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Photopolymerized micropatterns with high feature frequencies overcome chemorepulsive borders to direct neurite growth

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Abstract

Developing and regenerating neurites respond to a variety of biophysical and biochemical cues in their microenvironment to reach target cells and establish appropriate synapses. Defining the hierarchical relationship of both types of cues to direct neurite growth carries broad significance for neural development, regeneration, and, in particular, engineering of neural prostheses that improve tissue integration with native neural networks. In this work, chemorepulsive biochemical borders are established on substrates with a range of surface microfeatures to determine the potential of physical cues to overcome conflicting biochemical cues. Physical micropatterns are fabricated using photomasking techniques to spatially control photoinitiation events of the polymerization. Temporal control of the reaction allows for generation of microfeatures with the same amplitude across a range of feature frequencies or periodicities. The micropatterned substrates are then modified with repulsive chemical borders between laminin and either EphA4-Fc or tenascin-C that compete with the surface microfeatures to direct neurite growth. Behavior of neurites from spiral ganglion and trigeminal neurons is characterized at biochemical borders as cross, turn, stop, or repel events. Both the chemical borders and physical patterns significantly influence neurite pathfinding. On unpatterned surfaces, most neurites that originate on laminin are deterred by the border with tenascin-C or EphA4-Fc. Importantly, substrates with frequent micropattern features overcome the influence of the chemorepulsive border to dominate neurite trajectory. Designing prosthesis interfaces with appropriate surface features may allow for spatially organized neurite outgrowth *in vivo* even in the presence of conflicting biochemical cues in native target tissues.

Keywords

cochlear implant; neural prosthesis; photopolymerization; methacrylate; neural regeneration

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1. Introduction

While current neural prostheses, such as cochlear or retinal implants, restore rudimentary sensory perception, their performance is substantially limited by poor integration with the target neural tissue (Leach, *et al.*, 2010, O'Leary, *et al.*, 2009, Winter, *et al.*, 2007). In particular, sensory neural prostheses provide a limited number of effective, independent signal channels. The distance between the prosthetic electrodes and the targeted neural elements prevents precise stimulation of specific neural populations and thereby limits spatial resolution. For example, the distance between the electrodes in the scala tympani and the spiral ganglion neurons (SGNs) that they activate in the modiolus results in stimulation of SGNs over a broad frequency range and significant interaction of adjacent channels (Rubinstein, 2004, Shepherd, *et al.*, 2004, Sly, *et al.*, 2007). Thus, the precise, tonotopic, spatial organization inherent to the auditory system is poorly exploited by the implant (Wilson and Dorman, 2008). Increasing the number of effective signal channels provided by the prosthesis would substantially improve auditory performance outcomes as the device would more effectively utilize the tonotopic spatial organization within the cochlea (Pfungst, *et al.*, 2011, Rubinstein, 2004, Shannon, *et al.*, 2004).

To improve tissue integration and enhance the stimulatory specificity of neural prostheses, there is great interest in developing methods to promote regrowth of the sensory neurites into close proximity, or even contact, with the stimulating electrodes (Pettingill, *et al.*, 2007, Pfingst, *et al.*, 2011, Richardson, *et al.*, 2006, Roehm and Hansen, 2005, Shibata, *et al.*, 2011). Precisely directing neurites to specific electrodes in this manner would substantially increase the number of available perceptual signal channels. To be useful, neurite outgrowth must preserve the precise spatial organization of the nervous system such as the tonotopic neural organization in the cochlea (O'Leary, *et al.*, 2009, Roehm and Hansen, 2005, Wise, *et al.*, 2010). To direct neural processes, biomaterial scientists and biomedical engineers have designed or modified materials with chemical or physical micro- and nanoscale patterning that guide neurite outgrowth (Belisle, *et al.*, 2008, Klein, *et al.*, 1999).

Because favorable cellular interactions at the interface between a biological system and a material often require location specific signaling, photo-dependent processes such as photolithography and photopolymerization are increasingly exploited in biomaterial and tissue engineering studies and device fabrication due to their inherent spatial control (Ainslie and Desai, 2008, Eisenbarth, 2007, Fisher, *et al.*, 2001, Lin and Anseth, 2009). Utilizing the reaction control afforded by photo-initiation, our labs previously demonstrated that photopolymerized microtopographic features direct SGN neurite growth (Clarke, *et al.*, 2011, Tuft, *et al.*, 2013). Further, the ability of microfeatures to guide SGN neural-pathfinding depends on feature transitional slope, pattern complexity, and matrix stiffness (Tuft, *et al.*, 2013, Tuft, *et al.*, 2014a, Tuft, *et al.*, 2014b).

Cells generally and neurites specifically encounter and respond to a complex milieu of biochemical and biophysical signals in their microenvironment (Chou, *et al.*, 1995, Clark, *et al.*, 1990, Dalby, *et al.*, 2004, Karuri, *et al.*, 2006). Because these biochemical or biophysical cues may present guidance cues that conflict with intended growth patterns, it may be necessary to design guidance cues capable of overcoming such conflicting directional

signals. Accordingly, in this work, SGNs and TGNs are cultured on photopatterned materials that are modified with adsorbed biochemical borders that guide neurite growth. Neurite behavior is evaluated at the biochemical border to determine physical guidance cues that dominate neural process directional growth outcomes. Bioactive species used in the work include: laminin, an extracellular glycoprotein that facilitates neuronal adhesion and process outgrowth; tenascin-C (TnC), an extracellular glycoprotein that modulates neurite outgrowth and guidance; and EphA4-Fc, a chimeric peptide consisting of the chemorepulsive extracellular domain of EphA4 fused to the Fc portion of immunoglobulin. The adsorbed peptides form a distinct border across each sample that modulates neurite pathfinding. Importantly, physical guidance cues designed to have sufficiently narrow spacing overcome repulsive biochemical directional cues.

