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14. ABSTRACT

The primary goal of this collaborative research project was to develop next generation engineered nerve guide conduits (NGCs) with aligned nanofibers and favorable release kinetics of neurotrophic factors to help improve surgical outcomes after injuries involving peripheral nerves.

In the first two years of the work, we developed an improved version of the nanofiber NGCs with increased surface area of nanofibers and gradient loading of the neurotrophic factor GDNF (Glial cell derived neurotrophic factor). Furthermore, we evaluated their degradation rates in vivo. Next we tested the new nanofiber NGCs in vivo in rats to determine which combination of neurotrophic factors, surface modifications and nanofiber structures are better for peripheral nerve regeneration. Finally, once we identified the optimum combinations for new nanofiber NGCs using the rat peripheral nerve regeneration model, we evaluated the effects of such modifications in a large gap nerve repair in a large animal model using canine peroneal nerve. Our results show that relatively steep gradient of neurotrophic factor release, combined with longitudinally aligned nanofibers of a certain size organized in a S-shape conduit provide the best chance of improving peripheral nerve regeneration.

15. SUBJECT TERMS
Nanofiber nerve guides, nerve regeneration, neurotrophic factor, gradient loading

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1. INTRODUCTION:

Peripheral nerve injury is a common complication of complex tissue trauma and often results in significant disability in war injuries. Regeneration of peripheral nerves is often incomplete and in complex war injuries donor nerves are difficult to find for nerve repair. Nerve guide conduits (NGCs) made of biodegradable materials offer a potential solution to this problem. Based on our previous accomplishments in developing a nanofiber containing NGCs, the primary goal of this collaborative research project is to develop new nanofiber NGCs with improved nanofiber guidance cue and modulated trophic factor delivery capabilities that promise faster nerve regeneration and better functional recovery.

2. KEYWORDS:

Nerve guidance conduit, neurotrophic factor, GDNF, gradient, electrospun nanofiber, dog study

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: To engineer novel nerve guides with aligned nanofibers that provide contact guidance and modulated neurotrophic factor delivery *in situ*.

- Proposed date of completion: End of 2011
- Date of completion: End 2013

Aim 2: To assess nerve regeneration and functional recovery of nerve guides in a rat model, and optimize the nerve guide configuration.

- Proposed date of completion: End 2012
- Date of completion: Mid 2014

Aim 3: To demonstrate efficacy in a larger animal model using optimized nerve guide from Aim 2.

- Proposed date of completion: End 2013
- Date of completion: End 2015

Innovation: New nerve guide with nanofibers and modulated neurotrophic factor delivery promises faster regeneration and functional recovery.

What was accomplished under these goals?

Summary:

We were able to complete the major aims established at the onset of this project. With the first aim of the project, we utilized a live-cell imaging platform to investigate the effects of topographical and biochemical guidance on DRG neuron and human primary Schwann cell migration. Cells were cultured on aligned nanofiber substrates of different fiber diameters to determine the effect of fiber diameter on the speed and directional guidance of cell migration. From these experiments, we found that intermediate fiber diameters (700 nm to 2 µm) promoted both fast cell migration and suitably restrict the migration of cells along the axis of the fibers (Fig. 1a-d). Using a cell migration chamber setup developed for this project (Fig. e-h), we examined the effects of delivering GDNF gradients of varying steepness on influencing the migration dynamics of Schwann cells. Using this novel platform, we demonstrated the importance of gradient steepness as an important characteristic determining the ability for Schwann cells to detect and respond to a gradient. We found that gradient steepness influences both the migration speed and the directional guidance of Schwann cells, but that gradients which promote the highest speed were not necessarily those that also promote the greatest directional guidance. As the results of *in vitro* experiments could not directly predict which gradient steepness promotes the greatest overall functional recovery, it was important for us to screen different gradients in our *in vivo* small animal model to determine which gradient design was best for use in the large animal model.

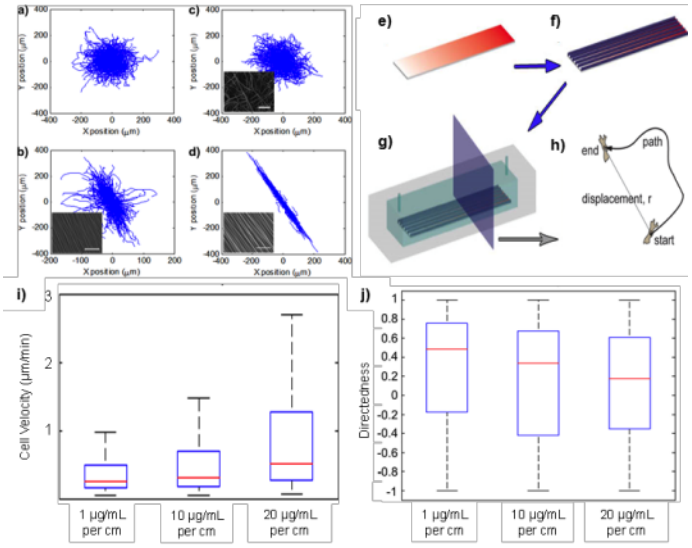

Figure 1. *In vitro* cell migration platform for investigating topographical and biochemical guidance effect of human primary Schwann cells. Migration pattern of cells on 2D control (a), random nanofibers (b), 180 nm aligned nanofibers (c), and 750 nm aligned nanofibers (d). Scale bars = 30 µm. *In vitro* live-cell migration chamber allowing for high-throughput cell tracking (e-f). Cell migration speed of cells exposed to GDNF gradients with increasing concentration ranges (i). Directedness, or migrational bias, of migration with a value of 1 indicating cells migrating perfectly in the direction of gradient, 0 being non-biased migration, and -1 indicating cells migrating in opposite direction of gradient (j).

With the design parameters obtained from results in aim 1, we successfully developed a nerve guidance conduit with which we could incorporate both topographical and biochemical guidance cues. We tested multiple configurations of the conduit, and through numerous *in vivo* small animal experiments screening the different configurations, we found the optimal configuration was an S-shaped conduit which provided both high contact area of aligned fibers to interact with

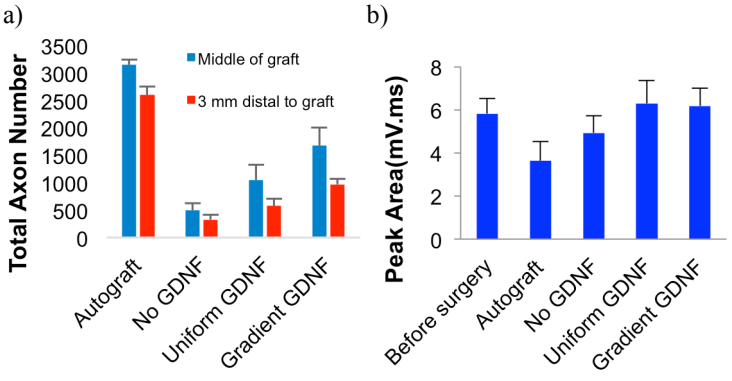

Figure 2. Histomorphometric and functional analysis of large animal nerve regeneration study. Total axon count (a) and EMG peak area (b).

ingrowing tissue, but also included two large luminal spaces within which new nerve tissue could grow. Using this NGC configuration, we tested the efficacy of NGCs containing aligned fibers and different gradients of GDNF to determine the final configuration to use for the large animal model in aim 3. With the experiments in aim 2, we determined that the NGC with the steepest GDNF gradient was most effective in promoting nerve regeneration in a rat sciatic nerve model, and this gradient condition was selected for testing in the large animal model.

With aim 3, we were successful in utilizing an NGC, which combined topographical guidance and biochemical guidance to enhance regeneration in a novel large animal peripheral nerve injury model. We discovered that although our conduits promoted the regeneration of a lower number of axons, the axons that regenerated exhibited higher functional output in the GDNF gradient group compared to that of the autograft control (Fig. 2), potentially indicating that the GDNF gradient improves the maturity of regenerating nerves. One potential problem we encountered in the large animal nerve repair model is that the composition of our NGCs made it susceptible to compression at sites of active movement across joints, somewhat limiting the success of our NGCs compared to the autograft in terms of number of axons regenerated. Nevertheless, our results demonstrated the promising potential of utilizing gradients of growth factors as a method for improving the regenerative efficacy of NGCs and gave us tremendous insight into new design parameters, which should be considered when developing NGCs for use in high-activity regions of animal and human limbs.

