Published in final edited form as:

Acta Biomater. 2014 December ; 10(12): 4939–4946. doi:10.1016/j.actbio.2014.08.032.

Retinal Ganglion Cell Polarization Using Immobilized Guidance Cues on a Tissue-Engineered Scaffold

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Abstract

Cell transplantation therapies to treat diseases related to dysfunction of retinal ganglion cells (RGCs) are limited in part by an inability to navigate to the optic nerve head within the retina. During development, RGCs are guided by a series of neurotrophic factors and guidance cues; however, these factors and their receptors on the RGCs are developmentally regulated and often not expressed during adulthood. Netrin-1 is a guidance factor capable of guiding RGCs in culture and relevant to guiding RGC axons toward the optic nerve head in vivo. Here we immobilized Netrin-1 using UV-initiated crosslinking to form a gradient capable of guiding the axonal growth of RGCs on a radial electrospun scaffold. Netrin-gradient scaffolds promoted both the percentage of RGCs polarized with a single axon, and also the percentage of cells polarized toward the scaffold center, from 31% to 52%. Thus, an immobilized protein gradient on a radial electrospun scaffold increases RGC axon growth in a direction consistent with developmental optic nerve head guidance, and may prove beneficial for use in cell transplant therapies for the treatment of glaucoma and other optic neuropathies.

Keywords

Retinal ganglion cell; Netrin; Axon guidance; Protein immobilization

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1. Introduction

Directing retinal ganglion cell (RGC) axons to extend toward the optic nerve head is a major challenge, both during normal development and also when considering RGC replacement therapy in injury or disease. During development, RGC axons in the retinal nerve fiber layer (RNFL) are directed to the optic nerve head through the optic nerve to targets in the brain, by several soluble and matrix-associated signals including ephrins, netrin-1, slit-1 and 2 and heparan sulfate. Disruption of any these factors can lead to improper guidance within the RNFL and optic nerve hyperplasia.[1–3] Netrin-1, a soluble protein responsible for guidance within the retina but also for branching and targeting in the cortex,[4, 5] has been shown to be expressed in the developing [6] and adult optic nerve, as well as in a response to injury such as axotomy [7] with the guidance factor regulated temporally through expression of the receptor DCC.[8] This protein has been of particular interest because it is sufficient on its own to guide RGC axons in vitro. [9] Relevant to our studies, guidance of RGCs by Netrin-1 can still be modulated by other signaling pathways owing to the activation of specific integrins [10] capable of reversing the attractive nature of Netrin-1 on fibronectin to a repulsive nature on laminin.[11]

Can similar guidance cues be incorporated into tissue engineering approaches being investigated for retinal repair? Recently, transplanted RGCs have been observed to project dendritic processes into the inner plexiform layer,[12] and stem cells transplanted directly onto the optic nerve head are able to project a process through the optic nerve head.[13] However, transplanted cells located away from the optic nerve head were unable to extend their axons radially as occurs with endogenous RGCs in the normal retina. In order to direct the axons of transplanted cells, nanofiber scaffolds created by electrospinning have been used to give a radial physical guidance cue for axon extension for transplanted RGCs; however, cells grown on this scaffold extended neurites either toward or away from the center where the optic nerve would be, or often in both directions. Tissue engineering of the peripheral and central nervous system has incorporated both soluble and immobilized neurotrophic factors through a variety of methods including microfluidic devices, diffusional gradients and chemical immobilization, to direct the neurite outgrowth of neurons in two and three dimensions. Because chemical immobilization creates a fixed protein pattern on a material surface which can be implanted, while soluble gradients cannot be readily translated in vivo,[14] methods for protein gradients have been formed through photolithography, either using a photomask [15] or through focusing of light on a actuating mirror,[16] micro-contact printing [17] and microstamping.[18]

Here we describe a method for polarizing the growth of RGCs cultured on an electrospun radial scaffold. By means of photolithography, Netrin-1 will be chemically immobilized in a linear gradient onto a fibronectin-coated scaffold. We will demonstrate that early postnatal primary RGCs cultured on scaffolds containing Netrin-1 show an increase in polarization during the growth of a single axon, better mimicking the growth seen in vivo. We will also demonstrate that cells cultured on scaffolds containing this protein gradient will show an increase in the guidance of the RGCs toward the center of the scaffold, mimicking the growth toward the optic nerve head found in vivo.