2. Materials and Methods

2.1. Photopolymerization of micropatterned substrates

Methacrylate thin films were photopolymerized on glass slides functionalized with the silane coupling agent 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich) to prevent polymer delamination. Prior to treatment with the coupling agent, slides were first cleaned and oxidized with O₂ plasma for 3 min at 30 W RF power (PDC-001 Harrick Plasma Expanded Cleaner, Ithaca, NY) while under 300 mTorr vacuum. Following removal from the plasma chamber, slides were immersed in a 1/100 v/v solution of the silane coupling agent and n-hexane (Sigma-Aldrich) overnight in a covered container at room temperature (~21°C). Each slide was then rinsed with fresh hexanes and dried in the fume hood before being placed in a sealed container. Functionalized slides were used immediately as a substrate for polymerization.

Mixtures of hexyl methacrylate (HMA, Sigma-Aldrich) and 1,6-hexanediol dimethacrylate (HDDMA, Sigma-Aldrich) were prepared with 1 wt% of 2,2-dimethoxy-2-phenylacetophenone (DMPA, BASF) as the photoinitiator. Copolymer compositions are represented as whole numbers (e.g. 40/60, 50/50) but each polymer fraction is 0.5 wt% less to account for incorporation of the photoinitiator. 20 µL of pre-polymer solutions were pipetted onto the center of a functionalized slide then covered with a 2.54 cm × 2.54 cm × 0.1 cm glass-chrome Ronchi rule photomask (Applied Image Inc., Rochester, NY) consisting of alternating transparent or opaque bands ranging from 10–50 µm in periodicity (P10–P50) for parallel patterns or with a cut untreated glass slide of the same dimensions for unpatterned samples (Fig. 1). Photopolymerization was performed with a high-pressure mercury vapor arc lamp (Omnicure S1500, Lumen Dynamics) filtered to emit 365 nm light intensity of 16 mW/cm². The curing module was equipped with an 8 mm aperture × 50 mm length beam homogenizing fused silica light pipe (Edmund Optics) and a collimating lens (RLQ-1, Asahi Spectra). Light intensity was measured with a Cole-Parmer Series 9811 radiometer. Microfeature amplitude was tuned by shuttering UV radiation at specific times thereby preventing further initiation events and resulting in rapid termination of the polymerization. Following the set exposure time, the photomask was removed from the polymer and the sample was washed with 95% ethanol to remove residual surface monomer. Rinsed samples were then post-cured for 10 min under the same light source and intensity

without the photomask and under ambient conditions to maximize monomer conversion without reducing surface channel amplitude due to trough back-filling.

2.2. Micropattern Characterization

Micropattern feature spacing and depth were measured by white light interferometry (Dektak Wyko 1100 Optical Profiling System, Veeco). Feature amplitude was measured as the difference between a maximum ridge value and an adjacent minimum groove value. For each composition and exposure time, average feature height was determined by measuring channel amplitude in nine different areas across the surface ($n = 3$). Feature spacing or periodicity was measured as the distance between the highest points on adjacent ridges and was consistent with photomask band spacing. Measurements and 3D images were generated using Vision software associated with the instrument.

Micropattern morphology was further characterized by scanning electron microscopy (SEM, S-4800, Hitachi). Conductive silver paint was applied to the bottom of glass substrates modified with micropatterned methacrylate thin films for mounting on aluminum SEM stubs to acquire top-down images. Each polymer surface was sputter coated with gold prior to examination by SEM. Electron accelerating voltage was set at 2 kV.

2.3. Creation of biochemical borders

Following the generation of unpatterned or micropatterned methacrylate thin-films, adsorbed biochemical borders were created on the surface to investigate their role in neurite pathfinding on physical guidance cues. To create biochemical borders, equal volumes of solutions with different bioactive species, e.g. one solution containing laminin (20 $\mu\text{g}/\text{ml}$, Sigma-Aldrich) and the other containing tenascin-C (TnC, 100 $\mu\text{g}/\text{ml}$, Millipore) or EphA4-Fc (10 $\mu\text{g}/\text{ml}$, R&D Systems), were separately deposited on a methacrylate polymer surface overnight at 4 °C. A glass coverslip for each solution was placed on the dispensed volume so as to cover half of the polymer film while aligning adjacent to the other coverslip. Protein solutions subsequently spread across the surface due to capillary forces between the glass coverslip and the methacrylate thin-film forming a boundary between the two proteins where the two cover slips join. Following adsorption, polymer films were rinsed three times with phosphate-buffered saline (PBS).

