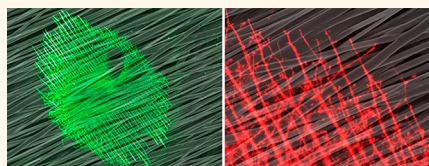


# Neurite Outgrowth on Electrospun Nanofibers with Uniaxial Alignment: The Effects of Fiber Density, Surface Coating, and Supporting Substrate

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**ABSTRACT** Electrospun nanofibers with uniaxial alignment have recently gained its popularity as scaffolds for neural tissue engineering. Many studies have demonstrated that the nanofibers could guide the neurites to extend along the direction of alignment, resembling the native hierarchy of the nerve tissue. However, the contact cues provided by the nanofibers can be far more complicated than just guiding the neurites to extend along them. In the current study, we used dorsal root ganglia as a model system to systematically investigate the interactions between neurites and uniaxially aligned nanofibers. We demonstrated, for the first time, that the neurites could not only project along the nanofibers, but also be directed to grow along a direction perpendicular to the aligned nanofibers, depending on the following parameters: (i) the density of nanofibers, (ii) the protein deposited on the surfaces of the nanofibers, and (iii) surface properties of the substrate on which the nanofibers were supported. We also investigated the pharmacological effect of myosin II inhibition on the nanofiber-guided growth of neurites by adding blebbistatin to the culture medium. Our findings offer new insights into the design of nanofiber-based scaffolds for nerve injury repair and will provide new guidelines for the construction of well-defined neuronal network architecture (the so-called neural circuits).



**KEYWORDS:** dorsal root ganglia · neural tissue engineering · neurite outgrowth · electrospun nanofibers · contact guidance

Electrospun nanofibers have been extensively explored as tissue engineering scaffolds for repairing both the central and peripheral nervous systems.<sup>1–4</sup> When used as uniaxially aligned arrays,<sup>5–7</sup> the nanofibers are supposed to guide the extension of the regenerated neurites from the proximal stump to the distal stump through the anisotropic cues. To this end, McDonald *et al.* demonstrated that uniaxially aligned nanofibers could better facilitate the axons to cross a 3-mm defect in a rat model of spinal cord injury than their random counterparts.<sup>8</sup> They attributed the enhancement in healing performance to the highly organized architecture of aligned nanofibers, which could largely mimic the structure of the native extracellular matrix (ECM) of the spinal cord. Bellamkonda *et al.* observed a similar pattern of outgrowth for the neurites of peripheral nerves in a rat model of sciatic nerve injury.<sup>9</sup> They reported

that only aligned nanofibers could successfully bridge a gap of 17 mm in a sciatic nerve, leading to significantly improved recovery of functions thanks to the reduced mismatches between the regenerated neurites and their footprints in the distal stump. Parallel to these *in vivo* studies, the notion that uniaxially aligned nanofibers can guide the extension of neurites along the direction of alignment has also been extensively examined *in vitro* by many research groups.<sup>10–13</sup> In all these and other studies, only parallel guidance, or neurite extension along the direction of fiber alignment, has been reported for uniaxially aligned nanofibers.

Despite its prevalence, the notion that neurites should merely extend parallel to the aligned nanofibers was challenged by Finkelstein *et al.*, who discovered that the neurites of the central nervous system preferentially grew perpendicular to the

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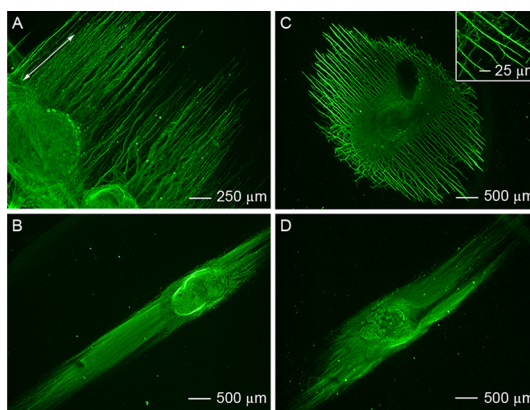
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alignment of poly( $\epsilon$ -caprolactone) (PCL) nanofibers at the interface between the implant and rat brain tissue.<sup>14</sup> In this case, perpendicular rather than parallel guidance was observed for the nanofibers. These authors, however, did not offer an explanation for the observed pattern of perpendicular outgrowth. A number of key issues remain to be addressed: for examples, why the uniaxially aligned nanofibers could not guide the extension of neurites along them? What are the factors that control the outgrowth pattern of neurites? Do the neurites have a similar function regardless of the outgrowth pattern?

