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Microfluidics-Based Laser Guided Cell-Micropatterning System

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Abstract

The ability to place individual cells into an engineered microenvironment in a cell-culture model is critical for the study of *in vivo*-relevant cell-cell and cell-extracellular matrix interactions. Microfluidics provides a high-throughput modality to inject various cell types into a microenvironment. Laser guided systems provide the high spatial and temporal resolution necessary for single-cell micropatterning. Combining these two techniques, the authors designed, constructed, tested, and evaluated 1) a novel removable microfluidics-based cell-delivery biochip and 2) a combined system that uses the novel biochip coupled with a laser guided cell-micropatterning system to place individual cells into both 2D and 3D arrays. Cell-suspensions of chick forebrain neurons and glial cells were loaded into their respective inlet reservoirs and traversed the microfluidic channels until reaching the outlet ports. Individual cells were trapped and guided from the outlet of a microfluidic channel to a target site on the cell-culture substrate. At the target site, 2D and 3D pattern arrays were constructed with micron-level accuracy. Single-cell manipulation was accomplished at a rate of 150 $\mu\text{m/s}$ in the radial plane and 50 $\mu\text{m/s}$ in the axial direction of the laser beam. Results demonstrated that a single-cell can typically be patterned in 20-30 seconds, and that highly accurate and reproducible cellular arrays and systems can be achieved through coupling the microfluidics-based cell-delivery biochip with the laser guided system.

1. Introduction

The ability to pattern cells at the single-cell level is essential in understanding the fundamental molecular mechanisms that regulate cell-cell and cell-ECM (extracellular matrix) interactions. These interactions, which play a significant role in cellular

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heterogeneity, occur via direct contact or by exchange of soluble-factors at distinct spatial and temporal points. To study these spatiotemporally specific interactions, cell-micropatterning techniques have been developed to create cell-culture models with a defined cell arrangement [1-5]. For populations of stem cells or cancer cells, for example, several techniques have been developed to achieve single-cell micropatterning to investigate cellular heterogeneity.

Microcontact printing (μ CP) was one of the first cell-micropatterning techniques [6]; to promote or inhibit cell adhesion, typical μ CP involved chemical modification of the substrate surface [7, 8]. Recently developed single-cell-micropatterning techniques are based on atomic force microscopy (AFM) [9], dielectrophoresis (DEP) [10, 11], microfluidic devices [12-14], and laser guided systems [15, 16].

Microcontact printing and AFM allow the user to tightly control the 2D surface properties of the substrate by creating intricate designs of immobilized proteins for controlling cell adhesion and growth. However, substrate modification alone does not allow for precise nonstatistical cell-micropatterning. DEP and microfluidics utilize fluid flow to move cell-suspensions through microchannels to pattern single-cells. To achieve single-cell micropatterning, microwells or auxiliary microfeatures must be incorporated into the system to immobilize microfluidics-delivered cells at each micropatterning site [17-19]. This enables high-throughput cell micropatterning, but places limits on substrate geometries and spatiotemporal resolution. Laser guided cell-micropatterning systems utilize optical forces to trap and guide cells in solution and place them at a specific site in the cell-micropatterning substrate with very high spatial (sub-micron) and temporal (seconds) resolution. In conventional laser guided cell-micropatterning systems, cell-suspensions are typically placed in the micropatterning chamber by nonspecific cell-solution delivery [20-22] or by an external hollow fiber attached to a micropump [23, 24]. Problems with these methods include low cell throughput, nonspecific patterning areas, air bubbles, and cell aggregation; each can lower the efficiency of laser guided cell-micropatterning.

To address these problems and enhance performance, a novel microfluidics-based, on-chip cell delivery system was designed as a removable component for the laser guided cell-micropatterning system. When this system (previously developed by our group [24]) is coupled with the microfluidics-based cell-delivery biochip, it provides unique features: 1) the capacity to simultaneously pattern two or more cell types. Thus, in contrast to the microcontact-printing technique, multiple treatments of the target surface specific to each cell type are not necessary; 2) the capacity to produce specific cell-micropatterning arrays and geometries with high spatial resolution that can easily be achieved in real time by precisely moving the microscope stages during cell deposition; and 3) the capacity to create 3D cell-pattern constructs by layering various cell types. Here, we report the design, construction, testing, and evaluation of the microfluidics-based cell-delivery biochip.

