Development of an Enhanced Microfluidic Platform for Three-Dimensional Neuron Cell Culture

John C.S. McCaw, Bryson M. Brewer, Lijie Yang, Deyu Li

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BRIEF. Here the development of a novel microfluidic device is described that enhanced media perfusion that can benefit neurons cultured in a three-dimensional matrix, attempting to model the *in vivo* microenvironment in the brain.

ABSTRACT. Primary neurons are a highly difficult cell type to culture as a result of their delicate structure and complex environmental needs. Due to the central role of neurons in the body and subsequent involvement in many diseases, reliable *in vitro* cell culture is necessary for understanding neuronal function and behavior. While modern microfluidic technology has provided improved methods for *in vitro* neuron culture, current approaches still do not provide precise modeling of the *in vivo* neuronal microenvironment. Notably, neurons reside in a three-dimensional environment *in vivo*; thus an accurate *in vitro* culture model should incorporate three-dimensional components. In this report, the optimization of a microfluidic device is described that enhanced media perfusion for neurons cultured in a 3D matrix that allowed dynamic imaging and can be applied to culturing neurons. Specifically, potassium hydroxide was used to etch an oxide coated silicon wafer beveled edges, which was then used with standard soft-lithography techniques to construct the microfluidic device. The resulting beveled edges yielded improved sealing in the device, and allowed for improved flow rate and perfusion for possible neuron culture. The device outlined was also a highly versatile model applicable for other microfluidic cell culture applications.

INTRODUCTION.

In the modern world, conditions affecting the brain and neurons are increasingly a research priority as more is understood about the brain and its functions. However, relatively little is known about the nature of these diseases and how to treat them. Several conditions have both scientific and public interest, most notably Alzheimer's Disease (AD), neuroepithelial cancers (cancers of the neural tissue and epithelial tissue supporting neurons), and Amyotrophic Lateral Sclerosis (ALS) [1; 2; 3]. Notably, 5.2 million Americans were reported by the Alzheimer's Association 2014 Alzheimer's Disease Facts and Figures report as living with AD, approximately 30,000 Americans were living with ALS in 2011 as reported by the ALS Association, and 20,919 incidences of neuroepithelial cancers were reported in 2011 by the National Program of Cancer Registries (NPCR) [4; 5; 2]. The Alzheimer's Association also reported that AD was the fifth-leading cause of mortality in Americans ages 65 and older and the leading cause of morbidity for this age range [4]. Furthermore, half of all ALS patients die within three years of diagnosis according to the ALS Association, and the Surveillance, Epidemiology, and End Results Program (SEER) reported that only approximately one-third of individuals with neuroepithelial cancer will survive past five years [5; 6]. Viewing the incidence and mortality rates of these diseases, it becomes readily apparent that in-depth research of neurons and neural microenvironments is merited to alleviate the high rates of incidence; however, current approaches to neuron research are in need of an effective *in vitro* system to more accurately model the *in vivo* brain microenvironment. Additionally, current research practices are costly and often do not take into account the complex three-dimensional microenvironment in the brain.

Neurons are a highly difficult cell type to culture as demonstrated by a study that found traditional culture methods produced population viabilities at 40-80%, with 80% being recognized as optimum by Xie, Markesbery and Lovell [7]. Additionally, another study focused on optimizing culture media achieved 60% survival in culture after four days, still below an optimum level [8]. One potential cause of this lowered viability is the difficulty of providing delicate neural cells with a sufficient amount of nutrients in a dish. Also, current cell culture methods are slow, requiring full plates or dishes of culture for each trial. As a result of the large scale of traditional culture methods, inaccuracies are encountered when monitoring any small-scale aspect, as well as a high cost per test. However, microfluidic devices offer greater control of nutrient delivery and environmental conditions and accordingly are strong candidates to improve neuronal cell culture vitality.

Microfluidic devices are mechanical devices applied to the microscale, allowing the minute control of small variables and environments. This field has seen near universal applications among disciplines, such as minute manipulations of cells in microbiology as discussed by Polacheck et. al. and the creation of a microscopic, room temperature microfluidic fuel cell created by Choban et. al. [9; 10]. This merit is due to the versatile ability of microfluidic devices in form and function. Specifically through the use of soft-lithography, devices can be constructed using polydimethylsiloxane (PDMS), which is inert, non-toxic and generally hydrophobic allowing it to create water holding channels, though its surface can be altered to create a hydrophilic state allowing it to bond with other pieces of PDMS. Through these factors, PDMS is shown to be a suitable material for use with cell culturing, in addition to its optical clarity, which allows for microscopy compatibility. In addition to these benefits, microfluidics reduce the size of the culture system and the number of cells needed, allowing for more affordable testing and greater accuracy of results. Finally, through the ability to incorporate various mechanical devices, which can manipulate environmental conditions or the cells directly, different cellular environments can be modeled allowing for more accurate culturing and experimentation.

