Active 3-D microscaffold system with fluid perfusion for culturing *in vitro* neuronal networks†‡

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This work demonstrated the design, fabrication, packaging, and characterization of an active microscaffold system with fluid perfusion/nutrient delivery functionalities for culturing *in vitro* neuronal networks from dissociated hippocampal rat pup neurons. The active microscaffold consisted of an 8 × 8 array of hollow, microfabricated, SU-8 towers (1.0 mm or 1.5 mm in height), with integrated, horizontal, SU-8 cross-members that connect adjacent towers, thus forming a 3-D grid that is conducive to branching, growth, and increased network formation of dissociated hippocampal neurons. Each microtower in the microscaffold system contained a hollow channel and multiple fluid ports for media delivery and perfusion of nutrients to the *in vitro* neuronal network growing within the microscaffold system. Additionally, there were two exposed Au electrodes on the outer wall of each microtower at varying heights (with insulated leads running within the microtower walls), which will later allow for integration of electrical stimulation/recording functionalities into the active microscaffold system. However, characterization of the stimulation/recording electrodes was not included in the scope of this paper. Design, fabrication, fluid packaging, and characterization of the active microscaffold system were performed. Furthermore, use of the active microscaffold system was demonstrated by culturing primary hippocampal embryonic rat pup neurons, and characterizing cell viability within the microscaffold system.

Introduction

Motivation

Most of the neurophysiological research on re-aggregate neuronal cultures to date has been performed with 2-D (planar) *in vitro* neuronal cultures on 2-D multielectrode arrays (MEAs).[1,2] However, the problem with single neuron or 2-D neuronal network studies is that every task performed by the brain requires the coordinated interaction of multiple, 3-D neuronal networks.[3–5] The primary motivation for studying 3-D neuronal cultures is to provide a spatial environment which closer approximates the *in vivo* environment of the brain (*i.e.*, 3-D cell matrix). It has been argued that 2-D *in vitro* neuronal cultures fail to properly mimic the behavior of 3-D *in vivo* neuronal networks. Furthermore, it has been demonstrated that 2-D cultures display differing electrical responses from their 3-D counterparts, which implies that 3-D cell cultures allow for more cell-to-cell interactions and, therefore, more accurately mimic actual *in vivo* cellular networks.[6,7]

3-D neuronal cultures can provide neuroscientists with a better model to study the nervous system than past 2-D neuronal cultures have provided.[8–11] However, *in vitro* culturing of 3-D neuronal networks has been difficult in the past, since *in vitro* cultures composed from dissociated neurons lack intact, functioning, circulatory systems. In the absence of a functioning circulatory system, numerous factors, such as a lack of nutrient distribution, gas exchange, and waste removal, contribute to rapid necrosis in the center of the cell culture.[12–14] Specifically, 3-D re-aggregate neuronal cultures have been performed with limited success due to the absence of a functioning circulatory system.

The lack of an appropriate cellular scaffold with circulatory system-like functionality has resulted in a stunted gathering of information on the functioning dynamics of 3-D neuronal complexes. Subsequently, biologists have been unable to obtain an adequate understanding of how the brain functions under normal, healthy conditions, and in response to traumatic or degenerative injury.[15] In turn, this lack of knowledge with regard to the functioning of 3-D neuronal networks has directly affected the clinical sector, resulting in insufficient treatments for traumatic brain injuries, neurodegenerative diseases, and paralysis, to name only a few.

The active microscaffold system presented in this paper enables the growth and analysis of re-aggregate *in vitro* neuronal networks, providing increased neuronal network

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‡ The HTML version of this article has been enhanced with colour images.

complexity (representative of a quasi 3-D culture) over planar neuronal cultures produced on standard 2-D MEAs. The neurons cultured on the active microscaffold system grew and formed networks on the differing planar surfaces of the microscaffold structure, thereby providing increased network complexity. Furthermore, the fluid perfusion functionality of the active microscaffold system allows for biochemical control of the extracellular environment, serving as an artificial circulatory system to enable the possibility of long-term 3-D *in vitro* neuronal cultures. Although *in vivo* experiments provide the best real-time response from living animals and inherently serve as 3-D tissue culture models, the benefit of *in vitro* studies lies in the fact that environmental conditions, stimuli, and injury to the cells/network can be controlled with an exact precision that is unattainable in a living animal. Additionally, an *in vitro* culture system eliminates complex variables such as immune response and inflammatory factors that arise within *in vivo* cultures.[14]

Background/significance

There has been a significant effort towards the development and characterization of scaffold materials for 3-D cell cultures.[9–11] Our work builds on the conventional concept of passive scaffolds for cell culturing by adding an active integrated fluid perfusion functionality directly into the microscaffold system. The active microscaffold's integrated fluid perfusion functionality mimics a functioning circulatory system to augment the study of current 2-D neuronal networks and works towards 3-D *in vitro* neuronal growth and network formation of the re-aggregate neuronal cells.