2 Materials and Methods

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research with all protocols approved by the Institutional Animal Care and Use Committee of both the University of Miami Miller School of Medicine and the University of California San Diego.

2.1 Electrospinning

Electrospinning was conducted as previously described.[19] Briefly, Poly-D L-lactic acid (PLA, Purac Biomaterials Inc., PDL20) was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Chem-Impex International Inc.) at a concentration of 6.6 % (wt/vol). The PLA solution was pumped by syringe pump (New Era Pump Systems Inc., NE-500) at a continuous feed rate of 2 ml/hr and ionized in a 20 gauge blunt-tipped needle (Hamilton) using a high voltage power supply (SpellmanHV, 230-30R). Scaffolds were created using a radial collector which was constructed from a 1 mm diameter copper wire acting as the central pin. A plastic cup, 1.8 cm in diameter, was coated around the outside and upper rim with aluminum foil mounted on the central pin, with both the central pin and cup connected to the same ground. Scaffolds were produced at 15 kV with a flow rate of 2 ml/hour and a collecting distance of 12 cm.

2.2 Protein Crosslinking

Recombinant Netrin-1 (R&D Systems) or BSA-FITC (Sigma) was crosslinked onto laminin- or fibronectin-adsorbed PLA scaffolds through three methods (Figure 1): direct chemical coupling, UV-initiated chemical coupling with the crosslinker initially attached to the soluble protein, and UV-initiated chemical coupling with the crosslinker initially attached to the scaffold.

Direct chemical coupling was achieved by activating the carboxylic acid groups of the adsorbed laminin or fibronectin with 500 μ L of a 1 mg/ml N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC, Thermo Scientific) solution for 15 minutes followed by the addition of 500 μ L of a 2 mg/ml Sulfo- N-hydroxysuccinimide (NHS, Thermo Scientific) solution, to increase the aqueous stability of the activated carboxylic acid. Primary amine groups from 500 μ L of a 0.1 mg/ml drop of protein reacted with the activated acid groups to form covalent crosslinks.

For UV-initiated crosslinking samples with the crosslinker initially attached to the soluble protein, 500 μ L of a 0.1 mg/ml protein solution was reacted overnight at 4°C with 2 mg/ml of the diazirine crosslinker sulfo-succinimidyl 6-(4,4'-azipentanamido)hexanoate (Thermo Scientific). The solution was added to a fibronectin-adsorbed scaffold and the sample exposed to 1 minute of 30 mW/cm² of a 351 nm laser beam from a Coherent Innova 90-6 argon-ion laser. The beam was expanded by a x10 telescope (CVI, Albuquerque, NM) and routed onto an etched filter/engineered diffuser (RPC Photonics Quote #RPD-ED-2012), which facilitated the gradient patterning of the beam. Samples were washed three times and then incubated until use in Neurobasal media.

For UV-initiated crosslinking samples with crosslinker initially attached to the scaffold, 500 μ L of a 2 mg/ml solution of the diazirine crosslinker was incubated for 3 hours at room temperature with a fibronectin-adsorbed scaffold. Following incubation, the crosslinker solution was removed and 500 μ L of a 0.1 mg/ml protein solution were added to the surface. Samples were exposed as before to the argon-ion laser beam through the etched filter. Samples were washed three times and then incubated until use in neurobasal media.