2. Materials and methods

2.1. Microfluidics-based laser guided cell micropatterning

2.1.1. System design—Figure 1 illustrates the overall structure of the microfluidics-based laser guided cell-micropatterning system used for single-cell micropatterning. It is composed of four modules: laser guidance optics, illumination and imaging optics, cell-micropatterning chambers (including the cell-micropatterning substrate and the microfluidics-based cell-delivery biochip), and the computer-controlled 3D microscope stages. The laser source (a 2W CW Ti:Sapphire laser tuned at 800 nm and operated in TEM00 mode) is coupled to a single-mode optical fiber and fed into the microscope through a fiber collimator. The collimated light is delivered, after passing through a dichroic mirror, into the back aperture of the objective (a 20× Mitutoyo long working distance objective, 0.42 NA), which is used as a laser-micropatterning and microscope-imaging lens. A green LED light source is used for illumination. The imaging beam is reflected by the dichroic mirror and fed through a tube lens into the charge-coupled device (CCD) camera that is mounted on a 3D optical stage. An infrared filter is positioned directly in front of the CCD camera to remove any randomly scattered laser beam.

The cell-micropatterning chamber was a petri dish (60 mm) modified with a 40 mm machine-cut hole in the center. A circular # 1 glass coverslip (50 mm, Fisher Scientific) was mounted on the bottom of the modified petri dish to seal it. The coverslip can be used as a viewing window or directly as the cell-culture substrate. To enclose the cell-patterning chamber, a petri dish lid was modified with a 40 mm machine-cut hole in the center and a circular 50 mm # 1 glass cover slip was mounted on top of it. The cell-micropatterning chamber was mounted on a motorized computer-controlled three-axis stage (Aerotech®). Movement of individual cells from the output ports of the microfluidics-based cell-delivery biochip to the target site was guided by movement of the stage relative to the stationary laser beam. The cell was trapped in the center of the beam and simultaneously guided to the desired location. Cell navigation was user- controlled with a standard wired Xbox-360 controller (Microsoft). The left joystick manually controlled stage movement in the X and Y axes, and the right joystick controlled movement of the Z axis. The relative vertical position of each joystick set the velocity and direction of the corresponding axes. The left trigger on the controller linked to an optical shutter, which turned the laser beam on or off. The representative positions of the A, B, X, and Y controller buttons were indicated by colored arrows overlaid via live-stream video onto the micropatterning environment, allowing user-navigation.

2.2. Microfluidics-based cell-delivery biochip and cell-micropatterning substrate

2.2.1. Microfluidics-based cell-delivery biochip fabrication—The microfluidics-based cell-delivery biochip and 3D engineered cell-micropatterning substrates were fabricated of polydimethylsiloxane (PDMS). The microfluidic biochip comprises three layers of PDMS (figure 2). The inlet reservoirs were fabricated by pouring a PDMS solution (two-part elastomer Sylgard 184 with a 10:1 base-curing-agent ratio, Dow Corning) into an empty 35 mm petri dish and baked (85 °C for two hours) on a hotplate to obtain a 10 mm thick membrane. The PDMS was cut into two 15-mm square blocks and a 6 mm hole was

punched (Harris Uni-Core, Sigma) into the center of the squares to form a reservoir. The top layer was fabricated by pouring the PDMS solution into a 60 mm petri dish and baking (85 °C for one hour) on a hotplate to reach a final membrane-thickness of 1 mm. A 25 mm square was cut out of the PDMS membrane and two 4 mm holes were punched (Harris Uni-Core, Sigma) to complete the top layer. The channel layer, with approximately 50 μ m deep microchannels, was made using standard photolithography and soft lithography processes. The 2D features for the flow channel (figure 3(A)) were designed with SolidWorks/AutoCAD software and printed to a monochrome photomask (CAD/Art Services, Inc.). A master mold on a silicon wafer was created through photolithography using photosensitive SU-8 epoxy (Microchem) and then placed in a petri dish. The same PDMS solution was poured into the dish to produce a layer (approximately 1mm thick) of membrane with a 50 μ m deep groove as the channel. Once the PDMS cured (85°C for one hour), the channel layer was peeled off the master mold.

Assembly of the microfluidic biochip started by plasma treatment of the inlet reservoir blocks and the top layer followed by baking on a hotplate (140 °C for one hour) to create an irreversible bond. After baking, the channel layer was plasma-treated and bonded to the top layer, creating a seal to form microfluidic flow channels. To complete the assembly, the entire microfluidic injection biochip was baked on a hotplate (140 °C for one hour). Following sterilization, the chip was ready for use in conjunction with the cell-culture substrate. The assembly of the cell-micropatterning chamber (top not shown) is illustrated in figure 2.

