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Anna Linnenberger, Robert R. McLeod, Tamara Basta, Michael H. B. Stowell, "Three dimensional living neural networks," Proc. SPIE 9548, Optical Trapping and Optical Micromanipulation XII, 95481O (28 August 2015); doi: 10.1117/12.2191406

SPIE.

Event: SPIE Nanoscience + Engineering, 2015, San Diego, California, United States

3D Living Neural Networks

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ABSTRACT

We investigate holographic optical tweezing combined with step-and-repeat maskless projection micro-stereolithography for fine control of 3D positioning of living cells within a 3D microstructured hydrogel grid. Samples were fabricated using three different cell lines; PC12, NT2/D1 and iPSC. PC12 cells are a rat cell line capable of differentiation into neuron-like cells NT2/D1 cells are a human cell line that exhibit biochemical and developmental properties similar to that of an early embryo and when exposed to retinoic acid the cells differentiate into human neurons useful for studies of human neurological disease. Finally induced pluripotent stem cells (iPSC) were utilized with the goal of future studies of neural networks fabricated from human iPSC derived neurons. Cells are positioned in the monomer solution with holographic optical tweezers at 1064 nm and then are encapsulated by photopolymerization of polyethylene glycol (PEG) hydrogels formed by thiol-ene photo-click chemistry via projection of a 512x512 spatial light modulator (SLM) illuminated at 405 nm. Fabricated samples are incubated in differentiation media such that cells cease to divide and begin to form axons or axon-like structures. By controlling the position of the cells within the encapsulating hydrogel structure the formation of the neural circuits is controlled. The samples fabricated with this system are a useful model for future studies of neural circuit formation, neurological disease, cellular communication, plasticity, and repair mechanisms.

Keywords: Tissue Engineering, Optical Tweezing, spatial light modulators (SLMs), micro-stereolithography

1. INTRODUCTION

Crosslinked polymers are often utilized to create synthetic hydrogel scaffolds for tissue engineering by encapsulating living cells that can then be studied to answer fundamental biological questions as well as to fabricate regenerative medical implants. Current approaches for fabricating these hydrogel constructs do not provide simultaneous 3D control of polymer structure and 3D placement of cells. 2D patterning of cells is commonly accomplished by lithographic deposition of an extra-cellular protein, for example poly-L-lysine is used to direct neuronal cell attachment and growth on planar electrode arrays [1,2,3]. However, 2D structures do not represent the actual 3D environment of living tissue and thus are limited in suitability for many basic biological studies and tissue engineering therapies. Do to this limitation, a variety of methods to construct complex 3D tissue scaffolds are under study, most derived from 3D printing [4]. While these methods result in 3D structures that define scaffold shape, they provide no control over the internal distribution of cells within the scaffold. The random distribution of cells limits the repeatability of studies and makes precise experiments involving cell-cell interaction impossible in a 3D scaffold environment. Alternatively, optical tweezing has been used to precisely arrange living bacteria in a polymerizable solution [5]. However, the depth of a high-NA optical tweezing system is limited by aberrations [6] to approximately ± 20 μm and thus does not provide true 3D structuring of cells.

Optical Trapping and Optical Micromanipulation XII, edited by Kishan Dholakia, Gabriel C. Spalding
Proc. of Vol. 9548, 95481O · © 2015 SPIE · CCC code: 0277-786X/15/$18 · doi: 10.1117/12.2191406

In this article, we investigate the use of holographic optical tweezing, to precisely arrange cells within layers, in combination with projection micro-stereolithography, to fabricate multilayered 3D structures encapsulating these cells. Cells are first arranged by optical traps in a liquid monomer mixture which is then locally crosslinked into a hydrogel by photopolymerization to permanently entrap the cells. The sample is translated laterally and the process is repeated to pattern arbitrarily large *x,y* dimensions. The finished layer is then moved away from the cover slip, causing inflow of fresh cells and monomer which will be formed into the next layer. Live cell lithography [5] thus provides micron-scale 3D control of both cellular distribution and gel structure to enable new forms of engineered live cell tissue scaffolds [7].

The goal of this research is to demonstrate the ability to precisely fabricate 3D living neural networks by controlling the position of the cells in the scaffold, and the channels available to guide growth. The long term goal is to utilize the platform for studies of neurological disease. Genome editing methods can be employed to produce CRISPR/CAS edited human induced pluripotent stem cell lines that can be used to produce optogenetically controlled human neuronal networks with well-defined disease states and/or genetic mutations. By utilizing the fabrication platform to precisely control interconnected networks of healthy and disease mutated cells we will be able to ask questions that are complementary to current research of neurological disease within animal models. However, our approach offers a greater level of control for studies of alterations to the physical structure or firing patterns of diseased and healthy neural circuits, and use of human iPSC derived neurons is advantageous for studies of human neurological disease.

