NEUROMUSCULAR SYNAPTOGENESIS IN AN OPEN CHAMBER MICROFLUIDIC DEVICE

J. Cheng[1*], T. Chang[1], N. Bhattacharjee[1], and A. Folch[1]
[1]University of Washington, Seattle, WA, United States of America

ABSTRACT
This paper reports on a microfluidic device with high spatiotemporal control used as an *in vitro* model of neuromuscular junction (NMJ) developmental biology. The device is assembled using three poly(dimethylsiloxane) (PDMS) layers and consists of micro-trenches for long-term culture of myotubes and an array of 32 identical focal stimulation jets to stimulate the myotubes. The device operates in an open-chamber format, allowing the cells to be cultured using conventional techniques, which significantly improves cellular viability and enhances protein expression for studying neuromuscular junction development.

KEYWORDS: Focal stimulation, Hydrodynamic focusing, Surface modification, Neuromuscular junction

INTRODUCTION
A major goal in neuroscience is to understand the development of synapses, the highly specialized membrane regions that mediate communication between neurons. The defining characteristic of the mature neuromuscular junction (NMJ) is the high surface concentration of acetylcholine receptor (AChR) clusters on the postsynaptic muscle terminal [1]. The embryonic development of the NMJ synapse is a spatiotemporally dynamic event in which initially diffuse acetylcholine receptor (AChR) patterns on the muscle membrane organize into intricate AChR "pretzel"-like patterns [1] in response to focal signals, most notably agrin, secreted by the innervating motor neuron axon. Intriguingly, all but one of the axons innervating a given myotube retract through a synaptic competition process [2, 3]. The rules by which the muscle cell weighs each signal (i.e. biochemical onset and concentration) in "selecting" one synapse over another remains, for lack of quantitative and spatiotemporally fine tools, poorly understood.

Traditional cell culture tools such as petri dishes and micropipettes do not allow spatiotemporal control of agrin stimulation of myotubes, which occurs at a sub-cellular micron scale *in vivo*. Previous studies have attempted focal agrin stimulation using agrin-coated beads [4] and agrin micropatterns [5], but these approaches have no control over the concentration, onset, or duration of agrin stimulation. Microfluidics offers a high degree of control of the cell microenvironment because transport phenomena such as diffusion and fluid flow velocity profiles are highly predictable and reproducible. Micropatterning and microfabrication allow single cells to be confined within well-defined geometries, allowing for more precise interrogation [6]. In this case, it is important to observe AChR dynamics in single elongated myotubes to avoid confounding factors from neighboring cells. Previous studies in the Folch Lab have demonstrated focal microfluidic agrin stimulation of micropatterned myotubes, effectively replacing the motor neuron with a microfluidic stream of agrin delivered to focal regions of the myotube surface [7-11]. However, these studies were limited to a single stream of focal agrin stimulation on myotubes, which is not representative of the synaptic competition process. Furthermore, experiments were done in a closed chamber format, causing cell death. We build upon the previous studies by designing a novel, user-friendly, highly reproducible open chamber device that will allow for sophisticated control of spatiotemporal and combinatorial variation of NMJ developmental stimuli.

THEORY
The key features of the device includes: 1) micro-trenches coated with a cell-adhesive protein for isolating myotubes and confined by a cell-repellant polymer coating outside the trenches; 2) microfluidic channels that deliver multiple orthogonal focal stimuli to the cells in an open chamber. The device **(Figure 1)** consists of two layers of PDMS: a bottom cell culture layer containing two microtrenches for culturing myotubes, and a top microfluidic layer containing 32 microchannels that deliver stimuli orthogonally to focal regions on the apical surface of the cells (*9*). Flow is driven by positive pressure and vacuum from the vacuum aspiration channel. This work builds upon a previously reported device from MicroTAS 2010 with PDMS surface modification to create a long-term coating of Matrigel, a basement membrane protein extract known to induce aneural AChR "pretzels" **(Figure 2)** [12]. The outside of the trenches is coated with a cell-repelling surfactant so as to confine myotubes within the trenches. C2C12 myoblasts are seeded in the two trenches. The trench geometry protects the cells from flow-induced shear stresses. The whole system is a conventional cell culture system open to the bulk cell culture media, which helps to maintain, over the long-term (> 2 weeks), constant gas and nutrient concentration, pH, and osmotic stability and allows for straightforward cell seeding.