Extensive research has been performed on 2-D *in vitro* neuronal networks; with MEAs for extracellular recording/stimulation of 2-D *in vitro* neuronal networks being well established.[7] Also, microfluidic technologies have been developed in order to selectively nourish and pattern 2-D *in vitro* neuronal cultures. Furthermore, integration of planar MEAs with microfluidic devices (porous MEAs) has combined the ability to gather electrical data from a 2-D neuronal network while extending the culture lifetime.[13,14]

The primary objective of our work was to develop an active 3-D microscaffold system for culturing *in vitro* re-aggregate neuronal cultures that have increased network complexity over the standard planar neuronal cultures performed on 2-D MEAs. The key features of the microscaffold system were the microtower arrays, cross-connects, and integrated microfluidic perfusion functionality for control of nutrient delivery at precise locations within the culture, thus enabling long-term viability of *in vitro* neuronal networks with increased complexity. The objective of the research was to enable *in vitro* culturing of re-aggregate neuronal networks through use of the active microscaffold system with nutrient perfusion functionality, thus providing neuroscientists with a superior *in vitro* model for studying neuronal networks than is currently available.

Methods

Design of the microscaffold system

The microscaffold system consisted of an 8 × 8 array of SU-8 microtowers that projected vertically from a 1.5 cm × 1.5 cm × 500 μm Si orifice plate substrate. Each microtower projected 1.0 mm or 1.5 mm above the Si orifice plate, and extended out the backside of the Si orifice plate by 1.5 mm. Additionally, adjacent microtowers were structurally joined on the topside by horizontal SU-8 cross-members in both the *x* and *y*-directions. A schematic of the active microscaffold system consisting of an 8 × 8 array of vertical microtowers with integrated cross-connects is provided in Fig. 1(a). The microscaffold consists of eight separate linear

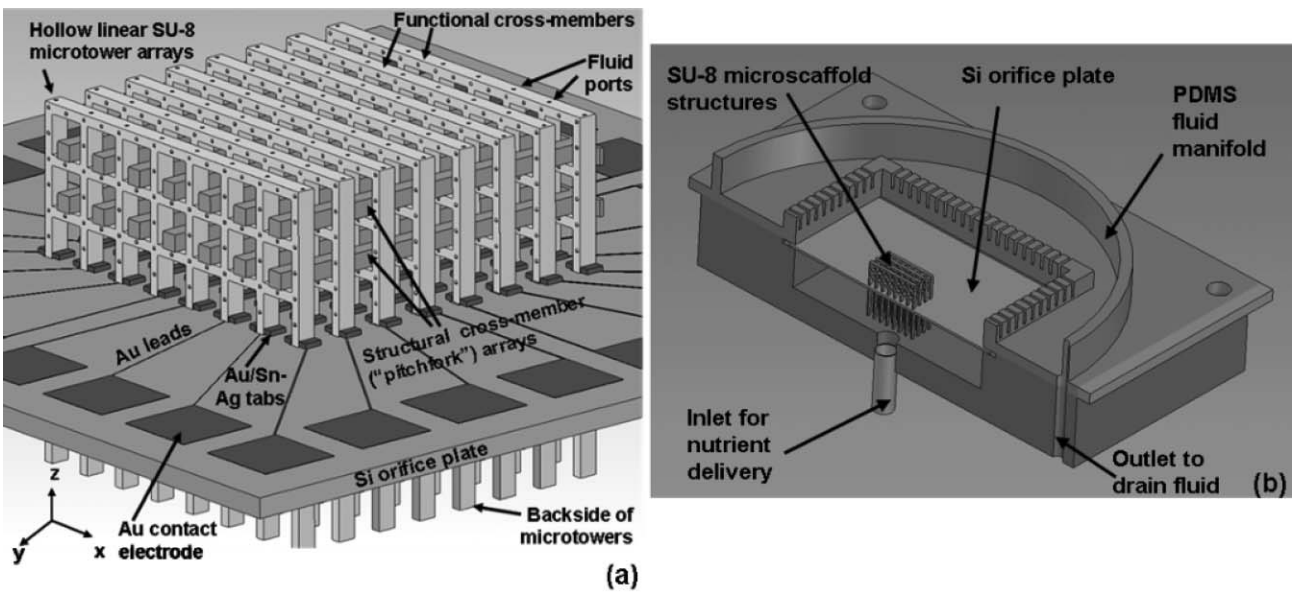

Fig. 1 Schematics showing the microscaffold system. (a) An 8 × 8 array of hollow microtowers with functional cross-connects along the *x*-direction and structural cross-connects along the *y*-direction. The microtowers in this schematic project 1.5 mm out the topside and backside of the Si orifice plate. (b) A 3-D cross-section of the microscaffold device encased in the PDMS fluid manifold. The fluid manifold allows for continuous perfusion of the cells growing within the active microscaffold.

microtower arrays packaged in succession, one adjacent to the next. Each microtower and functional cross-member contained a hollow fluid channel and multiple fluid ports to aid in fluid perfusion/nutrient delivery to the *in vitro* neuronal network growing on the topside of the microscaffold device.

