Published in final edited form as:

Acta Biomater. 2013 July ; 9(7): 7158–7168. doi:10.1016/j.actbio.2013.03.032.

Navigating neurites utilize cellular topography of Schwann cell somas and processes for optimal guidance

Cristina Lopez-Fagundo, Jennifer A. Mitchel, Ph.D, Talisha D. Ramchal, M.S.¹, Yu-Ting L. Dingle, M.S, and Diane Hoffman-Kim, Ph.D.

Department of Molecular Pharmacology, Physiology and Biotechnology, Center for Biomedical Engineering, Brown University, Providence RI, USA

Abstract

The path created by aligned Schwann cells (SCs) after nerve injury underlies peripheral nerve regeneration. We developed geometric bioinspired substrates to extract key information needed for axon guidance by deconstructing the topographical cues presented by SCs. We have previously reported materials that directly replicate SC topography with micro- and nanoscale resolution, but a detailed explanation of the means of directed axon extension on SC topography has not yet been described. Here, using neurite tracing and time-lapse microscopy, we analyzed the SC features that influence axon guidance. Novel poly(dimethylsiloxane) materials, fabricated via photolithography, incorporated bioinspired topographical components with the shapes and sizes of aligned SCs, namely somas and processes, where the length of the processes were varied but the soma geometry and dimensions were kept constant. Rat dorsal root ganglia neurites aligned to all materials presenting bioinspired topography after a 5 days in culture and to bioinspired materials presenting soma and process features after only 17 hours in culture. Key findings of this study were: Neurite response to underlying bioinspired topographical features was time dependent, where at 5 days, neurites aligned most strongly to materials presenting combinations of soma and process features, with higher than average density of either process or soma features; but at 17 hours they aligned more strongly to materials presenting average densities of soma and process features and to materials presenting process features only. These studies elucidate the influence of SC topography on axon guidance in a time-dependent setting and have implications for the optimization of nerve regeneration strategies.

Keywords

bioinspired materials; topographical cues; dorsal root ganglia (DRG); axon; time-lapse; alignment

© 2013 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

Corresponding Author: Diane Hoffman-Kim, Ph.D., Brown University, Box G-B 393, Biomed Center, 171 Meeting St., Providence, RI, 02912, Phone: (401) 863-9395, Fax: (401) 863-1595, dhk@brown.edu.

¹Present address: School of Medicine, Duke University, Durham, NC, 27710

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

After injury in the peripheral nervous system (PNS), Schwann cells (SCs) promote nerve regeneration by providing permissive cues, including extracellular matrix molecules, cell adhesion molecules, soluble factors, and topographical cues. SCs normally wrap themselves around axons, form a myelin sheath, and respond to injury by myelin extrusion, down-regulation of myelin genes, and proliferation and alignment in tubular Büngner bands that guide regenerating nerve fibers toward their targets [1]. While the directive effects of SCs on neurite outgrowth involve a variety of cues, and guidance likely occurs through multiple mechanisms [2], guidance by topography is emerging as an important aspect of successful nerve regeneration.

Contact guidance is the phenomenon by which the physical shape of a substratum induces migration, directional growth, and alignment of cells [3, 4]. Nerve guidance promoted by SCs at an injury site [5] provides an example of *in vivo* contact guidance, and has motivated a number of *in vitro* studies that, by testing different surface scale topographies and substrate types [6–9], have investigated the topographical features that elicit directional neuronal growth. The most common type of topography used in these studies consists of micro-grooves and plateaus where parameters such as groove depth and width and plateau height and width are varied. These topographical properties of material surfaces can play important roles in directing nerve growth [10], but they tend to over-simplify the topographies presented to cells *in vivo*. While contact guidance of cells by physical contours of a substrate was recognized in the earliest days of tissue culture [11], challenges still remain to clarify the specific features and dimensions that affect neurite outgrowth and alignment.

Nerve guidance is influenced by a myriad of cues presented by the local environment, and the biochemical complexity of live cells can make elucidating the contributions of individual cues difficult. Our lab has described a method to fabricate polymeric materials that replicate the topography of aligned SCs and can direct neurites [12–14]. While both cellular and geometric topographies can guide neurites, specifications of topographical features that encode critical contact guidance information have not yet been determined. We propose that the combination of features necessary for optimal neuronal guidance includes the features and dimensions of SCs. Here, by deconstructing the local topographical cues presented by SCs, we present a systematic analysis of the cellular topographical parameters that influence their contact guidance of axons. Analysis of neurite extension and alignment on “geometric bioinspired substrates” elucidates the parameters that are sufficient for contact guidance, as well as those that are optimal.

Materials and Methods

Abbreviation	Substrate Description
SA	SC somas with average length processes
SS	SC somas with short processes
SL	SC somas with long processes

Abbreviation	Substrate Description
SO	SC somas only
PO	SC processes only
RR	Relief replica

Fabrication of relief replicas (RR) of SCs

All cell culture reagents were from Invitrogen Life Science (Carlsbad, CA, USA), unless otherwise stated. SC from adult rat sciatic nerve (generous gift from Dr. Mary Bunge, University of Miami, Coral Gables, FL, USA) were cultured on tissue culture plastic flasks pre-coated with 100 µg/mL poly(L-lysine) (PLL; Sigma, St. Louis, MO, USA) in Dulbecco's modified eagle's medium (DMEM) with 10 % fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (base media) supplemented with 2 µM forskolin (Sigma), 10 µg/mL bovine pituitary extract (Sigma) and 2 µM heregulin (generous gift from Genentech, Vacaville, CA, USA) (SC media). Cells used in experiments were between passages 5 and 7. Cultured cells were maintained in a humidified medium at 37 °C with 5 % CO₂. Polymeric RR of SC were fabricated as previously described by Bruder et al. [12] with the optimized conditions described by Kofron et al. [15].

Design, fabrication, and characterization of geometric bioinspired substrates

A phase contrast micrograph of a fixed SC template was used to trace an outline of SC cell bodies (somas) with the Image J v1.36 (National Institutes of Health) Freehand Selection tool. An ellipse was fit to each traced soma with the Measure Function, noting the major and minor ellipse axes as the length and width of each cell, respectively. The length, width and spacing of the cellular extensions (processes) were measured with the Straight Line Selection tool and measure function. Average spacing between cellular processes and somas was also measured (Supplemental figure 1). Four samples were analyzed, three images of randomly chosen fields of view (FOV) were acquired per sample, and 100 cells were measured per FOV. Within each FOV, approximately 28% of the cells were measured. Cells' total surface projected area was measured with the measure function and approximated by Equation 1. White light interferometry (WIM) was used to determine the height of SC RR. Height profiles were obtained at 10x magnification, recorded by WIM with a Zygo New View 6000 3D profiler (Zygo Co., Middlefield, CT, USA), and analyzed with Gwyddion 2.0 software (Czech Metrology Institute, Brno, Czech Republic).

