REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	3/26/2007	Final Report (4/1/2006-12/31/2006)

4. TITLE AND SUBTITLE
A Microfluidics Approach to Investigate Axon Guidance

5. FUNDING NUMBERS
W911NF-06-1-0078

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
50530.1-LS-II

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The purpose of this STR project was to demonstrate and explore the capabilities of a novel microfluidic concentration-gradient generator, as it relates to developmental and regenerative neurobiology. The device, consisting of a series of alternating cell-culture chambers and reagent channels that are interconnected via micro-channels, establishes and maintains steady concentration gradients within a static cell-culture chamber for neuronal culture. Concentration gradients of both small molecules (pharmaceutical agents and second messengers) and macromolecules (neurotrophins and other proteins) can be easily achieved and quantified in this device. Thus, quantitative studies of neuronal polarization and axon pathfinding of neurons in response to micro-environmental cues can be accomplished. Ultimately, the device can be integrated with (potentially implantable) solid-state devices and subsequently deliver the appropriate concentration gradient and combination of guidance cues to specific axons. In so doing, we will be able to guide and interface the axons with the solid-state devices that will ultimately inject them with electrical stimuli. This will no doubt advance the next-generation prosthetics.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Microfluidics, chemotaxis, concentration gradient, neurons, axonal chemotaxis	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	**UNCLASSIFIED**	**UNCLASSIFIED**	**UL**

NSN 7540-01-280-5500

Standard Form 298 (Rev.2-89)
Prescribed by ANSI Std. 239-18
298-102

I. Statement of Problem Studied

Over a century ago, Cajal [1] hypothesized that gradients of chemical substances secreted from target tissue guide growth cones, in a manner very much similar to the chemotaxis of leukocytes. Since then, numerous investigators [2-7] have shown that axon pathfinding is indeed directed by the concentration gradient of such extracellular guidance cues as netrins [8-10], semaphorins [11, 12], and slits [13]. Furthermore, when the guidance cue concentration varies by many orders of magnitude, the growth cone can adapt and re-adjust its sensitivity to that guidance cue [14-17]. Recently, Ming et al. [18] have shown that a series of cellular events can even *tune* the sensitivity of signaling pathways to a guidance cue gradient, thereby greatly affecting the guidance of growth cones. Despite the intense work in understanding axonal chemotaxis, there are still numerous challenges one must overcome before one can realize guidance cue-based therapies for axonal regeneration after traumatic injury or disease. Such challenges include 1) determining the minimum gradient of a particular guidance cue needed to thus guide the axon; and 2) understanding the circumstances that can lead a guidance cue to attract or repel axons, since a single guidance cue molecule can be multifunctional, attracting or repelling axons at short or long range.

To advance the study of axonal chemotaxis so that effective therapeutic interventions based on guidance cues can be designed, we have developed a platform technology based on microfluidics. Microfluidic devices have been used to perform a variety of biological assays [19]. In addition to advantages including ease in fabrication, low consumption of costly reagents, short reaction times, the capability to perform parallel experiments, and portability, several phenomena occur only in microfluidic devices and not in larger-scale traditional devices [20, 21]. Among them is the rapid diffusion of the reagents. Because microfluidic channel dimensions typically range from 1 to 1000 um in width and height, diffusion becomes a viable method to mix fluids and control reaction rates. Furthermore, in microfluidic channels, the diffusion distance can be made very small (a few micrometers), thus diffusion-mediated chemical reactions can occur much more rapidly than those that occur in macroscopic reaction vessels.

The microfluidic-based device we have developed under this STIR is shown in Figure 1. As shown, it consists of alternating cell-culture chambers and reagent channels interconnected via micro-channels. Three cell-culture chambers (labeled as **A**, **B**, and **C** in Figure 1) are for culturing neurons, while four reagent channels (labeled as **1**, **2**, **3** and **4** in Figure 1) are injected with a specific guidance cue at different concentrations. The soluble guidance cue in the reagent channels can quickly diffuse through the interconnecting channels and establish specific and different concentration gradients in the three cell-chambers. By slowly and continuously driving the guidance cue to flow through the reagent channels, we can maintain the concentration gradient profiles in the cell-chambers for a period of days.

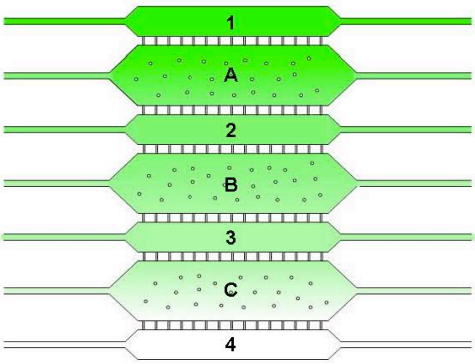

Figure 1: *Schematic of our microfluidic concentration-gradient generator. As shown, the device consists of several alternating chambers that are interconnected via a set of microchannels. Chambers **A**, **B**, and **C** correspond to culture chambers and chambers **1**, **2**, **3** and **4** correspond to reagent chambers.*

Because the cell-culture chambers remain static in our microfluidic concentration-gradient generator, a guidance-cue gradient could be initiated immediately after plating cells, thereby providing an opportunity for a systematic study of axonal guidance and neuron polarization. Concentration gradients of both small molecules (pharmaceutical agents and second messengers) and macromolecules (neurotrophins and

other proteins) are easily achieved in this system. Furthermore, the device is portable, optically clear, and stable over time; therefore, it is compatible with time-lapse microscopy protocols.