2.3 Immunostaining

Samples were fixed for 30 minutes with 4% paraformaldehyde (Electron Microscopy Sciences) diluted in phosphate-buffered saline (PBS, pH 7.2). Samples were washed 3x with fresh PBS and then blocked for 30 minutes with 10% goat serum (Invitrogen), with 0.2% triton X-100 (EMD Millipore) to permeabilize the cell membrane. RGCs were stained for β III tubulin (mAB, 1:500, Covance) and Netrin-1 (pAB 1:500, R&D Systems) overnight at 4°C, washed 3x with PBS and labeled with Alexafluor 546 goat anti-mouse IgG (1:500, Invitrogen) and Alexafluor 488 goat anti-rat IgG (1:500, Invitrogen). Samples were imaged using a TCS SP5 Inverted Confocal Microscope (Leica) with mosaic images stitched using LAF software (Leica).

2.4 Quantification of Netrin-1 Gradient

Immunostained samples were imaged at 0.5 mm intervals in both the horizontal and vertical directions from the center of the scaffold to the periphery using an Axio Observer.Z1 Inverted Fluorescence Microscope (Carl Zeiss). Samples were imaged in both the 488 and 546 channels with the same exposure settings used for each position on the same scaffold. Intensity of the fluorescence was quantified using ImageJ and the ratio of the intensity at 488/546 at each position from the center was averaged and plotted.

2.5 Retinal Ganglion Cell Isolation and Culture

Retinal ganglion cells (RGCs) were purified to >99% by sequential immunopanning as described previously.[20, 21] Briefly, retinas were isolated from early postnatal rat or GFP-positive mouse (Jackson Laboratory) litters (postnatal day 2–5), digested using papain and dissociated into single cells using mechanical trituration. After negative selection to remove macrophages and endothelial cells, RGCs were isolated by Thy1 reactivity. RGCs were cultured at 37°C, 10% CO₂ and 100% humidity in Neurobasal media supplemented with insulin, sodium pyruvate, penicillin/streptomycin, n-acetyl cysteine, triiodo-thyronine, forskolin, Sato, B27 and BDNF and CNTF growth factors at previously published concentrations.[20, 21]

PLA scaffolds were cut from radial collectors and sterilized for 20 minutes by UV irradiation. Next, scaffolds were equilibrated to culture conditions by incubating with fibronectin (2 μ g/ml, Trevigen) in Neurobasal medium (Invitrogen). All samples were seeded with 35,000 RGCs.

2.6 Analysis of Polarization

RGCs were stained for β 3 tubulin and the entire scaffolds imaged using confocal microscopy as described above. Imaged z-stacks were flattened using ImageJ software and

cells counted using the cell count plugin. Across the entire scaffold in each condition, all neurites extending from the RGC cell body greater than two cell bodies in length, previously characterized as axonal,[22] were analyzed. RGCs were categorized as polarized towards the scaffold center if the cell extended a single neurite towards the scaffold center ($\pm 10^\circ$), polarized not towards center for all RGCs extending a single neurite in the other 340° range, or bipolar if the neuron extended multiple neurites longer than two cell bodies in length, regardless of direction. RGCs whose neurites branched after extension from the cell body did not affect the categorization as polarized or bipolar.

3. Results

To verify that Netrin-1 concentrations used in soluble form in prior axon guidance experiments were sufficient for a biological response when chemically immobilized, netrin was immobilized in a random, non-gradient-forming method by EDC and NHS onto laminin-coated scaffolds. When compared to RGCs cultured on laminin-coated scaffolds which had been incubated in the Netrin-1 solution without first being activated by EDC and NHS (Figure 2A), RGCs cultured on these chemically immobilized scaffolds (Figure 2B) demonstrated an increase in the number of polarized RGCs, i.e., cells extending only a single axon (Figure 2C). In addition, RGCs cultured on these scaffolds extended shorter axons owing to the combination of Netrin-1 on laminin becoming a repulsive guidance cue. These results establish that the concentration of immobilized Netrin-1 was sufficient to elicit a biological response.