While modern microfluidic technology has provided improved methods and yields for *in vitro* neuron culture, current approaches still do not provide precise modeling of the *in vivo* three-dimensional neuronal microenvironment, a crucial aspect for the study of neural disease. These *in vivo* microenvironments contain additional elements foreign to traditional culture that are crucial for the accurate study of neural disease, such as confinement stimuli, stress caused by the flow of fluids, and from variable substrates in the body [9]. For example, with the device developed by Gao et. al., it was shown that pneumatic valves have great propensity in microfluidics, but the pneumatic valve developed was unable to provide a full three-dimensional neural microenvironment [11]. In a different three-dimensional microfluidic device, only astrocytes were cultured in a three-dimensional microenvironment while neurons were cultured parallel to the astrocytes on a two-dimensional surface [12]. Altogether, an optimal neuron culturing device requires full *in vivo* modeling including a three-dimensional matrix, the ability to conduct high-throughput testing, culture viability, and sufficient perfusion to allow for greater nutrient exchange.

In this report, the optimization of a thin-membrane valve equipped microfluidic device is described, which provided superior media exposure to a three-dimensional matrix while allowing dynamic imaging of the matrix activity. These conditions suggest an optimal environment for neuron culture and study. This device incorporated a main lower chamber that was separated into three regions by pressure chambers that inflated downward into it. These three horizontal channels were created through the layering of two main PDMS pieces, separated by a thin PDMS membrane that created the valve. Adding hydraulic or pneumatic pressure to the top chambers operated this valve. This produced two outside supporting channels on either side of an interior channel that was deep enough to allow

for the loading of a three-dimensional collagen matrix. These changes to the microfluidic device allowed for the formation of a functional matrix and improved perfusion into the matrix, suggesting an improved method for culturing neurons.

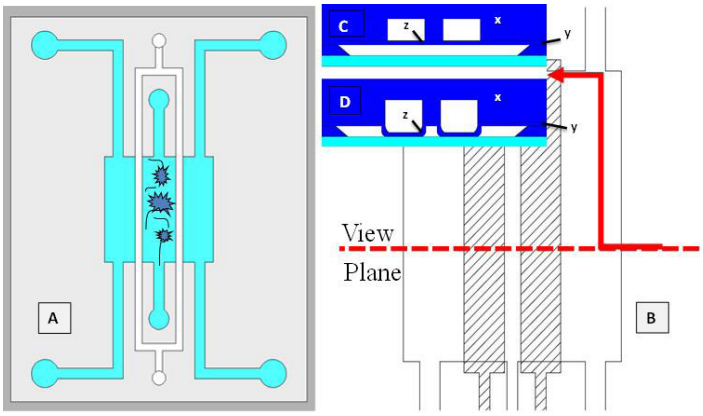

Figure 1. 1A is an overhead schematic of the membrane valve device (A). 1B is an exact drawing of the main culture chamber with pressure chambers above, lined (B). In red is a cross-cut of the diagram, shown in C and D. (C-D) This device incorporated a main lower chamber with three inlets and outlets that was separated into three regions by pressure chambers that inflated downward into the main chamber, all shown in Figure 1A in two planes (A). In light blue is the bottom cell culture chamber and channels and in white is the pressure chamber, and the cells in the center depict where neurons would be cultured. These three horizontal channels were created through the layering of two main pieces, x and y (C), separated by a thin membrane that created the valve, labeled z. Adding hydraulic or pneumatic pressure to the top chambers pushed this thin membrane (z) downward (D), producing two outside supporting channels on either side of an interior channel that was deep enough to allow for the loading of a three-dimensional collagen matrix.

MATERIALS AND METHODS.

Creation of the Silicon Mold.

To create the first silicon mold for the lower chamber, an SPR-220 negative photoresist was patterned onto a silicon wafer of <100> orientation that was coated on both sides with a silicon dioxide layer. Following this, a positive mask outlining was placed evenly on the coated oxide wafer and exposed to UV light. The wafer was then soft baked and developed using an SPR-220 developer. The final feature was rinsed with deionized water to remove excess photoresist and was dried with compressed nitrogen.

1-3 days later, the wafer was removed and the back of the wafer was covered with tape before it was placed in a room-temperature Buffered Oxide Etch (BOE) bath until the silicon dioxide not covered by the photoresist pattern on the front of the wafer was visibly removed. The BOE used was an Ammonium fluoride – hydrofluoric acid mixture from Sigma-Aldrich (40207 ALDRICH St. Louis, MO). The wafer was rinsed with DI water and left to sit in a DI water dilution bath for several minutes, and then was rinsed a second time with DI water. Finally, the wafer was rinsed with acetone and isopropanol and then dried with compressed nitrogen.