2.4. Neuron cultures

The use of animals and all protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. Dissociated SGN and TGN cultures from P3–5 rat pups were prepared as previously described (Hansen, *et al.*, 2001a, Hansen, *et al.*, 2001b, Roehm, *et al.*, 2008, Tuft, *et al.*, 2013) and plated onto patterned and unpatterned methacrylate thin-films. Neurotrophin-3 (NT-3, 50 ng/ml) and brain derived neurotrophic factor (BDNF, 50 ng/ml) were added to SGN cultures and NGF (50ng/ml) was added to TGN cultures to support neuronal survival and neurite growth. After 72 h, the cultures were fixed and immunostained with anti-neurofilament 200 (NF200) antibodies (1:400, Sigma-Aldrich), which detects neurites in SGNs and TGNs, as previously described (Hansen, *et al.*, 2001a). Alexa 488 conjugated secondary antibody (1:800, Invitrogen) was used to detect the primary antibody immunolabeling. Digital epifluorescent images were captured of the entire

polymer surface using the scan slide application of Metamorph software (Molecular Devices) on a Leica DMIRE2 microscope (Leica Microsystem) with a Leica DFC350FX digital camera.

2.4. Characterization of Neurite Behavior at Biochemical Borders

To characterize SGN and TGN neural outgrowth at biochemical borders, neurite responses at the borders were categorized into four distinct behaviors: cross, turn/parallel, repel, and stop. Neurites that extend greater than 10 μm past the border after approaching it from the opposite side are scored as a cross. Processes that reach and then subsequently reorient to align with the direction of the border are scored as turn/parallel. A neurite is scored as repel if it reaches the border and reverses direction, and as stop if it does not cross, turn, or repel after encountering the biochemical border. A minimum of 20 neurite encounters with the biochemical border were scored for each condition and each condition was repeated at least 3 times.

2.5. Statistics

Statistical analysis was performed using SigmaStat 3.5 software (Systat Software). Student t-test was performed to compare between two groups. Multiple groups were compared by performing a one-way ANOVA followed by a Holm-Sidak post hoc or, for data that were not normally distributed, an ANOVA on ranks Kruskal-Wallis analysis of variance on ranks and a Dunn's Method or Tukey Test multiple comparison procedure. Results are statistically significant if $p < 0.05$.

3. Results and Discussion

3.1. Biochemical Borders on Micropatterned Polymers

To evaluate the interplay between conflicting substrate bound biochemical cues and surface topographical features, borders of laminin and either EphA4-Fc or TnC were developed on photopolymerized topographies (Fig. 2). Our labs previously demonstrated photopolymerization as an effective method to fabricate and tune topographic guidance cues appropriate for neural process contact guidance (Clarke, *et al.*, 2011, Tuft, *et al.*, 2013, Tuft, *et al.*, 2014a). Selectively exposing the pre-polymer formulation to UV irradiance through a photomask inhibits local initiation events and slows polymerization rates beneath reflective bands. Consequently, raised features develop beneath transparent bands and surface depressions form beneath reflective bands yielding a globally patterned substrate (Fig. 1). For these studies, photomasks with parallel reflective and transparent bands with periodicities ranging from 10–50 μm are used to generate anisotropic ridge-groove topography. Meanwhile, feature depth or amplitude is precisely controlled on the micro- and nano-scale by modulation of irradiance exposure time for each periodicity (Fig. 1) (Tuft, *et al.*, 2013). In this study, a relatively shallow feature amplitude of $1.5 \pm 0.2 \mu\text{m}$ for each pattern periodicity was chosen as it would induce aligned neurite growth but still allow for directional changes upon the encounter of biochemical borders (Tuft, *et al.*, 2013). Unpatterned controls are generated by exposing photopolymer formulations to UV light beneath glass slides devoid of reflective chrome plating.

Neurites encounter a variety of biochemical and biophysical features that ultimately determine neurite trajectory including fixed or bound molecules that often present distinct borders as well as more gradual gradients of diffusible molecules. Here we elect to initially explore the interaction of surface topographical features with fixed biochemical borders. These borders are generally more potent than gradients, facilitate characterization of neurite responses at defined locations, and are more commonly used in designing nerve regeneration substrates. Thus we generate substrates that present growing neurites with a strong chemorepulsive signal that is in competition to topographic microfeatures of varying potency. To create biochemical borders, equal volumes of solutions with different peptides, e.g. one solution containing laminin and the other containing TnC or EphA4-Fc, are separately deposited on a methacrylate polymer surface. A glass coverslip for each solution is placed on the dispensed volume in such a way as to cover half of the polymer film while aligning adjacently to the other coverslip. Peptide solutions subsequently spread across the surface due to capillary forces between the glass coverslips and the methacrylate thin-film. A portion of the peptide from the solution remains adsorbed to photopolymer substrates following multiple wash steps as evidenced by immunofluorescence microscopy (Fig. 2). Only a slight bleed is observed between coverslips for a given peptide species indicating that the advancing growth cone encounters a true biochemical border rather than a gradient transition between the two bioactive peptides (Fig. 2A,B). The peptides used in this study all adsorb readily to unpatterned methacrylate films and evenly coat photopatterned topography (Fig. 2B,D).

To characterize neural outgrowth at biochemical borders, neurite responses were categorized into four distinct behaviors: cross, turn/parallel, repel, and stop (Fig. 3). Interestingly, neurites that grow parallel to the border must cross multiple ridges and grooves oriented perpendicularly to the biochemical border (Fig 3A). A neurite that is repelled grows back towards the soma (Fig. 3B), whereas neurites that stop fail to further grow in any direction (Fig. 3C). In addition to those with arrested growth, stop behavior, which was not common, could also represent neurites that had reached the border at the time the cultures were fixed.