Next, it was necessary to determine which method of photoinitiated crosslinking would immobilize the highest concentration of protein. To determine this, fluorescently labelled BSA-FITC protein was immobilized through two separate methods of UV-activated crosslinking. In the first method, the crosslinker was initially coupled to the scaffold through the NHS subunit and the soluble protein then crosslinked through the light-activated diazirine unit. In the second method this process was reversed, with the UV-crosslinker initially reacted to the BSA-FITC protein in solution through the NHS subunit, and then coupled to the surface through the diazirine subunit. When exposed to the same crosslinking conditions, samples in which the crosslinker was initially attached to the scaffold did not show an increase in fluorescence (corresponding to immobilized BSA-FITC) compared to absorbed BSA-FITC controls, which were exposed to BSA-FITC solution and UV irradiation without the UV crosslinker (Figure 3). Samples in which the crosslinker was initially coupled to the soluble BSA-FITC protein did show a significant increase in fluorescence when compared to controls ($p < 0.05$), though this increase was less than the increase measured using EDC/NHS chemical coupling. Small dots of fluorescence were observed during immobilization of this method which may represent the immobilization of polymerized BSA-FITC rather than a monolayer which would be formed using either the chemical method or method where the UV-crosslinker is initially crosslinked to the scaffold. In addition to the increase in fluorescence, small gas bubbles were produced during the reaction, presumed to be nitrogen, a byproduct of the diazirine reaction (not shown). Thus, we found that immobilizing the UV-crosslinker initially to the soluble protein produced a greater concentration of immobilized protein, and we used this method in our subsequent experiments to examine the biological effects on axon guidance.

Because prior work on axon guidance suggested that a linear gradient is required to align single axons in one direction,[23] it was necessary to establish that the protein could be immobilized in a linear gradient, its density increasing toward the center of the radial scaffold. An etched diffusing filter in combination with a beam telescope placed in the laser's path was used to smooth and expand the natural Gaussian intensity distribution of the 351 nm argon-ion laser.[24] The fraction of Netrin-1 which reacted with the UV-crosslinker was immobilized onto fibronectin-coated PLA scaffolds. The gradient was visualized through immunostaining and quantified by measuring the fluorescence intensity at 0.5 mm intervals from the scaffold center, taking intensity measurements at 448 nm for Netrin-1 labeling and at 546 nm for autofluorescence, to normalize the Netrin-1 concentration to the number of fibers at each position. The Netrin-1 concentration on experimental samples was observed to be linear, whereas samples without crosslinker showed an exponential decrease in Netrin-1 labeling away from the high fiber density central point of the scaffold (Figure 4). Values were taken along the orthogonal axes of each scaffold and the ratio of intensities averaged. Thus, using this filter and crosslinker approach we were able to generate a gradient of Netrin-1 on the scaffolds.

With this Netrin-1 gradient on the scaffold, we next asked whether we could increase the percentage of RGCs extending axons toward the scaffold center. Purified RGCs were seeded, and following two days of growth, samples with and without immobilized Netrin-1 were fixed and immunostained for β -tubulin, a marker for RGC neurite growth. Cells and their axons were categorized as polarized (i.e. having one axon only) with growth toward the center of the scaffold, polarized with growth away from the center of the scaffold, or bipolar. RGCs cultured on control fibronectin-coated scaffolds showed no statistical difference in the percentage of cells polarized toward the center, polarized away from the center, or not polarized because the cells extended axons in both directions (Figure 5). In contrast, RGCs cultured on scaffolds with immobilized Netrin-1 gradients demonstrated a statistically significant increase in the percentage of axons growing along the gradient toward the scaffold center, and a decrease in cells with axons growing away from the center and in cells extending axons in both directions (Figure 5). However, RGC axons on both control and Netrin-1 immobilized scaffolds did not enter or cross the central point of the scaffold (Fig 5C, 5F). Thus, culturing RGCs on electrospun scaffolds with a Netrin-1 gradient increased the percentage of cells extending an axon toward but not crossing the scaffold center, mimicking the centripetal axon growth observed in the retina *in vivo*.