The vertical SU-8 microtowers in the 8 × 8 array design had a center-to-center spacing of 600 μm, and the horizontal cross-members had a center-to-center spacing of 500 μm. Each of these SU-8 microtowers had a 120 μm width and an 85 μm depth. In addition, a hollow fluid channel (with a cross-sectional area of 70 × 23 μm^2) extended along the entire length of each microtower. Furthermore, each of the horizontal cross-members that ran parallel to the *x*-direction also contained a fluid channel of identical dimensions. However, the cross-members that ran orthogonal to the linear tower arrays were purely structural and contained no fluidic functionality. The fluid channels in the microtowers and functional cross-members served as a means of fluid/nutrient transport in the system. To facilitate fluid perfusion/nutrient delivery, there were numerous fluid ports (20 × 20 μm^2) along the length of each microtower and functional cross-member, which allowed for nutrient perfusion, gas exchange, and biochemical control of the extracellular environment throughout the neuronal cell culture growing on the topside of the microscaffold system. The total active volume of the microscaffold available for cell culture in the 8 × 8 microtower array format was 18.7 μL or 28.0 μL, depending on the height (1.0 mm or 1.5 mm, respectively) of the microtowers in the array.

Although characterization of the electrical stimulation/recording functionalities of the microscaffold are not included in the scope of this paper, electrodes were integrated into each microtower at varying heights to enable future electrical stimulation and recording of the neuronal network cultured on the microscaffold system. Two 15 × 15 μm^2 gold (Au) electrodes were positioned either along the length of each microtower or on the functional cross-members. Corresponding electrical leads were insulated within the microtower walls and connected to large structural Au tabs where the microtower arrays intersected the Si orifice plate. The Au tabs served a dual purpose: (1) to prevent the linear tower arrays from sliding further through the Si orifice plate upon packaging, and (2) to provide future electrical connectivity between the traces located on the vertical microtowers and those patterned on the horizontal Si orifice plate. Hence, to integrate electrical functionality of the microtower electrodes, it will be necessary to form a 90° bond between the electroplated Au tabs on the microtowers and the electroplated Sn–Ag tabs on the Si orifice plate, which connect to the Au traces on the Si orifice plate (see Fig. 1(a)). The large Au contact electrodes seen at the periphery of the Si orifice plate in Fig. 1(a) will allow integration of the future electrically functioning microscaffold system with the Multi Channel Systems preamplifier to obtain stimulation/recording data from the cultured neuronal network once electrical functionality has been established in the active microscaffold system.

In order to enable integration of the fluid perfusion functionalities of the active microscaffold system, a two-piece poly(dimethylsiloxane) (PDMS) (Dow Corning) fluid manifold was designed which sandwiched the Si orifice plate at its periphery. The schematic shown in Fig. 1(b) provides a 3-D cross-section of the PDMS fluid manifold and microscaffold system. The fluid manifold served as an interface between the microscaffold and the external fluid delivery system. Tubing for fluid delivery was attached to the inlet port at the base of the manifold *via* a syringe pump. The fluids first entered the bottom pool in the PDMS manifold, and then entered the fluid channel at the base of each microtower. The fluids then traveled up through each of the microtower lengths, through their functional cross-arms, and out the fluid ports to perfuse the neuronal network cultured on the top-side of the microscaffold system.

Fabrication approach

The microtower arrays were fabricated through a series of micromachining processes including photolithography, metal sputtering, electroplating, and wet and dry etching. The linear SU-8 tower arrays are first fabricated in a planar fashion on top of a Si wafer, as shown in Fig. 2.

First, a 1600 Å thick chromium (Cr) release layer was deposited on the Si wafer by dc sputtering in an argon (Ar) atmosphere. Next, the outer wall of the tower arrays was photolithographically patterned using a 5 μm thick SU-8 2005 layer. The first SU-8 layer was exposed with UV at 90 mJ cm^{-2}, post-exposure baked (PEB) on a hotplate at 65 °C for 1 min and 95 °C for 1 min, developed for 2 min in P-type thinner (Shipley) with mild agitation, rinsed in IPA for 2 min, and hard baked at 150 °C for 30 min to improve adhesion and durability. The outer wall of this first SU-8 layer contained 20 × 20 μm^2 fluid side ports and 15 × 15 μm^2 openings for electrodes. Next, an 1800 Å thick Au layer was dc sputtered and a thick (23 μm) AZ 4620 (Clariant) photoresist layer was

