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Isolated node engineering of neuronal systems using laser direct write

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Abstract

Current limitations to the engineering of ex vivo and in vitro neural environments are hampering the ability to understand underlying neurophysiology. High levels of spatial specificity, reproducibility and viability have been previously reported using laser direct write (LDW) to print cells. However, despite the significant need no one has yet reported laser assisted printing of primary mammalian neuronal cells, an inherently sensitive but critically important population. Herein, we describe the use of LDW to reproducibly and accurately pattern viable dorsal root ganglion (DRG) neurons and supportive cells capable of neural outgrowth and network formation. Our demonstrated ability to engineer and control distinct micro-environmental components unlocks the potential for high throughput experiments to both understand underlying physiology and investigate therapeutic interventions.

Introduction

Current limitations in ex vivo and in vitro environments are hampering the ability to understand underlying neurophysiology, as well as slowing critical advances in neurodegenerative diseases, even though technology to interface with neuronal systems has improved dramatically. The nervous system represents, arguably, the most complex environment, and while in vivo studies have contributed vastly to our understanding, restrictions arise when studying individual influences and perturbations to neuronal activity. Basic mechanisms governing pain, sensation, memory, cognition and behavior, as well as pathologies including Alzheimer’s, ALS, Multiple Sclerosis and neuropathic diseases all involve a multitude of cells, micro-environmental cues, and mechanisms. Unfortunately, the interactions of this complicated milieu are so numerous that parsing out individual factors has thus far proven difficult. The ability to replicate increasing levels of biomimetic complexity in vitro will be critical to the future progress of neurobiology, neuropathology, and therapeutic interventions.

In order to fully understand the micro-environmental and intercellular interactions that underlie the behavior of neuronal cells, researchers require both the ability to recapitulate complex cellular constructs and interface efficiently with those models. Besides leading to insights into the mechanisms of neuronal development and functionality, advanced in vitro studies have the potential to develop the next generation of breakthroughs in understanding neural disease states. Organotypic slice preparation is the most prevalent method for studying the activity of neurons in a near native environment [1–3]. Unfortunately, little control over the influence of individual micro-environmental factors is available. On the other end of the spectrum, random cultures of primary dissociated cells are commonly employed for mechanistic studies of the nervous system in order to incorporate the numerous cells responsible for neuronal function [4, 5]. This allows for increased control over cell type and environmental perturbations, but the stochastic spatial nature negates complex organization and is inherently limiting. Increasingly, tissue engineering techniques are being employed to engineer in vitro constructs which more closely mimic in vivo micro-environments as model systems for testing.

Numerous techniques exist to study functional metrics of these neuronal systems, though as the biological complexity increases, from single cell to
organized organotypic models, the ability to perform systematic electrophysiological experiments in a controlled manner decreases. Patch clamp techniques are the most commonly used method to gain intracellular access to neurons, and much has been learned of the influence of both individual ion channels and synaptic inputs in electrophysiological control [6–10]. Unfortunately, this process is both time limiting and destructive to cells, hindering its ability to investigate network level activity. Improvement to material design, throughput and automated data analysis for multi-electrode arrays have vastly accelerated their use for studies of neuronal activity in populations of cells [11–13]. In addition, recently developed optical methods of both recording and stimulating neurons allow for real-time control of neurons, spanning individual cells and system level applications. Optogenetic regulation of ion channels, calcium imaging and voltage sensitive dyes have shown widespread spanning in vivo to in vitro applications [14–16]. The utility of these more recent techniques, however, is limited in vitro by the types of systems available to researchers, as described above. The ability to engineer neurons into distinct patterns, while carefully controlling the microenvironment is lagging behind many other biological systems.

Many techniques are commonly employed to define cellular orientation which also combine the rapid throughput capabilities so important to successful in vitro studies. The patterning of adhesion molecules and hydrogels, whether through stamping, lithography, or inkjet deposition [17–22] have been important to defining studies of individual neuronal cell types. In addition, inkjet printing of neurons themselves has been successfully demonstrated to control spatial organization [23, 24]. Microfluidic arrays have been employed to define not only the location of neuronal cell types, but also control the directionality of axonal outgrowth [25]. Unfortunately, limitations exist on the accuracy of spatial control and reproducibility, as well as the specificity of distinct cell types within these models. Understanding the extremely nuanced interactions of the nervous system will require high levels of control over the interactions between individual cells. Laser direct write (LDW) offers all of the advantages of the previously described techniques, while also addressing high spatial specificity and reproducibility and control over the specific cell type introduced to the system [26, 27]. LDW has been previously utilized to print B35 neuroblastoma cells in both 2D and 3D patterns, which then formed local neuronal networks [28]. However, no known reports have demonstrated the use of mammalian primary neurons. Incredibly, in spite of the significant need to incorporate this advanced technology, LDW has not yet been employed extensively for neural-based applications.