Acquired measurements were used to generate different geometric bioinspired designs (Table 1A) using AutoCAD LT 2009 (Autodesk). Designs were prepared for substrates presenting SC soma features with average length process features (Soma Average, SA); SC soma features with short processes features (Soma Short, SS); SC soma features with long process features (Soma Long, SL); SC soma features only (Soma Only, SO); and SC process features only (Process Only, PO). Designs were printed on chrome on quartz masks (Advanced Reproductions Corporation, Andover, MA, USA) with a resolution of 1.6 µm. Silicon wafers of approximately 1 µm depth were generated using photolithographic techniques. Negative tone Nano SU-8 2 (Microchem Co., Newton, MA, USA) was spun

onto silicon wafers (Silicon Sense, Inc., Nashua, NH, USA) at 3,000 rpm to achieve an approximate depth of 1 μm , corresponding to the average height of SC RR as measured above. Photoresist-coated wafers were baked at 65 °C for 2 minutes and 95 °C for 10 minutes before selective polymerization with ultraviolet light. Wafers were exposed for 12 seconds (Karl Suss MJB3 UV300) and post-baked for 1 minute at 65 °C and 95 °C. Following baking, SU-8 developer (Microchem) was used to develop the wafers for 1.5 minutes. Poly(dimethylsiloxane) (PDMS) was poured onto the wafers, de-gassed, baked for 2 hours at 95 °C, cooled and peeled from the wafer. PDMS containing raised geometric bioinspired substrate designs was cut and used for cell culture experiments.

Feature heights were confirmed with WIM and analyzed with Gwyddion software. Pattern fidelity and reproducibility on PDMS were assessed with scanning electron microscopy (SEM) (Hitachi S-2700) using a voltage of 8 kV, 25° tilt and a beam current of 8.

The percent area occupied by raised soma (ellipse) features, raised process (rectangle) features, and unraised space was quantified. Using the mask designs for each substrate, a defined region was chosen and the amounts of soma and process features present within the region were assessed. Values of 36.1 μm and 13.5 μm were used as the major and minor axes of an ellipse. A value of 1.8 μm was used as the width of a rectangle and the length varied by substrate: 52.5 μm (SA), 17.7 μm (SS) and 105.0 μm (SL). Ellipses on SO substrates were 52.5 μm apart. Once the total area of ellipses was summed, it was divided by the total area of the defined region to give the percent area of soma features present on each substrate. Once the total area of rectangles was summed for the defined region, it was divided by the total area of the defined region to give the percent of process features present on each substrate. The total area of features was also measured by summing the area of ellipses and rectangles present in the defined region. The region occupied by raised edge on each substrate was also quantified for each substrate. The perimeters of ellipses and rectangles present in a defined region were summed and divided by the total area of the defined region, to give the relative edge for each substrate (Table 1B).

Dorsal root ganglia (DRG) neurons harvest and culture

DRG explants were extracted from the spinal columns of P1-P3 postnatal rats and incubated in 0.25 % trypsin-EDTA in HBSS for 45 minutes at 37 °C. Trypsin was removed and explants were suspended in base media supplemented with 50 ng/mL nerve growth factor (NGF). Dissociated cells were plated at 57,700 cells/cm² (high-density) or 14,400 cells/cm² (low-density) on PDMS substrates coated with 50 $\mu\text{g/mL}$ laminin (LN) in HBSS and cultured for 5 days or 17 hours. Cell media was changed every other day. NIH guidelines for the care and usage of laboratory animals were followed.

Immunofluorescent Staining

Cells were fixed with 2 % paraformaldehyde in PBS after 5 days or 17 hours of culture. Nonspecific staining was blocked for 1h at room temperature with 10 % normal goat serum (Jackson Immuno Research Laboratories, West Grove, PA, USA) and 1 % bovine serum albumin (Sigma) in 0.1 M PBS (working solution), with 0.1 % Triton-X (VWR, West Chester, PA, USA) (blocking solution). Anti-neuronal class III beta tubulin (β -III-tubulin)

primary antibody (Covance Research Products Inc., Berkeley, CA, USA), used to visualize neurites, was diluted 1:500 in working solution and placed on samples overnight at 4 °C. Samples were rinsed 3 times with PBS at room temperature and incubated with Cyanine-3-conjugated goat anti-mouse secondary antibody (Cy-3) (Jackson Immuno Research Laboratories) diluted 1:200 in working solution for 1 h. After rinsing with PBS, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) diluted 1:100 in PBS.

Imaging and Microscopy

Immunostained samples were imaged on a Nikon Eclipse TE2000-S microscope equipped with phase contrast and epifluorescence optics and a motorized stage. 12-bit images were obtained using a Hamamatsu Orca-ER camera, an Orbit shutter controller, and a Ludl stage controller, outputting to OpenLab v4.0.4 (Improvision, Lexington, MA, USA). For each condition, at least three samples were analyzed, and a minimum of ten images of randomly chosen FOV were acquired per sample.

Quantification of Neurite Alignment

The orientation of neurites on geometric bioinspired substrates was quantified by processing β -III-tubulin labeled images with Neurient, a custom automated neurite tracing algorithm in Matlab [16]. The algorithm takes confluent neurite images and produces traced image coordinates, which can be used to determine neurite alignment and orientation. The number of neurite segments directed relative to $\pm 10^\circ$ of the horizontal axis of the underlying pattern was determined.

Time-lapse Microscopy

DRG were plated on SL, SS, PO, SA and SO substrates at a density of 14,400 cells/cm² (low-density) or 57,700 cells/cm² (high-density) and cultured on the stage of a Nikon microscope with an enclosed custom humidified chamber to maintain 37 °C and 5 % CO₂. After cells adhered to the substrates for 3 hours, phase contrast images for each substrate condition were taken every 5 minutes over a 14 hour period of time at a magnification of 200x for the low-density cultures and 100x for high-density cultures.