4. Discussion

The inability of the adult mammalian retina to replace RGCs lost in optic neuropathies has motivated research in cell replacement or tissue engineering therapies, including intravitreal injection of stem cells [25] and isolated primary RGCs.[12] However, RGC transplantation is inherently more difficult than photoreceptor transplantation, as it requires the transplanted cells to integrate dendritically with their amacrine or bipolar cell presynaptic partners in the inner plexiform layer, extend their axons radially through the retina to the optic nerve, grow down the optic nerve and find their appropriate targets in the brain. Previously, we investigated the use of both PLL-PEG hydrogels [26] and PLA electrospun scaffolds [27] to study RGC survival and neurite growth and as a potential cell delivery vehicle. Unlike

hydrogels, PLA electrospun scaffolds allowed RGC axons to replicate the radial organization of native RGCs in the retina, but they did not promote the polarization or centripetally directed growth seen in the retina *in vivo*. Here, to mimic the *in vivo* guidance of RGC axons, a Netrin-1 gradient was immobilized onto the scaffold surface in order to polarize the outgrowth of the seeded RGCs. Netrin-1 was chosen for these first experiments because it can guide RGC axons without the need for other guidance factors,[9] and because Netrin-1 continues to be expressed at the optic nerve head and is partly responsible for the entrance of RGCs into the optic nerve.[28] Future experiments will be designed to determine whether these RGC-seeded scaffolds are able to enhance guidance towards, and into, the optic nerve head. The incorporation of Netrin-1 did require a fibronectin coating of PLA fibers rather than the laminin coating used previously[19, 26] as laminin has been shown to alter Netrin-1 from an attractive to a repulsive guidance factor.[11] This use of Netrin-1 was also compatible with scaffolding purified RGCs from early postnatal rats, an age at which the DCC Netrin-1 receptor is expressed.[8]

In our initial studies, we utilized the repulsive effect of immobilized Netrin-1 on laminin to determine if we were able to immobilize a therapeutically relevant concentration of the guidance protein. However, using the chemical crosslinking methods we also observed the biological effect of an increase in the percentage of polarized RGCs. Despite reports of forming protein gradients using the EDC/NHS chemical crosslinking method,[29] we did not observe any directional preference of the polarized cells, and RGCs polarized toward the scaffold center were sometimes directly adjacent to RGCs polarized away from the scaffold center. This could be due to use of the hydrophobic PLA rather than the hydrophilic gelatin as the base polymer for the electrospun scaffold, thereby affecting the ability of the guidance factor to form its gradient through wicking. To overcome this limitation, we used the technique of photolithography which allows control over the organization of the immobilized proteins.

Growth factors and other guidance factors have previously been photoimmobilized on 3D scaffolds in order to direct neuronal cell growth using azido- or benzophenone-reactive groups.[14, 30] More recently, diazirines have been used to immobilize proteins on scaffold[31] or sensor [32] surfaces owing to their increased biostability, specificity of the reaction with low background, and ability to react with C-H, O-H and N-H through the formed carbene ion.[33, 34] In most cases, the photoreactive crosslinker has been initially reacted with the scaffold surface rather than with the soluble protein. This is likely to reduce the tendency of the proteins to polymerize during UV irradiation, and to prevent the deactivation of proteins by the reaction occurring in solution instead of at the scaffold surface, where protein can be immobilized. However, in our system we did not observe an increase in fluorescence during the immobilization of BSA-FITC, perhaps because of molecular distance from the crosslinker and proteins upon UV initiation, and more sensitive measures of protein concentration such as bicinchoninic acid assays were not compatible with the adsorbed fibronectin on the PLA fiber surface, which is necessary for axon growth.

