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Facile micropatterning of dual hydrogel systems for 3D models of neurite outgrowth

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Abstract

Understanding how microenvironmental factors influence neurite growth is important to inform studies in nerve regeneration, plasticity, development, and neurophysiology. *In vitro* models attempting to more accurately mimic the physiological environment by provision of a 3D growth matrix may provide useful foundations. Some limitations of thick 3D culture models include hampered solute transport, less-robust neurite growth than on 2D substrates, and difficulty in achieving spatial control of growth. To this end, we describe a 3D dual hydrogel model for embryonic rat day 15 dorsal root ganglion tissue explant growth using a digital micro-mirror device for dynamic mask projection photolithography. The photolithography method developed allowed simple, reproducible, one-step fabrication of thick hydrogel constructs on a variety of substrates, including permeable cell culture inserts. The relationships between projected mask size, crosslinked hydrogel resolution, and gel thickness were characterized, and resolution was found generally to decrease with increasing gel thickness. Cell viability in thick (481 μ m) hydrogel constructs was significantly greater on permeable supports than glass, suggesting transport limitations were somewhat alleviated. The observed neurite growth was abundant and occurred in a spatially controlled manner throughout the 3D environment, a crucial step in the quest for a more effective biomimetic model of neurite outgrowth.

Keywords

Photolithography; Tissue engineering; Cell culture; Nerve guidance; Poly(ethylene glycol)

Introduction

Axonal path finding during development and repair occurs by integration of a multitude of both chemotactic and haptotactic signals. Exploring the factors mitigating neurite growth and guidance may lead to better understanding of targeting during development and may enable pathologies to be better recognized and addressed. Thus there is a critical need for tunable culture environments in which structural and molecular variables can be studied for advances in understanding axonal responses to environmental factors. In this report, we describe a quick and simple approach enabling spatial control of neurite growth within a 3D matrix as a constrained growth model that may serve as a basis for studying effects of the microenvironment on neurite growth.

Tissue culture neurite outgrowth experiments have been crucial tools for studying neurite growth responses to their microenvironment. Culture models with 2D growth configurations have shown the influence of chemotaxis,^{1–4} protein gradients,^{1,2} topography,^{3–6} and

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preferences for specific cell adhesion ligands over others.⁷ More recently, researchers have increasingly been turning toward 3D models that may more accurately mimic the physiological environment.^{8,9} Indeed, cells cultured in 3D matrices have shown distinct differences in cellular characteristics such as gene expression¹⁰ and morphology¹¹ compared to identical cells examined in 2D cultures. Neuronal cells cultured in 3D have also exhibited more biomimetic behaviors in terms of both cytoarchitecture and electrophysiological characteristics such as resting membrane potentials, calcium dynamics, action potential propagation, and voltage-gated ion channel functionality.^{12,13}

While there is a distinct advantage in studying neurite growth in 3D models, suitable materials and reproducible methods for tuning them as neurite growth matrices are still being developed.¹⁴ Several naturally-derived hydrogels, including agarose¹⁵ and hyaluronic acid,¹⁶ have been found to be effective for the culture of neurons and glia and promotion of neurite growth, as have some synthetic polymers, such as self-assembling peptide gels,¹⁷ and ionically-charged hydrogels.¹⁸ A number of methods to tailor the structural microenvironment of hydrogels as culture environments with distinct geometries are being developed, and have been recently reviewed.^{19,20} Such techniques include the use of microprinted photomasks,²¹ etched silicon masters,²² elastomeric stamps,²³ laser ablation,²⁴ and dynamic mask microstereolithography.²⁵ The ability to functionalize hydrogels with biomolecules is another important facet of the microenvironment to control. Functionalized 3D matrices have shown that axons respond differently to soluble versus immobilized factors and growth tends to depend more heavily on the magnitude of the gradient than on absolute concentration.^{26,27} Highly specific, spatial control of chemical modification has been demonstrated using multiphoton excitation^{28,29} and photolabile deprotection for biomolecule coupling.³⁰

Despite the potential of 3D neural culture systems for studying neurite growth, they are not without limitations. First, nutrient and waste transport can become problematic due to diffusion limitations in thick 3D gels. Also, uniform 3D environments do not allow for spatial control of growth, which can lead to random neurite outgrowth and make assessment difficult due to variability. Some 3D hydrogels could also hinder growth cone extension due to a lack of attachment ligands or an inability of cells to remodel the matrix. Some of these limitations may be responsible for an observed deficiency in neurite extension within hydrogels, which is not as extensive as on 2D substrates.³¹ Additionally, complications arising from imaging spatially unbound neurite growth through thick gels often make visualization of neurites difficult.