In the present work, we aim to unravel the role of uniaxially aligned nanofibers in guiding the outgrowth of neurites in an effort to optimize the design of the scaffolds for nerve repair. Dorsal root ganglia (DRG) were chosen as a model system for the investigation. The neurons in DRG are unique in that they only send one axon into the peripheral target tissue with the other axon being directed into the central nervous tissue. In addition to their divergent axonal trajectories, the two axons of a given DRG neuron differ with respect to their presynaptic morphologies and post-synaptic targets.<sup>15</sup> Although many previous studies have shown that the neurites from DRG closely conformed to the alignment of nanofibers during extension,<sup>16–18</sup> here we demonstrate, for the first time, that the neurites could also be projected along a direction perpendicular to the alignment. Furthermore, we mechanistically investigated the interaction between the nanofibers and the neurites. We examined the following parameters that may play a role in guiding the extension of neurites: the density of nanofibers, the protein coating on the nanofibers, and the surface chemistry of the supporting substrate for the nanofibers. We also investigated the pharmacological effect on neurite outgrowth by adding blebbistatin, a specific inhibitor to myosin II, to the culture medium. Combined together, our results bring new insights into the field of neural tissue engineering and offer fresh guidelines for the design of nanofiber-based scaffolds.

## RESULTS

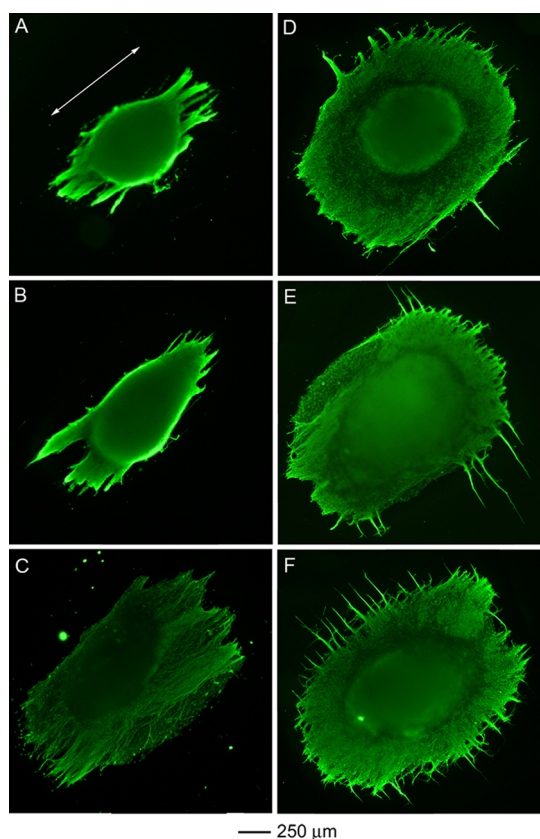
We first investigated the effect of fiber density on the outgrowth of DRG neurites. We collected the electrospun nanofibers across the gap of a U-shaped metal frame to generate a free-standing mat consisting of uniaxially aligned nanofibers. The mat was subsequently transferred onto a metal ring to obtain a suspended scaffold (Figure S1A). The fiber density was controlled by varying the collection time and then quantified using a scanning electron microscope (SEM). As shown in Figure S2, the density of fibers increased from approximately 100 to 3,000 fibers per mm when the collection time was increased from 1 to 30 min. When DRG were cultured on the free-standing scaffolds with a low fiber density (collected for 4 min),



**Figure 1.** Fluorescence micrographs showing the typical neurite fields of DRG cultured on free-standing scaffolds of uniaxially aligned nanofibers that were prepared by collecting for (A and B) 4 min and (C and D) 15 min, respectively. The surfaces of the nanofibers in (B) and (D) were coated with laminin prior to DRG culture. The arrow in (A) indicates the direction of fiber alignment and it applies to all other samples. The inset in (C) shows a blow-up of the splitting tips. All the samples were stained with anti-neurofilament 200.

the neurites tended to grow parallel to the direction of fiber alignment (Figure 1A). When the scaffolds with a low fiber density were coated by laminin, the neurite fields were essentially unchanged except that the neurites were further elongated (Figure 1B). However, when we switched to scaffolds with a high fiber density (e.g., collected for 15 min), neurites extending from the DRG tended to grow perpendicular to the fiber alignment (Figure 1C). In addition, the neurites had a propensity to split at their tips with the newly formed branches aligned along the underlying fibers (see the inset of Figure 1C). Surprisingly, when cultured on scaffolds with a high fiber density (collected for 15 min) and laminin coating, the resultant neurite fields presented a pattern of parallel growth (Figure 1D), similar to what was observed for the scaffolds with a low fiber density (regardless of laminin-coating).