3.2. SGN Neurite Behavior at Laminin:EphA4-Fc Borders

To generate substrates with an active biochemical border and varying potency of surface features unpatterned and micropatterned thin-films with a 1.5 μm channel amplitude and periodicities ranging from 10–50 μm were coated with laminin and EphA4-Fc (Fig. 2B). Laminin is an extracellular matrix (ECM) glycoprotein that facilitates cellular adhesion by binding trans-membrane integrin receptors and supports SGN neurite growth (Aletsee, *et al.*, 2002, Evans, *et al.*, 2007, Xu, *et al.*, 2012). EphA4 is a membrane tyrosine kinase receptor, expressed in the cochlear epithelium, that interacts with ephrin receptors to function as a chemorepulsive signal to SGN neurite outgrowth (Brors, *et al.*, 2003, Coate, *et al.*, 2012, Defourny, *et al.*, 2013). EphA4-Fc is a chimeric peptide consisting of the ligand binding portion of the EphA4 receptor and the Fc portion of immunoglobulin. This fusion results in a functional peptide that remains active when adsorbed onto substrate surfaces (Brors, *et al.*, 2003).

After coating the polymers with laminin and EphA4-Fc, dissociated SGNs are cultured on the substrate surface for 72 hrs in neurotrophic media. SGNs are immunostained with anti-neurofilament 200 (NF200) antibody, which labels all neurites arising from cultured SGNs (Jeon, *et al.*, 2011). SGN neurite outgrowth on unpatterned substrates extends randomly on both laminin- and EphA4-Fc-coated regions (Fig. 4A). Though EphA4-Fc has previously been identified as a repulsive cue, dissociated SGNs cultured solely on the EphA4-Fc-coated region displayed robust neurite growth with no obvious visual differences between SGNs cultured on the laminin-coated region. In contrast to wandering SGN neurite growth on unpatterned substrates, neural processes strongly aligned to parallel ridge-groove topographies generated by photopolymerization with the greatest alignment occurring on patterns with the narrowest feature spacing, i.e. low periodicity (Fig. 4B,C).

SGN neurites that originate on the laminin-coated side and encounter the laminin:EphA4-Fc border on unpatterned polymers extend across the border to the EphA4-Fc side only 31% of the time demonstrating the chemorepulsive influence of EphA4-Fc (Fig. 4D). The proportion of neurites crossing from the laminin-coated to EphA4-Fc-coated regions increases when SGNs are presented with decreasing periodicity and thus increasing physical guidance cues. Specifically, when SGNs are cultured on 50 μm periodicity and 1.5 μm amplitude parallel ridge-groove patterns, nearly 50% of the neurites are induced to cross the biochemical border to the EphA4-Fc side, ~20% more often than on the unpatterned substrates, although this is not statistically significant ($p=0.129$, one way ANOVA). It should be noted that the aspect ratio, i.e. height divided by width, of the 50 μm periodicity pattern is extremely low indicating that neurite growth is sensitive to even subtle changes in surface topography. A further, statistically significant, increase in biochemical border crossing is observed when using increasingly narrower features with both 33 μm and 20 μm periodicity patterns inducing 56% of the neurites that encounter the border from the laminin side to the EphA4-Fc side to cross ($p=0.008$ for P20 and P33). Significantly, a substantial increase in biochemical border crossing is observed when neurites are challenged with a 10 μm periodicity pattern ($p<0.001$). Nearly 90% of the SGN neural processes cross to the EphA4-Fc surface from the laminin surface while influenced by microfeatures that are more frequent and steep compared to greater periodicity patterns with lower slope in transitioning from the grooves to ridges.

When comparing neurite populations on unpatterned surfaces, a clear preference for growth on laminin is observed. For SGN neural processes that reach the biochemical border from the EphA4-Fc coated side, 68% readily cross without any other additive guidance cue which is a nearly 40% increase in neurite crossing compared to the opposite transition on unpatterned substrates ($p<0.001$, Student two-tailed t-test). As seen with the laminin to EphA4-Fc transition, micropatterns with relatively wide but shallow features increase neurite crossing from EphA4-Fc to laminin by approximately 10 – 12% with no significant differences in neurite crossing between the 50, 33, and 20 μm periodicity patterns ($p>0.05$). An even greater proportion of neurites are induced to cross the biochemical border when presented with a 10 μm periodicity pattern ($p=0.003$). It appears that the guidance strength of the physical pattern is not diminished by either bioactive signal presented at the biochemical border as 90% of the neurite population cross the border while approaching it from either side ($p=0.635$). Taken together, these results inspire confidence that physical

cues can be designed to overcome strong chemorepulsive cues that could disrupt spatially organized neural regeneration for *in vivo* applications.

To verify that the observed neurite behavior was due to the bioactive function of the laminin:EphA4 border rather than random process growth, SGN neurite behavior was characterized at arbitrary borders made on regions coated with only one bioactive species, i.e. a laminin to laminin or an EphA4-Fc to EphA4-Fc border (Fig. 4F,G). Accordingly, the neuronal soma and the entire length of the SGN neurite remain on regions coated only with laminin or EphA4-Fc. In both cases, the most striking difference between neurite behavior at the laminin:EphA4-Fc border and laminin:laminin and EphA4-Fc:EphA4-Fc borders occurs on unpatterned methacrylate thin films. The neurite proportion crossing the biochemical border between the two different bioactive species is less than 50% whereas on borders with the same species on either side greater than 90% of the neurites readily cross ($p < 0.006$, ANOVA).