Figure 3(A) schematically represents the microfabricated channel layer. The red highlighted region depicts the 50 μ m deep groove that formed the microfluidic channels (one inlet and two output ports). The central microfluidic channels, measured from the center of the inlet reservoirs (6 mm in diameter), were approximately 6 mm long (200 μ m wide). After branching, the width of each channel remained approximately 200 μ m. At the exit region, each microfluidic channel fanned out to a final width of 1000 μ m at the output port to reduce the influence of the total channel length (~17.5 mm) on the flow rate.

2.2.2. Cell-culture substrates—The 3D engineered PDMS membranes mounted on the glass bottom of the cell-micropatterning chamber and the glass bottom itself served as substrate materials for cell-culture experiments. Each surface was modified with a cationic polymer to promote cell attachment.

2.2.2.1. Glass coverslip: A CELL-VU® gridded glass coverslip (Millennium Sciences) and a 3D engineered substrate described above were used to validate single-cell placement with micron-level accuracy. The surfaces of the coverslips and the 3D culture substrate were modified (Section 2.2.2.3) to promote cellular adhesion. The 3D engineered substrate was used to highlight the versatility of the microfluidics-based laser guided cell-micropatterning system to create geometrically-constrained systems to study cell-cell interactions at the single cell level.

2.2.2.2. 3D engineered pattern substrate: A pattern master mold, as an example of typical applications, was created by modifying the fabrication procedure described in Section 2.2.1.

Due to the small features of the pattern design (e.g., the castle structure in figure 6), 10% v/v xylene (Fisher Scientific) was added to the 10:1 mixture of the PDMS solution to decrease viscosity and enhance its deposition into the mold microfeatures. The master mold was silanized with trimethylchlorosilane (TMCS, Supelco) for 30 minutes to assist in PDMS removal. Then, the PDMS mixture was spin-coated (500 rpm for 30 s) onto the master mold to generate an approximately 200 μm , uniformly thick membrane. The membrane was baked at 125 $^{\circ}\text{C}$ for 20 minutes to cure the PDMS. The membrane was removed from the mold and baked in a vacuum oven at 125 $^{\circ}\text{C}$ overnight. Then, a three-stage solvent oligomer extraction and a subsequent overnight vacuum drying process were performed to remove any residual solvent. The membrane was cleaned, sterilized, and activated by oxygen plasma before being mounted to the interior of the glass-bottom petri dish. Then, the entire dish was heated at 50 $^{\circ}\text{C}$ for 2 hours to create an irreversible bond.

2.2.2.3. Cell-micropatterning substrate surface modification: The surfaces of the cell-micropatterning substrates were modified by physisorption of a cationic polymer to improve cell attachment. Immediately after a 10 minute oxygen plasma treatment, 1.5 mL of 0.01% poly-L-lysine (PLL) solution (Sigma) was pipetted into the glass-bottom petri dish. The surfaces of the coverslips and PDMS membranes were allowed to soak in the PLL solution at room temperature for 24 hours. After 24 hours, the PLL solution was removed and the membrane was washed three times with phosphate buffered saline (PBS) solution to remove any unbound PLL.

2.2.3. Implementation of the assembled microfluidic biochip into the cell-micropatterning system—The microfluidic biochip was mounted on top of the cell-culture substrate (figure 2). Cell-micropatterning media was syringe-injected into the inlet reservoir to prime the microfluidic channels. Once stable gravitational force-induced microfluidic flow was established in the channels, cell-suspensions (e.g., 50×10^4 cells/mL) were added to the inlet reservoirs. The paths from the inlet reservoirs to the outlet ports were of equal length to ensure cell-suspension was evenly and equally delivered to each port. Based on a 5 mm fluid height in the inlet reservoir, COMSOL multiphysics simulations (figure 4) indicated an approximate flow rate in the branched channels of 500 $\mu\text{m/s}$ and approximately 90 $\mu\text{m/s}$ flow rate at the outlet ports.