2. FABRICATION SYSTEM

The fabrication system has been previously described [8]. In short, the holographic optical tweezing instrument (Cube, Meadowlark Optics) is shown in Fig. 1 [9]. Diffraction limited optical traps are formed using a 10 watt 1064 nm fiber laser (YLR-5-1064-LP, IPG Photonics) which illuminates a mirrored 512x512 pixel phase-only liquid crystal SLM (PDM512-1064 SLM, Meadowlark Optics) through a Keplerian beam expander. The phase patterns on the SLM are computed using the Lenses and Gratings algorithm [10,11] programmed in OpenGL and running on a graphics co-processor (Quadro FX 5600, nVidia). The SLM is imaged to the back focal plane of the 1.35 NA oil immersion microscope objective (UAPON 40XO340, Olympus) through a 4F imaging system. The field of view of the microscope objective is 550 μm, but the intermediate image of the sample is magnified such that the sample area and the area over which the SLM can efficiently optically trap are more closely matched. Thus, the microscope images 240 μm x 180 μm within the sample. The objective has at least 70% transmission from 405 nm to 1064 nm, making it possible to optically trap, image, and photopolymerize the sample in three distinct wavelengths simultaneously. This system is capable of simultaneously trapping up to 400 objects within a volume whose depth is limited by aberrations to approximately ±20 μm [5] without time sharing, in addition to correcting for aberrations as a result of the SLM and optical train [9].

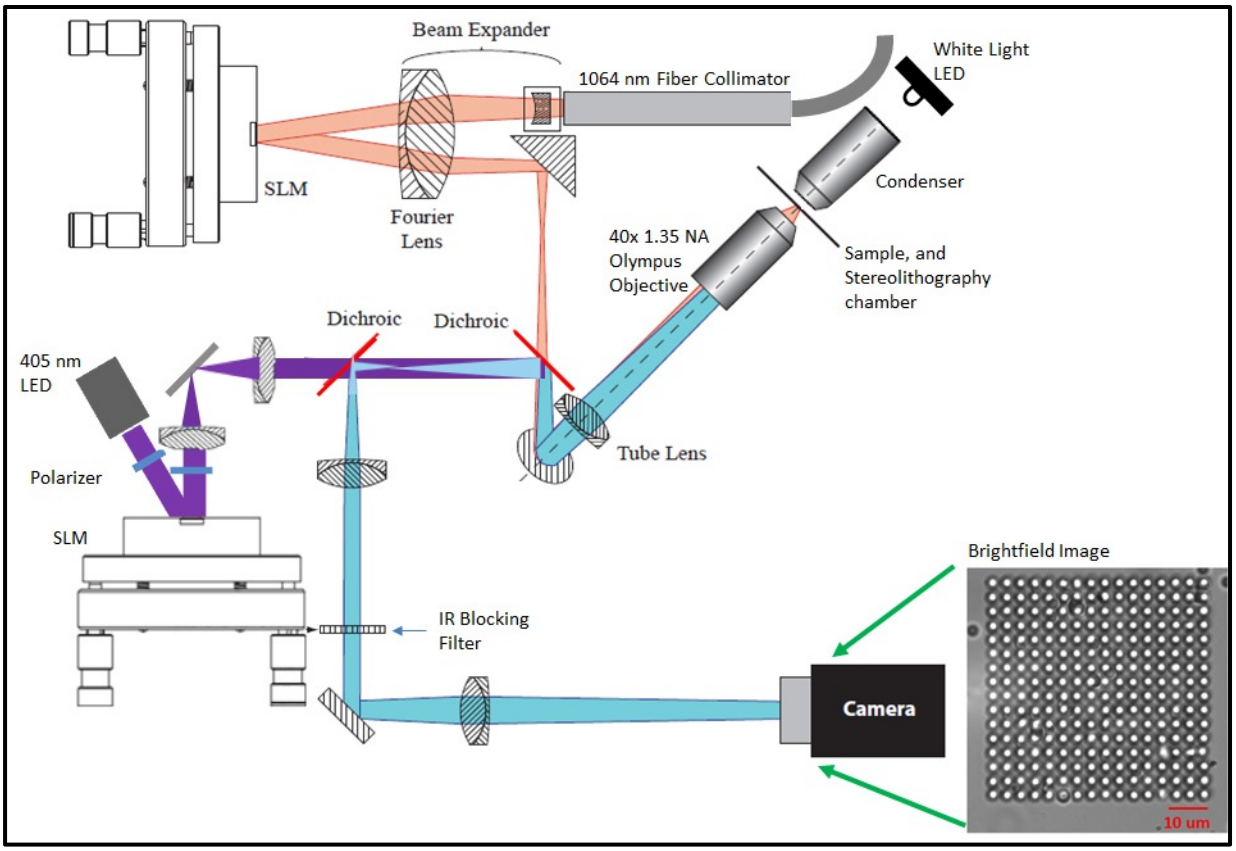

Figure 1 3D living neural network fabrication system consisting of a brightfield microscope, optical tweezing, and photolithography.

A 410 mW, 405 nm LED (M405L2, Thorlabs) is used as the light source to provide spatially uniform illumination with low coherence to suppress speckle noise and interference. The LED is polarized and used to illuminate a 512x512 pixel liquid crystal SLM (P512-405, Meadowlark Optics) employed as a programmable amplitude mask. The contrast ratio is greater than 200:1, ensuring that polymerization only occurs in the regions where pixels are turned on. A dichroic mirror (NT69-201, Edmund Optics) is used to introduce the 405 nm pattern into the imaging arm of the Cube, as shown in Fig. 2, such that the SLM plane is in focus at the sample. The imaging system de-magnifies the pixel pitch from 15 μm at the SLM to 0.67 μm at the sample.