The crossing proportion slightly increases at both homogenous borders when neurites are presented with topographical guidance cues. The increase is attributed to greater incidence of neurite growth in a direction that is perpendicular to the border which increases the chances that a neural process will be observed to cross. It should also be noted that the largest proportion of neurites to cross from laminin to EphA4-Fc or its reverse is 90% whereas the largest proportion of crossing neurites on laminin:laminin and EphA4-Fc:EphA4-Fc borders is 97% and 99%, respectively. For all border types, the highest incidence of neurite crossing occurs on 10 μm periodicity micropatterns. It is likely that the observed 10% discrepancy between hetero and homogenous peptide borders is due to chemorepulsion from the laminin:EphA4-Fc border. Even when extending from EphA4-Fc to laminin, neurites are less likely to cross the biochemical border than on homogenous transitions.

In addition to neurite crossing behavior at biochemical borders, neurite turn, repel, and stop responses further elucidate neural pathfinding behavior when processes are presented with physical guidance cues of various strengths (Fig. 3 and Supplemental Fig. 1A,B). For example, the ~70% of neurites originating on laminin that do not cross the laminin:EphA4-Fc border on unpatterned substrates are observed to split relatively evenly between the described turn, repel, and stop behaviors (Supplemental Fig. 1A). By contrast, SGN neurites that are presented with physical guidance cues are more often repelled by or stop at the border compared to unpatterned controls. The trend to repel or to stop at the border on micropatterned substrates increases with increasing feature frequency for neurites that originate on laminin (Supplemental Fig. 1A). As previously mentioned, the guidance strength of the photopolymerized topographic cues increases with decreasing pattern periodicity (Fig. 4D,E). The relative increase in the proportion of neurites that is repelled or stopped at the chemorepulsive border on micropatterned substrates is, therefore, most likely due to the inhibition of neurite turning on micropatterned substrates. In other words, a turn/parallel response requires that a neurite grows parallel to the biochemical border traversing over multiple ridge features since the topographical pattern lies perpendicular to the biochemical border. As these features become more frequent fewer neurites are able to

sustain growth over multiple topographic ridge/groove features and this reduction of turning/parallel behavior increases the relative percentage of neurites for the other scored behaviors.

Interestingly, for almost all pattern types as well as unpatterned samples, the most common SGN neurite behavior, other than crossing, is to stop after encountering the laminin:EphA-Fc border. It is unclear whether the observed stop behavior is due to an actual arrest in neurite outgrowth or if advancing neural growth cones are induced to pause at the biochemical border before transitioning to cross, turn, or repel behavior. A pause or a lag-time in neurite outgrowth at the bioactive border would lead to more frequent capture of the neurite terminus at the time the cultures are fixed and an increase the frequency of the neurite stop characterization. However, assuming that stop behavior represents arrested neurite growth in most cases, this is the most common neural process outcome other than crossing at the laminin:EphA4-Fc border. Arresting neurite outgrowth at spatially defined locations (e.g. at or near specific electrodes) represents a critical goal to improved integration of prostheses with neural tissue (Leach, *et al.*, 2010, O'Leary, *et al.*, 2009, Winter, *et al.*, 2007). Accordingly, designing material interfaces with biochemical stopping cues that work in conjunction with topographic patterning may potentially provide the necessary guidance as well as stopping signals if appropriately designed for a given neural niche.

The proportion of SGN neurites that does not cross from the EphA4-Fc to the laminin-coated side is significantly smaller than the proportion that does not cross the opposite transition for unpatterned substrates and for patterns with periodicities between 33 and 50 μm (Supplemental Fig. 1A,B) ($p < 0.011$, ANOVA). Accordingly, SGN neurite proportions for this group are all below 20%. Even for the small proportion of non-crossing neurites that meet the laminin:EphA4-Fc border from the EphA4-Fc side, stopping behavior is still the most common response. For unpatterned samples and for patterns with periodicities between 20 and 50 μm , the frequency of stopping behavior doubles from around 5% to 10% or higher. SGN non-crossing neurite behavior was not scored on homogeneous chemical borders as the vast majority of neurites ($> 95\%$) are observed to cross the border leaving prohibitively few neurites to be scored for other behaviors.

3.3 SGN Neurite Behavior at Laminin-TnC Borders

To further elucidate the hierarchical relationships of bioactive peptide borders and surface topographic features on neurite guidance, dissociated SGNs were cultured on unpatterned and micropatterned polymers coated with laminin and TnC (Fig. 5). TnC, like laminin, is an ECM peptide that is expressed in cochlear tissues (Whitlon, *et al.*, 1999). It likely acts as an important boundary formation molecule in the developing inner ear innervation and as a potential guidance molecule to direct SGN neurites towards cochlear implant electrodes (Kwiatkowska, *et al.*, 2016).

Initial, qualitative observations of SGN neural process outgrowth on laminin:TnC substrates illustrate similar trends seen for neurite extension on substrates modified with laminin:EphA4-Fc. Namely, process outgrowth spreads randomly on unpatterned substrates coated either with laminin or with TnC (Fig. 5A). Further, SGN neurite direction became increasingly aligned with anisotropic uni-directional microfeatures as pattern periodicity

decreased (Fig. 5B,C). Also like the laminin:EphA4-Fc system, SGN soma and neural processes appear healthy whether growing solely on laminin or TnC coatings.