2.3. Cell isolation and imaging

2.3.1. Cell isolation, fixation, and staining—Primary chick forebrain neurons (CFNs) and chick glial cells were used to demonstrate application of the microfluidics-based laser guided cell-micropatterning system. Embryonic Day 8 White Leghorn chicks were dissected, and CFNs were harvested according to the Heidemann protocol [25]; glial cells were harvested from Embryonic Day 14 White Leghorn chicks according to the Kentroti protocol [26]. Neurons and glia were provided with the same pattern medium: NeurobasalTM medium (Gibco®) (without L-glutamine), supplemented with 1% 1 $\times$ GlutaMaxTM (Gibco®), 1% antibiotic/antimycotic (10,000 units/mL penicillin G sodium, 10,000 $\mu\text{g/mL}$ streptomycin sulfate), 50 $\mu\text{g/mL}$ gentamicin, 2.5 $\mu\text{g/mL}$ amphotericin B (Sigma), and 2% B27 (Gibco®), to create cell-suspensions of 50×10^4 cells/mL. The CFNs were live-stained with Vybrant® DiI cell-labeling solution (Life Technologies). After laser

guided micropatterning, cell cultures were maintained in 2 mL of pattern medium for 1 hour at 37 °C and 5% CO₂ in an incubator to promote development and cell attachment to the substrate. Then, all pattern medium was removed and cells were fixed in 4% paraformaldehyde (Sigma) for 12 min. The fixation solution was removed, and cells were treated with 0.05% Triton X-100 (Sigma) for 5 min. The Triton X-100 solution was removed; diamidino-2-phenylindole (DAPI) nucleic acid stain (Thermo Scientific) was added, and the cells were left in the dark for 60 min. The DAPI solution was removed, and a mounting agent (ProLong® Gold Antifade Reagent, Invitrogen) was applied to finish the cell-preservation process.

2.3.2. Cell imaging—Phase-contrast and fluorescent images of laser micropatterned CFNs and glial cells (figure 5, 20× magnification) and a single CFN patterned onto a 3D engineered substrate (figure 6, 40× magnification) were obtained using a Zeiss Axiovert 200 M microscope. The stained nuclei and cell membranes of layered CFNs (figure 7) were imaged with a Nikon Eclipse Ti confocal microscope to illustrate the 3D-patterning ability of the microfluidics-based laser guided cell-micropatterning system.

3. Results

3.1. Operation of the microfluidics-based laser guided cell-micropatterning system

The complete microfluidic biochip (figure 2) was placed on top of various substrates to deliver cell-suspensions to the cell-micropatterning zone. A SolidWorks animation (movie I) and a real-time recording of the microfluidics-based laser guided cell-micropatterning process (movie II) are included as supplemental materials. Figure 3(B) shows the actual cross-section of a microfluidic channel. The fabricated channel had slightly distorted dimensions (top width of 210 µm, bottom width of 220 µm, and a channel height of 55 µm) in comparison with the designed rectangular cross-section (Section 2.2.1.)

A COMSOL multiphysics simulation of the flow rate through the microfluidic channel layer (figure 4) was conducted to determine the exit velocity of cell-suspensions from the microchannels. Figure 4 shows the simulation results based on the rectangular dimensions described in Section 2.2.1. The gravitational force and surface tension created by the amount of fluid in the reservoir (~1.4 mL, 5 mm height, and 6 mm diameter) provided an estimated hydrostatic driving pressure of ~45 Pa. The outlets were connected to a much larger reservoir so that the surface tension could be neglected, and the outlet pressure was treated as 0 Pa. Our data demonstrated that, given the simulation parameters, the flow-field velocity for cell delivery was not sensitive to the minor differences in the channel geometry between the designed and experimental microchannels. The simulation results indicated that the flow rate of cell-suspensions at the outlet ports was approximately 90 µm/s. The simulation results were supported experimentally (movie II), where cells had been experimentally estimated to leave the outlet ports of the microfluidic biochip at a flow rate between 50-90 µm/s. The flow rates produced were sufficient to keep cells in suspension and slow enough for the laser to trap and guide them from the outlet ports. Movement of the three-axis stage relative to the stationary laser beam allowed cells to be patterned to a predetermined target site on the cell-culture substrate. A laser power of 150 mW inside the culture chamber

allowed 150 $\mu\text{m/s}$ radial and 50 $\mu\text{m/s}$ axial cell-manipulation speeds, permitting cells to be patterned to areas on the substrate up to several millimeters (~ 5 mm) from the outlet port.

3.2. Cell-micropatterning experiments

3.2.1. Heterotypic 2D cell-micropatterning—To demonstrate the ability of simultaneous micropatterning of two heterotypic cell populations, the microfluidic biochip was utilized with two different homotypic cell-suspensions to create a 2D heterotypic cellular array. Figure 5 shows a combined fluorescence and phase-contrast image of a laser-micropatterned CFN array. Cells from two different microfluidic channels were simultaneously patterned onto a gridded glass coverslip. One channel was used to deliver DiI live-stained cells (red) and the other channel was used for non-stained cells. The cells were manually placed at the edges of a $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ square on the gridded glass coverslip. That is, we did not use the computer-controlled positioning algorithms that we reported in 2005 and 2006 to demonstrate submicron accuracy [27, 28]. In this study, we demonstrated with the gridded coverslip that the use of the microfluidic biochip does not adversely affect the resolution of laser guided cell-micropatterning. Rather, the method we report here eliminated the complications typically associated with placement of multiple cell types by laser cell-micropatterning; improving the performance of the laser guided cell-micropatterning system.