Neurite Tracking Analysis

Using a modified version of the Manual Tracing Plugin of Image J, at each time point of the 14 hour low-density time-lapse microscopy, the growing ends of the neurites were tracked (Figure 6A) and contact with a soma, a process or the space in between the features was recorded. It was also noted when a neurite was branching or interacting with other cells in the culture.

Velocity Analysis

Velocity was calculated by finding the displacement for each measured neurite step, i.e. the displacement between adjacent time points T and T-1 and $T = 5$ minutes, using Equation 2. Velocities for all steps per neurite were averaged to give average neurite velocity. To find the average velocity of neurites of one cell, the velocities of all the neurites on that neuron were averaged.

Feature Contact Analysis

At each time point of the 14 hour low-density time-lapse microscopy, the amount of time each neurite spent in contact with soma features, process features, and no features was measured and divided by the amount of time the neurite was tracked, which was determined by the lifespan of the neurite. The amount of time each neurite spent in each state was averaged per neuron and per substrate type to generate percent time contacting soma features, process features, and space. Three neurons were tracked for each substrate.

The percent time a neurite spent on a feature was normalized by the percent area of the corresponding feature. For example, the percent time spent contacting soma features on a SS substrate was normalized by the percent area of soma feature present on a SS substrate. The same procedure was done for process feature contact and space contact across conditions.

Alignment Analysis

The angle of a neurite's displacement was measured at each time point relative to $\pm 10^\circ$ of the horizontal (0°), and the vertical axis (90°). The angles were averaged to generate percent alignment of neurite steps for each neurite on a neuron, and percentages were averaged for each neuron.

Statistical Analysis

All data was compared within each condition and across conditions. The following statistical tests were performed. For $n > 3$, a homogeneity of Variance Test (Levene Statistics) was used to determine the variance of the groups. For equal variance in the groups, parametric tests (One-way analysis of variance (ANOVA) for 3 or more groups or Students t-test for 2 groups) were used to compare the means. Post Hoc analyses (Bonferroni) for equal variance in the population were also employed for multiple comparison of the means. For unequal variance in the groups, non-parametric tests (Brown-Forsythe for 3 or more groups or Welch's t-test for 2 groups) were used to compare the means. Post Hoc analyses (Games-Howell) for unequal variance in the population were also employed for multiple comparison of the means.

Results

Geometric bioinspired substrates presented the shapes and dimensions of aligned SC

Templates of aligned SC were generated as previously described by our laboratory [13]. SCs were grown to confluence on glass slides micropatterned with LN and fixed, generating a cellular template. To deconstruct the topography presented by SCs, somas and processes of cells within the template were measured. The width, length and height of SC somas and processes were measured [17], and somas were represented with ellipses while processes were represented with rectangles (Table 1A). With these measurements, five different topographical substrates were designed, to investigate which SC topographical features influence neuronal guidance.

Silicon wafers and PDMS containing geometric bioinspired designs were fabricated using photolithography and soft lithography techniques. Patterns were conserved on PDMS

(Figure 1A) and feature heights were 1.1 μm (Figure 1B), which was in accordance with the height of SC templates [16[18]]. Materials presented different amounts of raised surface area and edges. SL had 73.9% unraised area and 26.1% raised surface area, comprised of 17.3% raised soma features and 8.8% raised process features. SS substrates had 49.6% unraised area and 50.4% raised area, comprised of 46.5% raised soma features and 3.9% raised process features. PO substrates had 88.2% of unraised area and 11.8% of raised area, all of which came from raised process features. SA substrates had 65.0% of unraised area and 35.0% of raised area, comprised of 28.0% raised soma features and 7.0% raised process features. SO substrates had 72.0% unraised area and 28.0% raised surface area, all of which came from raised soma features (Table 1B). Substrates presented different amounts of raised edges, where SL had 13.5 mm/mm^2 , SS substrates had 14.3 mm/mm^2 , PO had 13.1 mm/mm^2 , SA had 13.6 mm/mm^2 and SO had 4.0 mm/mm^2 of raised edge, respectively. Polymeric replicas (RR) whose topographical features replicate those of native SCs (varying heights and spacings) [13] and which were previously shown to promote neurite guidance [12] were included as well in this study. Flat PDMS materials were used as controls.

DRG neurons aligned to geometric bioinspired topographical platforms after 5 days in culture

After 5 days culture, cells were healthy and prominent extension of neurites was observed. DRG neurites were randomly oriented on flat unpatterned substrates and showed alignment on all geometric bioinspired and RR substrates (Figure 2). On flat substrates neurites showed random growth (Figure 2M–N). On geometric bioinspired substrates, the percent of aligned neurite segments on SL ($44.0 \pm 0.8\%$) was significantly higher than the percent aligned on PO ($38.0 \pm 0.8\%$), SA ($37.0 \pm 0.6\%$), SO ($25.2 \pm 0.4\%$) and RR ($25.3 \pm 0.4\%$) (Figure 3). Neurite alignment was not significantly different between neurites on SL and SS substrates ($43.3 \pm 0.8\%$), between neurites on PO and SA substrates or between neurites on SO and RR substrates. Alignment of neurites on all the geometric bioinspired and RR substrates was significantly greater than alignment on flat controls ($17.5 \pm 0.3\%$) ($p < 0.05$, Brown-Forsythe test).

DRG neurites exhibited varied dynamic behaviors and aligned to geometric bioinspired substrates after 17 hours in culture

To investigate the early dynamic cellular behavior under time-lapse microscopy resulting in the neurite alignment found at 5 days, DRG cultures were observed for 14 hours, starting at 3 hours post-seeding to allow for cell attachment. Generally, neurites were highly motile, exhibiting exploratory, extension and retraction behaviors. In addition to interacting with topographical features, neurites also interacted with adjacent neurites and SCs in the culture. To complement the time-lapse study, which provided qualitative insights into early growth and alignment dynamics, alignment of substrates fixed at 17 hours, corresponding to the end of the time-lapse, were analyzed for alignment in the direction of the topography (Figures 4 and 5).

In cultures on SL substrates, neurites grew along the long axis of the soma features and parallel to the process features. They also used both the short axis of the soma features and the process features to cross into the adjacent row of parallel features. When neurites

encountered a process feature from a perpendicular approach, they traveled along it, crossed it or branched upon contact. After crossing or branching behaviors, the neurites continued to travel parallel to the process feature. Further, soma features appeared to serve as anchors to intermediate locations along the length of the neurite, such that the extending tip could be pulled into an adjacent channel formed by the feature rows. These dynamic interactions resulted in elongated neurite structures both in the channels and along the shallow diagonals between soma structures in adjacent rows (Figure 4A–B). Tracing analysis showed that $41.3 \pm 0.8\%$ of neurites were aligned at 17 hours in culture (Figure 5).