Large 3D scaffolds containing precisely positioned cells can be built from many individual layers through additive manufacturing [8]. A thin fluid layer is initially formed between the top and bottom windows by adjusting the *z* actuators. The glass spacer compensates for the mechanical height of the chamber, allowing the first layer to be near zero thickness. After a layer of cells encapsulated in a structured hydrogel is formed, the *z* actuators are used to raise both the upper window and the adhered gel layer, after which the process repeats. The result is a precise arrangement of cells with fine control of 3D position within each layer, and extended 3D position throughout the multi-layer scaffold.

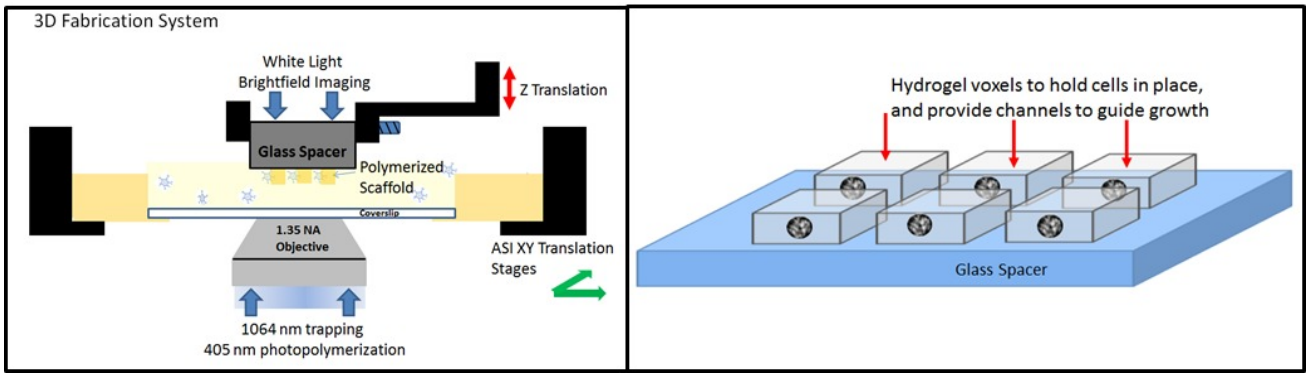

Figure 2 (left) A diagram of the sample chamber utilized to fabricate 3D living neural networks. The bottom of the sample chamber is a glass coverslip, and the top is a glass spacer that is mounted on a z translation stage. Liquid monomer and cells are injected into the sample chamber, and wick into the gap between the coverslip and glass spacer. Cells are lifted to the top of the sample chamber using optical tweezing. Permanent structures are formed through photolithography. (right) A diagram of a 2D structure is shown (flipped from the orientation within the sample chamber). Cells are held into a grid using hydrogel voxels, which additionally define channels to guide growth.

A series of brightfield images of fabricated 2D and 3D samples is shown in Fig. 3. To illustrate the ability to fabricate arbitrary 2D structures Fig. 3 a-c show an arrangement into the letters "CU", a square, and a circle about a center cell. An acellular 3D sample is shown in Fig. 4d. Two hydrogel grids are fabricated. Each hydrogel voxel is approximately 100 µm in diameter. At left a 3x3x2 layer grid was fabricated. Along the left column the second layer is aligned with the bottom layer. The spacing of the second layer is shifted along the middle and right columns such that the second layer is visible using a brightfield microscope. At right a 3x3x1 grid is shown. This illustrates that 3D patterning is possible, and that subsequent layers will adhere. In Fig. 4 e, f a 3D sample with cells is shown. The first layer consists of a 3x3 grid of NT2/D1 cells, and the second layer consists of a 2x2 grid of NT2/D1 cells.