For etching of the silicon mold to produce beveled edges, a wet potassium hydroxide (417661 SIGMA-ALDRICH St. Louis, MO) etch was performed. A 45% potassium hydroxide in water solution bath was heated to approximately 70-90°C and the wafer was submerged in the bath and allowed to etch for 1-7 hours, producing approximately feature heights ranging from 50μm to 210μm dependent upon etching time and temperature. Following this, the wafer was rinsed with DI water and dried with compressed nitrogen. The etching produces a 54.7° angle along all edges of the feature due to the <100> silicon crystal, which produced a desired bevel along all edges of the mold feature.

A second silicon mold was created for the pressure chamber piece by patterning SU-8 negative photoresist onto a silicon wafer in the method similar to that outlined above, and the baked SU-8 was used as the mold feature.

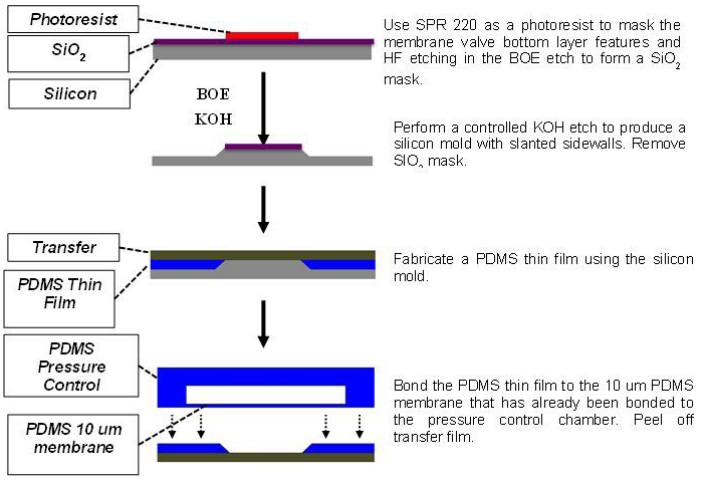

Figure 2. The process for fabricating the devices. It is important to note that the membrane was spin-coated on inactive plastic film while the PDMS thin film lower pieces were fabricated using a coated plastic transfer film.

Fabrication of the Devices.

A thin film was constructed of the bottom layer mold features by using a standard 1:10 PDMS solution (SYLGARD 184 Ellsworth Adhesives Germantown, WI), cured at 65°C for at least two hours with the coated transfer film weighed down on top. Thick bottom layers were also used, made using 1:10 PDMS cured at 65°C. The pressure control chamber was fabricated with a 1:10 solution of PDMS on a silicon mold, which was degassed and cured at 65°C. The membrane was fabricated by spin-coating degassed PDMS onto an inactive plastic film at 3000 RPM, then curing at 65°C. The membrane and pressure chamber were plasma treated and bonded to each other, producing the pressure control chamber. The pressure control chamber and bottom layer were plasma treated and bonded together so that they were aligned in the fashion of Figure 1.

Testing Efficacy of the Devices.

To test the efficacy, green dye was added to the central channel of the device and held in place by the pressurized membrane valve. First, the dye was held in the center chamber for five minutes and monitored throughout for leakage. Following this, the pressure in the pressure chambers was released, showing normal flow into the outer chambers. Finally, a collagen (Type 1 Rat Tail Collagen High Concentration from BD Biosciences) matrix loaded with polystyrene beads (SPHERO™ Polystyrene Particles (5% w/v) from Spherotech Inc.) was loaded in the central chamber of the device and allowed to polymerize at 37°C. The pressure valve was released and dye was loaded into one well of one of the outer chambers, demonstrating the perfusion of the dye across the collagen matrix. During these tests, images were taken at regular intervals to monitor the quality of the seal.

RESULTS.

Efficacy of the Seal Compared to Prior Devices.

Due to a sieve valve effect that occurs when attempting to seal rectangular channels with a deformable polymer membrane, previous devices such as that developed by Gao et. al. had difficulty holding pressure for large channel heights, a requirement to produce a three-dimensional microenvironment [11]. However, the new beveled device was shown to reduce this effect by forming a better seal with the channel sidewall (Figure 3A-C). Note that in Figure 3B and 3C, the small amount of leakage was likely due to diffusion through the thin water layer between the deflected PDMS membrane and the PDMS bottom layer. This leakage was minimal enough to be insignificant in the devices applications to three-dimensional culture, as the collagen matrix was still able to polymerize effectively.