Neurites on SS substrates traveled in a shallow zig-zagging manner in the space between adjacent feature rows, on top of single rows of alternating soma and process features, or along the diagonal axis created by the offset of soma features in adjacent rows. When neurites initially extended diagonally, the diagonal alignment became less apparent as the neurite tip extended horizontally and pulled the length of the neurite behind it. Many Schwann cells from the mixed DRG cultures appeared to align parallel to the feature axis, as well. The dynamic behavior of neurites on SS substrates resulted in horizontal and diagonal neurite structures, many of which resided on top of the raised features (Figure 4C–D). Tracing analysis showed $39.7 \pm 0.9\%$ of neurites were aligned at 17 hours in culture (Figure 5).

Neurites on PO exhibited two main types of dynamic behavior. Most neurites grew parallel to process features initially, followed in some cases by neurite branching. Branching occurred most consistently when neurites approached the process features from a perpendicular angle. Other neurites initially crossed process features perpendicularly, followed by horizontal growth. As with neurites on the SS substrates, as the extending tip continued to move parallel to the features, any initial vertical or diagonal orientation became less apparent as the length of the neurite was pulled straight behind the tip. Despite these dynamics observed for many cells, the majority of the neurite extension was horizontally aligned in the channels created by adjacent rows of process features. The response of growing neurites to the process features resulted in highly aligned cultures (Figure 4E–F). Tracing analysis showed that $46.0 \pm 0.8\%$ of neurites were aligned to the topography at 17 hours in culture (Figure 5).

Neurites on SA substrates traveled mostly in the horizontal direction, following the underlying topography. In particular, neurites extended parallel to process features and within the narrow channel formed by process and soma features from adjacent rows. Rather than crossing process features at perpendicular angles as observed on SL and PO substrates, neurites on SA tended to approach and cross process features at shallow angles. There appeared to be relatively more dynamic retraction of neurite tips on SA substrates compared to on other bioinspired topographies. As seen with SL substrates, soma features seemed to provide anchor points for intermediate locations along the length of the neurite. These dynamics resulted in aligned cultures (Figure 4G–H). Tracing analysis found that $44.7 \pm 0.9\%$ of neurites were aligned to the topography at 17 hours (Figure 5).

Neurite dynamics on SO substrates differed significantly from the other bioinspired substrates. In particular, growth appeared to be randomly oriented overall, and neurites

formed highly complex networks. Though neurites sometimes traveled parallel to the long axis of a soma feature, this aligned growth did not typically continue once the neurite encountered the space between topographical features. In addition, as they contacted the short side of the soma feature, neurites often branched, with one extension traveling parallel to the feature and the other extending in the diagonal or vertical direction. Together, these growth dynamics resulted in networked and randomly oriented morphologies on SO substrates (Figure 4I–J). Tracing analysis found that $20.5 \pm 0.3\%$ of neurites on SO were aligned parallel to the topography (Figure 5). (See supplemental videos, available online, to see representative time-lapse movies for each condition.)

Comparisons of alignment of traced neurites at 17 hours between conditions revealed that neurites were aligned on all process feature-containing geometric bioinspired and RR substrates (Figure 5). On flat and SO substrates, neurites showed random growth (Figure 4I–J, M–N). The percent of aligned neurites on PO and SA substrates was significantly higher than on SL and SS substrates, and neurites on all four process-containing materials exhibited significantly higher alignment than neurites on RR, SO, and Flat materials. Neurite alignment at 17 hours was not significantly different between neurites on SL and SS substrates, between neurites on PO and SA substrates or between neurites on SO and RR substrates. Alignment of neurites on all the geometric bioinspired substrates except SO was significantly greater than alignment on flat controls ($17.8 \pm 0.3\%$ alignment in an arbitrary horizontal direction) ($p < 0.05$, one-way ANOVA).

Neurites followed distinct paths on different geometric bioinspired substrates to become aligned at 17 hours

While time-lapse microscopy of cultures of 57,700 cells/cm² revealed interesting trends of cell behavior, time-lapse microscopy of lower density cultures (14,400 cells/cm²) was required to analyze initial interactions of neurites with topographical features for SL, SS, PO, SA and SO substrates. When DRG neurons were cultured under time-lapse microscopy, average neurite velocities were similar on the different substrates with 48 ± 6 μm/h on SL substrates, 48 ± 12 μm/h on SS substrates, 46 ± 6 μm/h on PO substrates, 33 ± 11 μm/h for SA substrates and 36 ± 2 μm/h on SO substrates. Neurites interacted with the distinct features differently and followed similar trends as those described from the 57,700 cells/cm² cultures. Representative images from these lower density cultures are shown in Figure 6.

Cells on SL substrates displayed horizontal outgrowth aligned in the direction of the underlying pattern. Neurites contacted soma and process features, as well as the unraised space between features. Neurites on SL substrates demonstrated the most continuously aligned outgrowth over time and appeared to grow parallel to the process features and the major axes of the soma features (Figure 6A, first row).

Cells on SS substrates demonstrated outgrowth in the direction of the underlying topography, but also exhibited some diagonal growth following the inherent diagonal axis of the offset soma orientation in the SS pattern. Neurites contacted processes and somas, as well as the space between features. They traveled within the defined space between rows of features, but also traveled across and on top of features. When neurites approached soma features they either branched if they perpendicularly approached the major or minor axis of

the soma, traveled along the edge of the ellipse, or turned in the direction of the curved feature. These behaviors manifested as net growth in the horizontal directions (Figure 6A, second row).

Cells on PO grew overall parallel to the process features presented by the pattern. Neurites interacted with consecutive processes and they did so primarily by reaching the next process perpendicularly, which in turn seemed to induce branching of the neurites and resulted in diagonal structures. On PO substrates, the tips of the neurites were often wide enough to travel in the center of the channel, while the edges of the tip were in contact with the process features (Figure 6A, third row).