Similar to neurite behavior on laminin:EphA4-Fc substrates, SGN neurite crossing of the laminin:TnC border is substantially lower for both unpatterned and patterned substrates for neurites originating on the laminin-coated side (Fig 4D,E and Fig 5D,E), indicating the chemorepulsive function of the border. However quantitative differences exist in SGN neurite crossing of the laminin:EphA4-Fc and laminin:TnC borders. For example, neurite crossing of laminin:EphA4-Fc borders increases substantially when dissociated SGNs are cultured on 1.5 μm amplitude and 50 μm periodicity photopolymerized topographic guidance cues – going from a 31% cross rate to nearly 50% (Fig. 4D). By contrast, when transitioning from laminin to TnC, SGNs presented with the same microfeature dimensions exhibit only a 4% average increase in crossing of the biochemical border compared to unpatterned surfaces (Fig. 5D), which is not statistically significant. Indeed, the proportion of SGN neurite crossing of the laminin:TnC border remains lower than 40% on both 33 and 20 μm feature periodicities, though the trend of increased border crossing at narrower periodicities becomes statistically significant on 20 μm periodicity micropatterns ($p < 0.001$, ANOVA). The guidance strength of topographical surface cues becomes more evident on 10 μm periodicity features as greater than 60% of neurites which reach the laminin:TnC border are induced to cross despite the strength of the biochemical border that prevents crossing on patterns with wider features and on unpatterned controls ($p < 0.001$, ANOVA). Thus, TnC appears to present a stronger chemorepulsive cue than EphA4-Fc for SGNs originating on laminin, yet surface features with narrow frequency spacing overcome this chemorepulsive cue.

SGN neurites cross from TnC to laminin-coated regions significantly more than going from laminin to TnC on substrates with a 50 μm periodicity pattern (Fig. 5D,E) ($p = 0.029$). Otherwise, although there is a clear trend towards increased crossing with narrower pattern periodicity the difference in neurite crossing is not statistically significant amongst the patterns. On unpatterned substrates, 21% of SGN neurites make the transition from TnC to laminin, similar to the percent crossing from laminin to TnC. With the widest periodicity micropattern, and therefore the weakest physical guidance strength, neurite crossing proportion increases nearly two fold to 37% which did not occur for neurites transitioning from laminin to TnC. Moreover, the proportion of neurites that cross from TnC to laminin-coated regions on 33 and 20 μm periodicity patterns is also significantly higher at 45% and 53% compared to 27% and 39% respectively for laminin to TnC transitions. While SGN neurites are increasingly likely to cross from TnC to laminin coated substrates with narrower features, it is noteworthy to observe that the proportion of SGN neurites that are induced to cross the biochemical border by 10 μm periodicity patterns is nearly the same (~60%) for laminin to TnC crossings as for the opposite transition. Taken together with the laminin:EphA4-Fc results, these data engender confidence that micropattern topography can be devised to overcome chemical cues that may disrupt spatially organized neurite growth.

Culture of dissociated SGNs on TnC alone does not substantially alter neurite pathfinding behavior as evidenced by cross results on homogeneous TnC:TnC borders (Fig. 5F). SGN neurites cross the homogenous border with a frequency greater than 90% for all patterned

conditions and for unpatterned controls. As with homogenous laminin:laminin and EphA4-Fc:EphA4-Fc coated regions, the proportion of neurites that cross TnC:TnC borders increases for SGNs presented with anisotropic photopolymerized guidance cues. For 33, 20, and 10 μm periodicity patterns SGN neurite crossing reaches an average of 99% indicating that TnC does not interfere with neural process growth cone guidance response to physical features.

Along with neural process crossing behavior, we characterized neurite turn, repel, and stop responses at the laminin:TnC border (Supplemental Fig. 2A,B). As previously described, the most common neurite behavior besides crossing for the laminin:EphA4-Fc system is stopping at the biochemical border. However, for laminin:TnC substrates, the most common neurite response, for all substrates other than 10 μm periodicity patterns, is to turn and continue to grow parallel to the border. This behavior is particularly evident on unpatterned substrates as 45% of non-crossing neurites turn to parallel the pattern when reaching the biochemical border from the laminin-coated side. Similarly, from the TnC coated side, 38% of non-crossing neurites also turn to parallel or align to the biochemical border.

Even the majority of non-crossing SGN neurites challenged with physical guidance cues on 50 and 33 μm periodicity turns at and aligns to the direction of the laminin:TnC border for neurites originating on the laminin side (Fig. 5G). For the 20 and 10 μm periodicity patterns, neurite behavior becomes more varied as there are even distributions between turn, repel, and stop responses. SGN neurites that encounter the TnC:laminin border from the TnC side do not exhibit any behavioral trends, other than the high proportion of turning on unpatterned and 33 μm periodicity samples, as the neurite distribution spreads relatively evenly among the characterized responses (Fig. 5H).

The significant tendency for SGN neurites to stop at the laminin:EphA4-Fc border is markedly diminished at laminin:TnC borders (Supplemental Fig. 1 and Supplemental Fig. 2, $p=0.001$, Mann-Whitney rank sum test). While stop behavior still occurs in at least 10% of the neurite population for each type of substrate, the proportion of neurite stops for each population does not make up the largest non-crossing proportion for any condition on laminin:TnC substrates. Contrasted with EphA4-Fc, TnC appears less likely to arrest neural process extension as may be occurring with EphA4-Fc. The disruption in SGN neurite crossing of the laminin:TnC border, even for neurites on effective physical guidance cues, suggests that transitioning from either TnC to laminin or vice versa presents a significant obstacle for advancing neural growth cones. Substantial increases in neurite turning and repeling behavior compared to neurite stopping further support this observation as neurites that turn or repel remain anchored to whichever adhesion molecule on which they originated.