Because of a low molar absorptivity, which we measured for sulfosuccinimidyl 6-(4,4'-azipentanamido)hexanoate as 68.3 (M*cm)^{-1} , it was necessary to use a 351 nm UV laser to provide the energy for carbene ion formation. The use of the UV laser to initiate the

chemical reaction allowed for patterning the reaction with light. In place of a normal photomask, a telescope and an etched filter were placed in the path of the laser beam to expand it to 1.5 cm in diameter and modify the Gaussian beam intensity profile into a quasi-conical distribution. While some variation at the nanoscale will still be observed during immobilization, at the microscale at which the growth cone of the RGC will sense the protein gradient, the slope of the protein gradient is sharp, creating a linear profile of immobilized protein on the surface of the scaffold. During analysis of the immobilized gradient, Netrin-1 was used and immunostained for rather than BSA-FITC, to ensure that the protein was still properly folded and its structure not obscured by the crosslinker following the immobilization process.

When RGCs were cultured on the Netrin-1-immobilized samples, it was observed that greater than 50% of RGCs were polarized toward the center of the scaffold compared to 31% of RGCs without immobilized netrin. While this increase is significant, it is not as large as we expected. This limited response could be due to the mechanism of Netrin-1 signaling, which has been demonstrated to be a result of mitochondrial dynamics[35] and increases in cAMP in the growth cone[11, 36]. However, one of the components in the defined RGC culture medium, forskolin, has been shown to also increase cAMP at the growth cone.[37] As forskolin strongly promotes RGC survival and axon growth,[22, 38] it may also affect polarization and Netrin-1 response. We might hypothesize that omitting it from the culture medium could increase the percentage of RGCs polarized toward the scaffold center even further, although testing this hypothesis would be severely hampered by reduced neuronal survival. Despite high concentrations of Netrin-1 found in both control absorbed samples and immobilized samples at the scaffold center, RGCs were not observed to enter or cross the central point of the scaffold. This inhibition is most likely due to the high density of random fibers found at the central core of the scaffold as we observed previously.[19] This observation emphasizes that neuron guidance is a balance between chemical and physical stimuli.[39]

Interestingly, we saw more of an effect on switching from bipolar to unipolar axon extension in the right direction toward the center than on decreasing the percentage of axons extending away from the center. In vivo, neurons are bipolar during a specific stage of development until extracellular cues trigger the designation of one of these neurites to form the axon while the other initiates the dendritic arbor.[40] The Netrin-1 gradient may act as a cue for the maturation of the seeded RGCs into a polarized form, which may ultimately lead to a more mature phenotype affecting their dendritic formation and electrophysiological properties. Both of these properties are known to be expressed in mature RGCs, and ultimately may drive the ability of RGCs to integrate into the host retina following transplantation.

5. Conclusions

The development of transplantation strategies for RGCs remains an important step toward the treatment of diseases of the optic nerve. We have shown that PLA electrospun scaffolds, while able to recreate the directionality of the RNFL, only directs 31% of seeded RGCs toward the center of the radial scaffold. Using a hetero-bifunctional diazirine crosslinker we

have demonstrated the ability to immobilize a gradient of Netrin-1 protein on an electrospun scaffold. With this protein-immobilized scaffold we have shown an increase to greater than 50% of RGCs that direct their axons toward the scaffold center. This represents an important step in increasing the number of RGCs whose axons will be guided to the optic nerve head when transplanted, and thence to their eventual targets in the brain.

Acknowledgments

We gratefully acknowledge support from the NEI (RC1-EY020297 JLG, P30-EY022589 to Shiley Eye Center, UCSD, and P30EY014801 to University of Miami) and unrestricted grants from Research to Prevent Blindness, Inc. We thank Gabe Gaidosh for assistance with confocal microscopy and Purac Biomaterials for their generous donation of medical-grade biomaterials.