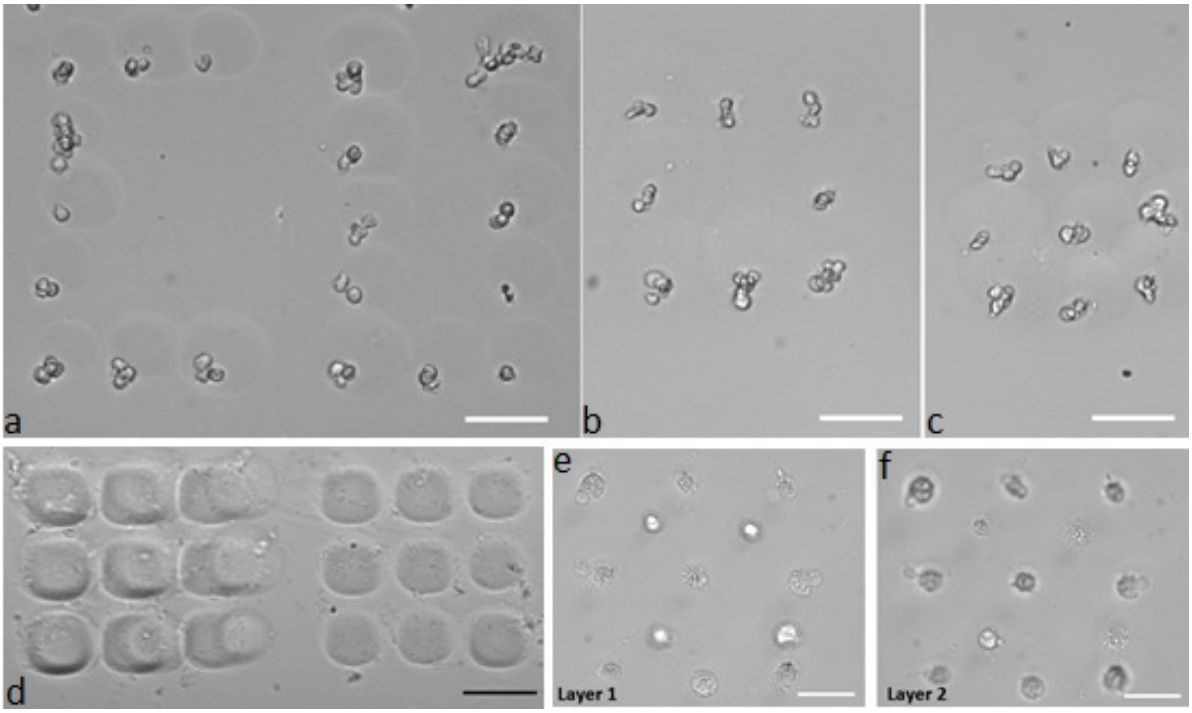

Figure 3 (a) 2D arrangement of NT2/D1 cells in hydrogel voxels to form the letters CU (b) square of NT2/D1 cells, (c) circle of NT2/D1 cells about a center cell (d) 3x3x2 grid of hydrogel voxels. The left column is co-aligned between the top and bottom layer. The second layer is purposely misaligned along the middle and left columns such that the two layers are more clearly visible. (right) a single layer 3x3 grid of hydrogel voxels. (e,f) 3D arrangement of NT2/D1 cells. The bottom layer consists of a 3x3 grid, and the top layer consists of a 2x2 grid. In each image the scale bar is 100 µm.

3. MATERIALS

Hydrogels are crosslinked polymers, which are often utilized to create scaffolds for cells. Recent research has focused on encapsulation of cells within thiol-ene photopolymerized PEG peptide hydrogels for studies of: 3D cell biology [12,13], the controlled release of therapeutically relevant proteins [14], directing stem cell differentiation [15], and promoting tissue regeneration [16]. Thiol-ene systems can be propagated by oxygen radicals, thus reducing the number of oxygen radicals present that can damage cells. Additionally, the thiol-ene propagation reaction rate is typically greater than that of (e.g.) acrylates, which improves the diffusion-limited resolution of polymer voxels.

In this work a degradable hydrogel was utilized (Fig. 5) consisting of 8-arm polyethylene glycol (PEG) Norbornene macromere, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) as photoinitiator [17], and a matrix metalloproteinase (MMP-9 CVPLSLYSGC, GenScript) sensitive peptide is utilized as a cross linker. The hydrogels were modified with RGD to promote cell adhesion and growth. The degradable thiol-ene hydrogel consists of cells suspended, at a density of $1x10^6$ cells/mL, in a 90% stoichiometrically balanced (1 ene to 0.9 thiol) monomer solution comprised of 10 wt. % 8-arm PEG-norbornene [18], 0.06 wt. % LAP, 3.2 wt. % PEG-dithiol, and 0.135 wt. % CRGDS. PC12 cells (ATCC # CRL-1721), NT2/D1 cells (ATCC # CRL-1973), and iPS cells require different adhesion promotors. For studies with PC12 cells, Poly-l-lysine (pre-diluted to 0.01%) was added at a concentration of 10 wt. %, and for studies with NT2/D1 cells and iPS cells Matrigel (pre-diluted according to manufacturer instructions) was added at a concentration of 40 wt. %. The remainder of the solution was growth media.

PC12 cells were maintained in DMEM/High Glucose medium (Invitrogen #11965) supplemented with 5% Horse Serum (Atlanta Biologicals #S12159), 5% Calf Serum (Atlanta Biologicals #J0016) and Pen-Strep (Invitrogen #15140). For differentiation PC-12 cells were plated on dishes coated with 0.001% poly-L-Lysine, adjusted to pH 8.5 with Borate Buffer, and differentiated in DMEM/High Glucose medium supplemented with 1% Horse serum, Pen-Strep and 100ng/ml NGF (Sigma #N0513).

NT2/D1 cells were maintained in ATCC-formulated DMEM (ATCC # 30-2002) supplemented with heat inactivated 10% FBS (Invitrogen #10082-139). Neuronal differentiation was accomplished by adding 10nM Retinoic Acid (Sigma #R2625) into the medium. To keep proliferation of NT/2D1 cells under the control 5uM AraC (Sigma #C1768) was added 3 days post differentiation.

iPS cells were maintained in TeSR-E8 Medium (Stemcell #05940) on dishes coated with Matrigel (Corning #354277).