Collagen Matrix and Perfusion Function.

For unobstructed testing, it was necessary for the pressure chamber to fully remove itself from the culture area. Figure 3D-G demonstrates that within 300 seconds, nearly all of the culture area has been filled with dye. Additionally, with a collagen matrix the dye was able to fully move through the matrix within 35 seconds (Figure 3H-M). This demonstrated effective perfusion throughout the culture matrix. Note that the polystyrene beads do not move during the cross-flow, shown in figures 3H-M, suggesting that the collagen matrix remained stable throughout the use of the device. Additionally, the rapidness of the flow indicated that a high rate of fluid flow was generated across the collagen matrix.

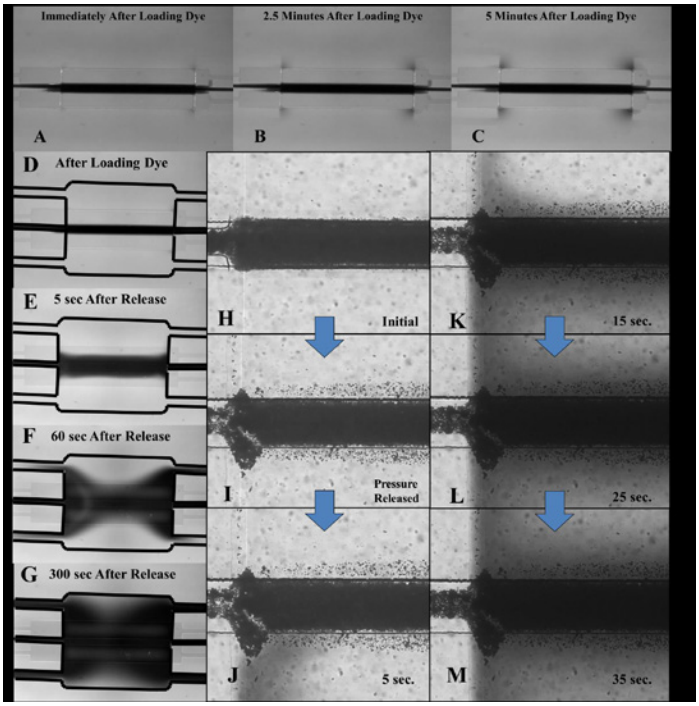

Figure 3. Images taken of the pressurized device with beveled edges immediately (A), at 2.5 (B), and 5 (C) minutes after dye was loaded into the central chamber. (D-G) Two dimensional perfusion after the release of pressure from the pressure control chamber is shown by green dye. Note that the edges of the bottom layer of the device have been outlined in the image for greater visibility. (H-M) A time-lapse of a collagen matrix in the center chamber of the novel device with dye cross-flow. Note that there was some expansion of polystyrene beads between A and B, but these did not move after this time and were likely lifted by the deflating membrane.

DISCUSSION.

Here the development of a microfluidic device that suggests future optimal culturing of neurons in a three-dimensional microenvironment is described. The versatile structure will allow for varied use of its multiple chambers. Specifically, the beveled-edge culture chamber was able to hold a seal for an extended period of time, allowing the use of a collagen matrix to create a more accurate model of *in vivo* conditions. The flow through bodily tissue could be modeled by the flow through a three-dimensional matrix generated in the device, further mimicking the *in vivo* microenvironment. Additionally, this central chamber could be used for the simple culturing of neurons in an optimum environment, as the enhanced perfusion allows for greater nutrient exchange in the culture. To further demonstrate the uses of the described device, neuron populations could be cultured in it to show improved viabilities over the limited traditional culture methods. The neuron populations could also be cultured in the central collagen matrix with astrocytes in the two side chambers and evaluated to show ability for co-culture. A chemoattractant could also be passed through one side of the device with a collagen matrix in the central chamber to generate a short concentration gradient that could be used to evaluate cellular migration and chemotaxis in a three-dimensional matrix. Beyond applications to neurons, this device could be used with other fragile cell types. Altogether, this device allows for many potential applications throughout cell biology, histology, and biochemistry due to its versatile design.

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(Brewer, Torricelli, Evege, & Price, 1993; Choban, Markoski, Wieckowski, & Kennis, 2004; Gao et al., 2011; Y. H. Kim et al., 2011; Polacheck, Li, Uzel, & Kamm, 2013; Xie, Markesbery, & Lovell, 2000.) when antibody-antigen complexes are incubated (37\u00b0C

John C. McCaw is a student at Hillsboro High School in Nashville, Tennessee. He participated in the Research Experience for High School Students (REHSS) program at Vanderbilt University.