3.4 TGN Neurite Behavior at Laminin-TnC Borders

SGNs are the target neurons stimulated by cochlear implant prostheses. Due to their importance in neural prosthetics, the behavior of their neurites in relation to biophysical and biochemical cues is the principal focus of these studies. To determine whether SGN neurite behavior is typical for other neuronal types, dissociated trigeminal ganglion neurons (TGNs) were also cultured on unpatterned and micropatterned substrates modified with laminin:TnC

borders as a comparison to SGNs (Fig. 6). Qualitative observations of TGN neurite outgrowth demonstrate similar behavior as SGN neurite growth on laminin:TnC-coated unpatterned and photopatterned polymer films (Fig. 6A–C). Specifically, TGN neurite extension proceeds randomly on unpatterned films on both laminin and TnC-coated areas (Fig. 6A). Further, on photopolymerized micropatterns, TGN neurite extension increasingly parallels microfeature anisotropy as pattern periodicity decreases (Fig 6B,C).

TGN neurite crossing behavior at the laminin:TnC border on both unpatterned and physically patterned polymer substrates strongly resembles SGN neural pathfinding (Fig. 5D,E and Fig. 6D,E). On unpatterned substrates, TGN neurites do not readily cross the laminin:TnC border from either side, though, the tendency to cross from the TnC to the laminin side is higher than the reverse transition with 28% and 19% crossing, respectively. Also similar to SGN neurite extension, the extent of TGN neurite crossing of the laminin:TnC border increases substantially as neurites are presented with physical guidance cues. However, the weakest physical cue used in this study, a 50 μm periodicity pattern and a 1.5 μm amplitude, provides only negligible change with a 4% and 2% increase in neurite crossing proportion when originating on either laminin or TnC-coated sides, respectively. TGN neurite crossing of the laminin:TnC border remains lower than 40% on both 33 μm and 20 μm feature periodicities except for TGN neurites originating on the TnC-coated side on 20 μm periodicity substrates. As the topographical surface guidance strength increases on 10 μm periodicity patterns, the majority of TGN neurites are induced to cross the laminin:TnC border when reaching it from either side. Similar to SGN behavior, 10 μm periodicity features cause approximately 60% of neurites that encounter the border to cross despite the presence of the biochemical border that prevents crossing on patterns with wider features and on unpatterned controls. Finally, TGN neurites, similar to SGN neurites, cross from TnC to laminin-coated regions more often than going from laminin to TnC (Fig. 6D,E). These similarities of TGN crossing behavior with SGN crossing behavior on the same topographically patterned and bioactively-coated substrates support the conclusion that photopolymerized topographies can be designed capable of guiding neurite growth even in the presence of contrary biochemical signals.

To further explore TGN neural pathfinding behavior and to compare it to SGN neural outgrowth, non-crossing TGN neurite responses are also scored as turn, repel, and stop responses when challenged with both biochemical and biophysical guidance cues (Supplemental Fig. 3A,B). Due to the repulsive influence of the laminin:TnC border, the majority of TGN neurites fail to cross the border unless TGNs are cultured on patterns with the strongest physical guidance cues (10 μm periodicity) (Fig. 6D,E). Like SGN neurites on unpatterned substrates and on micropatterns with weak guidance strength, the most prominent TGN neurite behavior is to turn and align to the border direction. Greater than 40% align to the border, rather than cross, for neurites that approach the border from the laminin side and greater than 30% behave similarly when reaching the biochemical transition from the TnC side (SFig. 3A,B). The 10% discrepancy in turning is likely due to increased favorability of TGN neuronal adhesion to laminin compared to TnC. However, the chemorepulsive effect is substantial for unpatterned substrates and for patterned substrates that have topographic cues with weak guidance strength (50 μm periodicity) as less than 30% of neurites cross for either condition and approaching the border from either direction.

The most common response of TGN neurites on unpatterned surfaces and surfaces with 50 μm periodicity micropatterns is to turn and align to the direction of the laminin:TnC border for neurites originating on either the laminin or TnC side (SFig. 3A,B). With increased micropattern frequency (33, 20 and 10 μm periodicity), non-crossing TGN neurite behavior becomes more evenly distributed between turn, repel, and stop responses. Thus, both TGN and SGN neurites on laminin:TnC substrates display a relatively even distribution of non-crossing behaviors on micropatterns with frequent features, while there is a greater tendency to turn and grow parallel to the border for neurites on unpatterned surfaces and patterned surfaces with a 50 μm periodicity. Only SGN neurites on laminin:EphA4-Fc substrates are observed to show an increase in stopping behavior as physical pattern guidance strength increases.