We sought a neurite outgrowth culture model that would combine a biomimetic 3D microenvironment with the spatial control of growth seen in 2D models. Towards this end, we adapted a “dynamic mask” projection photolithography technique for micropatterning photocrosslinkable hydrogels in thick configurations that can contain self-assembling hydrogels in specifiable geometric configurations. We use poly(ethylene glycol) dimethacrylate (PEG), a photocrosslinkable hydrogel which inherently inhibits cell adhesion and infiltration,^{32,33} together with Puramatrix, a synthetic peptide hydrogel, which has shown a propensity for cell migration and extensive neurite outgrowth.^{17,34} Using the dual hydrogel approach, we demonstrated simple and repeatable fabrication of thick micropatterned constructs that promoted robust neurite growth from tissue explants in a spatially defined manner that also minimized some limitations previously observed in 3D culture models.

Materials and Methods

Dynamic Mask Projection Photolithography

Hydrogel micropatterns were formed via projection photolithography in a manner similar to previous reports which used a digital micro-mirror device (DMD) for layer-by-layer microstereolithography.^{25,35} A DMD development kit (DiscoveryTM 3000, Texas Instruments, Dallas, TX) with USB computer interface (ALP3Basic) served as a dynamic mask by converting digital black and white images to micro-mirror patterns on the DMD array, in which individual mirrors may be turned “on” or “off” by rotating the angle of reflection from +12° to -12°, respectively. Ultraviolet (UV) light (filtered at 320–500 nm) from an OmniCure 1000 (EXFO, Quebec, Canada) Hg vapor light source was collimated with an adjustable collimating adapter (EXFO) and projected onto the DMD array. The reflected light was projected through a 4x Plan Fluor objective lens (Nikon Instruments, Melville, NY) with numerical aperture 0.13 and focused directly onto a photocrosslinkable hydrogel solution (Figure 1A). The iris of the UV light source was adjusted to maintain an irradiance output of 5.0 Watts/cm² as measured with a radiometer (EXFO). Hydrogel solutions were cured for approximately 55 seconds, inducing crosslinking through free radical chain reaction. Unlike the reports cited above, our method initiated crosslinking throughout the bulk with a single irradiation, negating the need for a layer by layer approach.

Formation of Dual Hydrogel Constructs

Hydrogel polymerization was performed as previously described for dynamic mask projection photolithography.³⁶ The photocrosslinkable solution was made by diluting poly(ethylene glycol) dimethacrylate (PEG) with average molecular weight (MW) 1000 Da (Polysciences Inc., Warrington, PA) to 10% (w/v) in either PBS or growth medium with 0.5% (w/v) Irgacure 2959 (I-2959) (Ciba Specialty Chemicals, Basel, Switzerland) as a photoinitiator. The concentration and molecular weight of PEG was chosen, based on previously published data, to both minimize cell adhesion and maximize hydrogel adherence to the polymerization surface.³³ Micropatterned PEG constructs were crosslinked directly onto one of three types permeable cell culture inserts: polyester, polycarbonate and collagen-coated PTFE Transwell® Permeable Supports (Corning Inc., Corning, NY) with 24 mm diameter membranes and 0.4 µm pores. Inner walls of the culture inserts, not the membranes themselves, were treated with Rain-X® (SOPUS Products, Houston, TX) to reduce meniscus effect of PEG solution. Each support was placed on the stage of an inverted microscope positioned directly below the lithography projection lens. After crosslinking, supports were rinsed, removing excess uncrosslinked PEG solution, and the micropatterned PEG remained attached to the surface. Hydration of PEG gel was maintained in buffered saline solution (4°C) if not used immediately.

A self-assembling peptide gel, Puramatrix (BD Biosciences, Bedford, MA), was diluted to 0.15% (w/v) in deionized H₂O prior to use, and was supplemented pre-gelation with 1 µg/mL soluble laminin (Invitrogen, Carlsbad, CA) when used for neurite outgrowth experiments. Both the concentration of Puramatrix and the addition of laminin were according to manufacturer's instructions for neural application. Using a pipette, this solution was carefully added to voids within the micropatterned PEG hydrogels. Contact with salt solution hydrating the PEG gel induced self-assembly of the Puramatrix, which remained confined within the PEG geometry. Puramatrix gelation was maintained by incubating at 37°C and 5% CO₂.