3.2.2. Cell-micropatterning onto a 3D engineered substrate—Fabrication of 3D features into a cell-culture substrate allows the geometric structures to influence cellular development. As an example, a 20 μm high PDMS castle was microfabricated to confine an individual CFN. The castle had eight radially aligned microchannels, which (made the PDMS castle an appearance of a circumferentially distributed triangles) allowed the confined neuron to develop dendrites and an axon that would extend through one of the eight microchannels. Figure 6 shows a single CFN that was laser-micropatterned onto a castle. At 16 hours, the development of the CFN demonstrated the influence that the microchannels have on the axonal projection. Once the extending axon reaches the end of the microchannel, an axonal pathfinding choice point is reached for studying regulations of axonal pathfinding (results not shown). Removal of the microfluidic injection biochip did not disturb the patterned cell; culture continued in the open environment. The patterned cell remained viable, and true single-cell isolation was achieved.

3.2.3. Building 3D cellular constructs—Figure 7(A) represents a side-view confocal image of laser-micropatterned cells. Stained nuclei (blue) that were presented at three distinct heights indicated three distinct cell layers. The bottom layer of cells was patterned from a glial cell-suspension reservoir. The middle and top layers were patterned from a CFN cell-suspension reservoir. CFN membranes were live-stained with DiI (red) to distinguish the various cell layers (figure 7(B)). As can be seen in figure 7(C), only cells from the CFN reservoir were present on the top layer. Despite the 1-hour culture period before the cells were fixed, axonal extension from one neuron to another was observed. These results show that viable 3D cellular constructs can be fabricated using the microfluidics-based laser guided cell-micropatterning system.

4. Discussion

4.1. Laser guided cell-micropatterning and microfluidics

The use of laser optics in cell patterning provides a means to create bottom-up engineered cell constructs and cell assays. Schiele and colleagues describe the many variations of laser-based direct-write techniques for cell printing [29]. Laser tweezers, as described by Ashkin [30] and laser-guidance systems share many similar characteristics. With an optical-tweezers system, Pine and colleagues reported lifting single cells plated onto a polyhydroxyethylmethacrylate (polyHEMA) surface and transferring them over a distance of 5 mm at a speed up to 200 $\mu\text{m/s}$ with a laser output power of 100 mW [22]. Similarly, we were able to guide cells over a total distance of 5 mm at a speed of 150 $\mu\text{m/s}$ in the radial plane and 50 $\mu\text{m/s}$ in the axial direction of the laser beam. Although the techniques can be applied to achieve comparable 2D cell-patterning results, laser tweezers utilize a high numerical aperture (NA) microscope to generate a strongly focused laser beam, whereas laser guidance employs a low NA microscope objective to produce a weakly focused laser beam. A highly focused beam creates a very strong trap, but it greatly reduces the effective working distance of the microscope (in Pine's system, it is 100 μm); thus, laser tweezers are not feasible for research in which a few cells need to be accurately positioned in a 3D construct or onto large features ($>100 \mu\text{m}$) of an engineered cell-culture substrate. For the microfluidics-based laser guided cell-micropatterning system, the vertical range of cell manipulation is at the level of a few centimeters (20 mm); it thus can be used to precisely move individual cells to a specific location in a 3D culture constructed in a petri dish.

Neither of the two conventional methods used to deliver cells for laser-guided cell patterning is without problems. The first method involves loading (e.g., by pipetting) cells into the cell-micropatterning chamber before laser guided cell-micropatterning [20-22]. This cell-delivery method requires complicated modification of the patterning surface to isolate reservoirs for multiple cell types. The second method, performed during laser cell micropatterning, involves pulsatile injection of small volumes of cell-suspensions into a chamber filled with culture medium [24, 31]. The injection setup consists of costly, mechanically manipulated syringes (one per cell type) loaded with a cell-suspension that is connected to the culture environment by a hollow fiber. Initially, the syringe and hollow fiber must be plumbed to remove air bubbles from the injection line; periodically, the position of the fiber must be adjusted to allow for coverage of the entire cell-patterning area. Between injection pulses, cell clumping and aggregation can clog the fiber. When this occurs, the fiber must be removed from the culture environment, cleared, and reinserted to continue administration of cells. Each time the system requires adjustment there is a risk of culture contamination. To increase the efficiency, sterility, and specificity of single-cell laser-guided patterning, we designed and implemented the microfluidic-injection biochip (Section 2.2.1). The continuous, gravity-driven flow of cell-suspension in the microfluidic channel provides constant cell delivery throughout the entire cell-micropatterning process, which typically lasts between one and two hours.