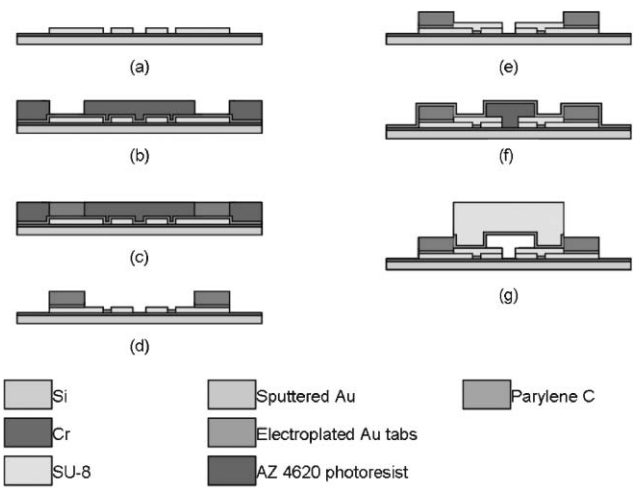

Fig. 2 Cross-sections of the planar fabrication process for the SU-8 microtower arrays. (a) A Cr release layer, followed by the first SU-8 layer. (b) An Au layer, followed by a 23 μm thick electroplating mold. (c) Thick (23 μm) Au tabs are electroplated. (d) The AZ 4620 electroplating mold is removed and the Au electrodes and leads are patterned. (e) The second SU-8 layer. (f) Sacrificial layer of AZ 4620 photoresist is defined, followed by deposition of a Parylene C layer. (g) The third SU-8 layer is patterned, the exposed Parylene C is etched away, and the sacrificial photoresist is removed.

spun on and patterned to form an electroplating mold. Au tabs 100 × 40 μm^2 were electroplated inside the mold on both sides of each SU-8 microtower to a thickness of 23 μm. Techni-Au 25-ES (Technic Inc.) electroplating bath and a platinum (Pt) mesh electrode were used to electroplate the Au microtower tabs (1.5 mA, 55 °C, 4.5 h). The photoresist electroplating mold was then cleaned off the wafer using acetone and isopropanol (IPA), and dried with N_2. The Au electrodes and corresponding leads (two per tower) were patterned and wet etched using a potassium iodide (100 g L^{-1}) and iodine (25 g L^{-1}) solution. The Au leads were subsequently insulated using a second SU-8 layer with a thickness of 5 μm. The second SU-8 layer was exposed with UV at 140 mJ cm^{-2}, post-exposure baked (PEB) on a hotplate at 65 °C for 1 min and 95 °C for 2 min, developed for 6 min in P-type thinner with mild agitation, rinsed in IPA for 2 min, and hard baked at 150 °C for 30 min to improve adhesion and durability. This second SU-8 layer was photolithographically patterned and also contained 20 × 20 μm^2 fluid ports to match the previous (first) SU-8 layer.

The hollow channel was defined using a 23 μm thick sacrificial layer of AZ 4620 photoresist. Next, a uniform 2.0 μm thick Parylene C coating was deposited by chemical vapor deposition (CVD) on the sample, which served to prevent mixing of the sacrificial resist with the top (third) SU-8 layer. The third SU-8 layer (SU-8 2025) was spin coated to a thickness of 75 μm and patterned to form the opposing outer wall of the SU-8 microtower. The third SU-8 layer was exposed with UV at 450 mJ cm^{-2}, post-exposure baked (PEB) on a hotplate at 65 °C for 1 min and 95 °C for 4 min, developed for 8 min in P-type thinner with mild agitation, and rinsed in IPA for 2 min. The third SU-8 layer was not hard baked since hard baking would have caused problems removing the AZ 4620 sacrificial layer from the fluid channels. The exposed Parylene C external to the array structure was etched away in an O_2 plasma using a reactive ion etch (Plasma Therm). Finally, the Cr release layer was wet etched in CR-7S Cr etchant (Cyantek) in order to release the completed SU-8 structures from the Si wafer. After removal from the Cr etchant, the released microtower arrays were rinsed in de-ionized (DI) water for 15 min and air dried.

3-D vertical packaging

Once the linear microtower arrays had been released from their original substrate by etching the Cr sacrificial release layer, a single microtower array was picked up by means of a vacuum hose and manually inserted into the through holes in the Si orifice plate. The large Au tabs at the base of each microtower served as mechanical stops to prevent the tower arrays from sliding further through the Si orifice plate. The linear arrays of microtower structures were individually packaged in succession, one adjacent to the next, thus forming a complete 8 × 8 array of vertical microtowers.

The Si orifice plates were fabricated by deep reactive ion etching (DRIE) in a Plasma Therm inductively coupled plasma (ICP) system. The through holes in the Si orifice plate were made using the Bosch process and had dimensions of 200 × 200 × 500 μm^3. When the SU-8 microtower arrays were first placed into the Si orifice plate, they had a slight tilt and were not positioned orthogonally to the Si orifice plate. Orthogonal alignment of the SU-8 tower arrays with respect to the Si orifice plate was accomplished with the aid of a tooling jig constructed by stereolithography (SLA). The SLA alignment jig contained a slit to hold the Si orifice plate, and also contained an array of comb-like teeth that slid in between adjacent packaged linear microtower arrays. The comb-like teeth on the SLA structure straightened out each packaged microtower array so that the vertically packaged linear microtower arrays formed 90° angles with respect to the Si orifice plate. Also, the structural design of the SLA jig forced the Au tabs at the base of each microtower to make contact with the Si orifice plate.