Herein, we describe a rapid technique to engineer isolated nodes of heterogeneous primary mammalian neuronal cells. Despite the significant need, we are unaware of any previous reports demonstrating laser assisted printing of primary mammalian neuronal cells, an inherently difficult but critically important population. We demonstrated the ability to vary the geometric organization of printed cells, and confirmed high levels of cell viability following LDW with sensitive primary neurons. In addition, the ability to monitor network formation and cellular maturation with time, as well as utilizing image analysis techniques following antibody staining, demonstrates the utility of this model to characterize neuronal systems. This technique is amenable to experiments characterizing the development and plasticity of neuronal networks, with potential as a rapid screen for pathological models and therapeutic interventions. The spatial control and design flexibility sets this technique up for a wide variety of uses within both the peripheral and central nervous system, affording researchers a novel tool to both understand and address key processes and disease states facing neurophysiology.

Methods

• Neuronal culture

All animal handling and cell harvesting protocols were implemented under guidelines set by NIH (NIH Publication #85-23 Rev. 1985) and the Institutional Animal Care and Use Committee (IACUC) of Tulane University. Embryonic day 15 (E-15) pups were obtained from timed-pregnant Long Evans rats (Charles River, Wilmington, MA). Spinal columns were isolated from embryos in Hank’s Balanced Salt Solution, from which dorsal root ganglia (DRG) were harvested and collected. Primary DRG cells were obtained through dissociation of DRGs by trypsinization (Thermo Fisher, Grand Island, NY) and mechanical separation. After dissociation, cells were then cultured in an incubator at 37 °C and 5% CO2 on collagen coated (BD) petri dishes for approximately one day in neurobasal medium supplemented with nerve growth factor, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (Thermo) to promote adhesion. After one day, FBS was replaced with B-27 and L-glutamine to encourage neuronal growth and discourage over proliferation of non-neuronal cell types [29]. Cells were again collected using trypsin after approximately 7 d in culture for LDW printing.

• Printing

Cell patterning through laser direct write used an excimer laser to interact with a gelatin material layer as the mechanism of action [30, 31]. An EX5 Excimer ArF laser pulse (193 nm) is used for deposition (GAM Laser, Inc., Orlando, FL), with fluence of 0.4 J cm−2 and pulse width of 8 ns. The size of cell patterned spot was controlled by
controlling the laser beam waist with an adjustable iris, giving spot sizes on the target of 19.5 to 22.0 μm, as measured on the print ribbon, for the radii in figure 1. Briefly, a heterogeneous mixture of dissociated DRG cells is seeded onto a gelatin coated quartz print ribbon at 2 × 10^6 cells ml^-1. Following a laser pulse, local evaporation of the gelatin occurs on the print ribbon, causing the transient formation of a vapor pocket. Targeted expansion of the pocket releases a droplet directly underneath the laser pulse, transferring the cell/gelatin mixture onto the receiving surface in a predefined pattern.

In order to target areas of the print ribbon with the desired density of cells, an image generated by a charge-coupled device camera is used to select the appropriate location on the ribbon prior to printing, checking for cell density and ensuring a successful print. The print ribbon is located directly above the receiving substrate used a custom coded program for control of Ensemble XY stages, while the print ribbon is controlled through a LabView graphic user interface.
interface, allowing for targeting of discrete groups of cells. The single pulse described above transferred the cells to the receiving surface, whereupon the surface was moved to the next location of interest. The general schematic of this system is shown elsewhere [31].

- **Fluorescent cell labeling**
  Cell viability was evaluated with a Live/Dead® assay (BioVision, Milpitas, CA) per manufacturer’s instructions. For both primary DRG and MDA-MB-231 human breast adenocarcinoma cells printed onto coverslips, 10x fluorescent images were taken of each spot 24 h after printing, for a total of 12 samples each. In addition, viability was analyzed for DRG cells which underwent all steps for LDW printing, other than the printing, as well as DRG simply pipetted onto coverslips, in order to further explore viability associated with printing primary mammalian neuronal cells. Cells staining positive for Live-Dye™ (live) and propidium iodide (dead) were counted manually using ImageJ (National Institutes of Health, Bethesda, MD). CellTracker™ Red CMTPX Dye (Thermo) was also used separately to visualize cells after printing, according to manufacturer’s instructions.