Hydrogel Adherence and Stability

Adherence of PEG hydrogels to cell culture inserts was assessed daily. PEG constructs were polymerized as described previously, and any PEG rectangles which detached from the substrate were counted as non-adherent. To assess the stability of Puramatrix gel formed within PEG, nine PEG constructs were crosslinked on each of four polyester inserts, and 1.75×10^7 beads/mL of fluospheres (Molecular Probes Inc., Eugene, OR) were added to Puramatrix prior to gelation within PEG voids. Inserts were placed in well plates, half of which were filled with 2 mL of PBS, sufficient to hydrate but not submerge the dual hydrogel constructs, while the other half were filled with 3 mL, fully submerging the constructs. Fluorescence microscopy was used to determine whether the Puramatrix remained inside the PEG constructs after 48 hours (Figure 2).

Hydrogel Thickness and Feature Size

Volumes of 350, 400, 450, 500 and 550 μ L of 10% PEG solution were added to polyester culture inserts, and four rectangular patterns at each volume were used to determine the relationship between volume and crosslinked PEG height. Samples were stained with Texas Red (Invitrogen) and thickness was measured using confocal imaging. To determine the minimum PEG feature size achievable, a series of both circular and rectangular photomasks (Figure 3B, C) were used to form patterned voids in PEG solutions over a range of thicknesses. The smallest size void achievable at each thickness was determined by decreasing the size of the mask until the void was not visible after crosslinking. Six samples of each shape were used to determine the average minimum void size at which point 100% of voids had formed. Circular void diameters were measured in the x and y directions, with rectangular void widths taken at the center and alternating left or right edges.

Tissue Harvesting and Culture

NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed. Embryonic day 15 (E-15) pups were removed from timed-pregnant Long Evans rats (Charles River, Wilmington, MA) and placed in Hank's Balanced Salt Solution. Spinal columns were isolated from embryos, from which dorsal root ganglia (DRG) were harvested and placed in Neurobasal Medium supplemented with nerve growth factor (NGF), 10% fetal bovine serum (FBS) and penicillin/streptomycin (P/S) (Invitrogen) to promote adhesion. After isolation, DRGs were placed on collagen-coated cell culture inserts and maintained in an incubator at 37°C and 5% CO₂ with B-27 and L-glutamine replacing FBS for growth medium.

Primary DRG neurons were obtained through dissociation of DRGs by trypsinization and trituration, followed by the subsequent removal of supportive cells utilizing fluorodeoxyuridine and uridine (3 day treatment). Cells were then suspended in Puramatrix according to the manufacturer's protocol at a concentration of 3×10^5 cells/mL. The cell suspension was added, at a volume sufficient to obtain ~480 μ m thick hydrogels, to either 24 well cell culture inserts or 24 well tissue culture plates (Corning), and self-assembly was initiated upon addition of growth medium (n=4). The constructs were incubated for 48 hours before testing cell viability.

Neurite Outgrowth in Dual Hydrogels

Collagen-coated PTFE cell culture inserts were soaked overnight in adhesion medium to hydrate the membrane. Four DRGs were then placed on the surface of an insert and allowed to adhere for two hours before the medium was replaced with 500 μ L of 10% PEG in growth medium as described above, without FBS. This volume could be adjusted to vary the thickness of the PEG constructs. The DMD was illuminated with a visible light source to aid

alignment of the projected mask with each adhered DRG. The visible light source was then replaced by the UV source and the PEG hydrogel crosslinked around the tissue explant. DRG-containing PEG constructs were washed 3 times with PBS to remove any uncrosslinked PEG solution. When applicable, modified Puramatrix was added to the void inside the PEG, and, in order to induce Puramatrix self-assembly, 1.5 mL of growth media was introduced beneath the insert. Constructs referred to as without Puramatrix were made as described above except without the addition of Puramatrix, thereby restricting DRGs to the 2D environment of the collagen-coated PTFE membrane. Constructs were maintained in an incubator at 37°C and 5% CO₂ for 7 days, and medium was changed after the 2nd and 5th days.

Constructs were prepared and visualized for morphology, viability, neurite outgrowth, and containment. If no neurites were visualized growing on or outside the PEG void, the sample was considered to have contained growth. This was done in identical PEG constructs both with and without Puramatrix added, and twelve trials for each condition were attempted for the five different PEG heights described previously. Trials were thrown out in cases of incomplete PEG polymerization or lack of DRG adhesion.