A mixture containing a 3:1 ratio of 184 PDMS and hexane was placed on the backside of the orifice plate to create a fluid seal at each of the through holes in the orifice plate. The PDMS mixture was cured in an oven at 70 °C for 40 min. Subsequently, the sacrificial AZ 4620 photoresist used to define each of the fluid channels was removed by a 1.5 h acetone soak, followed by a 15 min IPA soak. Scanning electron micrographs (SEMs) of the topside of a vertically packaged and sealed 8 × 8 microtower array are shown in Fig. 3 left. Additionally, Fig. 3 right provides SEMs of

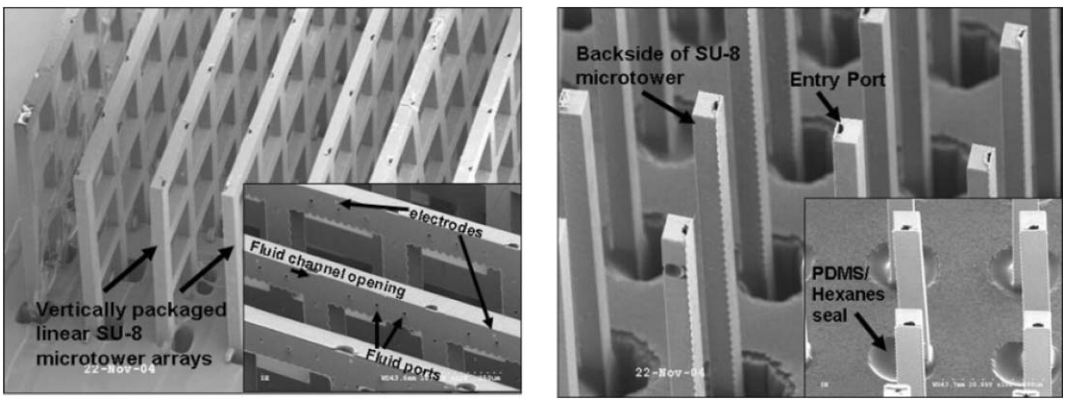

Fig. 3 SEM micrographs of the frontside and backside of the packaged microtower arrays. Left: Topside view of 8 linear SU-8 microtower arrays (1.5 mm tall) packaged and sealed into the Si orifice plate. The fluid channel openings, fluid ports, and electrodes can be seen in the inset. Right: Backside view of the packaged and sealed (inset) 8 × 8 SU-8 microtower array, showing the microtowers projecting 1.5 mm out from the backside of the Si orifice plate.

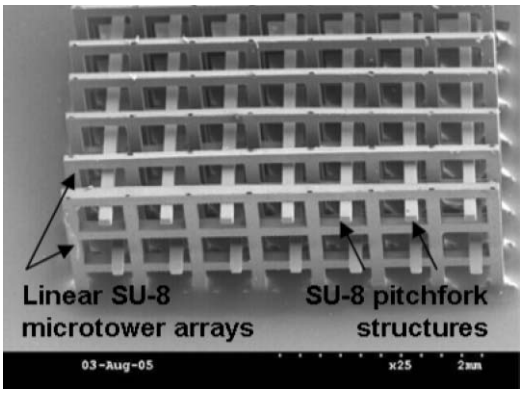

Fig. 4 SEM micrograph of the vertically packaged microtower arrays with the joining pitchfork structures that connect adjacent linear microtower arrays. These pitchfork structures are solid SU-8.

the backside of a vertically packaged and sealed 8 × 8 microtower array.

After the linear microtower arrays had been vertically packaged and sealed into the Si orifice plate, solid SU-8 "pitchfork" structures were slid into the packaged array of microtowers and sealed into place with PDMS. These "pitchfork" structures served as additional cross-members that connected adjacent linear microtower arrays to each other, and were implemented to allow the formation of neuronal networks between adjacent linear microtower arrays once dissociated neuronal cells had been plated on the active microscaffold device. An SEM micrograph of the vertically packaged microtower arrays with the joining pitchfork structures is provided in Fig. 4.

Biocompatibility

Neurons plated onto the SU-8 were cultured for 7 days and stained with Calcein-AM as a fluorescence measure of viability. On untreated SU-8, live cells averaged 240 ± 40 cells mm^{-2}. Following techniques for improved viability on SU-8, including prolonged heating, ultraviolet (UV) treatments, O_2 plasma, and IPA sonication,[16] this low viability increased to 1080 ± 70 cells mm^{-2} ($p < 0.001$). Other treatments, such as liquid CO_2, glow discharge, and a piranha etch, were experimented with to enhance the biocompatibility of the SU-8 without significant success.