4.2. 2D heterotypic cellular arrays

Tissues comprise various cell types that are spatially organized to achieve unique function through specific cellular interactions. Precise spatial arrangement of homotypic and heterotypic cells are essential for replicating the cell-cell interactions and functions observed in native tissues. For example, interactions between micropatterned homotypic cell pairs have been shown to influence cell structure, polarity, and proliferation [32, 33]. Numerous heterotypic cell-micropatterning systems have been devised to determine the nature of the interactions between certain cell types [34-36]. A thorough overview of the use of micropatterning to determine cell morphogenesis and function is provided by Théry [37]. However, many conventional methods to generate homotypic and heterotypic cell interactions focus on the culture or coculture of large numbers of cells that are not segregated into specific cell pairs. Figure 5 shows the micropatterning result of the microfluidics-based laser guided system in which individual cells were selected and simultaneously patterned into a 100 μm square to create a heterotypic cellular array. This demonstrates that the system permits the user to reproducibly control both the isolation of cell pairs and the distance at which they are separated. Advantages of this patterning method (which can be expected to increase single-cell viability) include: the capacity to evaluate the effect individual cells may have on each other without the influence of other cells; the decrease in overall micropatterning time; and the elimination of possible sources of bacterial contamination. The results of such micropatterning experiments may reveal previously unknown mechanisms related to cellular heterogenesis [38].

4.3. 3D cellular constructs

In vivo, precise spatial and temporal arrangement of different cell types is vital for normal tissue development. A fundamental aspect of recreating the native environment *in vitro* is organizing various types of cells with high spatial and temporal resolution. A number of methods have been used to create 3D constructs *in vitro*. One popular technique involves use of an inkjet printer [39, 40]. Despite this technique's robust ability to create cell layers for tissue constructs, there remains a lack of control over the precise arrangement of cells. Without a spatially defined structure, observing and recording the interaction between cell types or the migration of a cell from one place to another becomes less accurate.

Neuron-glia interactions play an incredibly important role for the proper development of the nervous system. Glial cells function in many ways to support neuron survival by: 1) maintaining the ionic environment surrounding the neuron, 2) modulating the rate of nerve signal propagation, 3) augmenting synaptic action by regulating neurotransmitter metabolism, 4) providing a scaffold for neuron migration or development, 5) secreting extracellular signaling molecules for axon guidance, and 6) aiding in the recovery of nerve cells due to injury or disease [41]. In the developing telencephalon, transient midline glial structures support the reciprocal growth of cortical axons to form the corpus callosum [42]. These cells serve as intermediate targets, also known as "guidepost cells," where molecular signaling molecules are secreted to influence axon pathfinding [43]. While glia monolayers serve as excellent growth substrates for axons, it has been shown that the beneficial growth properties for axons are dependent on spatial orientation and time [44]. Therefore, here we

used neuronalglial co-micropatterning as a realistic example to show our system's ability to create 3D spatiotemporal arrangements of heterogenic cell types.

We demonstrated the ability to create a 3D pyramidal structure of patterned glial cells and CFN neurons. Figure 7(A) shows the laser guidance system's capacity to create multiple layers (three in this case) of cells in a specific manner, resulting in a 3D construct. It can be seen that the pyramid structure lost cells from the second and third layers in the time between patterning and imaging. To preserve the structure of 3D-patterned cell constructs, each layer should include extracellular matrix-promoting cells to ensure proper adhesion of cells of interest. Despite the loss of some of the structure, cells were visible at three distinct levels providing evidence that patterned biological 3D constructs are possible using this method.

