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Charge and topography patterned lithium niobate provides physical cues to fluidically isolated cortical axons

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In vitro devices that combine chemotactic and physical cues are needed for understanding how cells integrate different stimuli. We explored the suitability of lithium niobate (LiNbO3), a transparent ferroelectric material that can be patterned with electrical charge domains and micro/nanotopography, as a neural substrate. On flat LiNbO3 z-surfaces with periodically alternating charge domains, cortical axons are partially aligned with domain boundaries. On submicron-deep etched trenches, neurites are aligned with the edges of the topographical features. Finally, we bonded a bicompartamental microfluidic chip to LiNbO3 surfaces patterned by etching, to create isolated axon microenvironments with predefined topographical cues. LiNbO3 is shown to be an emerging neuron culture substrate with tunable electrical and topographical properties that can be integrated with microfluidic devices, suitable for studying axon growth and guidance mechanisms under combined topographical/chemical stimuli. Published by AIP Publishing.

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There is a growing interest in micro/nano technologies developed for neuroscience research, particularly to study axonal growth and guidance.1 Among these, methods for generating micro/nano-topographical features received particular attention since axons tend to align with and accelerate on longitudinal structures, i.e., grooves, for certain combinations of groove width and depth.2–4 In fact, recent in vivo evidence5–7 led to the hypothesis that axially aligned micro-scale topography which incorporates submicron-scale features is required for the effective recovery of long (>20 mm) peripheral nerve defects.8

Topography with micron-scale feature sizes is typically created via standard photolithography on suitable masking layers, whose pattern is subsequently transferred to the substrate typically through an etching process.9 Features with submicron sizes, on the other hand, require a deep-UV or an electron beam lithography.10 3D-patterned substrates can be used to culture neurons directly or serve as molds for casting durable or biodegradable polymers such as polydimethylsiloxane (PDMS)11 or poly-lactic-co-glycolic acid,12 respectively, which then serve as neuron substrates. 3D neuron culture substrates can also be decorated with nano-patterned features, such as grooved fibers13 and Ti nanotube arrays.14

Axonal growth cones not only sense the mechanical properties of their microenvironment but also actively generate force by pulling on focal adhesions such that local topography affects guidance decisions.1 For example, combining topographical (e.g., 1 μm-wide and 400 nm-deep grooves) and biochemical (nerve growth factor) cues synergistically enhanced hippocampal axon length.2 Therefore, experimental models that provide independent topographical and biochemical cues offer additional insight on neural growth and guidance mechanisms. Microfluidic devices that fluidically isolate axons from their cell bodies or provide exclusive access to specific subcellular regions are now commonly used in neurobiology.15 Additionally, microfluidic devices are used to form gradients of chemotactic cues.16 In vivo, growth cones may be remotely distant from their cell bodies and typically perceive guidance cues as chemical gradients. Thus, microfluidic chips help create physiologically-relevant in vitro model systems. However, micro/nano-topographical cues are rarely integrated into microfluidic devices.17,18

Lithium niobate (LiNbO3) is a transparent ferroelectric material that has a spontaneous polarization, \( P_s = 0.71 \text{ C m}^{-2} \) along its z-axis, and is thus characterized by positive and negative bound polarization charges at the +z and −z crystal surfaces, respectively. Periodically poled LiNbO3 (PPLN) contains periodic ferroelectric domains (alternating in ±z), which are suitable for nonlinear optical applications. These domains can also be converted into topographical features due to their inherently different etch rates. Alternatively, topographical features can be created in monodomain substrates via photo- or electron beam lithography. Recent work showed that uncoated LiNbO3 surfaces are biocompatible with cell type specific effects: osteoblasts19 and mesenchymal stem cells13 exhibited increased proliferation and differentiation on charged LiNbO3. For stem cells—but not for osteoblasts—positively charged LiNbO3 was much more effective than negatively charged LiNbO3. In fibroblasts, LiNbO3 surface
charge was also shown to affect cell morphology and motility. LiNbO₃ has recently been shown to be suitable for manipulating and sorting biological material. Exploiting the bulk photovoltaic effect, electric field patterns can be induced by illuminating LiNbO₃, which can be used to pattern spores and pollen. Exploiting its pyroelectric properties, PPLN can also be used for generating permanent charge patterns on polymer membranes, which can then be used for cell patterning. Furthermore, photorefractive properties of iron-doped LiNbO₃ can be used for electrode-free immobilization of bacteria via light-induced dielectrophoresis. Finally, cell sorting can be achieved via acoustic waves by bonding LiNbO₃ to microfluidic devices.