The biocompatibility treatments that worked the best for culturing neurons were a 90 000 mJ cm^{-2} UV light exposure, followed by a 150 °C vacuum oven bake for 3 d, a 60 s O_2 plasma treatment, and a 60 s IPA sonication. The UV light exposure and the vacuum oven bake helped to fully cure and hard bake the SU-8, while the O_2 plasma treatment rendered the SU-8 surface hydrophilic to promote cell adhesion. Finally, the 60 s IPA sonication removed any remaining photoresist residues in the microtowers, and significantly improved cell survival on the SU-8 microtower arrays. The biocompatibility treatments mentioned above were are all performed after the SU-8 microtowers were vertically packaged and sealed into the Si orifice plate, but prior to packaging the device into the PDMS fluid manifold.

Biological experiments

New methods were developed to ensure that the microtowers in the interior of the arrays received similar poly-D-lysine (PDL) rinsing treatments and neuron applications as the microtowers around the periphery of the array. To prepare the microscaffold for neurons, the microtower assemblies were soaked in 70% ethanol (EtOH) overnight for sterilization. A vacuum exposure followed by re-application of EtOH ensured interior access of the EtOH. While wet, three cycles of rinsing in sterile water were followed by rinsing three times in 100 μg mL^{-1} PDL (Sigma). The last solution was left on the structures for at least 4 h or overnight. Immediately before plating neurons, culture medium (Neurobasal/B27/0.5 mM glutamine/25 μM glutamate, Invitrogen) was rinsed through the arrays five times.

Hippocampal neurons were isolated from embryonic day 18 rats (BrainBits LLC) according to established procedures.[17] Neurons isolated by digestion of the brain tissue in papain (Worthington, 2.0 mg mL^{-1} in Hibernate E (BrainBitsLLC)) were re-suspended in culture medium and applied to the arrays at 500 cells mm^{-2} of structure surface area in a 35 mm D culture dish. After 10 min in the incubator (Forma, 5% CO_2, 9% O_2), another batch of neurons was again applied to the arrays. After 30 min in the incubator, 3.0 mL of medium was added to the Petri dish to completely cover the arrays and associated neurons.

Briefly, the culture medium was exchanged with Neurobasal. Cells were loaded with Calcein-AM (5 μg ml^{-1}, Molecular Probes) for 1 h at 37 °C. After a rinse with warm Hibernate E low fluorescence (BrainBitsLLC) to preserve control of pH at ambient CO_2, neurons were imaged under a 20 × /0.95 NA Olympus water immersion objective. Viable cell fluorescence was excited with blue light. Green fluorescence was collected on a Q imaging Retiga CCD camera. Z-stacks were collected and deconvolved with Image Pro + software. Additionally, SEM analysis was performed on fixed neurons on the unpackaged SU-8 microtower arrays after 17 DIV.

Results

A PDMS fluid manifold, which serves as an interface between the microscaffold and the external fluid delivery system, was designed and fabricated to house the Si orifice plate and the packaged microtower arrays. The PDMS fluid manifold was designed in two pieces (a top piece and a bottom piece), which were created using 3-D paraffin wax molds. The wax molds were built in a 3-D rapid prototyping wax printer (Thermojet, 3-D Systems). Schematics of the top and bottom wax mold pieces and photographs of the respective PDMS structures created from those wax molds are shown in Figs. 5(a) and (b).

The bottom PDMS manifold piece contained a recess into which the 1.5 cm × 1.5 cm × 500 μm Si orifice plate fit and was sealed into place with PDMS. Next, the top and bottom PDMS manifold pieces were aligned and sealed together with PDMS. A 3-D cross-section of the fluid manifold integrated with the microscaffold is provided in Fig. 1(b). Tubing for media delivery was attached to the inlet port at the base of the manifold. The fluids first filled the pool at the bottom of the manifold, and then traveled up through each of the tower

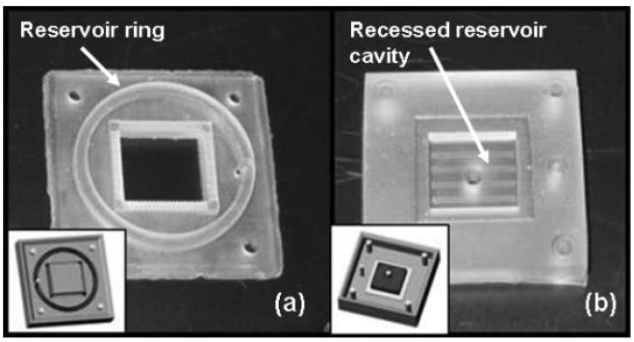

Fig. 5 PDMS fluid manifold. (a) Photograph of the top PDMS manifold piece. Inset shows a schematic of the 3-D wax mold for the top manifold section. (b) Photograph of the bottom PDMS manifold section. Inset shows a schematic of the 3-D wax mold for the bottom manifold piece.

lengths, through the tower cross-arms, and out of the fluid ports to perfuse the 3-D neuronal network proliferating within the active microscaffold system.