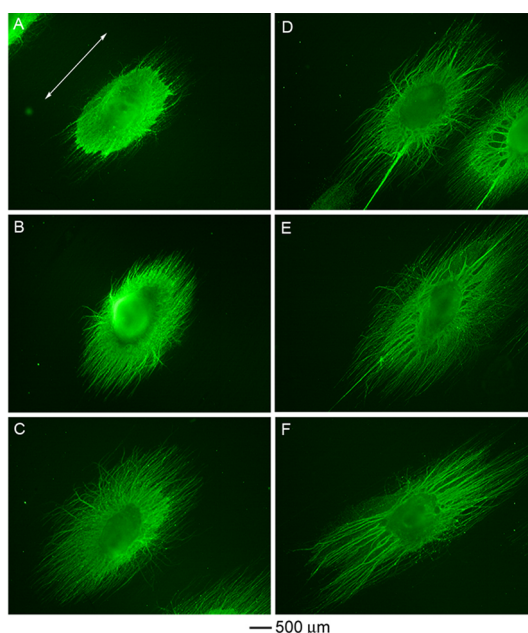
We then immobilized a set of scaffolds with different fiber densities on glass coverslips (Figure S1B) and used them for DRG culture to understand the effect of a supporting substrate on the outgrowth of DRG neurites. It was found that the neurites extending from DRG grew parallel to the direction of fiber alignment when the fiber densities were relatively low, with the collection time equal to and shorter than 4 min (Figure 2A–C). The neurites tended to form bundles and grow perpendicular to the direction of fiber alignment when the fiber collection time was equal to or longer than 8 min (Figure 2D–F). Branching was also observed for the neurites. In comparison, the neurites were observed to grow along the direction of fiber alignment when DRG were cultured on scaffolds that were fabricated with a collection time of 15 min, transferred onto glass coverslips, and then coated with poly(L-lysine) (PLL) and laminin from solutions with different concentrations (Figure 3). As the concentration of laminin



**Figure 2.** Fluorescence micrographs showing the neurite fields of DRG cultured on scaffolds of uniaxially aligned nanofibers that were supported on bare glass coverslips. The nanofibers were collected for (A) 1, (B) 2, (C) 4, (D) 8, (E) 15, and (F) 30 min, respectively. The arrow in (A) implies the direction of fiber alignment and it applies to all other samples. All the samples were stained with anti-neurofilament 200.

solution used for the coating process was increased, the length of the neurites increased (Figure 3B–F). Figure S3 shows a quantitative analysis of the average neurite lengths when the DRG were cultured on nanofibers coated with laminin in different concentrations. All the laminin-coated groups were significantly different from the PLL only group, as indicated by the asterisk marks above the bars. We also cultured DRG on scaffolds with different fiber densities that were supported on PEG-coated glass coverslips (Figure S1B). At very low fiber density, the neurites could barely perceive the existence of the fibers and thereby exhibited a haphazard pattern (Figure 4A) similar to those cultured on random nanofibers (Figure S4). At a fiber collection time of 2 min or longer, the neurites also formed bundles, but they tended to grow along a direction perpendicular to the nanofibers (Figure 4B–F). Neurite branching became more significant for samples as the fiber collection time was increased (Figure 4E,F).