- **Antibody staining**
  Specimens evaluated with immunocytochemistry were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min. Neurites were visualized using mouse monoclonal [2G10] neuron-specific beta III tubulin primary antibody (1:100; Abcam, Cambridge, MA). Presynaptic vesicles were labeled with rabbit anti-VGLUT2 primary antibody (1:100; Bioss, Woburn, MA), followed by Alexa Fluor® 568 preadsorbed goat anti-mouse immunoglobulinG (H + L) secondary antibody (1:100; Abcam). Cell nuclei were stained with Diamidino-Phenylindole (DAPI) nucleic acid counterstain according to manufacturer’s instructions (Molecular Probes, Eugene, OR). Antibody tagging steps were carried out in PBS with 0.1% Triton-X 100% and 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) at room temperature for an hour and a half, followed by three ten minute washes in PBS with 0.1% Triton-X 100 at room temperature.

![Figure 2. Cell viability analysis. (A) Representative image of printed dissociate DRG cells. (B) Representative image of printed 231 cells. (C) Analysis comparing DRG cells plated with pipette, DRG cells on print ribbon, printed DRG cells and printed 231 cells. (n = 12, *p < .05, ***p < .0001).](image)
• Neuronal characterization
Following antibody staining, image analysis was performed to characterize the engineered in vitro environment. In order to count DAPI labeled cells and neurons and to quantify the length of neurite growth image analysis was performed using ImageJ [32]. DAPI cells and neurons were counted with the image-based tool for counting nuclei (ITCN) plugin for ImageJ [33]. Images were first adjusted for threshold, converted to binary and then despeckled to remove noise. ITCN requires an estimated width for regions of interest and minimum distance to adjacent cells. To confirm that the width and adjacent distance estimates were correct, ITCN was used on a small section of each image and then confirmed by manual cell counting. ITCN was consistently within ±2% of the manual count. To quantify migration of cells outside of the original print, a region of interest (ROI) was defined for the printed area and ITCN was used outside of that ROI. Neurite length was quantified using the Simple Neurite Tracer plugin for ImageJ [34].

• Statistical analysis
To determine significance between two groups, a two-tailed Student’s t-test of equal variance (p < 0.05) was used. Equality of variance was calculated using Levene’s test (p < 0.05). To test differences between three or more groups, Minitab (Minitab, State College, PA) was used for one-way ANOVA followed by Bonferroni post-hoc analysis (p < 0.05). Unless otherwise specified, data are presented as mean ± standard deviation.

Results

• Laser direct write
Dissociated DRGs were successfully transferred using LDW in multiple patterns, demonstrating the potential to generate versatile multi-cellular heterogeneous populations of primary cells, including neurons. Array patterning can be varied for orientation (2 × 2 versus 3 × 3, etc), distance between nodes, and diameter of printed area, depending on the application (figure 1). Figure 1(A) depicts cell tracker red labeled neurons directly after printing, detailing the initial print orientation. The radius of printed cell nodes, 219.2 ± 13.3 or 272.7 ± 20.5 μm, and internodal spacing, 410.1 ± 69.5 or 275.1 ± 31.0, represented a user defined choice (figure 1(D)). The number of cells per printed node was also adjustable depending on radius of printed area and original density of print ribbon, with values of 38.5 ± 3.7 or 73.3 ± 15.8 cells per print for the settings described.

• Cell viability
Cell viability was examined for dissociated DRG cells after printing, with comparisons to MDA-MB-231 cells, as well as for DRG cells plated on coverslips with a pipette and DRG cells adhered to the print ribbon without LDW printing. Printed DRGs were 84.9% ± 4.7% alive, while printed 231s were 95.6% ± 3.44% living, DRGs plated using a pipette were 89.3% ± 2.0% viable, and finally DRGs on the print ribbon without exposure to the laser were 86.4% ± 3.3% live. Statistically, printed 231s had higher percentages of living cells (p < .0001) compared to all DRG conditions, while only pipette plated DRG were higher than LDW printed DRG (p < .05) (figure 5).

• Neuronal network formation

Figure 3 shows the discrete time-lapse formation of the neuronal network. By following the phase imaging of a single print, the extension of neurites within and between islands of cells is apparent, as well as proliferation and cell migration outside of the original print area. Initially, after 1 d in vitro (DIV1), only a few
neurites are visualized extending between islands, with connections mainly formed within individual islands themselves. Neurites are observed connecting islands to adjacent cells after DIV3, and after DIV7 numerous neurite extensions are apparent throughout the printed area, with individual islands losing their distinct organization due to cell migration.