The fluid was delivered to the inlet port *via* a syringe pump. Initial characterization of the fluidic system was performed by imaging the flow of de-ionized (DI) water dyed with food coloring through the microscaffold/fluid manifold package. Since the SU-8 was transparent, it was possible to observe the fluidic path of the colored DI water and determine whether there were any leaks in the PDMS/Si orifice plate seal. Furthermore, fluid functionality of the microscaffold/fluid manifold package was validated by running a mixture of polystyrene beads (1.0 μm in diameter, Molecular Probes) and DI water through the functional microtower cross-arms and out of the fluid ports along the length of the towers.

Microscope images of a completed 2-D (planar) released microtower array with the sacrificial photoresist removed from the fluid channels (thus forming the hollow fluid channels) are provided in Fig. 6. Fig. 6 shows a microtower array with two Au electrodes and insulated leads per microtower. The 15 × 15 μm^2 Au electrodes are exposed on the horizontal cross-members, and the corresponding insulated Au leads run horizontally along the length of each cross-member and vertically down the length of each microtower to connect to the large electroplated Au tabs at the base of each microtower. Fig. 6 shows the microtower array from the side which originally faced the Si substrate during fabrication (the first SU-8 layer), which is the side upon which the 20 × 20 μm^2 fluid ports and 15 × 15 μm^2 holes for each exposed electrode were fabricated. Also, due to the transparent properties of the SU-8, it is possible to see the hollow fluid channels, fluid ports, and Au electrodes and leads in Fig. 6. The inset in Fig. 6 shows a close up of an SU-8 microtower and functional cross-member, providing a clear image of the hollow fluid channels (width of 70 μm) and fluid ports (20 × 20 μm^2) centered along the hollow channels. The microtowers in this microscope image have a width of 120 μm and the functional cross-members have a width of 110 μm. Additionally, the keg wheel pattern seen along the periphery of the SU-8 microtower structure provided additional structural integrity at the interface between the overlying and underlying SU-8 layers.[18]

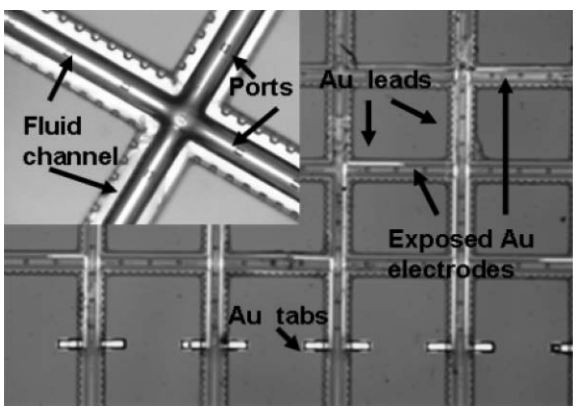

Fig. 6 Microscopes images of a completed 2-D (planar) microtower array with 2 Au electrode and leads per microtower. The sacrificial photoresist has been removed, thus forming hollow fluid channels along the length of each microtower and functional cross-member.

Fig. 7 provides a stereoscope image of an 8 × 8 array of 1.5 mm tall microtowers and pitchfork structures packaged and sealed into a 3-D format, thus yielding the active microscaffold system. The microtowers in Fig. 7 extended 1.5 mm out of both the topside and backside of the 500 μm thick Si orifice plate. The microtowers are each 120 μm wide and have a center-to-center spacing of 600 μm. The functional cross-members are 110 μm wide and have a center-to-center spacing of 500 μm. Fig. 7 also depicts the two Au electrodes and leads per microtower, the large Au electroplated tabs at the base of each microtower, and the fluid ports that run along the length of each microtower and functional cross-member.

Once the linear microtower arrays were released from their original Si substrate, they were vertically packaged and sealed into the Si orifice plate with PDMS. Subsequently, the structural cross-members (or pitchforks) were manually inserted in between the packaged microtower arrays, resting upon the functional cross-members, and were also sealed in place with

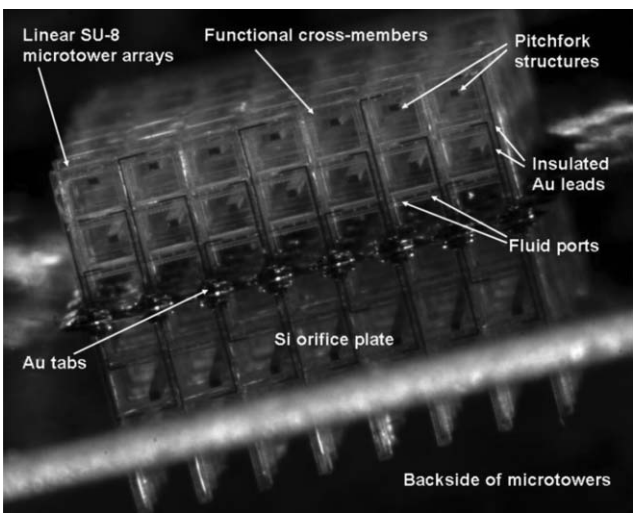

Fig. 7 Stereoscope image of an 8 × 8 array of 1.5 mm tall microtowers and pitchforks packaged and sealed into a 3-D format, thus yielding the active microscaffold system.