The fine protrusions of the axonal growth cone are commonly known as filopodia, which are thin, finger-like structures acting as antennae for the cell to probe the local environment and they are filled with tight, parallel bundles of filamentous (F)-actin.<sup>19</sup> F-actin is

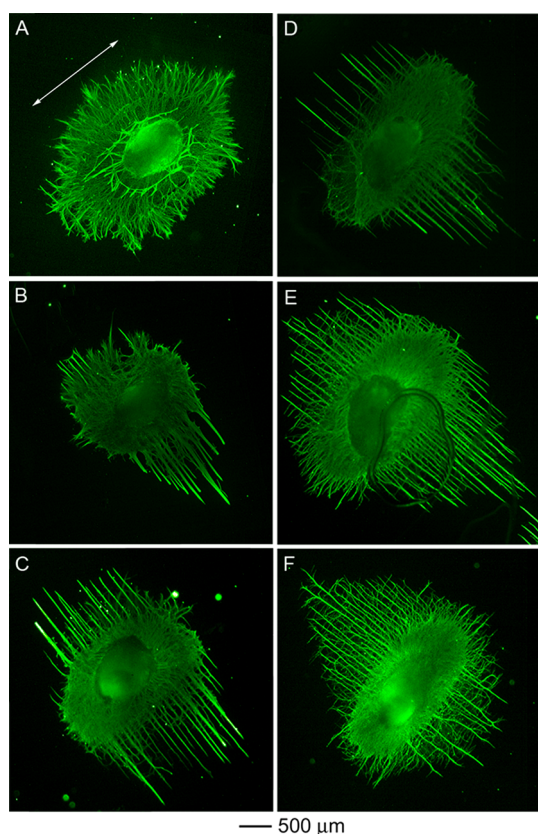


**Figure 3.** Fluorescence micrographs showing the neurite fields of DRG cultured on nanofibers that were deposited on glass coverslips and then coated with different chemical species. The fiber collection time was 15 min and the scaffolds were coated (prior to DRG culture) with (A) PLL and (B–F) PLL and then laminin from solutions with different concentrations: (B) 26, (C) 52, (D) 104, (E) 208, and (F) 516  $\mu\text{g/mL}$ . The arrow in (A) implies the direction of alignment for the underlying nanofibers, and it applies to all other samples. All the samples were stained with antineurofilament 200.

important for axon outgrowth and guidance.<sup>20</sup> We stained DRG cultured on scaffolds made of uniaxially aligned nanofibers with rhodamine-phalloidin, a probe with high affinity toward F-actin. For the sample prepared with a fiber collection time of 1 min (Figure 4A), long filopodia was detected, which could be attributed to the randomly distributed neurites (Figure 5A). Unexpectedly, no filopodia structure was observed for neurites perpendicular to the fiber alignment (Figure 5B–E). This result was contradictory to the previous findings that cortical neurons would fail to elaborate neurites if the formation of filopodia was blocked.<sup>19,20</sup> In contrast, filopodia structure was observed on the branched neurites (Figure 5E), resembling those extending parallel to the fiber alignment (Figure 5F).

To better understand the role of uniaxially aligned nanofibers in guiding neurite extension, we investigated the pharmacologic effect of blebbistatin, an inhibitor to myosin II,<sup>21</sup> on the outgrowth of DRG neurites. Most of the neurites extending from DRG cultured on free-standing scaffolds of uniaxially aligned nanofibers (with a collection time of 15 min) in the presence of blebbistatin (50  $\mu\text{g/mL}$ ) mainly presented parallel outgrowth, with very few neurites growing perpendicular to the direction of fiber alignment within a certain period of time (Figure 6A). For the