While we were largely able to complete our three primary aims, we limited our investigation of two design parameters listed in the initial project proposal, 1) incorporation of cell-adhesive ligands onto the surface of aligned fibers, and 2) modification of fiber degradation by blending gelatin into fiber composition. We limited the investigation of the cell-adhesive ligands primarily because we wanted to limit our research focus on investigating the effects of fiber diameter and gradient characteristics on cell migration, and testing different adhesive ligands would have introduced excessive experimental complications. Additionally, instead of pre-coating the aligned fibers in our NGCs with adhesive ligands, we instead relied on the endogenous adhesive ligands secreted by the injured nerve stumps to which the NGCs were sutured in the *in vivo* animal models. While we did investigate the effect of modifying fiber degradation rate on nerve regeneration, we found that NGCs containing blended fibers reduced the regeneration potential of NGCs compared to NGCs containing PCL-only fibers. Thus, we chose to utilize PCL-only fibers for the small animal and large animal experiments in aims 2 and 3.

A more detailed research design, methods and results section is below.

Detailed Research Design, Methods and Achievements

The primary goal of this project was to develop an alternative to autologous nerve grafts used in repair of peripheral nerve injuries in war and civilian life. Based on our previous experience and preliminary data we proposed the following specific aims:

Specific Aim 1: To engineer nerve guides with aligned biodegradable nanofibers that provide contact guidance cue and modulated neurotrophic factor delivery to regenerating axons and Schwann cells;

Specific Aim 2: To assess nerve regeneration rate and functional recovery in a rat model of nerve repair, and to optimize nerve guide configurations;

Specific Aim 3: To demonstrate efficacy in a large animal model using optimized nerve guide from Aim 2, as a prerequisite for clinical translation.

The following section summarizes our findings in relation to our initial aims and discusses the significance and impact of our work.

Specific Aim 1: ***To engineer nerve guides with aligned biodegradable nanofibers that provide contact guidance cue and modulated neurotrophic factor delivery to regenerating axons and Schwann cells.***

One major advantage of our nanofiber nerve guides is the modular design, which allows the nanofiber-induced contact guidance and neurotrophic factor delivery system to be optimized independently. On nanofiber development, we examined the effects of nanofiber diameter on axonal outgrowth and Schwann cell migration in an *in vitro* culture model. In parallel, we developed the hydrogel loading methods and investigated the effects of varying process parameters on the release kinetics and loading capacity of neurotrophic factors glial derived neurotrophic factor (GDNF). Utilizing *in vitro* cell migration platforms, we screened for which nanofiber and gradient conditions would provide optimal nerve cell growth and guidance. The outcome of this Aim provided us with a set of nanofiber NGCs with tunable physical configurations and neurotrophic factor loading levels and gradient profiles for in vivo evaluation in Specific Aim 2.

Sub-Aim 1A: *To prepare electrospun nanofiber matrix with controlled nanofiber diameter and evaluate effect of fiber diameter and alignment on axonal outgrowth and Schwann cell migration in an in vitro culture model.*

We have successfully demonstrated that rat dorsal root ganglion neurons (DRGs) cultured on aligned nanofiber substrates exhibit preferential extension in the direction of fiber alignment and that extension varies based on fiber diameter. DRGs cultured on 480-nm aligned fibers showed the highest degree of directional growth preference. Additionally, DRGs grown on the 480 nm were notably longer than those on the 260-nm aligned fibers and on the random fiber substrates, providing evidence that the proper selection of fiber diameter and alignment will enhance not only the directional preference of axonal growth, but also the speed at which the axons will extend. These results demonstrate that the incorporation of aligned nanofibers within the lumen of a nerve guide will likely provide directional contact guidance for regenerating axons and Schwann cells, increasing the speed of axonal and Schwann cell migration and providing an improved regenerative outcome compared to random fiber conduits.

In addition to measuring DRG outgrowth, we investigated the migration of human primary Schwann cells cultured on nanofiber substrates of differing alignments and diameters. Throughout the course of this project, we made significant improvements in our live-cell microscopy and MATLAB-based cell migration, which allowed for high-throughput cell tracking

studies of dissociated Schwann cells migrating on a wide range of fiber sizes and configurations. Studies were conducted by culturing primary human Schwann cells with labeled nuclei on nanofiber-coated coverslips in 24-well plates, observing cell migration using live-cell imaging microscopy, and analyzing cell migration with our MATLAB-based programs. Our results demonstrated that aligned fiber substrates provided enhanced topographical restriction of the migration of cells along a single axis (Fig. 1d) compared to 2-dimensional substrates (Fig. 1a) and random fiber substrates (Fig. 1b). We hypothesize that this topographical restriction is an important factor in the promotion of uniaxial migration of nerve outgrowth and will aid in faster peripheral nerve functional recovery where nerve outgrowth occurs along linear paths. For these single-cell studies, the ability for aligned fibers to restrict the migration direction of cells was shown to be fiber-size dependent. Our results confirmed that small nanofibers (180 nm, Fig. 1c) were less able to restrict cell migration direction than larger fibers (Fig. 1d), and cells were better able to migrate onto neighboring fibers. This transmigration appeared to become limited as fiber size increases, indicating a preference for the cell processes to extend along single fibers once fiber size becomes suitably large.

We also demonstrated that cell migration speed is dependent on fiber diameter, with cell migration rate increasing as fiber diameter increases (Fig. 1e). Based on our results, cells migrating on aligned fiber substrates with fiber diameters of less than 1 μm exhibited migration speeds lower than that of two-dimensional substrates. Fibers larger than 1 μm in diameter exhibited cell migration velocities that were comparable or faster than two-dimensional substrates. However, although the speed of migration was higher on larger fibers, cell migration direction fluctuated more on larger fibers, and thus they exhibited lower migration persistence compared to small fibers and 2D controls (Fig. 1f). This demonstrated that fibers of intermediate diameter (700 nm to 2 μm) might provide the best combination of directional restriction and migration speed.

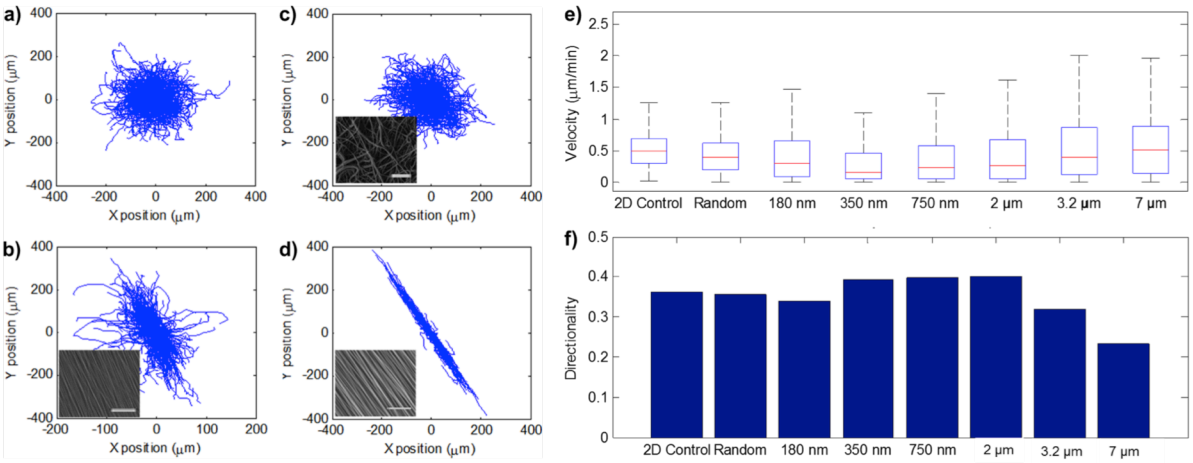

Figure 1. Fiber size-dependent migration of human primary Schwann cells on polycaprolactone nano/micro-fibers. Migration pattern of cells on 2D control (a), random nanofibers (b), 180 nm aligned nanofibers (c), and 750 nm aligned nanofibers (d). Scale bars = 30 μm. Cell migration speed (e) and migration persistence (f) of cells on varying substrates. For all figures, n > 450 cells for each group.