5. Conclusions

A microfluidics-based laser guided cell-micropatterning microscope was developed to increase the efficiency of using laser guidance to manipulate cells *in vitro*. A removable microfluidic biochip was fabricated and implemented into the laser guided cell-micropatterning system to allow the user to select a single cell from a cell-suspension and guide it to a target site on a cell-culture substrate. With this system, small numbers of cells can be patterned with the high spatial accuracy needed for systematic study of cell-cell interactions in an open culture environment. Simultaneous patterning of heterotypic cell types into 2D and 3D cellular arrays can be achieved to create arrangements and structures that mimic cellular interactions *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Alom Ruiz S, Chen CS. Microcontact printing: A tool to pattern Soft Matter. 2007; 3:168–77.
2. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. Nat. Biotechnol. 2008; 26:120–26. [PubMed: 18026090]
3. Guilak F, et al. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell. 2009;517–26.
4. De Silva MN, et al. Micro-patterning of animal cells on pdms substrates in the presence of serum without use of adhesion inhibitors. Biomed. Microdevices. 2004; 6:219–22. [PubMed: 15377831]
5. De Silva MN, et al. Two-step cell patterning on planar and complex curved surfaces by precision spraying of polymers. Biotechnol. Bioeng. 2006; 93:919–27. [PubMed: 16358279]
6. Kumar A, Whitesides GM. Features of gold having micrometer to centimeter dimensions can be formed through a combination of stamping with an elastomeric stamp and an alkanethiol “ink” followed by chemical etching. Appl. Phys. Lett. 1993; 63:2002–04.

7. Mrksich M, et al. Using microcontact printing to pattern the attachment of mammalian cells to self-assembled monolayers of alkanethiolates on transparent films of gold and silver. *Exp. Cell Res.* 1997; 235:305–13. [PubMed: 9299154]
8. Oliva AA, et al. Patterning axonal guidance molecules using a novel strategy for microcontact printing. *Neurochem. Res.* 2003; 28:1639–48. [PubMed: 14584818]
9. Staii C, et al. Positioning and guidance of neurons on gold surfaces by directed assembly of proteins using atomic force microscopy. *Biomaterials.* 2009; 30:3397–404. [PubMed: 19342092]
10. Faenza A, et al. High yield patterning of single cells from extremely small populations. *Anal. Chem.* 2013; 85:3446–53. [PubMed: 23418883]
11. Rosenthal A, Voldman J. Dielectrophoretic traps for single-particle patterning. *Biophys. J.* 2005; 88:2193–205. [PubMed: 15613624]
12. Hsiao AP, et al. Microfluidic device for capture and isolation of single cells. *Proc. Soc. Photo Opt. Instrum. Eng.* 2010; 7759:77590W–90W-9.
13. Wheeler AR, et al. Microfluidic device for single-cell analysis. *Anal. Chem.* 2003; 75:3581–86. [PubMed: 14570213]
14. Yun K-S, Yoon E. Micro/nanofluidic device for single-cell-based assay. *Biomed. Microdevices.* 2005; 7:35–40. [PubMed: 15834518]
15. Ma Z, et al. Laser guidance-based cell micropatterning. *Cell and organ printing.* 2010:137–59.
16. Odde DJ, Renn MJ. Laser-guided direct writing of living cells. *Biotechnol. Bioeng.* 2000; 67:312–8. [PubMed: 10620261]
17. Skelley AM, et al. Microfluidic control of cell pairing and fusion. *Nat. Methods.* 2009; 6:147–52. [PubMed: 19122668]
18. Di Carlo D, et al. Dynamic single cell culture array. *Lab on a Chip.* 2006; 6:1445–49. [PubMed: 17066168]
19. Frimat J-P, et al. A microfluidic array with cellular valving for single cell co-culture. *Lab on a Chip.* 2011; 11:231–37. [PubMed: 20978708]
20. Barron JA, et al. Laser printing of single cells: Statistical analysis, cell viability, and stress. *Ann. Biomed. Eng.* 2005; 33:121–30. [PubMed: 15771266]
21. Guduru SK, et al. Analysis of neurite outgrowth for a laser patterned neuronal culture. 2nd International IEEE EMBS Conference on Neural Engineering. 2005:336–39.
22. Pine J, Chow G. Moving live dissociated neurons with an optical tweezer. *IEEE T. Biomed. Eng.* 2009:561184–88.
23. Pirlo RK, et al. Laser-guided cell micropatterning system. *Rev. Sci. Instrum.* 2011; 82:013708–08-6. [PubMed: 21280838]
24. Ma Z, et al. Laser-guidance-based cell deposition microscope for heterotypic single-cell micropatterning. *Biofabrication.* 2011; 3:034107. [PubMed: 21725149]
25. Heidemann SR, et al. The culture of chick forebrain neurons. *Meth. Cell Biol.* 2003; 71:51–65.
26. Kentroti S, Vernadakis A. Differential expression in glial cells derived from chick embryo cerebral hemispheres at an advanced stage of development. *J. Neurosci. Res.* 1997; 47:322–31. [PubMed: 9039654]
27. Bakken DE, et al. Laser micropatterning of polylactide microspheres into neuronal-glial coculture for the study of axonal regeneration. *Macromol. Symp.* 2005; 227:335–44.
28. Pirlo RK, et al. Cell deposition system based on laser guidance. *Biotechnol. J.* 2006; 1:1007–13. [PubMed: 16941447]
29. Schiele NR, et al. Laser-based direct-write techniques for cell printing. *Biofabrication.* 2010; 2:032001. [PubMed: 20814088]
30. Ashkin A. Optical trapping and manipulation of neutral particles using lasers. *PNAS.* 1997; 94:4853–60. [PubMed: 9144154]
31. Pirlo RK, et al. Biochip/laser cell deposition system to assess polarized axonal growth from single neurons and neuron/glia pairs in microchannels with novel asymmetrical geometries. *Biomicrofluidics.* 2011; 5:013408–11.
32. Nelson CM, et al. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating rhoa. *Mol. Biol. Cell.* 2004; 15:2943–53. [PubMed: 15075376]