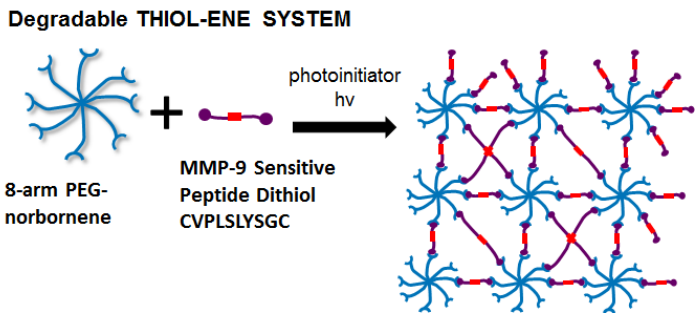

Figure 4 A diagram of the structure of thiol-ene hydrogel. Photoinitiator enables crosslinking between the 8 arm PEG-norbornene and a matrix metalloproteinase (MMP) sensitive peptide dithiol. Cells that secrete MMP-9 are able to break the crosslinks in the gel allowing mobility for replication and differentiation.

4. RESULTS

4.1 Hydrogel Biocompatibility

To verify biocompatibility of the cells with the hydrogel, randomly organized samples of NT2/D1 or iPS cells are encapsulated in hydrogel structures approximately 1 inch in diameter. Bubbles within the hydrogel are utilized as reference marks to monitor cell development over the span of a week. As shown in Fig. 6, NT2/D1 cells readily degraded the hydrogel, allowing for cell mobility, and replication, indicating biocompatibility with the hydrogel material. Alternatively iPS cells do not show signs of mobility, suggesting that the cells do not express MMP-9 and cannot degrade the hydrogel (Fig. 7). It is not yet known if the cells will be able to degrade the gel after they have differentiated. If it is found that degradation is desired it is possible to use a peptide dithiol with an alternative MMP degradable sequence [19].

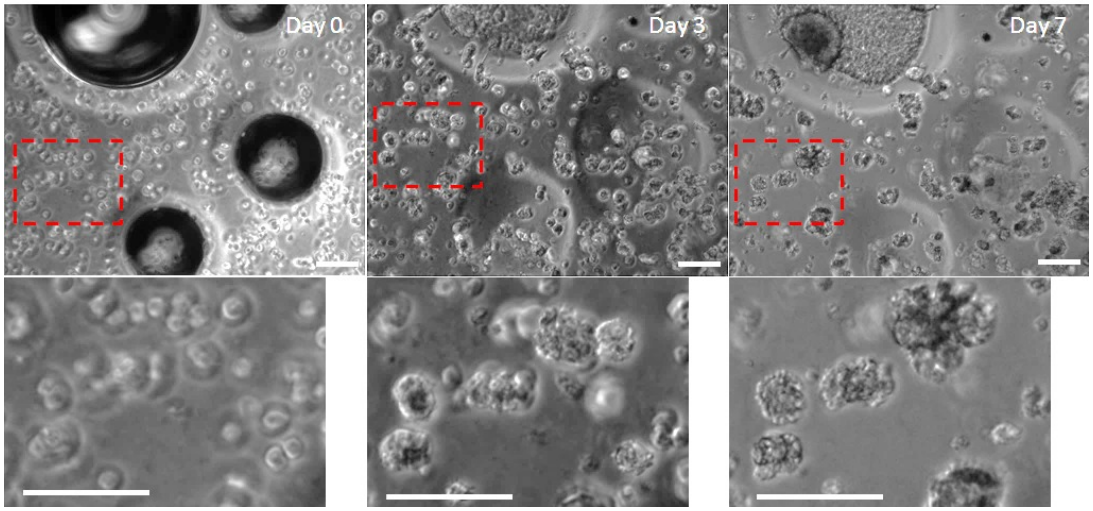

Figure 5 A sample was fabricated with a random organization of NT2/D1 cells growing in the degradable gel. Several air bubbles in the gel made a convenient landmark in the sample to monitor the cells behavior over the span of 7 days. An area is marked in red along the top images noting a section of the image that is magnified in the bottom row. This region highlights cell mobility in the gel. Scale bars are 100 µm.

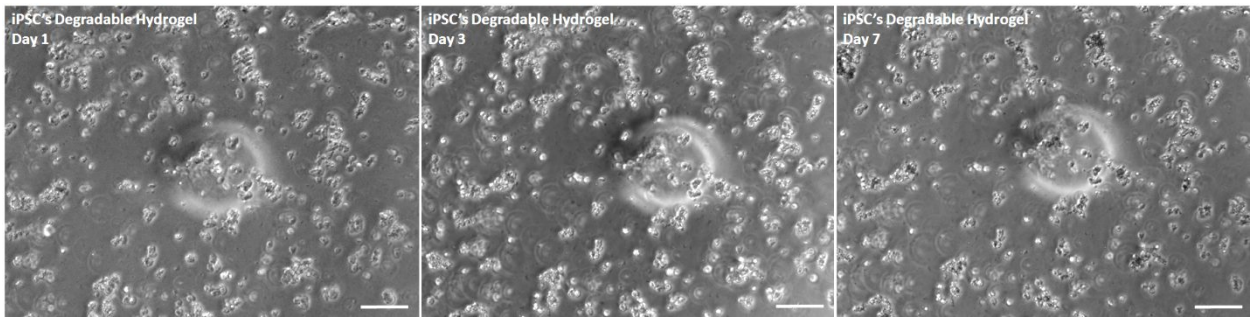

Figure 6 A sample was fabricated with a random organization of iPSC cells growing in the degradable gel. An air bubble in the gel made a convenient landmark in the sample to monitor the cells behavior over the span of 7 days. As can be seen there is no significant change in the cell position (in contrast to Fig. 6 where significant cell mobility was seen). Scale bars are 100 µm.