Specimen Preparation and Visualization

Live specimens were evaluated for viability with a Live/Dead® assay (Invitrogen) per manufacturer's instructions. For cell suspensions in Puramatrix, wide field fluorescent images were captured at multiple focal planes throughout the depth of the gel in 3 different areas of each hydrogel specimen. Standard deviation projections were then analyzed for cell viability in both cell culture inserts and tissue culture plates by counting calcein AM (live marker) and ethidium homodimer-1 (dead marker), giving a total of 12 samples per condition. Specimens evaluated with immunohistochemistry were fixed in 4% paraformaldehyde for 2 hours. Cell nuclei were stained with DAPI Nucleic Acid Stain (Molecular Probes) per manufacturer's instructions. Neurites were stained using mouse monoclonal [2G10] to neuron specific β III tubulin primary antibody and goat-anti-mouse IgG- H & L (CY2) secondary antibody, and dendrite staining was carried out using rabbit polyclonal to MAP2 primary antibody and donkey-anti-rabbit IgG Dylight 594 secondary antibody (AbCam, Cambridge, MA). Each step was carried out in PBS with 0.1% Saponin and 0.2% BSA overnight followed by 3 washes in PBS with 0.1% Saponin. Bright field and conventional fluorescent images were acquired with a Nikon AZ100 stereo zoom microscope (Nikon, Melville, NY) equipped with fluorescence cubes, while confocal images were acquired using a Zeiss LSM 510 Meta microscope (Zeiss, Oberkochen, Germany). Average depths of β III labeled structures were calculated from confocal images to measure the distance between the first and last focal plane containing fluorescence (n=7). Image processing was performed with Image J (National Institutes of Health, Bethesda, MD) and V3D software (Howard Hughes Medical Institute, Ashburn, VA), used to visualize confocal image stacks in 3D³⁷. The proportion of neurite growth over the depth of the gel was quantified from pixel counts of manually thresholded confocal slices. Confocal slices were binned in 10% increments of total depth, and the measured fluorescence of binned slices was compared to the total measured for each Z-stack to give the proportion of neurite growth throughout the depth of the construct (n=3). The VolumeJ plugin was used to create depth coded Z-stack projection of neurite growth. Confocal z-stacks were acquired through the maximum depth of visible neurite growth (186 μ m) with 3.0 μ m thick slices (1024×1024×63) for both Puramatrix and non-Puramatrix containing constructs. The Z Code Stack function with spectrum depth coding LUT was used to add color, and the stacks were merged using a standard deviation z projection. Lastly, Z-stacks were despeckled to remove background noise. Cryogenic scanning electron microscopy (Cryo-SEM) was performed by freezing specimens in slushed liquid nitrogen and imaging with a Hitachi

S4800 Field Emission SEM (Hitachi, Krefeld, Germany) and Gatan Alto 2500 Cryo System (Gatan, Warrendale, PA) at 3kV and -130°C .

Statistics

Statistical analysis was performed using a student's t-test of equal variances. Statistically significant differences were determined using the requirement that $p < 0.05$. Equality of variance was calculated using Levene's test ($p < 0.05$). Unless otherwise specified, data are presented as mean $\pm$ standard deviation.

Results

Dual Hydrogel Construct Formation

Dynamic mask projection photolithography reproducibly produced thick hydrogel micropatterns, ~ 200 to $500\text{ }\mu\text{m}$, with specifiable geometries in a simple, one-step procedure. To illustrate the versatility of this approach, a number of thick hydrogel constructs of varying sizes and shapes were crosslinked one after another directly on permeable cell culture inserts in less than one minute with 5.0 Watts/cm^2 incident on the DMD, as shown in Figure 1B and C. Crosslinked hydrogels showed some loss of fidelity as compared to the original mask, which is evident in Figure 1D and E, in which corners became rounded, slightly changing proportions of the micropatterns formed. This effect, as well as the micropattern resolution achievable, was found to depend heavily on hydrogel thickness. These relationships between photomask size, hydrogel feature size, and thickness are summarized in Table 1 and further discussed in Section 3.3.