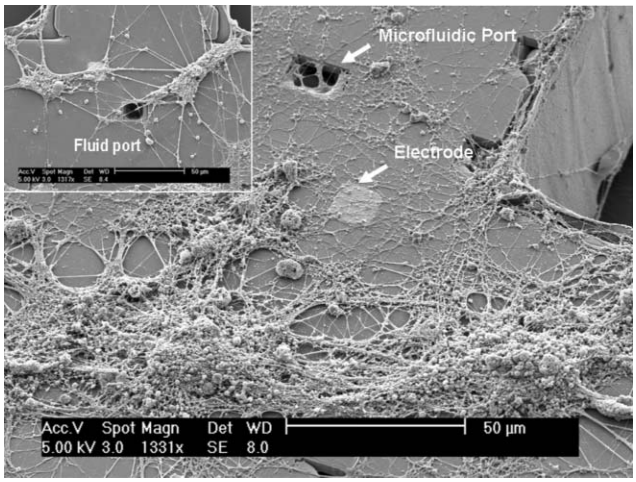

Fig. 8 SEM micrograph of neuronal growth on an SU-8 microtower. Electrodes and fluid ports can be seen.

PDMS. Next, the sacrificial photoresist was removed using a 1.5 h acetone soak followed by a 15 min IPA rinse to form the hollow fluid channels. The sacrificial photoresist could be removed either before or after the microtowers were vertically packaged, but it was found that removing the sacrificial photoresist after the packaging and PDMS sealing process helped to better maintain the integrity of the hollow fluid channels. If the sacrificial photoresist had been removed from the microtower arrays prior to the packaging and PDMS sealing process, it was found that the PDMS sealing process was more likely to cause blockages in the hollow fluid channels.

With a neuron plating density of 50 000 cells cm^{-2}, significant growth of neurons on the microtower arrays was observed after four to six days in culture. Cell viability was confirmed using live/dead cell fluorescent staining, and SEM analysis was also performed to observe cell distribution and network formation on the different planar surfaces of the microtower structures. The qualitative evaluation of neuronal growth was made by the adhesion of branching somata and neurites. During the 17 d of culture on the unpackaged SU-8 microtower arrays, neurons adhered strongly to the microtower surfaces and abundant neurite branching was observed on the differing planes of the microtower arrays. An SEM micrograph of neuronal growth at 17 DIV on an SU-8 microtower is provided in Fig. 8, where it can be seen that the fluid ports and electrodes did not seem to hinder cell growth. As can be seen in Fig. 8, the neurons grew well on both SU-8 insulation/structural material and on the exposed Au electrodes ($15 \times 15\ \mu m^2$). Interestingly, cells occasionally grew so as to partially cover the fluid ports ($20 \times 20\ \mu m^2$). The neural fibers tended to grow along the edge of the microtower structures, which implies that electrodes positioned along of the edge of the microtowers should be considered in future microtower designs.

Conclusions

The active microscaffold system is an enabling technology that assists in the growth and complexity of *in vitro* re-aggregate neuronal networks. The structural design of the microscaffold system (a 3-D microtower array with integrated cross-connects that join adjacent microtowers) allows for increased neuronal network formation from re-aggregate cells, and the integrated fluid perfusion/nutrient delivery capabilities of the microscaffold allow biochemical control of the extracellular environment by assisting in nutrient delivery, gas exchange, and waste removal at various depths within the culture. One important biological set of questions revolves around the dependency of cell growth, differentiation, and mix of cell types on the delivery of local fluid flow to simulate a vasculature. Further, the neuronal networks cultured on the active microscaffold system exhibit an increased complexity compared with neuronal networks cultured on standard planar 2-D MEAs. Therefore, the neuronal networks cultured on the presented active microscaffold system are likely to have different functional/computational properties from the standard planar 2-D neuronal cultures cultured on commercial 2-D MEAs.

Future work will analyze the increased complexity of the multiple planar neuronal networks cultured on the active microscaffold system, which will be investigated by recording electrical activity from the neuronal network using the embedded microtower electrodes in the active microscaffold system. A similar structure made of PDMS was used to control the cell organization in 3-D,[19] and although this application was for fibroblasts, it underscores the experimental importance of understanding the response of tissues to 3-D microstructures that will always, at least partially, interfere or influence their *in vitro* organization. Future work also includes integration of the microscaffold system with neural processing chips and the Multi Channel Systems preamplifier in order to electrically stimulate/record from 59 sites while simultaneously delivering nutrients/aeration to the *in vitro* neuronal network cultured on the active microscaffold system.

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