Cells on SA substrates demonstrated an overall alignment in the direction of the underlying pattern. Neurites on SA substrates were highly exploratory and interacted with both process and soma features coupled with branching and retracting. They traveled along the long axis of the somas or used them to cross to the next line of features. The neurites also contacted spaces and neurites appeared more aligned when they were between the long axes of a soma and a process (Figure 6A, fourth row).

Cells on SO substrates demonstrated different neurite outgrowth dynamics, with neurites growing in all directions during initial stages of outgrowth. The SO substrate was the only substrate to present a discontinuous feature pattern; in response, neurites grew within the space between repeated soma features, as well as on top of soma features. However, the neurites were still influenced by the presence of repeated aligned soma features. When contacting a soma feature, neurites branched, turned in the direction of the curvature, or continued to grow along the edge of the feature. Outgrowth on SO substrates was more complex than on process containing substrates, on SO neurites grew without a distinct direction until they contacted soma features, at which point their behavior was affected. (Figure 6A, last row). Interestingly, when the percent of neurite steps aligned to the vertical axis during the 14 hr time-lapse study was evaluated, neurites on SO showed significantly more steps in the vertical direction ($12.3 \pm 0.6\%$) than neurites on all other substrates. (See supplemental videos, available online, to see representative time-lapse movies for each condition.)

At 17 hours, corresponding to the end of the time-lapse microscopy, fixed, low-density DRG cultures were randomly oriented on flat unpatterned substrates and showed alignment on all process feature-containing geometric bioinspired and RR substrates. On flat and SO substrates, neurites showed random growth (Figure 7). The percent of aligned neurite segments on SL ($46.7 \pm 1.4\%$) was not significantly different than the percent aligned on PO ($42.8 \pm 1.2\%$) and SA ($50.7 \pm 1.5\%$) (Figure 7). Neurite alignment at 17 hours was not significantly different between neurites on SL and SS substrates ($42.8 \pm 1.2\%$), between neurites on PO and SA substrates or between neurites on SO ($20.5 \pm 0.3\%$) and RR ($21.7 \pm 0.4\%$) substrates but neurites on SS aligned significantly less than those on PO and SA substrates. Alignment of neurites on all the geometric bioinspired materials except SO substrates was significantly greater than alignment on flat controls ($16.9 \pm 0.5\%$) ($p < 0.05$, one-way ANOVA test).

The outgrowth of neurons on the different substrates as measured by the number of neurites at 17 hr was similar across conditions, ranging on average from 2.33 to 5 neurites per cell.

Neurites spent different amounts of time on soma and process features

Neurites on SL substrates spent significantly more of their time ($64.2 \pm 5.3\%$) contacting space and process features ($25.6 \pm 4.5\%$) than soma features ($10.1 \pm 1.1\%$) (Figure 8A). Neurites on SS substrates spent significantly more time contacting soma features ($46.5 \pm 3.4\%$) and space ($37.1 \pm 3.3\%$) than process features ($15.1 \pm 3.5\%$). Neurites on PO spent similar portions of their time contacting process features ($58.2 \pm 9.3\%$) and space ($41.4 \pm 9.3\%$). Neurites on SA spent similar amounts of their time contacting soma features ($27.9 \pm 1.6\%$), process features ($30.7 \pm 6.0\%$) and space ($39.8 \pm 6.8\%$). Neurites on SO substrates spent significantly more time contacting space ($72.1 \pm 3.5\%$) than soma features ($27.9 \pm 3.4\%$).

Comparing across substrates, neurites on SS spent significantly more of their time on soma features than neurites on SL, SA and SO. Neurites on SA spent the same amount of their time on soma features as neurites on SO substrates. The percent time neurites spent on process features was significantly different between SS and PO and between SL and PO ($p < 0.05$, Welch's t-test). Neurites on SO spent significantly more of their time contacting space than neurites on SS and SA substrates (Figure 8A).

In order to take into account the differences in areas presented by the different substrates (Table 1B), we defined a unitless interaction parameter, where:

When the interaction parameter is greater than 1, this is a stronger interaction that could be accounted for by random growth.

Neurites on SL substrates had significantly higher interaction parameters with process features (2.9 ± 0.55) than either soma features (0.58 ± 0.06) or space (0.87 ± 0.07) (Figure 8B). Neurites on SS substrates had significantly higher interaction parameters with process features (3.88 ± 0.90) than either soma features (1.00 ± 0.07) or space (0.75 ± 0.07). Neurites on PO had significantly higher interaction parameters with process features (5.00 ± 0.78) than space (0.47 ± 0.10). Neurites on SA also had significantly higher interaction parameters with process features (4.39 ± 0.86) than either soma features (1.00 ± 0.06) or space (0.61 ± 0.11) ($p < 0.05$, one-way ANOVA). Neurites on SO substrates had similar interaction parameters with soma features (1.00 ± 0.12) and space (1.0 ± 0.05), $p < 0.05$ by Student's t-test. Comparing across substrates, neurites on SA and SL substrates had significantly higher interaction parameters with soma features than neurites on SL did. Neurites on PO had a significantly lower interaction parameter with space than those on SO.

Discussion

Our overall goal was to gain understanding of axon guidance in the environment of SC topography. Without presenting cues from live cells, we developed a biomaterial system to vary topographical feature parameters and clarify critical cues. Various approaches are being developed to provide injured nerves with bridges that contain exogenous aligned SC and/or

that recruit and align host SC, in order to enhance regeneration [18–20]. We designed the materials of this study not to directly replicate *in vivo* conditions, but rather to deconstruct the local environment surrounding axons. These materials take on additional relevance when we consider the myriad possibilities for optimizing therapeutic bridges presenting aligned SC.

A specific objective was to analyze the SC topographical features that present key guidance information to navigating neurons. The novel materials fabricated in this work were designed to present a set of topographical cues inspired by SC shapes, dimensions and spacing, measured from an aligned and confluent SC monolayer template. By utilizing chrome mask-based photolithography and PDMS-based soft lithography, the SC inspired features were generated with single micron resolution. With these materials, we set out to analyze the relative strength of neurite response to the following parameters: topographical features versus flat; geometric versus biomimetic features; process features; lengths of process features; and soma features.