Gelation of Puramatrix occurred upon injection of Puramatrix solution into the voids of PEG hydrogels hydrated with buffered salt solutions. This *in situ* gelation was confirmed by addition of fluorescent microparticles (Invitrogen) to the Puramatrix solution before injection into the PEG voids. When suspended in Puramatrix and injected into PEG hydrogel structures, both of which were devoid of salt to prevent peptide self-assembly, microparticles were observed to disperse. In contrast, as shown in Figure 2, microparticles remained confined to PEG structures when injected in the presence of salt solution, suggesting peptide self-assembly and gel formation took place *in situ* upon contact with salt solution, as reported for this type of gel.¹⁷ Figure 2 also demonstrates the gel appeared to contract upon gelation, slightly pulling away from the borders of the PEG gel. This sometimes led to the complete detachment of the Puramatrix gel from the PEG gel, but, as discussed in the following section, Puramatrix remained inside of the PEG given correct conditions.

Hydrogel Adherence and Stability

PEG hydrogel constructs remained adherent to both polyester and collagen treated PTFE inserts throughout the length of our experiments. In fact, the hydrogel constructs adhered throughout the length of a three week test (data not shown). These results are consistent with those seen by Moeller, et al.³³

Puramatrix containing fluorescent microspheres was injected into the micropatterned voids of PEG structures crosslinked onto permeable culture inserts and allowed to gel via self-assembly. When the inserts were placed in the wells of a 6-well plate, Puramatrix remained in 100% of the constructs throughout the 48 hour experiment when hydrated with a volume (2 mL) of PBS insufficient to completely submerge the constructs, as confirmed in Figure 2. However, Puramatrix frequently detached from within the PEG structures when completely submerged in PBS (3 mL): $13 \pm 13\%$ remained after the initial equilibrium, and only $10 \pm 11\%$ of the constructs still contained Puramatrix after 48 hours (Figure 2). A statistically

significant difference in detachment existed between the two volumes both immediately and after 48 hours.

Hydrogel Thickness and Feature Size

The minimum void size achievable over a range of PEG thicknesses was characterized by crosslinking both circular and rectangular voids within different volumes of PEG solution added to culture inserts. PEG gels were stained and imaged with confocal microscopy to determine the relationship between the volume of PEG solution added and resulting crosslinked gel thickness. Volumes of 350, 400, 450, 500, and 550 μL of 10% PEG solution added to polyester culture inserts resulted in crosslinked structures with the following average thicknesses, respectively: $233 \pm 24 \mu\text{m}$, $368 \pm 32 \mu\text{m}$, $433 \pm 19 \mu\text{m}$, $481 \pm 14 \mu\text{m}$ and $543 \pm 9 \mu\text{m}$. The minimum void size achievable at each thickness was taken to be the size for which 100% of specimens formed a measurable void. The average widths of these voids for each of the five experimental thicknesses of PEG described above were obtained for both circular and rectangular voids. As shown in Table 1, the smallest features formed were circular voids $62 \pm 6 \mu\text{m}$ in diameter and rectangular voids $85 \pm 4 \mu\text{m}$ wide, both at a thickness of $233 \pm 24 \mu\text{m}$. For both circular and rectangular voids, as the thickness of PEG increased, the minimum feature size also increased, representing a decrease in resolution (Table 1). The trend was similar for both types of void features, but it may have been more linear for rectangular channels than for circular wells (Figure 3A). In all cases, a pronounced beveling effect was apparent due to the nature of the optics used. As seen in Figure 3D and E, the void size was smallest at the membrane surface, where the light is focused, and widened toward the top of the gel.

Cell Viability

Cell viability in $\sim 480 \mu\text{m}$ thick Puramatrix hydrogels was evaluated in both cell culture inserts and tissue culture plates. As shown in Figure 4, the average proportion of live cells was $89.4 \pm 4.0 \%$ when cultured in permeable inserts, but only $83.5 \pm 3.4 \%$ in rigid plates, representing a statistically significant difference ($p < 0.001$).

Neurite Outgrowth in Dual Hydrogels

The PEG thickness necessary to constrain neurite growth was investigated by culturing DRG explants in constructs with increasing thicknesses. Containment was measured here because it was crucial to the ability of our system to function reliably as an in-vitro model. Impartial polymerization frequently occurred with $233 \mu\text{m}$ thick PEG, leading to unusable constructs. Additionally, throughout the polymerization process, some DRG detached from the surface of the membrane, leading to a lower than expected number of trials for analysis. For the constructs containing Puramatrix, a distinct increase in the containment of neurites was seen as gel thickness increased (Table 2). At a thickness of $233 \mu\text{m}$, no constructs limited the growth of neurites. The rates of containment for the subsequent heights of 368 , 433 , 481 and $543 \mu\text{m}$ were: 10%, 22.2%, 63.6% and 87.5. Overall, higher percentages of containment were seen in constructs lacking Puramatrix. Neurites appeared able to grow over the sloping PEG walls at certain thicknesses in both groups (Figure 5A, B), but in constructs without Puramatrix there was more efficient containment at similar heights (other than $534 \mu\text{m}$) and more effective containment by a lower height than in PEG with Puramatrix (Table 2).