4.2 Live Cell Lithography Viability

2D grids of PC12 cells, NT2/D1 cells, and iPS cells are fabricated to verify the cells ability to replicate after patterning over the span of 1 week. The majority of the cells are seen to survive and replicate until the space between hydrogel voxels is confluent (Fig. 9-10). It is found that cells viability is higher if cell clusters are arranged as opposed to single cells, particularly for NT2/D1 cells and iPS cells. With iPS cells shedding is prevalent, and it is expected that approximately 60% of the cells should shed over the span of 1 week. As shown in Fig. 10, a significant amount of shedding is observed over the first 3 days, but limited shedding is observed over the remaining 4 days that the samples

are maintained. In samples with iPS cells, cells appear to be adhering to the top of the hydrogel structure, and are replicating to fill the channel along the right portion of the structure (Fig. 10). This appears to result in a loss of organization, but this is expected when the cells are replicating as opposed to differentiating.

These tests demonstrate many key milestones in the project. First, cells can be handled out of a sterile environment without leading to contamination, which is particularly challenging for the iPS cells which are sensitive to antibiotics. Second, cells can be suspended in liquid monomer prior to structuring, without significant impact to cell viability. Third, cells can be exposed to optical traps for the span of < 1minute without impact to viability. Fourth, the hydrogel structures do provide a biomechanical boundary that will guide growth, and do not alter cell viability.

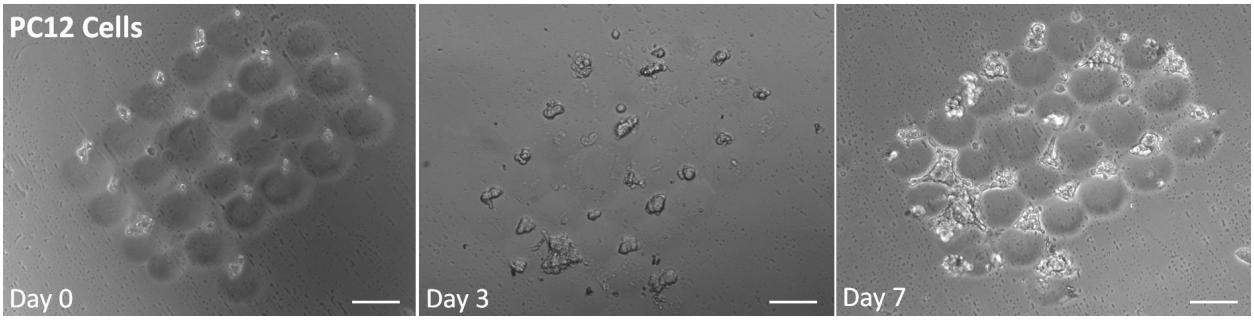

Figure 7 Grid of PC12 cells organized and held in place with hydrogel voxels. Over the span of 7 days the cells replicated and become confluent in the gaps around the hydrogel structures. Scale bar 100 µm.

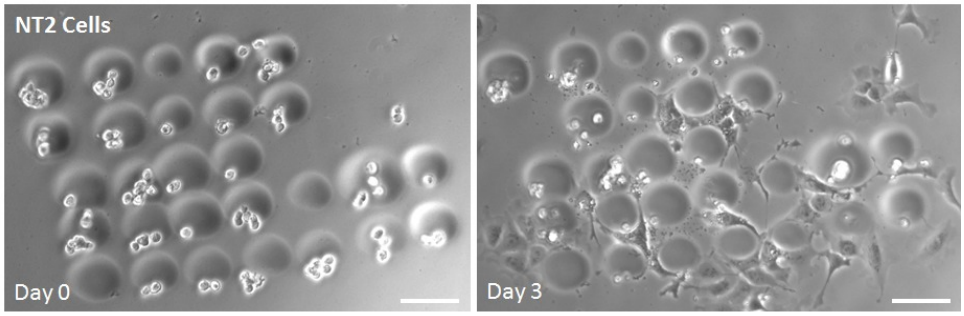

Figure 8 Grid of NT2/D1 cells, which likewise replicate to fill the gaps between the hydrogel structures. Scale bar 100 µm.