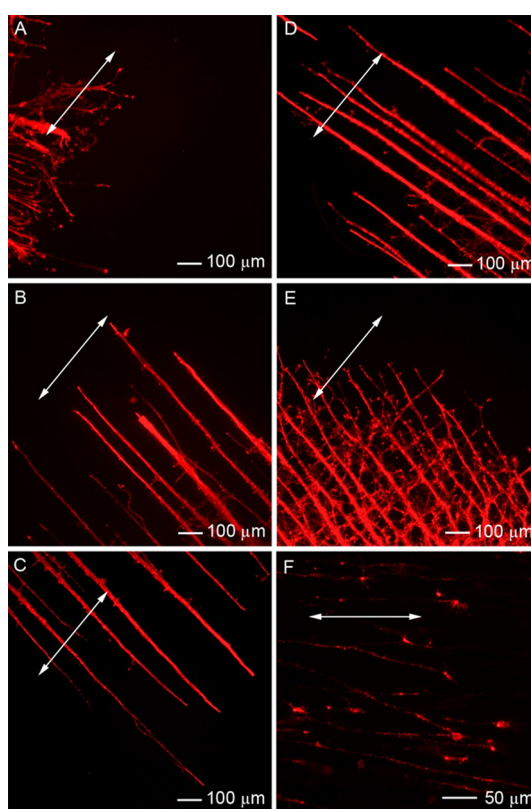




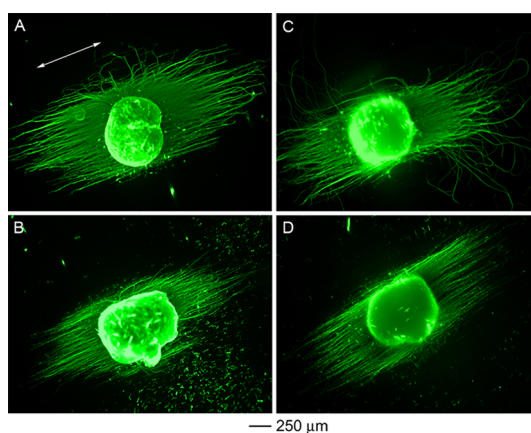
**Figure 4.** Fluorescence micrographs showing the neurite fields of DRG cultured on aligned nanofibers that were deposited on glass coverslips that were precoated with PEG. The fiber collection time was (A) 1, (B) 2, (C) 4, (D) 8, (E) 15, and (F) 30 min, respectively. The arrow in (A) implies the direction of alignment for the underlying nanofibers, and it applies to all other samples. All the samples were stained with antineurofilament 200.

same scaffold but with laminin coating, most of the neurites still exhibited parallel growth while some of the neurites in the vicinity of DRG main body displayed a random distribution (Figure 6B). However, when DRG were cultured on uniaxially aligned nanofibers (with a collection time of 15 min) supported on PEG-coated glass coverslips, the neurites initially grew parallel to the direction of fiber alignment and then began to defasciculate: the branched neurite grew along the perpendicular direction or became disorganized after a certain period of time (Figure 6C). When the scaffolds were coated with laminin, the neurites only showed parallel growth (Figure 6D). For all these cases, the neurites showed a tendency to form bundles (fascicles) in the presence of blebbistatin.

In an effort to obtain a clear picture about the interactions between neurites and uniaxially aligned nanofibers, DRG cultured on the nanofiber scaffolds were dehydrated and then imaged using an SEM. Fascicles were formed for neurites outgrowing from DRG cultured on scaffolds consisting of fibers with a collection time of 4 min and deposited on PEG-coated glass coverslips formed. They interacted with uniaxially

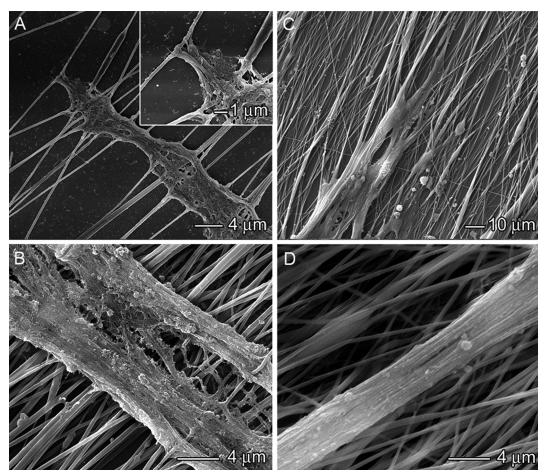


**Figure 5.** No filopodia structure was observed for neurites growing perpendicular to the fibers. Fluorescence micrographs showing F-actin staining of neurites extending from DRG that were cultured on nanofibers supported by PEG-coated glass coverslips. The fiber collection time was (A) 1, (B) 4, (C) 8, (D) 15, (E) 30, and (F) 30 min, respectively. The fibers were coated with laminin in (F). The arrows indicate the direction of alignment for the underlying nanofibers. All the samples were stained with rhodamine phalloidin.



**Figure 6.** Fluorescence micrographs showing the effect of blebbistatin on neurite extending from DRG cultured on free-standing, aligned nanofibers (A) without and (B) with laminin coating, and aligned nanofibers deposited on PEG-coated glass coverslips (C) without and (D) with laminin coating. The fiber collection time was 15 min. All culture experiments were conducted in the presence of blebbistatin (50  $\mu\text{g/mL}$ ). The neurites extending from DRG mainly outgrow parallel to the direction of fiber alignment and formed fascicles. All the samples were stained with antineurofilament 200.