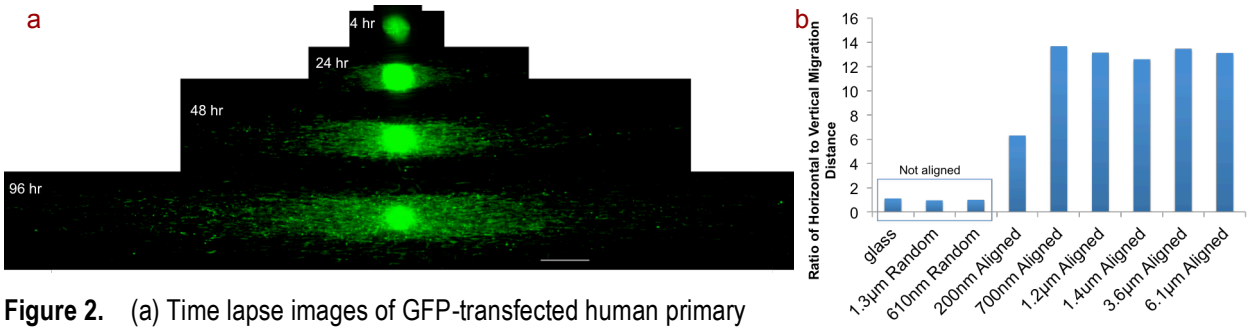

Figure 2. (a) Time lapse images of GFP-transfected human primary Schwann cells migrating out spheroid over 96 hours. Spheroid seeded onto 1.4-μm aligned PCL fibers oriented horizontally in the image. The scale bar is 500 μm. (b) Averaged aspect ratio of spheroid migration groups at 48 hours. The aspect ratio is the horizontal migration distance divided by vertical distance travelled.

To supplement our findings from the single-cell Schwann cell migration studies, we cultured spheroids of human primary Schwann cells on random and aligned fiber substrates. This study was designed to investigate how fiber diameter and alignment influenced the outgrowth of Schwann cells from a group of cells at a single point source (such as the proximal and distal stump of a damaged nerve). We monitored the outgrowth of GFP-transfected Schwann cells over the course of 4 days using time-lapse imaging. Schwann cells cultured on aligned fiber substrates exhibited significant restriction of migration direction along the axis of fiber alignment (Fig. 2a). Further analysis of the migration demonstrated that a fiber diameter of at least 700 nm was required to substantially restrict the migration of Schwann cells along a single axis (Fig. 2b). Combined with the results from the single-cell migration study, fibers of intermediate diameters (700 nm to 2 μm) were concluded to promote suitable cell migration speed while capably restricting migration along the fiber axis and were therefore exclusively utilized for the remaining studies in aims 1 through 3.

Sub-Aim IB: *To optimize the hydrogel processing protocol and growth factor loading method and investigate the effect of hydrogel composition and process parameters on the release kinetics and loading capacity of neurotrophic factors.*

(a) Characterization of the hydrogel-based gradient generation platform. To promote an additional driving force for regenerating nerves across the entirety of a nerve gap, we developed a method of delivering gradients of neurotrophic factors in a hydrogel-based delivery platform which promotes unidirectional growth of neurites and Schwann cells.

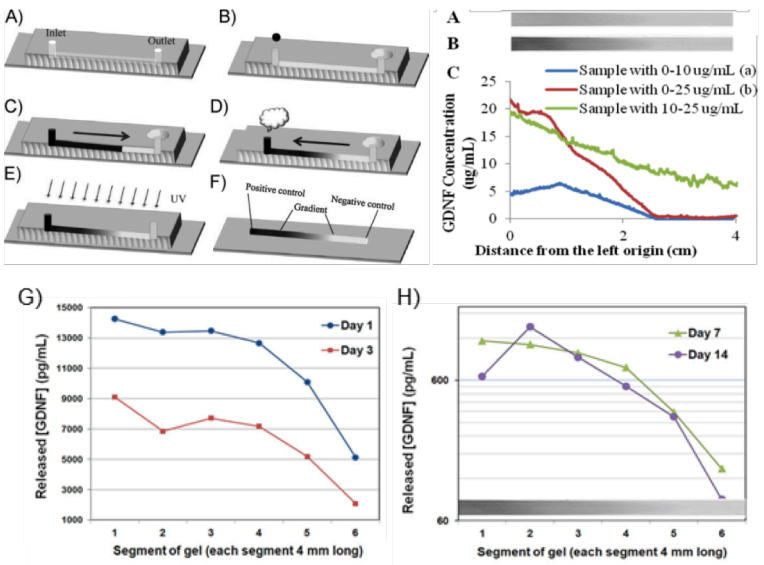

Figure 3. Gradient generation method (A–F) and characterization and concentrations of the released GDNF from the hydrogel encapsulated with known amount of GDNF (G, H). The released GDNF was detected by ELISA; and the inset is a gel encapsulated fluorescently-labeled GDNF gradients in methacrylated gelatin hydrogel.

The method we utilize generates a methacrylated gelatin hydrogel film that contains multi-centimeter long GDNF gradients of controllable length, concentration range and steepness. This method allows for the ease of loading multiple neurotrophic factors sequentially or simultaneously. Changing the GDNF concentration in the pre-fill or inlet solutions changed not only the concentration range of the gradient but also the steepness of the gradient. Gradient characteristics could also be controlled through variation of other parameters such as time between inlet injections and number of injections. We tested the release rate of the loaded GDNF, and showed that GDNF is released from the hydrogel layer in gradient fashion (Fig. 3) for at least 14 days.

Using this platform with a high degree of control over GDNF temporal and spatial gradient, we tested the effects of multiple gradient parameters on directional axon guidance, axon growth speed, and Schwann cell directional growth and migration. We also optimized the hydrogel crosslinking density to ensure that the processed gelatin hydrogel is sufficiently robust to be incorporated in the NGC. Our NGC design allows for the different components of the NGC (aligned nanofibers, gradient hydrogel) to be manufactured separately and reconstructed into the complete NGC. This modular design is integral in our ability to separately control the different topographical and biochemical cues we deliver and will be discussed in further detail in aims 2 and 3.

(b) Migration guidance of Schwann cells in response to GDNF gradients. In order to test the migration of human primary Schwann cells in response to gradients delivered by GDNF gradient-loaded hydrogels, we developed a novel cell migration chamber design to incorporate both the gradient hydrogel and aligned nanofibers. We placed sheets of aligned nanofibers on hydrogels loaded with GDNF gradients and encapsulated the construct in a PDMS channel (Fig. 4a–d). Dissociated human primary Schwann cells were seeded onto the fiber/hydrogel construct, monitored using time-lapse microscopy, and the migration was analyzed using a MATLAB-based tracking and analysis program. Utilizing this migration chamber setup we developed, we have a platform with which we can control both biochemical and topographical cues and monitor thousands of cells in real-time as they respond to the provided cues (Fig. 4e–g). This platform gives us a powerful tool for determining which cues are most effective in enhancing neural cell migration and promoting directional migration guidance.