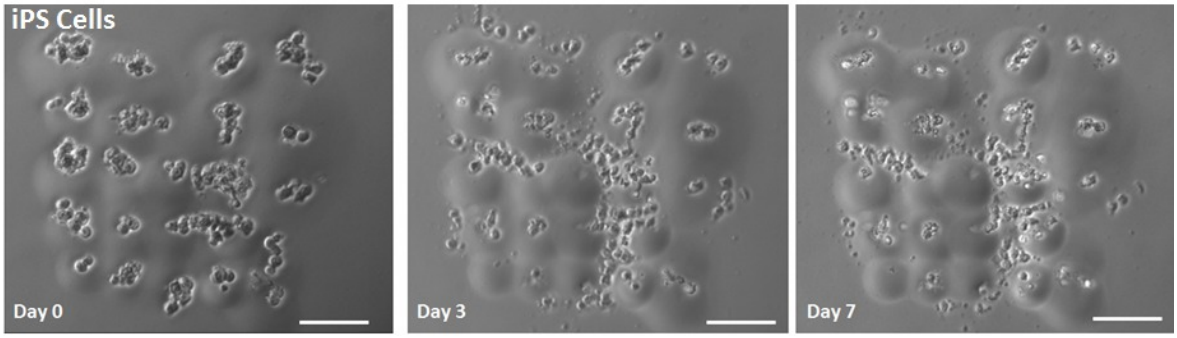

Figure 9 iPS cells incubated for 7 days. Scale bar 100 µm.

4.3 Differentiation

With many of the cells surviving, and replicating within and around the hydrogel structures, the focus shifted to differentiation. In the first sets of samples cells are structured such that they are only partially submerged within the hydrogel voxels. The intent was to use the hydrogel to hold the cells in place, yet give the cells enough room to differentiate without being confined by the hydrogel. As shown in Fig. 11, the cells were found to readily break free of

the hydrogel and migrate in the sample. Although the cells still grow along the edge of the hydrogel, the loose structures fabricated did sufficiently confine the cells to ensure that the cell body remain in the same general location. Because the cells are capable of degrading the hydrogel, cells can be further submerged within the gel to increase confinement, and interconnectivity can be determined by controlling the proximity of the cells to each other.

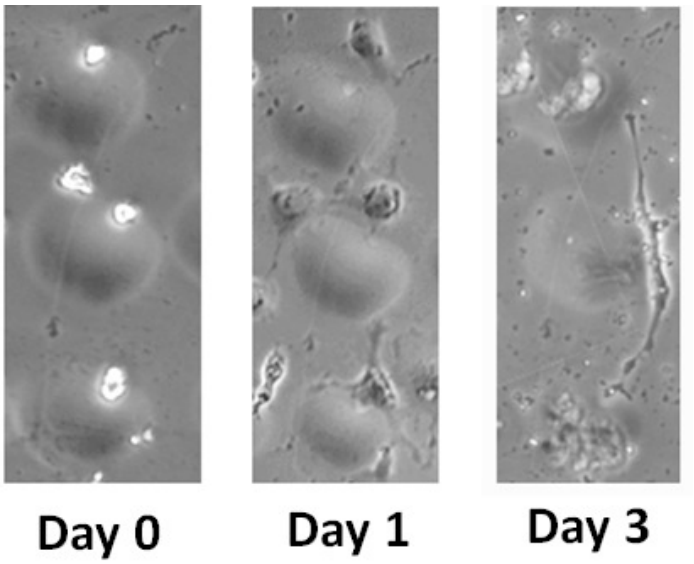

Figure 10 NT2/D1 cells organized along the edge of hydrogel voxels. The cells are incubated in differentiation media. On day 0 the cells are partially submerged in the hydrogel. By day 1 the cells break free from the hydrogel, and grow around the perimeter of the gel. By day 3 the cells at the top and bottom of the grid have migrated away from the gel. Current samples are not sufficiently confining to hold the cells in an organized pattern for long term studies.

Previously fabricated samples set an expectation for NT2/D1 differentiation on glass (Fig 11a), but it was not known how the cells would differentiate when entirely encapsulated in hydrogel, and if they would be able to degrade the hydrogel after differentiation. To address this, randomly organized samples of NT2/D1 cells are encapsulated in a large hydrogel structure approximately 1 inch in diameter, and are incubated in differentiation media containing retinoic acid and araC for 10 days. Bubbles are used as a reference points to track cell development. After 7 days fine structures are seen, potentially leading toward interconnected cells, as shown in Fig. 11 b and c.

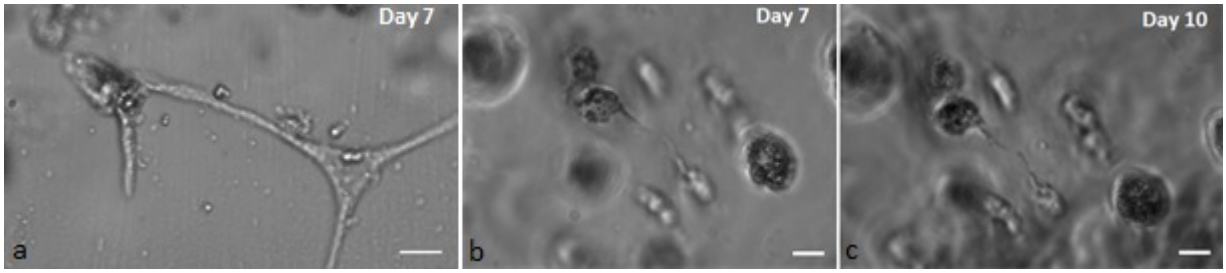

Figure 11 (a) NT2/D1 cells that migrated away from the hydrogel structure, and differentiated on glass after 7 days in differentiation media (b) Randomly organized NT2/D1 cells entirely encapsulated within the degradable hydrogel incubated in differentiation media with araC. After 10 days incubation cells began to degrade the hydrogel and interconnect. Scale bar = 10 µm.