Here, we investigate the potential of LiNbO₃ as a neuron substrate, providing charge and topography patterns to fluidically isolated axons, where the combined effects of chemotactic, charge, and topography cues can be studied in controlled microfluidic environments. We show that neurites align with boundaries of charge and topography patterns, and that these patterns can be embedded in the axon microenvironment, towards a better understanding of axon growth behavior.

Primary embryonic mouse cortical neurons were harvested as previously described. The culture medium consisted of DMEM-Glutamax supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin solution (P/S), and B₂₇ neuron supplement (all from Gibco). At 3 days in vitro (DIV), the culture medium was replaced with Neurobasal (Gibco) supplemented with 1% P/S and B₂₇. We first cultured neurons at 2 × 10⁴ cells-cm⁻² on z-plane-parallel cut congruent LiNbO₃ wafers (CasTech, Fuzhou, China) with uniform charge and on periodically poled, z-cut LiNbO₃ (Gooch & Housego, Palo Alto, CA) with a charge pattern with ∼30 µm domain period. Prior to characterization and cell culture, substrates were cleaned by consecutive rinsing with acetone, isopropanol, and de-ionized (DI) water. Atomic and piezoresponse force microscopy (AFM/PFM) were performed to probe surface topography and charge, respectively. For AFM, MFP-3D (Asylum Research, Santa Barbara, CA) was operated in the amplitude modulation mode with cantilevers having a typical resonant frequency of ∼330 kHz and a spring constant of ∼42 N m⁻¹ (PPP-NCH; Nanosensors, Neuchatel, Switzerland). For PFM, DPE-XSC11 D (MikroMasch, Wetzlar, Germany) cantilevers having a typical force constant of 42 N m⁻¹ and a resonant frequency of 350 kHz were used. PFM amplitude images show the sample deformation under the applied AC voltage as measured by the tip displacement and the corresponding cantilever deflection. PFM phase images show the phase lag of the tip oscillation with respect to the modulation voltage and indicate the direction of ferroelectric polarization and thereby the sign of the corresponding polarization charge at the surface.

Neurons did not grow unless the substrates were coated with 2.5 µg/ml poly-L-lysine (PLL; Sigma, Wicklow, Ireland) in Dulbecco’s phosphate buffered saline (DPBS; Lonza, Basel, Switzerland) overnight and rinsed 3× with DPBS. Cells were aldehyde-fixed on 3–6 DIV, stained to reveal β₃-tubulin, F-actin, and nuclei, and mounted on microscope slides as previously described. Neurite length was quantified from tubulin staining using the NeuronJ plug-in in ImageJ (NIH, Bethesda, MD). Cell clustering was quantified by dividing the number of clusters to the number of nuclei. A neurite length cut-off of 60 µm (1.5× median neurite length of control neurons grown on PLL-coated glass coverslips) was used before calculating the axon/soma ratio. Substrates were recovered and reused following imaging by carefully removing them from microscope slides and sonicating first in 2% PCC-54 detergent (Thermo-Fisher Scientific, Waltham, MA) in DI water, subsequently in DI water, and finally in isopropanol (20 min each). As shown in Figure 1, neurons exhibited increased clustering and decreased axonal connectivity on substrates with uniform charge, suggesting sub-optimal PLL coverages. Interestingly, the direction of the charge (i.e., negative or positive) did not affect the outcome, similar to the effects of charged LiNbO₃ on osteoblast proliferation and mineral accumulation. Figure 2 shows neurons cultured on charge-patterned PPLN substrates. On these substrates, axons appeared to recognize and align with domain boundaries, reminiscent of the avoidance of domain boundaries by fibroblasts on a periodically poled lithium tantalate (PPLT). To check if there was a preferential deposition of the positively charged PLL to negatively charged domains, we coated the substrate with fluorescein isothiocyanate-conjugated PLL (FITC-PLL; Sigma); however, no preferential deposition was detected (Fig. 2(a), inset). In future work, it will be important to elucidate the role of surface cleaning and functionalization in addition to the surface charge on the attachment and proliferation of different cell types.