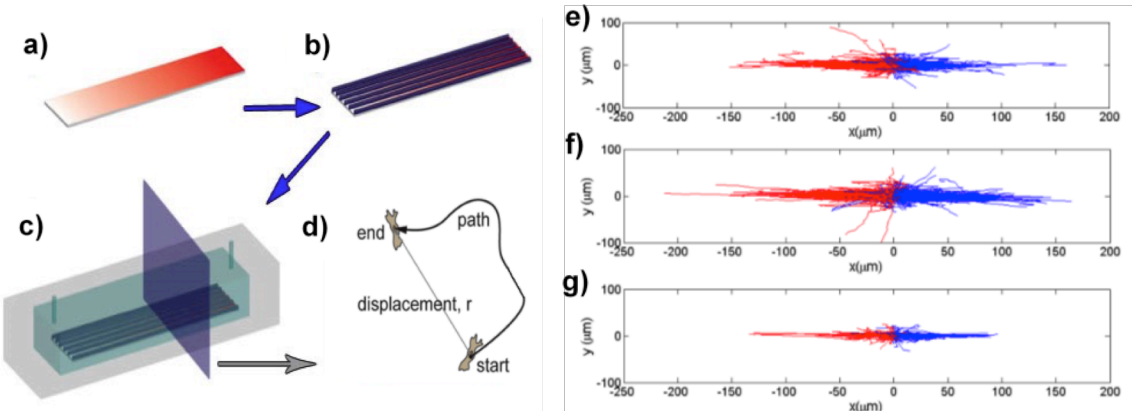

Figure 4. *In vitro* live-cell migration chamber allowing for high-throughput cell tracking. Gradient hydrogels with controlled gradient characteristics are generated (a) and aligned nanofibers are placed over the hydrogel (b). PDMS migration chamber is placed over the hydrogel/fiber construct (c) and cell migration is tracked from point of origin and along the entire trajectory (d). This method was used to monitor cell migration of primary human Schwann cells exposed to 0-1 μg/mL (e), 0-10 μg/mL (f), and 0-20 μg/mL (g) GDNF gradients, simultaneously tracking over 1000 cells per sample.

As our primary neurotrophic factor of interest for *in vivo* studies in aims 2 and 3 is GDNF, most of our gradient-based studies have been focused on the effects of varying GDNF gradient characteristics on Schwann cell migration kinetics and directional bias. Specifically, we were interested in determining how GDNF gradient concentration range affected the migration velocity and directional bias of Schwann cells, and found that markedly different cellular responses occur when increasing the concentration range of the GDNF gradients. Using our newly developed migration chamber platform, we were able to show that Schwann cell migration velocity increased significantly as the concentration range of GDNF gradient increased (Fig. 5a). This indicated that GDNF provided a concentration-dependent pro-migratory cue. However, as GDNF gradient concentration range increased, directional migration bias decreases (Fig. 5b), demonstrating that when gradient concentration ranges were too high, cells were less able to sense the direction of the gradient profile. These results demonstrated the importance of designing gradients that balance pro-migratory cues with sufficient directional guidance, considerations that were important for the incorporation of gradients into *in vivo* nerve guides.

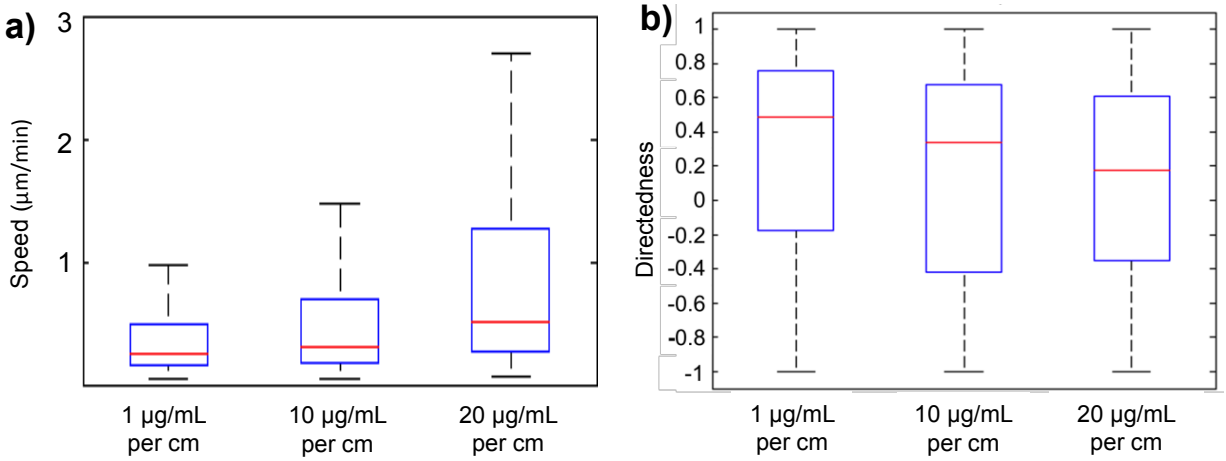

Figure 5. Primary human Schwann cell migration in response to varying gradients of GDNF. Cell migration speed of cells exposed to GDNF gradients with increasing concentration ranges (a). Directedness, or migrational bias, of migration with a value of 1 indicating cells migrating perfectly in the direction of gradient, 0 being non-biased migration, and -1 indicating cells migrating in opposite direction of gradient (b). Cell number > 1000 for analysis.

From these data, we have determined the importance of the steepness of the GDNF gradient in the ability for the Schwann cells and neurons to sense and respond to the gradient. Although concentration ranges effective *in vitro* may be different than what is effective *in vivo*, we can utilize this knowledge to inform the design of our *in vivo* gradients, emphasizing that we examine different gradient steepness rather than varying total concentration.

Sub-Aim IC: *To construct nanofiber NGCs with optimum nanofiber and neurotrophic factor configurations.*

The precise configuration of the NGC will be discussed in Aim 2. From the electrospun aligned fiber migration experiments, we have determined that NGCs should contain fibers of 700-nm to 2-µm diameters, the size range which best promoted both migration speed and migration guidance. Although we cannot directly extrapolate the *in vitro* Schwann cell gradient guidance results to the concentration range to be used for *in vivo* study, we confirmed that the gradient

steepness plays a major role in the ability for the cells to detect and respond to the GDNF gradient. Therefore, small animal studies in Aim 2 focused on investigating the effect of gradient steepness on nerve regeneration in preparation for the large animal study in Aim 3.

Significance

The information we obtained from our *in vitro* aligned fiber and GDNF gradient experiments gave us important insight into the design criteria, which must be used for the *in vivo* experiments in Aims 2 and 3. Fiber diameter and alignment was shown to play a major role in the migration guidance of Schwann cells. Small diameter fibers (< 500 nm) did not provide sufficient migration guidance and reduced cell migration speed. Large diameter fibers (> 3 μm) promoted faster Schwann cell migration, but exhibited a reduction in the ability to restrict migration directionality. This information gives us a range of fiber diameters, which yielded balanced and optimal migration rate with higher degree of directionality for Schwann cells and likely also for regenerating axons in a NGC configuration. The data obtained from the gradient guidance study suggested the importance of gradient steepness Schwann cell migration and neuronal outgrowth. While a specific steepness configuration might increase cell migration, it may not be optimal for promoting unidirectional guidance. We utilized this information to design experiments in Aim 2 to screen different gradient profiles for their *in vivo* regenerative efficacy.

Specific Aim 2: ***To assess nerve regeneration rate and functional recovery in a rat model of nerve repair, and to optimize nerve guide configurations***

The primary purpose of this aim was to screen and evaluate the nerve guidance conduit (NGC) configurations using the fiber and gradient compositions tested in Aim 1 in preparation to studies in the large animal model of peripheral nerve injury and repair. As it was not possible for us to try all experimental compositions in a large animal model, we used the rat 15-mm gap defect in the rat sciatic nerve injury model to identify the best combination of fiber composition and neurotrophic factor loading. The rat sciatic nerve transection and repair model used in this aim followed a standard protocol that we have used extensively in previous studies. The details of the experimental procedure and evaluation methods are described below. All groups tested in this specific aim are listed in Table 1.

Table 1. All groups tested in rat sciatic nerve injury model in Aim 2.