In an effort to balance void size resolution and pattern fidelity with neurite containment, subsequent neurite growth experiments were carried out in constructs with an average PEG thickness of $481 \mu\text{m}$ ($500 \mu\text{L}$ solution). Constructs monitored for cell viability after 5 days showed an overwhelming amount of live cells, with an extremely small portion of dead cells located in the DRG itself (Figure 6A). After fixation and staining at 7 days, neurites and

migrating cells were constrained by the geometry of the PEG hydrogel, as suggested by dual labeling with β III tubulin and DAPI (Figure 6B). Neurite outgrowth was consistently robust and all labeled structures were concentrated inside the Puramatrix portion of the dual hydrogel (Figure 6). Additionally, MAP2 antibody labeling suggests that a substantial portion of the neurite growth in the constructs appeared to be dendritic (Figure 6E). Growth appeared to occur first along the boundary between the two gels, as is evident in Figure 6A, in agreement with reports of similar preferential growth between the interfaces of two materials.^{31,38} However, behind the neurites extending along the channel, growth was seen filling in the inner space between the PEG (Figure 6A). Images of leading neurite growth in the 3D bulk of the Puramatrix showed a tendency to grow in random directions (Figure 6D). In direct contrast, we found that neurites growing along the surface of the cell culture insert oriented themselves obliquely, apparently closely following the fibers of the insert membrane (Figure 6F). Outgrowth was observed to fan out at the bifurcation point with no apparent preference in direction. A considerable amount of branching and fasciculation was observed, which was especially apparent at the leading edge of growth (Figure 6D). By approximately seven days, outgrowth was seen throughout the length of the channels. Significantly more growth was observed in Puramatrix filled constructs, as compared to the apparently abortive limited growth seen in constructs without Puramatrix (Figure 7B, C). Confocal imaging confirmed that neurite growth occurred in three dimensions, as shown in Figure 7. The average thickness of β III labeled structures was $159.8 \pm 23.9 \mu\text{m}$ thick in Puramatrix-containing constructs, while the average thickness in constructs without Puramatrix was $85.4 \pm 38.6 \mu\text{m}$, a difference which was found to be statistically significant (Figure 8A, $p < 0.005$). Figure 7D represents an example of growth in a construct lacking Puramatrix, where growth appeared crowded and neurites grew to a maximum height of $54.0 \mu\text{m}$. Neurite growth in constructs without Puramatrix was visualized growing along the membrane of the collagen-coated PTFE, with no growth occurring in the PEG itself. Alternatively, Figure 7E demonstrates neurite growth in a dual hydrogel construct, with notably less neurite crowding observed, and individual neurites growing through Puramatrix in multiple focal planes, reaching a maximum height of $120.0 \mu\text{m}$. Figure 8B further demonstrates that neurite growth was not confined to either the membrane or the top surface of the Puramatrix, as $7.3 \pm 2.9 \%$ and $4.9 \pm 1.3 \%$ of total growth was seen in the bottom and top 10% of the slices, respectively. Unlike the neurites, DAPI staining indicated migrating cells were not influenced to migrate into Puramatrix, remaining confined near the support surface (Figure 7A), although previous research suggests that glial cell migration and neurite growth often occur together.^{30,39}

Discussion

Understanding how microenvironmental factors influence neurite growth is important to inform studies in nerve regeneration, plasticity, development, and neurophysiology. Neurite growth within 3D culture models may more accurately represent neural growth *in vivo*. While 3D culture environments may represent a more biomimetic approach, implementation may be hampered by transport limitations and a lack of spatial control of growth. The 3D dual hydrogel system described in this paper was found to be simple, reproducible, and highly effective for constraining and spatially controlling neurite outgrowth from embryonic tissue explants. This approach could be useful as part of an overall strategy to study microenvironmental effects on neurite extension *in vitro*.