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Figure 1.

Netrin-1 immobilization strategies

Netrin-1 was immobilized to the surface of radial scaffolds through 3 different methods: chemical crosslinking using EDC and NHS reacted first with the scaffold and then the activated scaffold with Netrin-1 (A); UV-crosslinking by reacting the bi-functional UV-crosslinker first with the scaffold surface through its NHS subunit and then with Netrin-1 through the diazirine subunit when activated with 365nm light (B); and UV-crosslinking by reacting the bi-functional UV crosslinker first through its NHS subunit with Netrin-1 and then with the scaffold surface through the diazirine subunit when activated with 365nm light (C). While the use of the UV-crosslinker offers more control for the formation of a gradient, reacting the crosslinker first with the surface (B) can lead to a lower concentration of

immobilized Netrin-1 should the surface be activated with UV light when the protein is not a favorable distance or should multiple bonds be made to a single protein, while reacting crosslinker first with the Netrin-1 protein can lead to polymerization of the protein should they react with other Netrin-1 proteins prior to reacting with the scaffold surface.

Figure 2.

Seeding on Netrin-1-immobilized scaffolds led to a higher percentage of polarized RGCs. RGCs seeded on control PLA scaffolds (A) and Netrin-1 immobilized scaffolds (B) were stained for $\beta 3$ tubulin and analyzed for axon polarization and were classified as polarized or bipolar (C). A significant increase in the percent of polarized RGCs was observed on cells seeded on Netrin-1-immobilized scaffolds (mean $\pm$ standard error of mean; * $p < 0.05$ by unpaired Student t-test). Scale bar A, B: 200 μ m A',B': 75 μ m.

Figure 3.

Protein crosslinking to scaffold surface through different strategies.

Compared to control samples which were exposed to UV irradiation but did not contain crosslinker (A), BSA conjugated with FITC was immobilized using three different techniques, using EDC/NHS chemical coupling (B), UV-initiated coupling with crosslinker attached to the soluble protein (C), and UV-initiated coupling with crosslinker attached to the scaffold surface (D). Total fluorescence was measured in 3 unbiased selected images from each scaffold and normalized to control samples (E) (mean $\pm$ standard error of mean; * $p < 0.05$ by one way ANOVA coupled with post hoc analysis by Fisher's LSD test). Scale bar 50 μm .

Figure 4.

Netrin-1 immobilized using UV-initiated crosslinking created a linear concentration gradient on nanofiber scaffolds.

Netrin-1 was immobilized by UV-initiated crosslinking and quantified by immunostaining. Samples were imaged at 0.5 mm intervals from the scaffold center to the periphery. Total 488 nm fluorescence (f_{488}) at each position was divided by autofluorescence in the 546 nm channel (f_{546}) to standardize the immobilized Netrin-1 to the fiber density at each position. The ratio (f_{488}/f_{546}) at each position was normalized to the ratio at the scaffold center. Control samples were exposed to the same UV irradiation but did not include crosslinker. Scale bar 50 μ m.

Figure 5.

Netrin-1-immobilized scaffolds increased polarized RGC axon growth toward the scaffold center.

When compared to RGCs cultured on control scaffolds without immobilized Netrin-1 (A–C), RGCs cultured on Netrin-1-immobilized scaffolds (D–F) showed a significant shift from bipolar (arrow heads) to polarized cells, as well as an increase in RGCs with polarized growth toward the scaffold center (thick closed arrows); some RGCs polarized away from the center (thin open arrows). In both control scaffolds (C) and Netrin-1-immobilized scaffolds (F) RGC neurites were unable to extend across the central point of the scaffold

(white dashed circle). (* $p < 0.05$ by one way ANOVA with post hoc analysis by Fisher's LSD test, NS: not significant) Scale bars: A and D 300 μm ; B and E 100 μm ; C and F 500 μm .