	Groups	# rats	Fiber Distribution	Aligned Fiber Density	Fiber Diameter (nm)	Composition % of Gelatin (PCL is Remainder)	GDNF Loading (ng/tube)
Tier 1	1	8	spiral no fiber				0
	2	8	Single Layer	medium	760	0	0
	3	8	Spiral	medium	760	0	0
	4	8	Spiral	high	760	0	0
	5	8	Spiral	low	760	0	0
Tier 2	6	6	Spiral	medium	760	0	20
	7	6	Spiral	medium	760	0	200
	8	6	Spiral	medium	760	0	2000
Tier 3	**9**	6	Fibrin Spiral	medium	**1200**	0	600
	10	6	**Fibrin Spiral**	medium	760	0	600
	11	6	Fibrin Spiral	medium	**400**	0	600
	12	6	Fibrin Spiral	medium	760	**90**	600
	13	6	Fibrin Spiral	medium	760	**80**	600
Tier 4	14	4	**Microfibers**	medium		0	~600
	15	4	**Fringe Spiral**	medium	1200	0	~600
	16	4	**"S" open design**	medium	1200	0	~600
Tier 5	17	4	S design	medium	1200	0	Uniform
	18	4	S Design	medium	1200	0	Shallow Gradient 60-180
	19	8	S Design	medium	1200	0	Steep Gradient 1-240
	20	8	1 layer	medium	1200	0	Steep Gradient 1-240

Sub-Aim 2A *To evaluate the effect of nanofiber diameter and degradation rate on nerve regeneration*

In this aim, we have tested multiple iterations of NGC conduit design to determine the optimal fiber composition and fiber/hydrogel configuration. All NGCs tested composed of a conduit containing aligned nanofibers, a hydrogel containing GDNF (uniform or gradient), and a porous outer tube composed of PCL (Fig. 6). This sub-aim primarily investigates the effect of fiber composition (density, material choice, diameter) and the configuration of the fibers and hydrogel within the conduit. Two conduit designs were utilized (the spiral conduit and the S-shaped conduit) in order to determine the configuration that could promote the greatest regeneration.

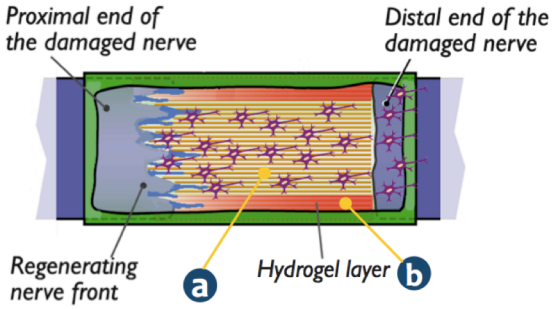

Figure 6. Design of the nanofiber nerve guides incorporating the nanofiber guidance cues (a) and neurotrophic factor gradient loaded in the hydrogel layer (b) between the outer membrane and nanofibers.

(a) Design and testing of novel gelatin-based spiral conduit. The first iteration of conduit design consisted of alternating layers of fiber and hydrogel in a novel spiral configuration in the lumen of a tube (Fig. 7). The rationale of this design was to maximize the contact of neurons and Schwann cells with aligned fiber surfaces. In the open lumen design that we have used in the past, the aligned fibers were only located on the outer surface of the lumen with a large open luminal space with no fiber presence. The spiral design was intended to increase the exposure of fibers to cell contact by dispersing the fibers throughout the conduit by using a hydrogel film as a spacer layer between fiber layers.

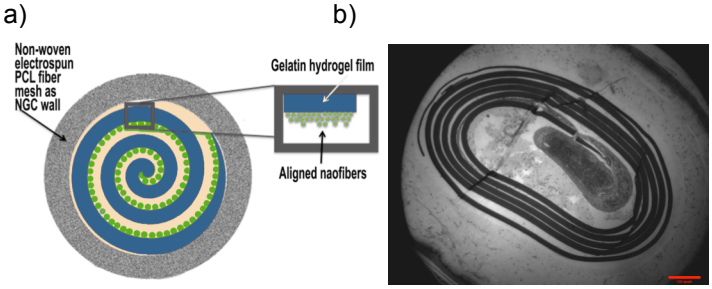

Figure 7. Spiral conduit composed of interchanging layers aligned nanofibers and gelatin hydrogel film. Schematic of conduit design (a). Micrograph of sectioned and stained Group 3 conduit, after 8 weeks in vivo. Slice taken from section of conduit 5-8mm away from proximal end of conduit.

The groups for the first two tiers of animal studies (Groups 1–8, Table 1) were designed to test whether the spiral design, with its increased area of aligned nanofibers, would improve regeneration as compared with the older NGC design wherein the aligned fibers were only in a single layer on the inner luminal wall of the conduit. Here we also examined whether there was a dose response to the nanofiber number by varying the density of nanofibers per unit area of conduit by laying down different amount of fibers. The fibers were spun to a density of 0, 1×, 2×, and 3×, relative to one another (Table 1, aligned fiber density column). The nanofiber size was maintained at 760 nm, within the intermediate diameter range of our fiber production capabilities.

Several trends were observed from this first round of experiments. The control groups with no aligned fibers (Group 1) or the single layer of aligned fibers (Group 2) showed poor regeneration in almost every rat. Rats with spiral design conduits (Groups 3–5) exhibited better regeneration than rats in the single-layer and non-fiber designs, but we witnessed significant variation from rat to rat within the groups. There was not any obvious trend between the groups with higher densities (2× and 3× fiber densities) of aligned nanofibers (Groups 3 and 4). Although the

results were promising, the initial groups demonstrated some unexpected problems with the spiral NGC design. For all of the spiral groups, the hydrogel and fiber sheet compacted outwards against the wall of the tube rather than being uniformly distributed throughout the lumen of the tube. This may have been caused by hoop stress experienced by the films due to the high crosslinking density necessary to form the hydrogel. Additionally, due to the high crosslinking density, the gelatin-hydrogel layer degraded much slower than anticipated and demonstrated limited cell infiltration. Combined with the compaction of the hydrogel and fiber outward against the wall of the tube, this limited the effective surface area of fibers, which the regenerating nerve can come in contact with. Thus, although the spiral design was intended such that nerve tissue could grow throughout the spiral layers, growth was only seen within the central void created by the outwardly compacted spiral, limiting the potential area of the spiral conduit significantly. Because of this factor, although axonal density in limited region was better in the gelatin-based spiral conduits compared to the single-layer design, the overall regeneration outcomes in the spiral conduits remained generally poor in all of the Groups 1–5 as listed in Table 1.

Due to limitations in the regeneration of the gelatin-based spiral groups, Groups 6–8 including GDNF as the primary variable parameter, originally designed to show whether there was any optimal loading level of GDNF within the conduit, with 20 ng, 200 ng, or 2 µg of GDNF per conduit, were largely inconclusive. As such, we were unable to determine the optimal GDNF loading using this conduit design.

(b). Fibrin-based spiral conduit design and testing. As an alternative to gelatin hydrogels in the spiral conduit, we prepared low-density fibrin hydrogels using diluted Tisseel (clinically used tissue adhesive) as the source of fibrin gel, considering translational steps in the future. By replacing the gelatin layer with fibrin hydrogel, the conduit design was improved in many ways. The spiral layers showed lower propensity to compact against the outer walls, increasing the surface area of aligned nanofibers available to interact with the regenerating axons. Testing the fibrin-based spiral conduits *in vivo* (Groups 9–13) demonstrated and improvement compared to gelatin films, with layers of nanofibers being better distributed within the nerve luminal space in fibrin groups. There was a great improvement in the functional electrophysiological results over the previous tier of experiments. However, much like in the gelatin-based spiral conduit, few neurons grew within the fiber/hydrogel layers, instead growing within the open lumen in the center of the conduit or between the spiral sheets. This limited effective space within which re-generating nerve tissue grows and limited the potential for functional recovery using this design.

(c). Effect of fiber composition on degradation and nerve regeneration. In addition to modifying the composition of the hydrogel, we incorporated gelatin into the fiber composition in order to modify the degradation kinetics of the fiber substrate and determine whether the degradation rate of the substrate influences nerve regeneration. Gelatin was mixed into the PCL solution prior to electrospinning the fibers, followed by crosslinking of the fibers using vapor-phase glutaraldehyde. By blending 80% or more gelatin into the PCL fibers, the degradation rate of fibers was dramatically increased compared to lower ratios or pure PCL (Fig. 8). However, when we incorporated the blended fibers in spiral conduits, the increased degradation rate resulted in reduced regeneration. From

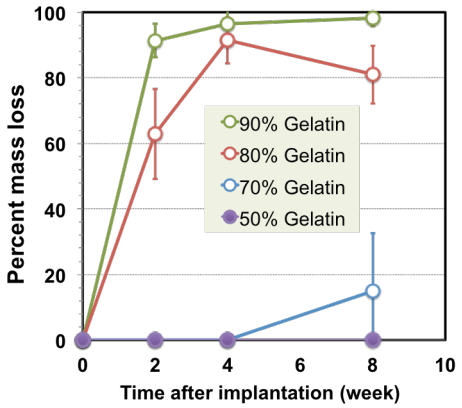

Figure 8. Effect of nanofiber composition on *in vivo* degradation rate.

these experiments, we concluded to use only pure PCL fibers for the aligned fiber substrates in the remaining experiments for Aims 2 and 3.