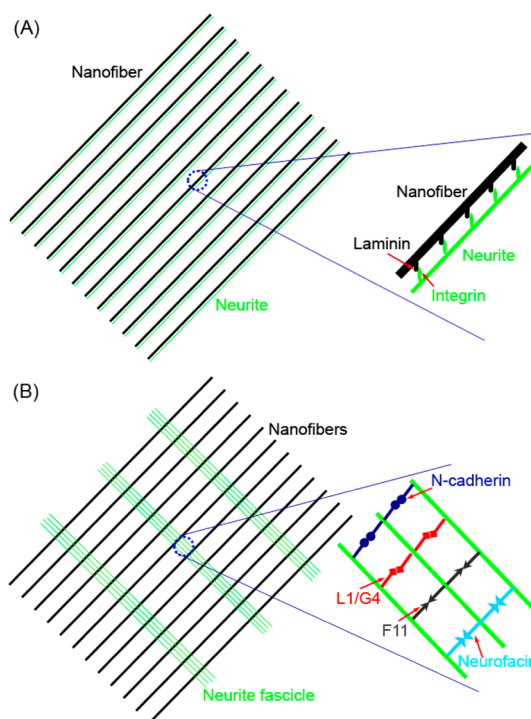




**Figure 7.** SEM images showing the morphology of neurites on uniaxially aligned nanofibers that were deposited on PEG-coated glass coverslips. The fiber collection time was (A) 4 and (B–D) 15 min, respectively. Compared to the sample in (B), the nanofibers were coated with laminin in (C) and there was blebbistatin (50  $\mu\text{g/mL}$ ) in the culture medium of (D).

aligned nanofibers orthogonally (Figure 7A). The neurites could cross over the gap (roughly 1–5  $\mu\text{m}$ ) between adjacent fibers. Also, it seems that the neurites could grasp the nanofibers and part of them even extended into the space underneath the fibers (Figure 7A, inset). The neurites showed a similar growth behavior when DRG were cultured on scaffolds consisting of fibers with a collection time of 15 min and supported on PEG-coated glass coverslips (Figure 7B). In comparison, when the scaffolds were coated with laminin, the neurites did not form bundles and grew parallel to the fiber alignment (Figure 7C). In this case, it is difficult to differentiate the neurites from fibers since they were along the same direction and the neurites did not form fascicles. After adding blebbistatin, the neurites also formed fascicles, but they were aligned parallel to the direction of fiber alignment (Figure 7D). These observations indicate that neurites sensed the nanofiber scaffold as a three-dimensional and flexible substrate and could interact with individual fibers in a number of different ways: that is, the neurites could cross over a fiber from above, the neurites could pass by a fiber from below, the neurites could grow along an individual fiber, and the neurites could grasp and pull fibers together.

We also examined the interactions between neurites and nanofibers during neurite outgrowth using time lapse imaging. The extension of neurites from DRG was imaged every 15 min. When DRG were cultured on scaffolds composed of fibers with a collection time of 4 min and deposited on PEG-coated glass coverslips, the neurites continuously explored the appropriate direction for extending. The neurites could grasp and pull the fibers, and elongate perpendicular to the direction of fiber alignment (Movie S1). At the same time, we



**Figure 8.** A strong interaction between neurites and nanofibers could lead to parallel growth of neurites along the fibers while those neurites interacted with fibers poorly showed perpendicular growth. Schematic illustration of two different modes of neurite outgrowth on uniaxially aligned nanofibers: (A) parallel growth, and (B) perpendicular growth.

noticed that cells migrated out to the regions surrounding the DRG explant. When DRG were cultured on laminin-coated samples, the neurites extended along the long axis of fibers and cells also migrated out along the direction of fiber alignment (Movie S2).

## DISCUSSION

Uniaxially aligned electrospun nanofibers have been previously used to guide the outgrowth of DRG neurites. However, previous studies were largely restricted to the observation of a parallel growth pattern, which could be attributed to the use of scaffolds with low fiber densities or certain surface chemistries for the supporting substrates. In the present work, we cultured DRG on scaffolds composed of uniaxially aligned nanofibers with different combinations of fiber density, surface chemistry, and supporting substrate. In addition, serum-free culture media (neurobasal media with N2 supplement and 30 ng/mL NGF) was employed, which could be essential to exclude the complexity associated with serum deposition on the nanofibers. In the present work, we demonstrated, for the first time, that DRG neurites could exhibit not only parallel outgrowth but also perpendicular outgrowth when cultured on scaffolds consisting of uniaxially aligned nanofibers.