33. Desai RA, et al. Cell polarity triggered by cell-cell adhesion via e-cadherin. *J. Cell. Sci.* 2009; 122:905–11. [PubMed: 19258396]
34. Bhatia SN, et al. Controlling cell interactions by micropatterning in co-cultures: Hepatocytes and 3t3 fibroblasts. *J. Biomed. Mater. Res.* 1997; 34:189–99. [PubMed: 9029299]
35. Felton EJ, et al. Heterotypic cell pair co-culturing on patterned microarrays. *Lab on a Chip.* 2012; 12:3117–26. [PubMed: 22739471]
36. Tsuda Y, et al. Heterotypic cell interactions on a dually patterned surface. *Biochem. Biophys. Res. Commun.* 2006; 348:937–44. [PubMed: 16901464]
37. Théry M. Micropatterning as a tool to decipher cell morphogenesis and functions. *J. Cell. Sci.* 2010; 123:4201–13. [PubMed: 21123618]
38. Altschuler SJ, Wu LF. Cellular heterogeneity: Do differences make a difference? *Cell.* 2010; 141:559–63. [PubMed: 20478246]
39. Boland T, et al. Application of inkjet printing to tissue engineering. *Biotechnol. J.* 2006; 1:910–17. [PubMed: 16941443]
40. Xu T, et al. Inkjet printing of viable mammalian cells. *Biomaterials.* 2005; 26:93–99. [PubMed: 15193884]
41. Nedergaard M, et al. New roles for astrocytes: Redefining the functional architecture of the brain. *Trends Neurosci.* 2003; 26:523–30. [PubMed: 14522144]
42. Shu T, Richards LJ. Cortical axon guidance by the glial wedge during the development of the corpus callosum. *J. Neurosci.* 2001; 21:2749–58. [PubMed: 11306627]
43. Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. *Science (New York, N Y).* 1996; 274:1123–33.
44. Faissner A. Axon guidance by glia. *Encyclopedia of neuroscience.* 2009:1063–72.

Figure 1.
Diagram of the microfluidics-based laser guided cell-micropatterning system.

Figure 2.
An exploded assembly and overall diagram of the microfluidics-based cell-delivery biochip and cell-culture substrate.

Figure 3. (A) 2D schematic of the microfluidic biochip flow-channel layer (depth is 50 μm). (B) Phase contrast image (20 $\times$) of a microchannel cross-section. Scale bar 50 μm .

Figure 4.
COMSOL simulation for the flow rate of cell-suspensions through the microfluidic biochip.

Figure 5. Fluorescence and phase contrast combined image (40x) of a laser-micropatterned CFN array. The two DiI live-stained cells (red) were from one microfluidics-cell-delivery channel, the remainders were from the other channel. Scale bar 25 μm .

Figure 6.

A single CFN at 16 hours: It was selected from the microfluidic cell-delivery channel and laser-micropatterned onto a PDMS-based cell-culture substrate.

Figure 7.

3D construct of glial cells (Bottom Layer) and CFNs (Middle and Top Layer). (A) DAPI-stained (blue) confocal side-view image showing different heights of cell nuclei. The top layer of cells is highlighted in orange, the middle layer is highlighted in yellow, and the bottom layer is highlighted in white. (B) Side-view confocal image with DAPI-stained (blue) nuclei and DiI-stained (red) CFN membranes. (C) Top-down view of the top layer of patterned CFNs with DAPI-stained (blue) nuclei and DiI-stained (red) cell membranes.