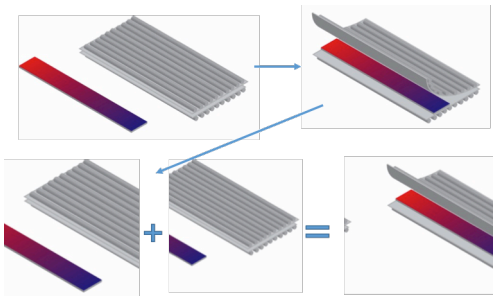

Figure 9. S-shaped conduit design. Hydrogel containing GDNF is placed between two aligned fiber sheets, formed into an S-shape, and placed within a porous outer conduit.

(d). S-shape conduit design and characterization. To overcome the limitations of the open lumen and spiral designs, we began testing an S-shaped conduit (Fig. 9) based on the conduit first used by a collaborator at Hopkins, Dr. Andres Hurtado in spinal cord repair. The S-shaped conduit was designed to have more open area compared to the spiral design conduit, but more fiber surface area than the open lumen design. The use of this design was inspired by the histological results of Groups 1–13. While having poor regeneration overall, the areas of regenerating nerve tissue could often be found in sections of the nerve guide where luminal spaces of greater than 100-µm diameter had formed between hydrogel and fiber layers. The "S" design intentionally incorporates the formation multiple lumens within a single conduit. Additionally, the central curve of the S-shape has some inherent elasticity, and provides additional strength in resisting compressive stresses that the nerve guide might endure *in vivo*, further protecting the regenerating nerve from impingement from movement or ingrowth of surrounding tissues. A hydrogel containing growth factors can be incorporated between layers of aligned fibers in this design, allowing growth factors to be released near the nanofibers. The S-shaped conduits exhibited significant ingrowth of tissue into the two main lumens of the conduit (Fig. 10), and had much better regeneration than that of the spiral conduits. Due to the significant improvement in regeneration, we chose to proceed to use the S-shaped conduit design as the configuration for the remainder of the small animal and large animal studies in Aims 2b and 3.

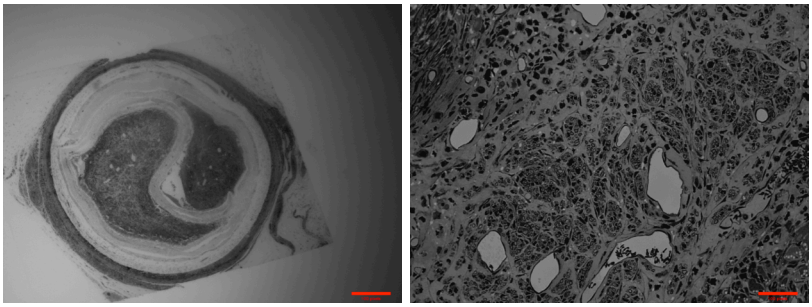

Figure 10. Transverse cross-section of midpoint of group 19 nerve guide at the 4week timepoint. This nerve guide had the "S": design with a steep gradient. Right: increased magnification of the left fascicle.

Sub-Aim 2B *To identify the optimum neurotrophic factor combination(s) on nerve regeneration.*

Once the optimum NGC configuration and degradation rate were identified in Aim 2A, we used those parameters when evaluating the optimal neurotrophic factor combination to be used in the final nerve guides in the large animal study.

(a). Comparison of effect of gradient steepness on regeneration in rat sciatic nerve model. Following the results in Aim 1 highlighting the importance of gradient steepness in Schwann cell response and the optimization of NGC configuration in Aim 2a, we investigated the effect of GDNF gradient steepness on regeneration in a rat sciatic nerve model. Based on the NGC configuration optimization in Aim 2a, we utilized the S-shape conduit for this study. To incorporate GDNF gradient hydrogel films into an S-shaped conduit, we generated a

methacrylated gelatin film containing the desired GDNF gradient (Fig. 4a–f), placed it between two aligned nanofiber sheets, rolled the sheets into an S-shape, and placed the sheets/film within a porous tube (Figs. 9, 10). We tested the four following groups (#17–20, Table 1): S-shaped conduit with no GDNF, S-shaped conduit with uniform GDNF, S-shaped conduit with shallow GDNF gradient, and S-shaped conduit with steep GDNF gradient. Conduits were placed in a 7-mm rat sciatic nerve injury model, nerve function was tested and nerves harvested after 4 weeks.

From this experiment, we concluded that the steep GDNF gradient promoted significantly greater regeneration of axons compared to that of the other three groups (Fig. 11) and increased the functional recovery of the sciatic nerve after 4 weeks (Fig. 12). The shallow gradient exhibited axon regeneration comparable to that of the uniform GDNF group, indicating that the shallow gradient steepness was not sufficient to influence nerve regeneration. Additionally, the shallow gradient and uniform GDNF groups exhibited very poor functional recovery (Fig. 12), indicating the potential presence of the "candy store" effect that is typically seen when delivering uniform concentrations of growth factors within NGCs. The improvement in recovery in the steep gradient showed the great potential in utilizing gradients of growth factors as a method of improving the recovery of damaged peripheral nerves. This experiment highlighted that the mere presence of a gradient will not be sufficient to improve nerve regeneration unless the gradient is of necessary steepness to be detectable by the regenerating nerve. Based on these results, we chose the steepest gradient condition as our sole gradient condition for the large animal study.

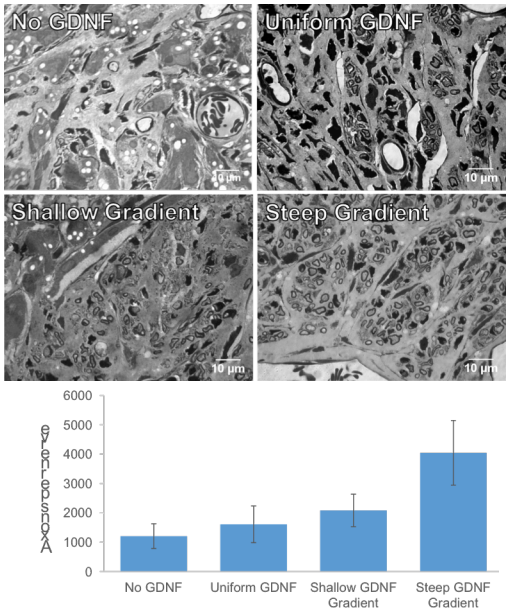

Figure 11. Histology of 4-week rat sciatic nerve regrowth. Axons per nerve count for each of the four groups were shown in the bottom panel.

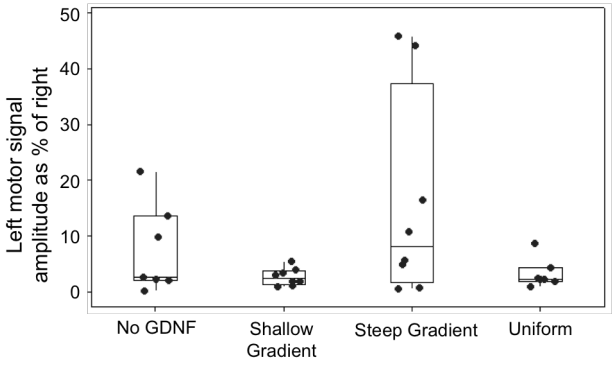

Figure 12. Left motor signal amplitude measured by functional EMG.