Neurites aligned to topographical features and not to flat materials, and they aligned more strongly to geometric materials incorporating process features than to biomimetic materials. Key findings from this study include the following: (1) The strongest neurite alignment to SS and SL at 5 days suggested that optimal topographies for axon guidance at this time were combinations of soma and process features, with higher than average density of either soma features (SS) or process features (SL); (2) The ability of neurites in a high-density culture at 5 days to align to topographies presenting soma features alone (SO) suggested that soma topography was sufficient for axon guidance by this time; and (3) The strongest alignment to PO and SA at 17 hours suggested that neurite-substrate alignment mechanisms changed over time. In addition, the weaker neurite alignment in high-density versus low-density cultures at 17 hours suggests that cell-cell interactions, likely including SC-neuron interactions, play a role in influencing alignment.

Topography for axon guidance

It was not until recently that the guidance capability of SC topography was demonstrated, with the development of biomimetic materials that replicate SC topography at the micro- and nanoscales [13]. Furthermore, recent findings have described enhanced growth and alignment of DRG neurons on these materials mediated not by live SC but by the biomimetic materials, highlighting the importance of SC topography [14]. In the present study, the specifications of SC topographical features that encode critical contact guidance information have been explored, and can be considered in light of other studies of topography and cell function.

Previous studies of nerve response to plateau-groove topographies provide a context for the present studies of nerve growth on process features. A variety of neuronal types have shown neurite alignment to grooved topographies [7–9, 21–23]. *Xenopus* spinal cord neurons can align to grooves as shallow as 14 nm and as thin as 1 μ m, but many neural cells have shown preferences for larger dimensions. Chick DRG neurons showed alignment on grooves that were 100 μ m wide and 150 μ m deep [8] while rat hippocampal neurons grew parallel to underlying patterns when patterns were at least 4 μ m wide and 130 nm deep [22]. Neurite

alignment to the PO materials of this study (1.8 μm width, 1.1 μm depth) was in agreement with these previous studies.

Previous studies had suggested that neurites would align most strongly to the present study's PO materials, which contained continuous plateau-groove features for neurites to follow. Notably however, neurites across all the experimental conditions aligned with similar strength to PO and to materials whose process features were interrupted by soma features (SA). Further, by 5 days, neurites were able to align to discrete soma features (SO) with the same spacing as SA. Interestingly, this alignment was similar in strength to that on biomimetic materials (RR) replicating SC topography. The importance of soma features for neurite guidance resonates with studies of 2 μm width with 1.5 μm gap pillar arrays, in which hippocampal neurons grew from pillar to pillar [22]. SO materials had larger diameters and spaces than materials of those previous studies [24, 25], and had the same spacing as SA materials in this study.

One goal of the initial design of this study was to test the effects of different process length features. After observing neurite alignment to soma features, we noted that these materials also present different densities of soma features. Neurite alignment by 5 days was strongest on materials presenting combined soma and process topography, but with either longer processes and the soma features more spread out (SL); or shorter processes and the soma features closer together (SS) than average (SA). Interestingly, when the cellular density was kept constant and the culture time was reduced to 17 hrs, neurite alignment was strongest to materials presenting process features only (PO) and SA. Notably, neurites aligned to a higher degree on all process-containing geometric bioinspired substrates when compared to the replica substrates (RR) regardless of the culture time and density. This is consistent with previous work from our group [17], where substrates generated lithographically from bioinspired traces of cells were more efficient at guiding neurons than their replica counterparts. It is possible that neurites are more likely to keep to a path when presented with a continuous height (i.e. bioinspired substrates) than when presented with varying heights and spaces (i.e. replica substrate). While previous studies have proposed a positive role for feature edge in neurite guidance [17, 26, 27], here we did not observe such a correlation. We suggest that at 5 days, neurite guidance was influenced by feature density and culture time, whereas at 17 hours neurite guidance was influenced primarily by the nature of the underlying topographical features and not by the cellular density of the culture, since neurite alignment of high- and low- density cultures exhibited similar trends of alignment at this time.

How do neurite-topography interactions lead to neurite alignment?

We were interested in examining more closely the neurite-topography interactions that underlie some of the unexpected neurite alignment results we observed; therefore, we chose to observe neurite growth under time-lapse microscopy. Following the results from the 5-day experiment, our 14-hour time-lapse studies allowed us to analyze how early neurite interactions with soma topographical features initiate neurite alignment, and how early neurite interactions with combinations of soma and process features of different process feature lengths and soma feature densities initiate optimal neurite alignment.

Time-lapse microscopy revealed highly dynamic early neurite interactions with the topographical features (See supplemental movies). Our initial analysis showed that in many cases, neurites spent most of their time contacting the features that were most dense on each substrate. By defining a neurite interaction parameter, we were able to normalize neurite contact for the amounts of feature and space area. This illuminated several interesting trends for early neurite growth. Interaction parameters with spaces were similar across substrates, and were in the range of 1.0, corresponding to a theoretical random interaction value. On all substrates, neurites' interaction parameters with features were higher than with spaces, suggesting that the raised topographical features were more dominant cues than the channels created by them.

In this initial phase of growth, neurite interactions with soma features on SO were greater than random. These neurites branched, extended at multiple angles, but did not show any significant alignment preference during the first 14 hours. Neurite interactions with process features on all process-containing substrates were also greater than random. Neurite interactions with soma features on these substrates were not higher than random interactions.

When we consider together the complementary results from the 14 hour timelapse studies and the 5 day studies, we find the following: neurites aligned to materials presenting soma topographies alone by initially exploring the features but not aligning to them during the first 14 hours. By 5 days, it is likely that non-horizontally-aligned neurites had retracted or reoriented, and/or more horizontally-aligned neurites had extended. Neurites aligned to materials presenting combinations of soma and process topographical features initially within the first 14 hours aligning most strongly to PO and SA. When more cells were present, neurites were able to align to soma feature-containing substrates, and aligned most strongly to SL and SS. By 5 days, this alignment was enhanced by additional neurite extension. It is likely that in both cases, later alignment involved orientation to underlying topography, to neurites that were already present, and to the live SC that are also present in DRGs. Of note, DRGs' live SC have previously been shown to participate in guidance by topography [12].

Conclusions

We have generated a set of materials inspired by SC topography and used them to analyze the neurite response to distinct SC topographical features. Axons *in vivo* encounter migrating SC, SC with process of varying lengths, and SC of distinct shapes, depending on their fates [28]. These materials allowed us to model components of those conditions *in vitro*. In considering how SC guide axons *in vivo*, we hypothesize that SC processes provide continuous guidance features for navigating axons, while SC somas present points of intermittent guidance, for which continuous axon-soma contact is not required. Since substrate stiffness has been shown to affect neurite extension and branching (for review, [29]), it would be interesting in future experiments to vary parameters such as feature geometry, spacing, and material stiffness.