We next cultured neurons on microtopographical features created via reactive ion etching on y-cut LiNbO₃. Etched samples were prepared from 500 µm-thick y-cut substrates (CasTech). A 160 nm Cr layer was deposited on the samples using an E-beam sputtering system (Auto 306 HV; Edwards, Crawley, UK), which acted as a hard mask. Spin-coated Microposit S1813 photoresist (Shipley, Marlborough, MA) was patterned via a photomask (Photronics, Brookfield,
CT), and developed using Microposit MF319 (Shipley). An Inductively Coupled Plasma-Reactive Ion Etching machine (ICP RIE; Plasmalab 100; Oxford Instruments, Abingdon, UK) was used to transfer the pattern first to the Cr mask and then to the LiNbO₃ sample using Cl₂/O₂ and SF₆/Ar plasma combinations, respectively. The RIE etch time was adjusted to achieve 550 nm-deep features. The etched patterns consisted of 1D parallel microgrooves with 40 µm-spacing and widths varying from 2 to 10 µm, and 2D 10 µm-wide hexagonal pits in a hexagonal array with a period of 31 µm.

Axon alignment to microgrooves was quantified from the F-actin staining images using ImageJ. Multi-step image processing was conducted to exclude the cell body signals. Using the processed image, F-actin staining intensities were measured on the edges of grooves—a pair of 1.3 µm-wide stripes for each groove—and compared with identical stripes immediately adjacent to the groove edges (outside grooves). Two major observations were made: (1) axons typically elongated parallel to the microgrooves, preferentially aligning with their edges, as evidenced by approximately 1.2× higher neurite staining on groove edges relative to other areas (Fig. 3); (2) fine neurites appeared to follow hexagonal pits (Fig. 4). These observations are consistent with the literature, where the tendency of neurites to elongate along edges⁹,¹¹ and channels¹ has been repeatedly demonstrated. Groove or ridge width appears to be the main factor determining the alignment of neurites to topographical features, where 200 nm and 500–1000 nm widths have been shown to be ideal for dorsal root ganglia neurons⁴ and differentiating PC12 cells,³ respectively.

Finally, to test microfluidic integration with microtopography, we bonded a bicompartamental neuron culture device to etched LiNbO₃ substrates via oxygen plasma-mediated surface activation (PE-100 plasma system; PlasmaEtch, Carson City, NV). Fabrication of PDMS (Sylgard 184; Dow Corning, Midland, MI) microfluidic devices was previously...
described. The devices consist of two compartments—somatic and axonal—interconnected by parallel microchannels that permit axonal growth. The “hexagonal pits” pattern was manually aligned with the axonal compartment during bonding such that axons emanating from the microchannels encountered an array of pits. Bonded devices were filled with DI water and sterilized with a 30 min exposure to UV light. Both compartments were incubated with 10 μg mL⁻¹ PLL in DPBS overnight at 37 °C and rinsed with DPBS prior to cell seeding. Neurons were seeded in the somatic compartment with a seeding density of approximately 8 × 10⁵ cells cm⁻². Cells were allowed to attach for 5 min, and subsequently, all wells were filled with the culture medium. Devices were placed in Petri dishes and 1 ml of 0.1% ethylenediaminetetraacetic acid (EDTA; Sigma) in DI water was added to the side of the dish to minimize evaporation during incubation (37 °C, 5% CO₂). Immunocytochemistry in these devices followed the same protocol with minor adaptations as described previously. Substrates that were bonded to microfluidic chips were also recovered and reused. Substrates were first sonicated in detergent solution for 30 min, and the chips were carefully removed by pushing from edges. Following separation, substrates were cleaned using the same protocol as for non-bonded substrates. Any remnant PDMS was scraped off using a scalpel, and the cleaning procedure was repeated. Topographical features did not interfere with device bonding or fluidic isolation, and axons appeared to avoid the pits (Fig. 5). Analysis of axon paths suggested that most axial segments were parallel to or made 30° angles with the direction of microchannels, suggesting that the axons navigated through the hexagonal pits pattern by forming point contacts with the edges of the pits and avoiding the 3D topography.

Overall, this work demonstrates the culture of neurons on uniform LiNbO₃, etched LiNbO₃, and PPLN, and expands on previous studies on the growth of other cell types on uniform LiNbO₃ (Ref. 19) and PPLT. The results point to promising research directions exploiting LiNbO₃ as a neural substrate. Potentially, these can be combined with further control and sensing capabilities afforded by the photonic and piezoelectric properties of this material, already widely used for the nonlinear optical frequency-conversion of conventional lasers and to generate standing and traveling surface acoustic waves for sorting particles or cells in flow channels. Moreover, the topography patterning and microfluidic integration capabilities demonstrated with this work indicate LiNbO₃ as a promising material template for developing highly complex in vitro neuron culture devices, which allow controlling not only the surface topography but also the local chemical microenvironment.

In summary, LiNbO₃ is an emerging neuron culture substrate with optical, electrical, and topographical properties that can be engineered to design complex experimental model systems, potentially capable of stimulating and sensing neurons at the subcellular level in a topographically and chemically defined microenvironment.

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