Significance

The results of the experiments conducted in this Aim also provided important insight into the NGC design parameters necessary to allow for maximum nerve tissue ingrowth and regeneration. From the selection of a NGC configuration, we learned that using an S-shaped conduit that combined the large luminal spaces with higher aligned fiber surface area provided a significant improvement in regeneration compared to open lumen and spiral conduit designs. When designing a gradient for *in vivo* delivery, we learned the importance of gradient steepness in consideration of gradient design. With these understandings, we moved to finalize the experimental design for the large animal study.

Specific Aim 3: ***To demonstrate efficacy in a large animal model using optimized nerve guide from Aim 2, as a prerequisite for clinical translation.***

The primary goal of this specific aim was to test the efficacy of optimized nanofiber nerve guide in a canine model of peripheral nerve injury and repair. Peripheral nerve repairs conducted in small rodents do not always translate into successful regenerative therapies in humans partly because of the significant differences in regeneration across small versus large gap repairs and differences in regeneration in small versus large animals. This aim was intended to utilize a large animal model as more clinically appropriate nerve regeneration paradigm to test the effectiveness of our optimized NGC. In this Aim, we tested the hypothesis that *"nanofiber nerve guides with optimized neurotrophic factor gradient incorporation provides a nerve regenerative substrate that matches and even surpasses autologous nerve grafts."*

Based on our findings from Aims 1 and 2, and given the effect to reduce the use of experimental animals, we chose three experimental groups to test in the canine model: S-shaped conduit without GDNF, S-shaped conduit with uniform GDNF, and S-shaped conduit with steep gradient of GDNF. These groups were tested against an autograft control. NGCs were designed as described in Aim 2, with the length increased for implantation in a 2-cm nerve gap and aligned fibers of 1.2-μm diameter. In brief, a 17-mm nerve section was excised from the common peroneal nerve of a beagle and the 2-cm NGC or an autograft was sutured to the proximal and distal stumps. The animal was observed for 7 months. After 7 months, functional recovery outcomes were measured using EMG and the nerve grafts were harvested for histomorphometric analysis.

From the nerve axonal counts, the autograft group had the best axonal regeneration of the four groups (Fig. 13a). The gradient of GDNF significantly improved the axonal regeneration compared to no GDNF and uniform GDNF groups, but the total axon count was significantly less than in the autograft, both in the middle of the nerve graft and distal end of the regenerated nerve. The major contributing factor for the decreased nerve counts in these groups was that the NGC appeared to be compressed in all experimental groups except for in the autograft. The nerve section selected for surgery was located in the ankle of the dog, which is a region of limited area and high mobility, potentially causing impingement of the NGCs, which were designed to be soft and flexible. This design criterion caused the NGCs to be very compressible, reducing their efficacy in this high-movement region of the dog hindlimb. As this was a novel large animal model, we did not discover this issue until the completion of the study, but we will use this information to inform the design of NGCs for future use.

Although the axon count was lower in the NGCs, the axon density in the tissue that regenerated was closer to that of the autograft, especially in the group containing the GDNF gradient. In this group, the axon density in the middle of the graft and distal to the graft was comparable to that of the autograft (Fig. 13b). This indicated that while the total nerve tissue area for each of the S-shaped NGCs was lower than

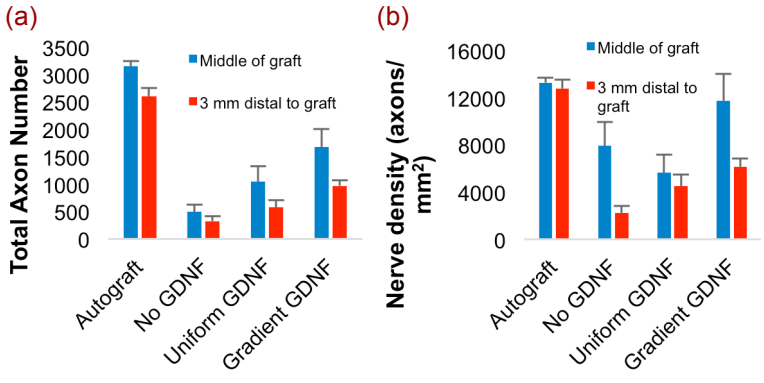

Figure 13. Total axon count in large animal model. Axon count in the middle of the nerve graft (a) and axon count in the distal nerve stump 3 mm from the end of the graft (b).

that of the autograft, the nerve tissue that regenerated was of comparable composition to that of the autograft. This indicates that by utilizing a more mechanically robust outer conduit to prevent a reduction in nerve area from impingement, our engineered NGCs have the potential to promote robust regeneration similar to that of the autograft.

Additionally, although the axon count was lower in our experimental groups, the functional recovery of the nerve was greater in the GDNF gradient group compared to the autograft and approached the latency and amplitude values of the nerve prior to surgery (Fig. 14). These data suggest that, although the total axon count was lower in the GDNF gradient group compared to the autograft, the neurons in the GDNF-gradient group had better signal transduction than the neurons in the autograft. We hypothesize that such a finding indicates that the neurons in the GDNF gradient group were potentially more mature than those in the autograft, which would allow for greater signal transduction in the presence of fewer axons. We are currently investigating further whether there are differences in axon diameter and myelination between the GDNF gradient and autograft groups. Our data demonstrates the exciting potential for gradients of growth factors to improve the functional recovery of nerve injuries though delivery in engineered NGCs. Further studies will need to be conducted to determine differences between gradients of different types of growth factors, but our research platform allows for such studies to be easily conducted.

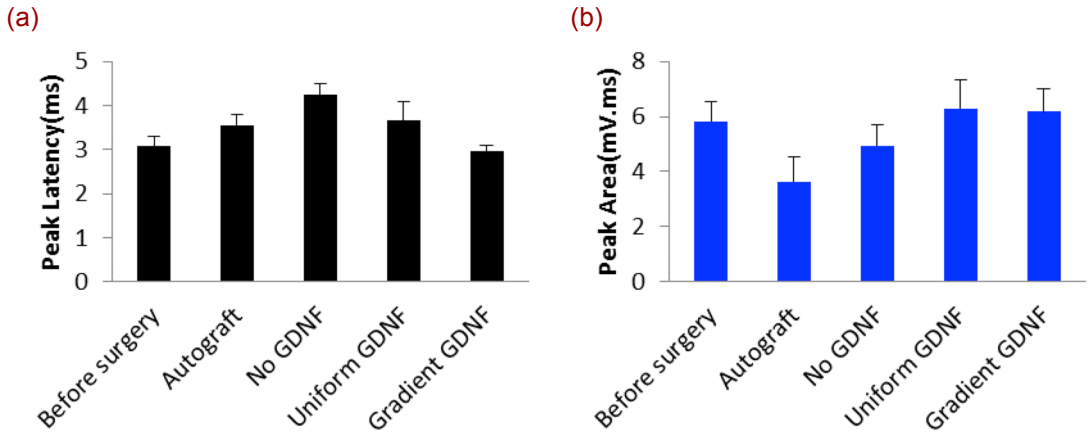

Figure 14. Functional EMG data of autograft and NGC groups in large animal model. Peak latency (a) and peak area (b).

Additional outcome measures used in the study

In addition to the main outcome measures of number of axons regenerated (nerve morphometry – Figure 13) and electrophysiology (Figure 14), we also looked at the walking behavior of the dogs (Task 3c) and histopathological evaluations for inflammation and scar formation (Task 3f). As seen in Figure 15, the animals did not display a significant change in the force they applied to the walking platform (Tekscan). Nevertheless, there was a statistically significant increase in the pressure applied to the walking platform in dogs in which the repair was done using the gradient GNDF NGC at earlier time point of 2 weeks and this was sustained at 2 months. The uniform GDNF group was similar to the gradient GNDF NGC group at 2 months.