In addition to furthering the understanding of neuron-glial interactions and axon guidance, this work can advance efforts for nerve repair after injury. For example, regeneration

strategies may increase axon alignment and precision of reconnection by incorporating discrete, repeating ellipse topography into longitudinal nerve guidance channels, fibers, or other bridge technologies. Finally, the types of cell topographies investigated with this approach are limited only by the cells available to be analyzed, and thus future investigations could be easily expanded to explore cell-cell interactions that underlie such phenomena as cell differentiation, wound healing, and transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Jennifer Pally for her help with pilot experiments. This work was supported by NIH IMSD R25GM083270-01 and NSF Graduate Research Fellowship to CLF; NSF REU to TR; NSF GK-12 Graduate Fellowship to YL; and NIH R01 EB005722-01, NSF Advance and Career Awards to DHK.

References

1. Stoll G, Müller HW. Nerve injury, axonal degeneration and neural regeneration: basic insights. *Brain Pathology*. 2006; 9:313–25. [PubMed: 10219748]
2. Thompson DM, Buettner HM. Neurite outgrowth is directed by schwann cell alignment in the absence of other guidance cues. *Annals of biomedical engineering*. 2006; 34:669–76.
3. Newgreen DF. Physical influences on neural crest cell migration in avian embryos: contact guidance and spatial restriction. *Developmental biology*. 1989; 131:136–48. [PubMed: 2642426]
4. Hoffman-Kim D, Mitchel JA, Bellamkonda RV. Topography, cell response, and nerve regeneration. *Annual review of biomedical engineering*. 2010; 12:203.
5. Bunge RP, Fernandez-Valle C. 3 Basic biology of the Schwann cell. *Neuroglia*. 1995:44.
6. Li N, Folch A. Integration of topographical and biochemical cues by axons during growth on microfabricated 3-D substrates. *Experimental cell research*. 2005; 311:307–16. [PubMed: 16263111]
7. Yao L, Wang S, Cui W, Sherlock R, O'Connell C, Damodaran G, et al. Effect of functionalized micropatterned PLGA on guided neurite growth. *Acta Biomaterialia*. 2009; 5:580–8. [PubMed: 18835227]
8. Houchin-Ray T, Swift LA, Jang JH, Shea LD. Patterned PLG substrates for localized DNA delivery and directed neurite extension. *Biomaterials*. 2007; 28:2603–11. [PubMed: 17324456]
9. Johansson F, Carlberg P, Danielsen N, Montelius L, Kanje M. Axonal outgrowth on nano-imprinted patterns. *Biomaterials*. 2006; 27:1251–8. [PubMed: 16143385]
10. Tsuruma A, Tanaka M, Yamamoto S, Fukushima N, Yabu H, Shimomura M. Topographical control of neurite extension on stripe-patterned polymer films. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2006; 284:470–4.
11. Harrison RG. The reaction of embryonic cells to solid structures. *Journal of Experimental Zoology*. 1914; 17:521–44.
12. Bruder JM, Lee AP, Hoffman-Kim D. Biomimetic materials replicating Schwann cell topography enhance neuronal adhesion and neurite alignment in vitro. *Journal of Biomaterials Science, Polymer Edition*. 2007; 18:967–82. [PubMed: 17705993]
13. Bruder JM, Monu NC, Harrison MW, Hoffman-Kim D. Fabrication of polymeric replicas of cell surfaces with nanoscale resolution. *Langmuir*. 2006; 22:8266–70. [PubMed: 16981733]
14. Richardson J, Rementer C, Bruder JM, Hoffman-Kim D. Guidance of dorsal root ganglion neurites and Schwann cells by isolated Schwann cell topography on poly (dimethyl siloxane) conduits and films. *Journal of Neural Engineering*. 2011; 8:046015. [PubMed: 21673394]

15. Kofron C, Hoffman-Kim D. Optimization by Response Surface Methodology of Confluent and Aligned Cellular Monolayers for Nerve Guidance. *Cellular and Molecular Bioengineering*. 2009; 2:554–72. [PubMed: 20625538]
16. Mitchel J, Martin I, Hoffman-Kim D. Neurient: An Algorithm for Automatic Tracing of Confluent Neuronal Images to Determine Alignment. *Journal of neuroscience methods*. 2013
17. Kofron CM, Liu YT, López-Fagundo CY, Mitchel JA, Hoffman-Kim D. Neurite Outgrowth at the Biomimetic Interface. *Annals of biomedical engineering*. 2010; 38:2210–25. [PubMed: 20440561]
18. Bunge MB. Novel combination strategies to repair the injured mammalian spinal cord. *The journal of spinal cord medicine*. 2008; 31:262. [PubMed: 18795474]
19. Calancie B, Madsen PW III, Wood P, Marcillo AE, Levi AD, Bunge RP. A guidance channel seeded with autologous Schwann cells for repair of cauda equina injury in a primate model. *The journal of spinal cord medicine*. 2009; 32:379. [PubMed: 19777858]
20. Matsas R, Lavdas A, Papastefanaki F, Thomaidou D. Schwann cell transplantation for CNS repair. *Current Medicinal Chemistry*. 2008; 15:151–60. [PubMed: 18220770]
21. Miller C, Jeftinija S, Mallapragada S. Synergistic effects of physical and chemical guidance cues on neurite alignment and outgrowth on biodegradable polymer substrates. *Tissue Engineering*. 2002; 8:367–78. [PubMed: 12167224]
22. Rajnicek A, Britland S, McCaig C. Contact guidance of CNS neurites on grooved quartz: influence of groove dimensions, neuronal age and cell type. *Journal of Cell Science*. 1997; 110:2905. [PubMed: 9359873]
23. Clark P, Connolly P, Curtis AS, Dow JA, Wilkinson CD. Topographical control of cell behaviour: II. Multiple grooved substrata Development. 1990; 108:635.
24. Dowell-Mesfin NM, Abdul-Karim MA, Turner AMP, Schanz S, Craighead HG, Roysam B, et al. Topographically modified surfaces affect orientation and growth of hippocampal neurons. *Journal of Neural Engineering*. 2004; 1:78–90. [PubMed: 15876626]
25. Hanson JN, Motala MJ, Heien ML, Gillette M, Sweedler J, Nuzzo RG. Textural guidance cues for controlling process outgrowth of mammalian neurons. *Lab Chip*. 2008; 9:122–31. [PubMed: 19209344]
26. Berry CC, Campbell G, Spadicino A, Robertson M, Curtis ASG. The influence of microscale topography on fibroblast attachment and motility. *Biomaterials*. 2004; 25:5781–8. [PubMed: 15147824]
27. Smeal RM, Rabbitt R, Biran R, Tresco PA. Substrate curvature influences the direction of nerve outgrowth. *Annals of Biomedical Engineering*. 2005; 33:376–82. [PubMed: 15868728]
28. Jessen KR, Mirsky R. The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience*. 2005; 6:671–82.
29. Moore SW, Sheetz MP. Biophysics of substrate interaction: Influence on neural motility, differentiation, and repair. *Developmental Neurobiology*. 2011; 71:1090–101. [PubMed: 21739614]

Figure 1.