4.1 Conclusions

Advancing growth cones respond to an assortment of physical as well as biochemical cues in their micro-environment. For neural tissue engineering, these physical and biochemical cues can be leveraged to precisely guide neural regeneration. Data presented in this work, based on a novel competition assay design, identifies appropriate physical microfeature dimensions that induce neurite alignment across conflicting biochemical directional cues. Specifically, biochemical directional cues are readily overcome when physical guidance cues are designed to have sufficiently narrow spacing at a given amplitude from ridge to groove of 1.5 μm . Designing prosthesis interfaces with physical cues that induce spatially organized neurite outgrowth even in the presence of conflicting biochemical cues will likely be necessary for *in vivo* applications with inherent biochemical signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic of the photopatterning process. Photopolymerizable methacrylate monomer is illuminated through a photomask and polymerization is selectively initiated in exposed regions resulting in raised microfeatures across the surface. Periodicities of 50 μm (left) and 10 μm (right) are depicted with corresponding top down view SEM images shown below.

Figure 2.

Fluorescence images of biochemical borders on unpatterned and micropatterned HMA-co-HDDMA polymers. Shown are chemical borders of laminin (red) and EphA4-Fc (green) on unpatterned (A) and micropatterned (B) substrates, and of laminin (red) and tenascin C (no stain) on unpatterned (C) and micropatterned (D) substrates. The represented micropatterns have dimensions of 50 μm periodicity and 1.5 μm amplitude (B) and 20 μm periodicity and 1.5 μm amplitude (D). Borders were established by placing adjacent coverslips over respective protein solutions on polymer substrates. The yellow line in (A) and (B) is an artifact from mixed fluorescence at the border.

Figure 3.

Neurite behavior at biochemical borders. The images represent categorized neurite behaviors at biochemical borders on micropatterned substrates. **A–C)** Neurite cross, turn/parallel (A), repel (B), and stop (C) behaviors are shown. The biochemical border between laminin and tenascin C is represented as a yellow dashed line. Regions coated with laminin and tenascin C are demarcated with a yellow L and T, respectively. Dissociated neurons are stained with anti-NF200 antibody (red). The pattern for each image is set horizontally and has a periodicity of 20 μm and a channel amplitude of 1.5 μm.

Figure 4.

SGN neurite crossing behavior at the biochemical border between laminin (Lam) and EphA4-Fc on micropatterned polymer substrates. **A–C**) Immunofluorescence images of dissociated SGNs cultured on unpatterned (A), 50 μ m periodicity (P50) (B), and 20 μ m periodicity (P20) (C) parallel micropattern substrates. SGNs are stained with anti-NF200 antibody. The biochemical border is marked with a dashed yellow line and the laminin and EphA4-Fc sides are marked with a yellow L or E, respectively. **D–G**) Proportion of neurites that cross the biochemical border from laminin to EphA4-Fc (D), from EphA4-Fc to laminin (E), from laminin to laminin (F), and from EphA4-Fc to EphA4-Fc (G). SGN neurites cross

the biochemical border significantly more when cultured on patterns with narrow features (i.e. low periodicities) compared to unpatterned substrates and wide features. Neurites are much more likely to cross from EphA4-Fc coated substrate to a laminin coating than from laminin to EphA4-Fc even on unpatterned surfaces. Numbers in the columns (N) represent the total number of neurites scored for each condition. * $p < 0.05$, one-way ANOVA followed by a Holm-Sidak post hoc compared to unpatterned surfaces. All patterned substrates have a microfeature amplitude of 1.5 μm .

Figure 5.

SGN neurite crossing behavior at the biochemical border between laminin (Lam) and tenascin C (TnC) on micropatterned polymer substrates. **A–C)** Immunofluorescence images of dissociated SGNs cultured on unpatterned (A), 50 μm periodicity (P50) (B), and 20 μm periodicity (P20) (C) parallel micropattern substrates. SGNs are stained with anti-NF200 antibody. The biochemical border is marked with a dashed yellow line and the laminin and tenascin C sides are marked with a yellow L or T, respectively. **D–F)** Proportion of neurites that cross the biochemical border from laminin to tenascin C (D), from tenascin C to laminin (E), and from tenascin C to tenascin C (F). Numbers in the columns (N) represent the total number of neurites scored for each condition. SGN neurites cross the biochemical border significantly more when cultured on patterns with narrow features (i.e. low periodicities) compared to unpatterned substrates and wide features. * $p < 0.05$, one-way ANOVA followed by a Holm-Sidak post hoc compared to unpatterned surfaces. All patterned substrates have a microfeature amplitude of 1.5 μm .

Figure 6.

TGN neurite crossing behavior at the biochemical border between laminin (Lam) and tenascin C (TnC) on micropatterned polymer substrates. **A–C)** Immunofluorescence images of dissociated SGNs cultured on unpatterned (A), 50 μm periodicity (P50) (B), and 20 μm periodicity (P20) (C) parallel micropattern substrates. TGNs are stained with anti-NF200 antibody. The biochemical border is marked with a dashed yellow line and the laminin and tenascin C sides are marked with a yellow L or T, respectively. **D,E)** Proportion of neurites that cross the biochemical border from laminin to tenascin C (D) and from tenascin C to laminin (E). Numbers in the columns (N) represent the total number of neurites scored for each condition. TGN neurites cross the biochemical border significantly more when cultured on patterns with narrow features (i.e. low periodicities) compared to unpatterned substrates and wide features. Numbers in the columns represent the total number of neurites scored for each condition. SGN neurites cross the biochemical border significantly more when cultured on patterns with narrow features (i.e. low periodicities) compared to unpatterned substrates and wide features. * $p < 0.05$, one-way ANOVA followed by a Holm-Sidak post hoc compared to unpatterned surfaces. All patterned substrates have a microfeature amplitude of 1.5 μm.