Our approach to photolithography proved beneficial due to the simplicity of the design and the versatility of fabrication. The use of a DMD eliminated the need to fabricate masks, allowing instead for the use of simple, adaptable black-and-white images. The UV exposure could be adjusted, if necessary, to influence crosslinking kinetics or address phototoxicity issues. Through the use of dynamic photomasks and projection optics, there was no need for

a mask to be in direct contact with the polymerization medium, allowing production of thick hydrogel structures, fully exposed to air, in a single step with flexibility in choosing the polymerization surface. The use of cell culture inserts also allowed us to overcome some of the transportation limitations for thick explant cultures (discussed further below), as well as permitting crosslinking around previously adhered DRG explants.

The feature sizes achieved by our setup were comparable to, or even showed improvement over, previously published work, even with thicker hydrogels ($>300\text{ }\mu\text{m}$).^{21,25} Additionally, with still thicker gel depths, $>500\text{ }\mu\text{m}$, feature sizes were sufficient for our neurite growth models. As demonstrated by Lu and colleagues,²⁵ employing a layer-by-layer approach produced intricate geometries of PEG structures with feature sizes near the order of $50\text{ }\mu\text{m}$. Though our feature sizes were slightly larger, by negating a layer-by-layer approach we decreased the time necessary for total polymerization, an important consideration when incorporating cells or tissue explants. Beebe, et al. describe a photolithography method providing comparable feature sizes with spacing of $\sim 100\text{ }\mu\text{m}$ obtainable, given sequential polymerization.²¹ However, we improved upon these results, as we were able to simultaneously polymerize our shapes, where they saw partial polymerization between objects with a spacing of $<250\text{ }\mu\text{m}$. Finally, while the DMD development kit may be specialized and costly, our use of a standard microscope objective suggests that a commercially available DMD-microscope could be employed, which may make this approach to micropatterning attractive and readily adaptable.

The two commercial hydrogels we employed were effective for neurite growth, and our model could be easily adapted to include other hydrogels. Crosslinked PEG consistently adhered to the permeable inserts, likely because the prepolymer liquid seeped into pores of the transparent membranes, with physical adhesion occurring upon polymerization. At a 0.15% concentration, Puramatrix alone is mechanically unstable, and it is barely more viscous than water prior to self-assembly via beta sheet formation,¹⁷ so it requires carefully controlled conditions to be effective. The PEG hydrogel on a permeable support served as a structural barrier to contain and shape Puramatrix as well as protect peptide beta sheets from breaking apart. Use of permeable cell culture inserts in our dual hydrogel model also served to improve cell viability as seen in the increased numbers of live cells in Figure 4 and the proportion of viable cells in Figure 6A, suggesting transport limitations associated with thick constructs required to contain neurite growth were likely alleviated due to oxygen diffusion through the gels from below *as well* as the sides and top. The viability experiment supports the advantage of using cell culture inserts over tissue culture plates as the polymerization surface for 3D constructs.

We believe that our methods represent a more biomimetic manner in which to create 3D constructs for the study of neurite growth. Comparison of Puramatrix functionality as a 3D environment for cell encapsulation has been previously demonstrated with other popular hydrogels.⁴⁰ Agarose has been frequently used as a 3D matrices to investigate neurite growth, although modification of agarose is necessary to obtain significant axon extension.¹⁵ Matrigel has shown promise as a 3D matrix for neural growth and neuronal-astrocyte co-cultures,¹³ but the presence of incompletely defined ECM proteins and cytokines in Matrigel may complicate studies of molecular neurite guidance. Puramatrix has previously been shown to support neurite growth and co-cultures with or without additional components. In one recent advance, Xu, et al. describe another method in which to print 3D neural sheets up to $\sim 1\text{mm}$ thick.⁴¹ However, none of the previous examples of 3D neural cultures exhibit directed growth, thereby negating any sort of spatial control and limiting study of axonal projections. We believe that our presentation of the peptide gel within a micropatterned construct represents a novel way to promote growth in a 3D environment within a constrained geometry. Our approach incorporates ease of use, fabricated spatial

control of abundant neurite growth, along with the ability to produce a “blank slate” scaffold, allowing for the selective incorporation of growth factors to inform future studies of neurite growth and guidance.