In addition, fluorescent labeling of distinct printed nodes demonstrated a more detailed representation of the same trend in network development. Through DAPI (blue) and β III tubulin (green), neurite extensions are easily distinguished allowing neurons and supportive cells to be visualized separately (figures 4(A)–(C)). At DIV3 (figure 4(A)), neurons and supportive cells remain largely within the original print area, while neurites have begun to interact within individual nodes as well as overlapping with those nearby. By DIV5 (figure 4(B)), all nodes have neurite extensions reaching between each other, and supportive cells have begun to migrate throughout the network. Finally, at DIV7 (figure 4(C)), both neuronal and supportive cell migration has spread and neurites extend throughout the network. Analysis of 28 printed nodes were performed at DIV7 (table 1). 125.5 ± 42.2 cells were visualized per print, with 12.5% ± 10.1% staining positive for neuronal markers. On average, 25.1% ± 8.6% of all cells and 20.7% ± 12.1% of neurons had migrated outside of the original printed area.

By tracing the length of total neurite extension and averaging over the number of printed neurons, neurite outgrowth of approximately 88.6 ± 53.7 μm length was measured. In addition, 50.2% ± 23.2% of neurons expressed markers for VGLUT2 expression. Using high magnification, synaptic expression of VGLUT2 (red) is seen in a subset of neurites, particularly at the leading edge of extensions, and overlap between neurites is apparent (figures 4(D)–(F)). Examples of neurons both lacking and co-expressing the glutamate transporter are depicted.

**Discussion**

Previous research has demonstrated the viability and utility for LDW printing of cell lines for a variety of applications. Studies of human adipose-derived stem cell interactions with living microvasculature [35], influences of size, density and spatial patterning on embryoid bodies [36], cancer cell dynamics and angiogenic influence [37, 38] as well as cell laden microbead patterning [31, 39] have all powerfully demonstrated the unique and valuable characteristics of LDW printing. The versatility of LDW extends to precise control over the compositional makeup and spatial organization (within 5 μm) of cells printed onto a variety of surfaces [30]. The high accuracy and

![Figure 4. Immuno]{http://www.dummyobserver.com/dummydata/dummydata.png}
reproducibility with the potential for high throughput capability, along with the ability for computer aided design and manufacturing (CAD/CAM), and capacity to visualize both cells and printing substrate prior to transfer, provides an unprecedented level of control in the engineering of tissue micro-environments [40]. In addition, visually assisted control over cell type, including multiple distinct cell phenotypes arranged concurrently on a single print [37], and cell density, variable down to single cell resolution [41] allows for precise control of the engineered micro-environment. Past reports have documented the minimal effects of LDW on cell viability [42–44], but to our knowledge, no one has yet reported laser assisted printing of primary mammalian neuronal cells, a sensitive and critical population.

Viability analysis demonstrates the difficult nature of primary mammalian dissociated DRG cells, in comparison to cancerous cell line, MDA 231 cancer breast cells (figure 2). The spatial organization of DRG cells through LDW led to the vast majority, 84.9%, of transferred cells surviving the process (figure 2(C)). However, this was statistically lower than 231s undergoing the same process, which survived at a 95.6% rate. Printed DRG cells compared to those plated on identical coverslips using a pipette, with no entrapment in gelatin or exposure to the laser pulse, survived at a slightly higher percentage, 89.3%. Because there was no statistical difference between DRG seeded onto the print ribbon and those physically printed off the ribbon, it appears the techniques for ribbon seeding itself induces some cell death. This is not surprising as the cells spend approximately 15 min without media and 10 min outside of the incubator between ribbon seeding and printing. Again, 231 cells survived at a higher rate than all variations with DRG cells, almost certainly because of the robust nature of these human breast adenocarcinoma cells. These results are also in line with previously reported results using LDW [45]. Nevertheless, an acceptable number of DRG cells survived, including neurons, and retained the ability to extend neuronal processes.