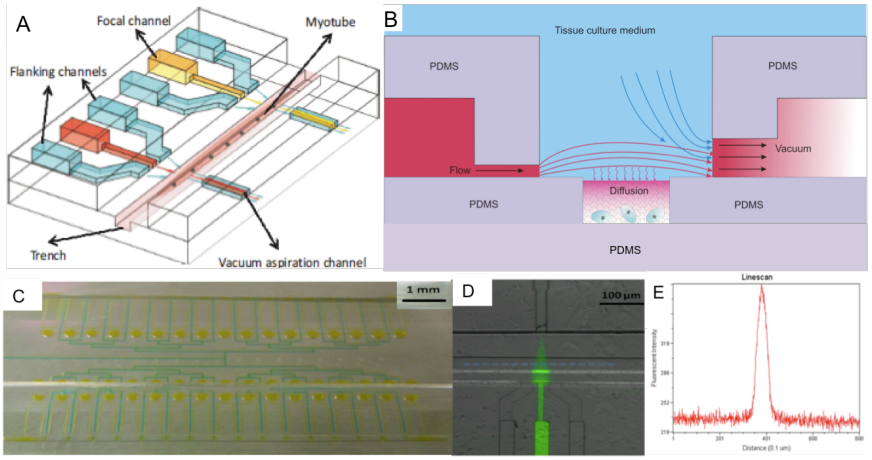

Figure 1: A) Schematic of microfluidic device with focal channels delivering stimulus, (aided by hydrodynamic focusing from flanking channels and driven by positive pressure and vacuum aspiration) to myotubes in trenches. (B) Cross-sectional view of the device. (C) A macroscopic photographic top view of the device shows 32 identical streams of stimulant (i.e. agrin) represented by yellow food coloring dye. (D) A top view of a single stream as shown by BSA- Alexa 488. (E) Linescan of the focal stream at the myotube trench ~20 µm (blue dash line in D), shown as fluorescence intensity of BSA- Alexa Fluor 488.

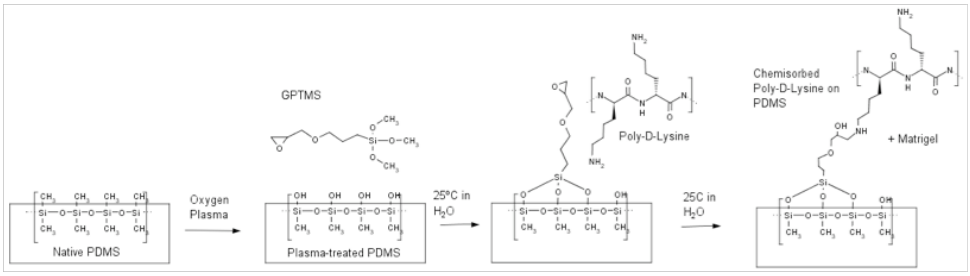

Figure 2: Chemical modification of PDMS surfaces with epoxysilane (GPTMS) to covalently immobilize Poly-D-lysine and Matrigel for long-term cell culture.

EXPERIMENTAL

Device Fabrication: The overall device consists of a top PDMS microfluidic layer bonded to a bottom PDMS cell trench layer. The microfluidic device is defined by three layer photolithography using the SU-8 photoresist (Microchem): Layer I (20 µm height) defines the three converging focal (20 µm width) and flanking (40 µm width) channels (32 channels total) delivering stimulant, Layer II defines the microchannels (50 µm height and 50 µm width) feeding the converging channels, and Layer III defines the exclusion molding layer (295 µm height) with holes for inlets and outlets for tubing for fluid input and vacuum aspiration. The three-layer structure is then molded from the master with a 10:1 ratio of PDMS prepolymer to curing agent (Sylgard 184) and bonded by oxygen plasma (20 s at 10 W and 75 mTorr) with the trench layer.

