samples.

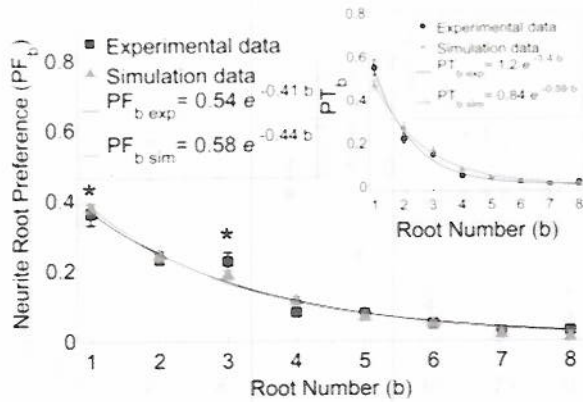


Fig. 3. Percent of neurofilament area occupied within a fixed distance of the first eight roots is shown for both experimental (black squares) and simulation (gray triangles) data. Data are fit to exponentials indicated with  $r^2$  values for experimental and simulation data that are respectively 0.955 and 0.996. Experimental  $PF_b$  was significantly larger for the labeled  $b$  when compared to the next root number (unpaired two-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 standard errors of the mean (SEM).

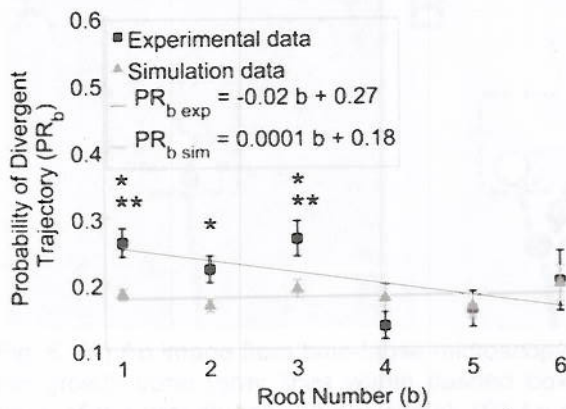


Fig 4. Probability of neurites choosing a divergent instead of a straight trajectory for the first six roots showed a linear trend for experimental and simulated data. Experimental (black squares) and simulation (gray triangles) data fit within the predicted 95% confidence intervals of their respective linear curves. Experimental  $PR_b$  was significantly larger for the labeled  $b$  when compared to  $b = 4$  (unpaired two-tailed t-test,  $*P > 0.05$ ,  $N = 20$ ). In addition, experimental  $PR_b$  was significantly larger for the labeled  $b$  when compared to  $b = 5$  (unpaired two-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. Error bars are SEM.