Using LDW technique to print DRG cells afforded high levels of control over the initial cellular environmental setup and subsequent cell migration, as well as the observation of network development and spatial reorganization through time. This sets the stage for the use of the system described herein as a platform for studies of neuronal development, synaptogenesis and network plasticity that is applicable to a wide variety of neuronal and supportive cell types, depending on the desired model. Figure 1 details the characteristics of spatial orientation of prints. Arrays of an almost infinite structure of different node orientations are possible, with two by two and three by three arrays shown in figures 1(A) and (B). In addition, radius of print and proximity of individual prints, or internodal distance, are variable (figure 1(C)), and the density of cells can be adjusted as needed (figure 1(D)). All of these factors could potentially influence the characteristics of the developing neuronal network, and in combination with mechanistic external cellular perturbations through genetic manipulation, biomolecular cues, or pharmacological intervention, effects can be studied both from a system wide and individual cell perspective. To demonstrate this, we quantified the baseline network characteristics after DIV7, with the cells allowed to “mature”. Due to the observed low percentage of neurons to supportive cells, 12.5% ± 10.1%, in our cultures, we optimized our ribbon seeding density and print sizes to ensure a high number of cells per print, with 73.3 ± 15.8 cells immediately after printing and 125.5 ± 42.2 at DIV7. After 7 d in culture, we saw a high rate of migration outside of the original print areas. This was expected as the cells reorganize themselves during network formation, resembling natural developmental processes. Printed neurons were demonstrated to be viable following printing, reinforced by the robust outgrowth of neurites throughout our cultures, averaging 88.6 ± 53.7 μm of neurite extension per neuron. Expression of presynaptic glutamate transporters, visualized through VGLUT2 staining, was frequently seen at the leading edges of axonal growth cones and where neurites overlapped, with 50.2% ± 23.2% of neurons staining positive. It is important to point out that rates of expression seen using our methods differ slightly from some reported in vivo values, though much discrepancy is apparent in the literature [46–48]. This is not unexpected, as culture conditions have been previously demonstrated to affect neuronal phenotype [49, 50].

The ability to spatially organize dense islands of neuronal cells, which retain the ability to develop and interact with adjacent nodes, has far reaching applications. Current efforts within the Environmental Protection Agency, including Tox21 and ToxCast, have been charged with understanding the potential toxic effects of existing and novel chemicals, and the developing nervous system represents one of the most vulnerable biological systems. Current methodology is limited in throughput, but the model system described herein has the potential to screen high numbers of neuronal cells for detrimental effects of exposure to agricultural and environmental agents. High levels of variation seen between cultures manifested itself in high standard deviation values in our study, representing the inherent biological stochasticity and underlying the need for high numbers of studies to determine statistical significance. In a similar vein, the pharmaceutical industry has an increased need to further test for the efficacy and safety of large numbers of novel compounds preclinically, in order to decrease high levels of attrition during clinical trials, which cost hundreds of millions of dollars and put human patients at risk. VGLUT2 expression described above has been linked to nociceptor regulated pain response in vivo [51–53], and though methods to alter this expression were not examined here, direct potential
therapeutic applications in pain management and neuropathic diseases could be studied using this system [54, 55]. Lastly, novel in vitro methods will be critical to help researchers understand biological mechanisms underlying a variety of disease states, as well as those governing cognitive and developmental processes [22, 56–58].

The model described herein has obvious potential for a variety of applications, and by integrating levels of biomimetic complexity, our platform has the potential to become an integral tool in neurophysiological research. Though examining the development of a stochastic network with time is very important to many questions in basic neurobiology and neuropathology, the ability to further control axo-nal outgrowth and synaptogenesis would lend itself to more targeted studies. Optogenetics has also recently been utilized as an effective method to direct axonal extension, affording additional control over network formation [60]. Additionally, the inclusion of a wider variety of cell types, particularly those defining critical tracts in the central nervous system (e.g., corticothalamic, pyramidal) or important fiber types in the peripheral nervous system (e.g., pain, sensory) would expand potential applications to a variety of areas of interest. Though outside the scope of this study, integration of electrophysiological techniques, including multielectrode arrays, or voltage sensitive dyes, would allow for the inclusion of functional outputs as another critical metric [59].

Conclusion

The engineered micro-environment described above represents an important step in neurophysiology. We demonstrated the ability to control numerous spatial aspects of the system, including print radius, print density, internodal distance, and geometric orientation through LDW cell printing. The process is amenable even to sensitive primary mammalian neuronal cells, allowing for near real time monitoring of neural network development. Printed cells maintained high levels of viability, sent out neuronal processes and were able to migrate and proliferate as expected. The ability to analyze high numbers of individual cell populations simultaneously will be beneficial to future experiments, with direct applications in developmental biology, neuropathology, environmental toxicology and drug discovery.

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