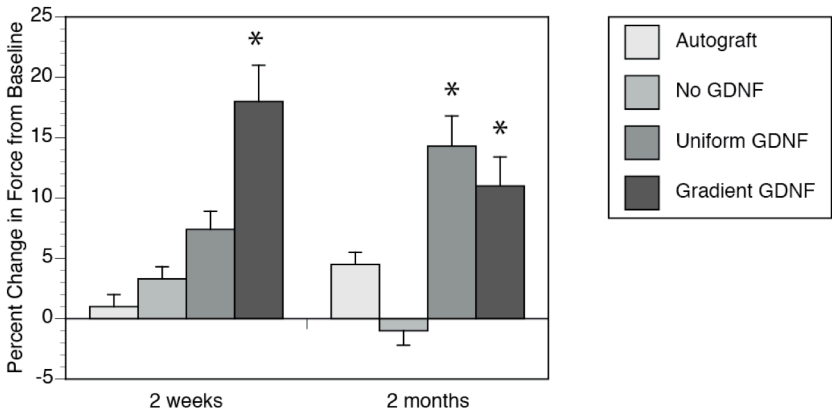

Figure 15: Percent change in force applied to the Tekscan Walking Platform in the operated limb at 2 weeks and 2 months post-op (normalized to body weight).* denotes $p<0.05$ compared to baseline.

In addition to the above studies, we examined the grafted nerve segments for evidence of inflammation and scar tissue formation using standard histopathological stains. There were no significant differences among all 4 groups in terms of scar formation or presence of inflammatory cells.

Significance

In Aim 3, we demonstrated the exciting potential for gradients of GDNF to improve the functional recovery of large gap nerve injuries. Functional recovery following repair in our canine model approached pre-surgical values and were generally better than values found in the nerve autograft. However, the compaction of our NGCs due to the soft and flexible conduit design limited their overall regenerative potential by reducing the available nerve area through which the regenerative nerve tissue can grow. In spite of this, the functional recovery exhibited by the GDNF gradient NGC demonstrates that the axons regenerated in the presence of a GDNF gradient may be more mature than those in an autograft. Future studies will investigate the effect of the composition of the outer conduit wall as well as determine the influence of gradients on axon outgrowth rate and Schwann cell maturity.

What opportunities for training and professional development has the project provided?

Significant opportunities to present research at academic conferences. Conferences attended by graduate students funded by this research:

- Kellin Krick: BMES 2013-2015, Society for Neuroscience 2012, TERMIS 2014-2015
- Russell Martin: Society for Neuroscience 2012, TERMIS 2014

Conference presentations offer the trainees ample opportunities to develop ability to communicate information to people outside their areas of expertise.

Opportunities for graduate student mentorship of undergraduate students. PI's provide numerous undergraduate students the opportunity to assist in completion of various research projects under the mentorship of graduate students and post-docs. This provides an excellent opportunity for the graduate students to develop their leadership and mentorship skills.

How were the results disseminated to communities of interest?

Two years of research partnership with local high schools to provide mentorship to female high school students interested in the STEM fields. Two summers of Research Experience for Undergrads programs, providing research mentorship of students from other universities.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Numerous findings were made throughout the completion of this project. First, we demonstrated the influence of aligned fiber diameter on the migration of human Schwann cells. There exists an optimal range of fiber diameters within which Schwann cells exhibit both fast and directional migration. Delivering a gradient of growth factors to neurons and Schwann cells promotes directional growth of the cells, and the steepness of the gradient plays a major role in the ability for the cells to sense and respond to the gradient. We have developed a method to deliver the gradients in a film, allowing us to produce cylindrical nerve guidance tubes which contain both aligned fibers and gradient delivery. These tubes can be used to bridge nerve gap injuries, as an alternative to nerve grafts. Through the experiments conducted during this project, we determined the optimal configuration for the fibers and gradient films and began testing the efficacy of the nerve guides in rat and dog studies. From these animal studies, we were able to demonstrate that delivering growth factors in a gradient was dramatically more effective in promoting regeneration compared to conduits without growth factors or with uniform concentrations of growth factors. Our report demonstrated the potential of utilizing gradient delivery as a method of improving nerve regeneration and as an alternative to the nerve autograft.

What was the impact on other disciplines?

Our discoveries made while determining the optimal configuration of the nerve guidance conduit will aid future design of NGCs which can incorporate both topographical and biochemical guidance. Additionally, our findings demonstrate the importance of the mechanical properties of the conduit in maintaining the viability of the nerve guide implanted in high-activity areas of the body.

What was the impact on technology transfer?

No technology transfer has directly resulted from this project. However, the findings influence the future design of our NGCs, emphasizing the importance of fiber surface area and open lumens in engineered nerve guidance conduits. Additionally, informs the use of gradients of growth factors as an effective method of improving the regenerative efficacy of engineered NGCs.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Delays in completion of aims 1 and 2 due to unforeseen issues with conduit design and manufacturing lead to delays of completion of aims 2 and 3 into an additional 4th and 5th year of study. Obtained no-cost extension for completion of study.

Changes that had a significant impact on expenditures

Nothing to report. Obtained no-cost extensions.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

No human subjects were used for this study.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

Krick K, Tammia M, Martin R, Höke A, Mao HQ. Signaling cue presentation and cell delivery to promote nerve regeneration. Current Opinions in Biotechnology, 22(5): 741-746 (2011). Published. (Federal Support acknowledged)

Additional manuscripts outlining work in aims 2 and 3 are under preparation and will be submitted soon.

(Not directly supported by this grant, but very much relevant to overall aims of enhancing peripheral nerve regeneration using topographical cues:
Ren YJ, Zhang S, Mi R, Liu Q, Zeng X, Rao M, Hoke A, Mao HQ. Enhanced differentiation of human neural crest stem cells towards the Schwann cell lineage by aligned electrospun fiber matrix. Acta Biomater. 2013 Aug;9(8):7727-36. PMID: 23628775
Jiang X, Mi R, Hoke A, Chew SY. Nanofibrous nerve conduit-enhanced peripheral nerve regeneration. J Tissue Eng Regen Med. 2012 Jun 15. PMID: 22700359)

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

-Martin R, Mi R, Mullen B, Ginn A, Höke A* and Mao HQ*. Optimization of nanofiber configuration in nerve guidance conduits, Poster Presentation at the Society for Neuroscience Annual Meeting, New Orleans, October 2012
-Krick KD, Khademhosseini A, Höke A* and Mao HQ*. Neurotrophic factor gradient generation for directional peripheral nerve growth guidance and regeneration, Poster Presentation at the Society for Neuroscience Annual Meeting, New Orleans, October 2012
-Krick KD, Khademhosseini A, Höke A* and Mao HQ*. Neurotrophic Factor Gradient Delivery for Migration Guidance of Schwann Cells, Oral Presentation at the BMES Annual Meeting, Seattle, September 2013
-Krick KD, Huang Y-J, Martin R, Searson P, Khademhosseini A, Höke A* and Mao HQ*. Neurotrophic Factor Gradier Delivery to Direct Schwann Cell Migration, Poster Presentation at the BMES Annual Meeting, San Antonio, October 2014
-Krick KD, Huang Y-J, Martin R, Searson P, Khademhosseini A, Höke A* and Mao HQ*. Engineering Neurotrophic Factor Gradients to Direct Human Schwann Cell Migration on Aligned Electrospun Fiber Matrix, Poster Presentation at the TERMIS Annual Meeting, Washington DC, December 2014
-Krick KD, Siu I-M, Höke A*, Brushart T, and Mao HQ*. Neurotrophic Factor Gradient Generation within Hydrogel Sheets for Differential Growth Guidance of Motor and Sensory Neurons, Poster Presentation at the TERMIS Annual Meeting, Boston, September 2015
-Krick KD, Siu I-M, Höke A*, Brushart T, and Mao HQ*. Gradient Generation Platform for Schwann Cell and Neuron Migration Guidance in 2D and 3D Cultures, Poster Presentation at the BMES Annual Meeting, Tampa, October 2015

- **Website(s) or other Internet site(s)**

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Nothing to report.

- **Technologies or techniques**

Novel large animal model was established by this project. We will publish the methodologies in upcoming publications.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Development of cell tracking and analysis software based in MATLAB and Python programming languages. Program will be reported in upcoming manuscripts.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Ahmet Höke (PI), MD/Ph.D., No change
Hai-Quan Mao (PI), Ph.D., No change
Ruifa Mi, MD/Ph.D. (research Associate), No change
Russell Martin (Graduate Student), No change
Kellin Krick (graduate Student), No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: N/A