Organized grids of NT2/D1 cell clusters are fabricated, and incubated in differentiation media with araC and retinoic acid. The number of cells in the clusters varied, with some cells near the edge or partially exposed from the hydrogel. Within three days cells at the top of the hydrogel begin to interconnect, as shown in Fig. 14. Cells entirely encapsulated within the hydrogel are slower to interconnect because they must degrade the hydrogel as they grow, but are also

differentiating. One of the benefits of work with NT2/D1 cells is that differentiation does not require transfection, as is required with the iPS cells. However, differentiation is slower, on the scale of 8 weeks. The slow rate of cells interconnecting is aggravated by the need to heavily confine the cells in hydrogel in order to maintain organization. Alternatively iPS cells are capable of differentiating in 2 weeks, but the ability to pattern iPS cells after transfection without altering differentiation has yet to be demonstrated.

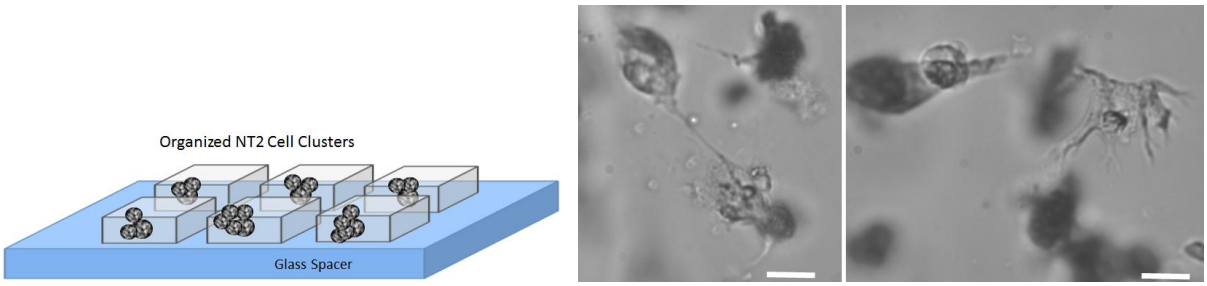

Figure 12 (left) To improve cell viability, cell clusters are organized as opposed to single cells. The cells near the top of the hydrogel have more mobility to interconnect, and whereas cells encapsulated in the hydrogel must degrade the hydrogel and are slower to interconnect. (right) **cell differentiation at the top of the hydrogel structure after 4 days incubation in differentiation media. Scale bar = 20 um**

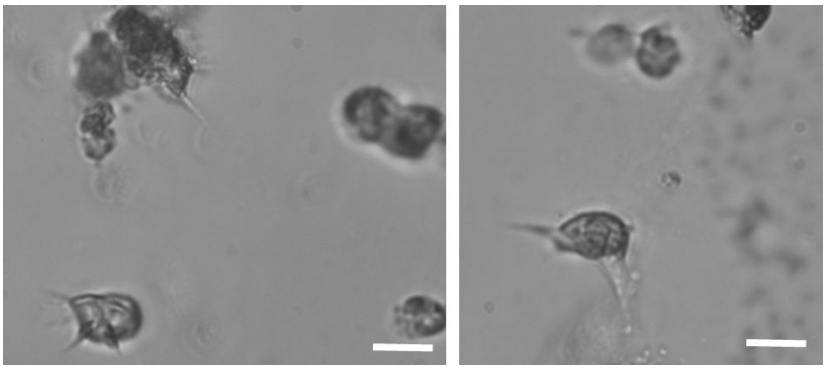

Figure 13 Cell differentiation of encapsulated organized NT2/D1 cells, after 4 days incubation in differentiation media. Scale bar = 20 um

5. CONCLUSIONS

In this work we demonstrate a system that merges holographic optical tweezing with micro-stereolithography to pattern PC12, NT2/D1, and iPS cells in hydrogel structures. A degradable hydrogel was selected to allow the cells mobility, yet confine the cells to an organized structure. Samples of cells organized into grids are incubated for up to a week. Cells are seen to replicate until the channels in the hydrogel structure are confluent. Samples of randomly organized cells encapsulated in hydrogel are incubated in differentiation media containing araC and retinoic acid show signs of differentiation within 10 days. Brightfield images suggest that cells are potentially interconnected, but further studies must be done to validate this. Samples of organized cells are shown to differentiate and interconnect on the top of the hydrogel structure within 4 days, and likewise show signs of differentiation and axonal growth within the hydrogel within the span of 7 days.

Further work must be done with NT2/D1 cells to conclusively demonstrate interconnectivity of cells within hydrogel. Additionally, work completed thus far indicates the fabrication method is likely biocompatible with iPS cells. However, iPS cells must be transfected to differentiate to human neurons, which will be the topic of future research.

ACKNOWLEDGMENTS

The authors would like to acknowledge the support of NIMH Grant number 1R43MH102946-01, which made this research possible.

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