Geometric bioinspired PDMS platforms reproduced feature shapes and dimensions as designed. (A) Phase contrast micrographs show the fidelity to the designs and the reproducibility of the patterns; SC RR substrate is shown for comparison. Geometric bioinspired patterns were conserved on PDMS as shown with scanning electron micrographs (insets). (B) White light interferometry oblique image (top) provided a three dimensional view of a representative SA geometric bioinspired substrate; the black line across the pattern represents the cross-section measured to obtain a height profile of this substrate (bottom). The height of the platform was $1.098 \pm 0.017 \mu\text{m}$. Scale bar = $50 \mu\text{m}$.

Figure 2.

DRG neurites growth was oriented on geometric bioinspired substrates after 5 days in culture. Corresponding phase contrast (left column) and epifluorescent images (right column) of DRG cultured for 5 days on LN-coated PDMS substrates presenting: (A–B) SL topography, (C–D) SS topography, (E–F) PO topography, (G–H) SA topography, (I–J) SO topography, (K–L) RR topography; or (M–N) flat PDMS. Neurites were stained with anti-class III beta tubulin (right column). Scale bar = 110 μ m.

Figure 3.

Quantitative analysis demonstrated neurite alignment on all geometric bioinspired substrates. Graph of percent neurites aligned within 10.0° of the horizontal axis for high-density DRG cultured for 5 days on LN-coated substrates presenting Flat or SL, SS, PO, SA, SO, RR topographies. Mean ± SEM are presented. * indicates $p < 0.05$ by Brown-Forsythe test.

Figure 4.

DRG neurite growth was oriented on some geometric bioinspired substrates after 17 hr in culture. Corresponding phase contrast (left column) and epifluorescent images (right column) of DRG cultured for 17 hr on LN-coated PDMS substrates presenting: (A–B) SL topography, (C–D) SS topography, (E–F) PO topography, (G–H) SA topography, (I–J) SO topography, (K–L) RR topography; or (M–N) flat PDMS. Neurites were stained with anti-class III beta tubulin (right column). Scale bar = 110 μ m.

Figure 5. Quantitative analysis demonstrated neurite alignment on some geometric bioinspired substrates. Graph of percent neurites aligned within 10.0° of the horizontal axis for DRG cultured for 17 hr on LN-coated substrates presenting Flat or SL, SS, PO, SA, SO, RR topographies. * indicates alignment significantly greater compared with SO, RR, and Flat. + indicates alignment significantly greater compared with SL and SS. # indicates alignment significantly greater than Flat. Mean $\pm$ SEM are presented.

Figure 6.

(A) Neurites followed distinct paths on each type of substrate to become aligned. Phase contrast images of DRG neurons growing under time-lapse microscopy on SL (first row), SS (second row), PO (third row), SA (fourth row), and SO (last row) substrates. White lines are superimposed on the neurites for better visualization. (B) Trajectories of neurite growth over the course of the time-lapse microscopy shown in (A) were superimposed on one corresponding phase contrast image for each substrate; the changing color of each trajectory represents time elapsed. Scale bar = 50 μm.

Figure 7.

Low density timelapse and endpoint studies demonstrated alignment in response to bioinspired topography at early times. Graph of alignment percent neurites aligned within 10.0 of the horizontal axis for DRG cultured for 17 hr days substrates presenting Flat or SL, SS, PO, SA, SO, RR topographies. * indicates alignment significantly greater compared with SO, RR, and Flat. + indicates alignment significantly greater compared with SS. # indicates alignment significantly greater than Flat. Mean $\pm$ SEM are presented.

Figure 8.

Neurites spent different amount and proportions of time contacting different features. (A) Graph shows the percents of time neurites spent contacting soma features, contacting process features and contacting space. (B) Graph shows percents of time as in (A), normalized by the percent area of somas, of processes, and of unraised space present on each substrate. n=3 cells for each substrate. Black bars, percent time contacting soma features. Gray bars, percent time contacting process features. White bars, percent time contacting space. For (A)* indicates percent time significantly greater than soma and process features. + indicates percent time significantly greater than process features. ^ indicates percent time

significantly greater than space. # indicate significantly greater than soma features. For (B), § indicate an interaction parameter significantly greater than soma feature and space. ▲ indicate an interaction parameter significantly greater than space. Mean $\pm$ SEM are presented.

Table 1

(A) Geometric bioinspired designs included ovals and/or rectangles that represent SC somas and processes, respectively. (B) Substrates contained different portions of their areas composed of specific features, and different portions of their areas composed of edge.

(A)						
Description	Mask design	Process length	Process width	Soma length	Soma width	Space between rows
Soma Long (SL): SC somas with long processes		105.0 μm	1.8 μm	36.1 μm	13.5 μm	13.5 μm
Soma Short (SS): SC somas with short processes		17.7 μm	1.8 μm	36.1 μm	13.5 μm	13.5 μm
Process Only (PO): SC processes only		1.0 μm	1.8 μm	36.1 μm	13.5 μm	13.5 μm
Soma Average (SA): SC somas with average length processes		52.5 μm	-	36.1 μm	13.5 μm	13.5 μm
Soma Only (SO): SC somas only		-	1.8 μm	-	-	13.5 μm

(B)					
	SL	SS	PO	SA	SO
% area of soma features	17.3	46.5	n/a	28.0	28.0
% area of process features	8.8	3.9	11.8	7.0	n/a
% area of features	26.1	50.4	11.8	35.0	28.0
% area of space	73.9	49.6	88.2	65.0	72.0
relative edge (mm/mm ²)	13.5	14.3	13.1	13.6	4.0