On the basis of the experimental results, we can propose two models to account for the outgrowth of

DRG neurite on uniaxially aligned nanofibers, which are schematically illustrated in Figure 8. In the first model, uniaxially aligned nanofibers coated with laminin could interact strongly with the neurites through transmembrane receptors such as neuronal integrins expressed by DRG neurons. The neurites could adhere to the fibers very well and initiate cdc42 and Rac G-protein pathways, leading to the formation of filopodia and lamellipodia (Figure 5F) elongated along the long axis of the uniaxially aligned fibers (Figure 8A, Movie S2). This scenario is supported by a comparison of the results in Figures 1C and 3F. By depositing laminin on the surfaces of the nanofibers with a relatively high density, the projection of the neurites would switch from perpendicular to parallel outgrowth. In the second model, the interaction between the neurites (*i.e.*, N-cadherin, L1/G4, F11, and neurofascin) was much stronger than the interaction between neurites and fibers. The neurites tended to form bundles or fascicles due to their poor adhesion to the pristine fibers (Figure 8B).<sup>22</sup> This scenario is supported by the results in Figures 1A and 4C. When cultured on pristine, free-standing fibers with a relatively low fiber density, the neurites preferred to extend along the fiber alignment. If the fibers were deposited on PEG-coated coverslips, however, the neurites switched to perpendicular outgrowth. Due to the repelling effect of the PEG, the neurites kept exploring the microenvironment around them, protruding, and retracting until they found the right direction to pursue (Movie S1). Since it would be the easiest for the neurites to grasp and pull the fibers along the perpendicular direction,<sup>23</sup> the neurites tended to project forward in a direction perpendicular to the fiber alignment. Also, owing to the nonadhering effect of the PEG coating, the neurites tended to form fascicles to diminish their interactions with the substrate. These results are in agreement with previous arguments that axonal fasciculation and defasciculation are likely controlled by adhesion-based mechanisms.<sup>24</sup>

Growth cones rich in actin microfilaments have the potential to interact with myosin II, a member of the myosin family of molecular motors.<sup>25</sup> It has been

reported that myosin II isoforms played an important role in cellular adhesion through modulation of the interaction between integrin receptors and the ECM.<sup>26</sup> Inhibition of myosin II would result in impairment of neuronal growth cone motility due to its involvement in retrograde flow and actin filament organization.<sup>27,28</sup> It was also reported that when growing on laminin, inhibition of the myosin II activity reduced the extension rate of axon.<sup>29,30</sup> In the current study, inhibition of myosin II in the presence of blebbistatin in the culture medium might have weakened the interaction between the neurites and the laminin-coated nanofibers. The interaction among the neurites became stronger and the neurites tended to form bundles, although the direction of neurite extension was still parallel to the long axis of the nanofibers (Figures 6B,D and 7D). Thus, the parallel growth of neurites was largely independent of myosin II. In contrast, the perpendicular growth of neurites was dependent on myosin II to a certain extent. When myosin II was inhibited, the original perpendicular growth of DRG neurites would change to parallel growth during the initial period of neurite extension (Figures 1C, 4E, and 6A,C). After reaching a certain length, the occurrence of neurite branching and turning eventually led to perpendicular and/or disorganized growth (Figure 6A,C).

## CONCLUSIONS

In summary, we have demonstrated that the direction of DRG neurite growth on uniaxially aligned, electrospun nanofibers could be either parallel or perpendicular to the direction of fiber alignment. The direction is determined by a set of parameters including fiber density, surface chemistry of the fibers, and surface property of the supporting substrate. We also demonstrated that myosin II could play an important role in the perpendicular contact guidance of neurite outgrowth on uniaxially aligned nanofibers. By contrast, myosin II does not appear to be necessary for the parallel contact guidance. This work will have significant implications for the design of nanofiber-based scaffolds for nerve repair applications.