Device Operation: The device contains three ports, two inlets for injection and one vacuum aspiration port, in order to generate an array of 32 identical focal stimulation jets **(Figure 1)**. Bovine Serum Albumin (BSA) conjugated with Alexa Fluor 488 (Invitrogen) is used to visualize and pattern focal plumes on the PDMS substrate and thus ensure the delivery of the molecules to the bottom of the trench. This is also used to determine the reliability and stability of the focal streams.

PDMS Surface Modification and Cell Seeding: The PDMS trench layer is covered with a PDMS slab and treated with oxygen plasma (60 s at 60 W and 670 mTorr) to selectively form silanol groups [Si-OH] within the trenches (leaving the trench outsides hydrophobic). The trench insides are then infused with 3-glycidoxypropyltrimethoxysilane (GPTMS) (1% v/v), decorating the surface with epoxy groups, which allows the covalent binding of poly-D-lysine (PDL) (0.1 mg/mL) via its amine groups. The PDL-coated trenches were then gently rinsed with PBS before coating with a 1:50 mixture of growth factor-reduced Matrigel Matrix (BD Biosciences) or laminin (0.1 mg/mL) in DMEM, and allowed to adsorb overnight at 37°C. Subsequently, the trench outsides are treated with a cell-repelling surfactant, Pluronic F127 (0.2% v/v). Cells are top loaded at a concentration of 1.7×10^5 cells/mL and allowed to attach into the trenches for 20 minutes at 37°C. The myoblasts fuse within the trenches into myotubes with differentiating media (DMEM with 2% horse serum and 1% penicillin and streptomycin) over ~7 days [13] after which the AChR "pretzels" can be labeled with fluorescently-conjugated bungarotoxin.

RESULTS AND DISCUSSION

Device Operation: A linescan of the focal stream at the myotube trench shows a narrow band about ~20 μm wide, shown as fluorescence intensity of BSA- Alexa Fluor 488 in Figure 1 E.

PDMS Surface Modification and Cell Seeding: We have observed robust long-term myotube maturation, growth and expression of AChR clustering "pretzels" – conditions that will allow for sophisticated, high-throughput experiments with microfluidic delivery of neurochemicals to cultured myotubes **(Figure 3)**. Furthermore, we find that thinner trench dimensions of 15-20 μm width and 40 μm height allows confinement of single myotubes that express robust AChR "pretzels."

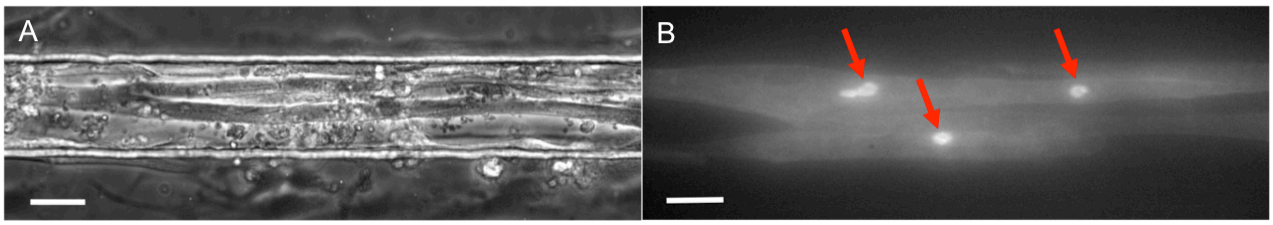

Figure 3: Mature myotubes at Day 11 after seeding confined within covalently-modified PDMS 40 μm wide microtrenches (A) under phase contrast microscopy and (B) AChR clusters stained with bungarotoxin-Alexa Fluor 488 with red arrows indicating agrin-independent AChR "pretzel"-shaped clusters. Scale bar = 20 μm.

CONCLUSION

We have demonstrated a microfluidic device with high spatiotemporal control of focal stimulation of cultured myotubes. Cells can be patterned inside trenches using PDMS surface modification and shown to survive and express AChR patterns in culture. Subsequently cells can be focally stimulated with 32 orthogonal streams of neurochemical stimulant. Future studies will prove focal stimulation of myotubes with Cell-tracker and agrin, and more sophisticated neurochemical experiments.

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CONTACT

*J. Cheng, tel: +1-206-6169036; joncheng@uw.edu