The approach we describe, though effective for its intended purposes, was not without some limitations. First, the minimum attainable feature size was limited by the resolution of our system and was observed to decrease with increasing PEG thickness. Similar to observations described by Beebe, et al. with beveling in deep channels (150–300 μm),²¹ we noticed that PEG voids narrowed throughout their depth (Figure 3D, E). This phenomenon would not be problematic except for the possibility that it enables neurite growth, presumably due to the adsorption of proteins from the medium to the PEG surface, over the sloped sides of the voids, limiting containment. If necessary, these effects could perhaps be overcome by choosing optics with lower numerical aperture or higher UV transmission, or by adjusting the focus to reverse the direction of the beveling. Second, while we hypothesized that Puramatrix gelation *in situ* would lead to physical entanglement of the two gels, the Puramatrix was frequently observed to detach from the PEG void, even while maintaining its shape (Figure 2). Maintenance of the height of the media combined with careful handling avoided detachment, although this may not be ideal. Another possible solution could be choosing crosslinked gels with larger mesh sizes to encourage entanglement or using photocrosslinkable semi interpenetrating networks,^{42,43} rather than two different hydrogels. Finally, staining for β III tubulin revealed growth did not occur throughout the entire height of the PEG constructs. It is likely that the depth of Puramatrix is considerably less than that of PEG because of the minimal volume ($<1 \mu\text{L}$) added to prevent Puramatrix overflowing the PEG void, as well as contraction during the self-assembly process. Production of thicker PEG constructs could increase the depth of neurite growth, though this would inherently further limit the attainable feature size.

We chose a bifurcating pathway suggestive of nerve growth, and future studies will take advantage of this design by allowing us to explore axon guidance molecules and analyze the growth pattern at a divergent point in the growth process. The model was relatively simple in regards to providing specific structural guidance cues, namely the presentation of a cell permissive region, surrounded by a cell restrictive border, facilitating neurite containment; future work will merge the ability to physically constrain growth with molecular signaling. More specific to the findings reported herein, we also hope to further investigate the migration of supportive cells, the fasciculation of neurites and the phenomenon of preferential growth along the hydrogel borders. The ultimate informative value of our dual hydrogel system may lie in maximizing and directing neurite growth using combinations of biomimetic signaling.

Conclusions

The dual hydrogel model laid out in this paper was effective for promoting neurite outgrowth in a spatially defined manner within a 3D environment. Using a DMD for dynamic mask projection photolithography, we were able to fabricate thick hydrogel constructs quickly, easily, and on a variety of support substrates. Photocrosslinked hydrogels consistently adhered to permeable supports, and the self-assembling peptide gel formed within the space provided. The two hydrogels remained stable and intact in culture conditions for the duration of the culture period, though care in handling was required to prevent separation of the gels. The relationships between projected mask size, crosslinked hydrogel feature size, and gel thickness were characterized, and resolution found generally to decrease with increasing gel thickness. Cell viability in thick hydrogel constructs was significantly greater on permeable supports than glass supports, suggesting that solute transport limitations were at least partially alleviated. The thick constructs were effective for

containing neurite growth from tissue explants. Neurite growth was robust and occurred in a spatially controlled manner throughout the 3D environment of the Puramatrix, a crucial observation in the quest for a more effective biomimetic model of neurite outgrowth.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Table 1

Comparison between projected mask size and minimum void size formed at thicknesses tested.

Volume (μL)	Thickness (μm)	Projected UV Size (μm)		Minimum Feature Size (μm)		% of Projection	
		Circle	Channel	Circle	Channel	Circle	Channel
350	233 ± 24	220	130	62 ± 6	85 ± 4	28% ± 3	65% ± 3
400	368 ± 32	290	160	92 ± 5	118 ± 5	32% ± 2	73% ± 3
450	433 ± 19	360	190	145 ± 11	141 ± 4	40% ± 3	74% ± 2
500	481 ± 14	400	230	174 ± 10	159 ± 5	44% ± 2	69% ± 2
550	543 ± 9	430	290	206 ± 12	190 ± 7	48% ± 2	65% ± 3

Table 2

Neurite growth containment as a function of hydrogel thickness.

	Volume (μL)	Thickness (μm)	n	% Contained
Puramatrix	350	233 ± 24	6	0.0%
	400	368 ± 32	10	10.0%
	450	433 ± 19	9	22.2%
	500	481 ± 14	11	63.6%
	550	543 ± 9	8	87.5%
Non-Puramatrix	350	233 ± 24	5	40.0%
	400	368 ± 32	10	30.0%
	450	433 ± 19	6	83.3%
	500	481 ± 14	10	90.0%
	550	543 ± 9	8	87.5%