## METHODS

**Fabrication of Electrospun Nanofibers.** The setup for electrospinning and the collectors for obtaining random and aligned nanofibers are similar to what we used in previous studies.<sup>2,9,18</sup> The polymer solution (20% w/v) used for electrospinning was prepared by dissolving poly( $\epsilon$ -caprolactone) (PCL, Sigma-Aldrich, St. Louis, MO) in a mixture of dichloromethane (DCM) and dimethylformaldehyde (DMF) (Fisher, Pittsburgh, PA) at a volume ratio of 80:20. Two different types of collectors, a glass coverslip and a U-shaped stainless steel frame, were employed to obtain random and uniaxially aligned nanofibers, respectively. The aligned fibers were transferred onto glass coverslips and then glued using Silastic Type A Medical Adhesive

(Dow Corning, Midland, MI) around the edges. The scaffolds for DRG culture were placed in different wells of a 24-well TCPs culture plate and then sterilized with ethylene oxide gas.

The electrospun nanofibers were coated with laminin (Millipore, Temecula, CA) as the following: the sample was immersed in a 0.1% poly-L-lysine (PLL) (Sigma-Aldrich, St. Louis, MO) solution for 1 h at room temperature, followed by washing with phosphate buffered saline (PBS) buffer (Invitrogen, Carlsbad, CA) three times. The sample was then immersed in a laminin solution (concentrations ranging from 26 to 516  $\mu$ g/mL) and incubated at 4 °C overnight. Prior to DRG seeding, the scaffold was rinsed with PBS buffer three times.

The nanofiber scaffolds were characterized using a scanning electron microscope (SEM, Nova 200 NanoLab, FEI, Hillsboro, OR).

To avoid charging, the scaffold was sputtered with platinum for 40 s in vacuum at a current intensity of 40 mA after the sample had been fixed to a double-side conductive tape on a metallic stud. The accelerating voltage was 15 kV.

**DRG Culture.** Embryonic day 8 (E8, stage HH35-36) chicks were removed from the eggs and decapitated. DRG were dissected from the thoracic region and collected in Hank's buffered salt solution (HBSS). The DRG were then seeded onto the nanofiber scaffolds (1 DRG per scaffold) and incubated for 6 days in a modified neurobasal (NB) medium supplemented with 1% ABAM, 1% N-2 supplement (Invitrogen), and 30 ng/mL rh $\beta$ -nerve growth factors (NGF) (R&D system, Minneapolis, MN). Six DRG were used for each experimental group.

**SEM Characterization of the Cultured DRG.** The DRG were fixed in 3.7% formaldehyde for 30 min and then dehydrated in ethanol with a series of concentrations (30%, 50%, 70%, 90%, 95%, and 100%), followed by drying in vacuum. Finally, the samples were coated with platinum for 40 s using a sputter prior to imaging by SEM. The accelerating voltage was 15 kV.

**Time Lapse Imaging.** DRG were seeded on nanofiber scaffolds and placed on the stage of an optical microscope. The setup was then placed in an incubator. DRG neurite outgrowth on the scaffolds were imaged every 15 min using Panasonic WV-BP130 attached to a Nikon microscope with FlashBus FBG (Nikon, Melville, NY).

**DRG Immunostaining.** After incubation for 6 days, the DRG were immunostained with anti-neurofilament 200 (Sigma-Aldrich). Briefly, the DRG were fixed in 3.7% formaldehyde for 45 min and permeabilized by 0.1% Triton X-100 for 30 min. They were blocked by treating with PBS containing 5% normal goat serum (NGS) (Invitrogen) for 1 h. Primary antibody diluted with PBS (containing 2% NGS) was applied to the samples by incubation overnight at 4 °C. The anti-neurofilament 200 marker was detected using AlexaFluor 488 goat anti-mouse IgG (1:200; Invitrogen) secondary antibody. After staining, fluorescence micrographs were captured using a QICAM Fast Cooled Mono 12-bit camera (Q Imaging, Burnaby, BC, Canada) attached to an Olympus microscope with Capture 2.90.1 (Olympus, Center Valley, PA). The average lengths of extending neurites were calculated from fluorescence images using custom-designed image processing software constructed in MATLAB (MathWorks, Inc.). The average length of neurite extension was calculated as the average distance between the elliptical curve identifying the border of the DRG cell mass and the elliptical curve identifying the leading edge of the neurite field along a line oriented radially from the center of the DRG cell mass. Mean values and standard deviation were reported ( $n = 6$ ). Statistical analysis was performed using the  $t$  test by analysis of variance at a 95% confidence level.

**Conflict of Interest:** The authors declare no competing financial interest.

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**Supporting Information Available:** Additional experimental data and movies (.avi). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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