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

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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 lengths 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 5 days in culture and aligned to bioinspired materials presenting soma and process features after only 17 h in culture. The key findings of this study were: neurite response to underlying bioinspired topographical features was time dependent, with neurites aligned most strongly to materials presenting combinations of soma and process features at 5 days, with higher than average density of either process or soma features, but at 17 h 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.

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1. 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

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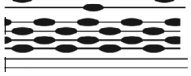
Table 1A
Geometric bioinspired designs included ovals and/or rectangles that represent SC somas and processes, respectively.

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 cm	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

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.

2. Materials and methods

2.1. Fabrication of relief replicas (RRs) of SCs

All cell culture reagents were from Invitrogen Life Science (Carlsbad, CA, USA), unless otherwise stated. SCs 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^{-1} 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^{-1} penicillin and 100 μg ml^{-1} streptomycin (base media) supplemented with 2 μM forskolin (Sigma), 10 μg ml^{-1} 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% CO_2. Polymeric relief replicas (RRs) of SC were fabricated as previously described by Bruder et al. [12] with the optimized conditions described by Kofron et al. [15].

2.2. 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 (Fig. S.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, ~28% of the cells were measured. Cells' total surface projected area was measured with the measure function and approximated by Eq. (1). White light interferometry (WIM) was used to determine the height of SC RR. Height profiles were obtained at 10× magnification, recorded by WIM with a Zygo New View 6000 three-dimensional (3-D) profiler (Zygo Co., Middlefield, CT, USA), and analyzed with Gwyddion 2.0 software (Czech Metrology Institute, Brno, Czech Republic).

$$\text{Total area} = \text{Area}_{\text{soma}} + (2 \times \text{Length}_{\text{processes}} \times \text{Width}_{\text{processes}}) \quad (1)$$

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 ~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 3000 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 min and 95 °C for 10 min before selective polymerization with ultraviolet light. Wafers were exposed for 12 s (Karl Suss MJB3 UV300) and post-baked for 1 min at 65 °C and 95 °C. Following baking, SU-8 developer (Microchem) was used to develop the wafers for 1.5 min. Poly(dimethylsiloxane) (PDMS) was poured onto the wafers, degassed, baked for 2 h 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 (Hitachi S-2700) using a voltage of 8 kV, 25° tilt and a beam current of 8.

The percentage 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 percentage area of soma features present

Table 1B
Substrates contained different portions of their areas composed of specific features, and different portions of their areas composed of edge.

	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

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