The impact of our proposed work is many-fold. First, our platform technology, based on microfluidics, provides the first real opportunity to control precisely the concentration gradient of a guidance cue in order to investigate the critical (or minimum) gradient necessary for growth-cone guidance. Second, our microfluidic chambers provide an *in vivo*-like culture environment under which neurons can grow. Third, our technology can be easily multiplexed so that we can 1) identify new guidance cues; and 2) investigate the role of multiple and interacting guidance cues on modulating growth-cone behavior. Fourth, and most importantly, an effective therapeutic scheme based on guidance cues and their concentration gradients can be designed using our microfluidics technology. Here, we envision that our microfluidics can ultimately be integrated with (potentially implantable) solid-state devices and subsequently deliver the appropriate concentration gradient and combination of guidance cues to specific axons. In so doing, we will be able to guide and interface the axons with the solid-state devices that will ultimately inject them with electrical stimuli. This will no doubt advance the next-generation prosthetics. Overall, our microfluidic concentration-gradient generator can greatly expand the types of experiments that can be performed in developmental and regenerative neurobiology.

II. Summary of the Most Important Results

The microfluidic concentration-gradient generator device shown in Figure 1 enables us to establish different guidance cue concentrations and gradients in a relatively short time (from seconds to minutes) and to maintain the gradient indefinitely. In addition, the cell-culture chambers shown in Figure 1 provide *in vivo*-like culture conditions under which we can ultimately 1) direct the polarization and guidance of embryonic rat hippocampal neurons with brain-derived neurotrophic factor (BDNF) and cAMP gradients; and 2) determine the minimum gradients of BDNF and semaphorin3A (Sema3A) needed to guide embryonic *Xenopus* spinal cord growth cones. Key to our device is that the cell-culture chambers remain static, thereby allowing shear-sensitive neurons, such as embryonic rat hippocampal neurons and *Xenopus* spinal cord neurons, to thrive.

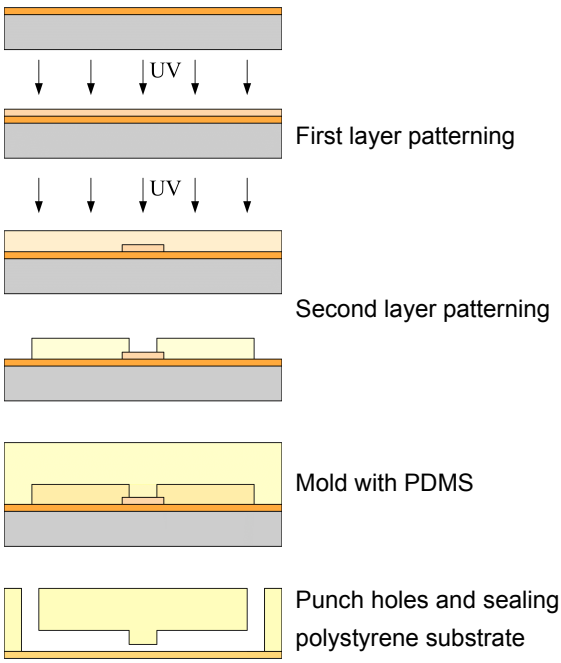

Figure 2: *Schematic of the process used to fabricate the microfluidic concentration-gradient generator. Standard photolithography is used to pattern the different layers.*

Device Description and Fabrication

The microfluidic concentration-gradient generator we have developed is shown in Figure 1. The generator is designed to have three cell-chambers of size 2000 μm × 1000 μm × 50 μm (L x W x H) (labeled as **A**, **B**, and **C** in Figure 1) for culturing neurons, and four reagent channels of size 2000 μm × 500 μm × 50 μm (L x W x H) (labeled as **1**, **2**, **3**, and **4** in Figure 1). We use standard photolithography and soft-lithography techniques to fabricate the device [22]. In more detail, we lithographically create a negative master of the microfluidic cell-culture chambers and reagent channels, which is subsequently cast into a poly(dimethylsiloxane) (PDMS) slab (Figure 2), a well-known

biocompatible material that has been used for a variety of cell-culture applications [23-26]. We create the master in two steps, each involving patterning SU-8 photoresist on a silicon substrate to form the negatives of the cell-culture chambers, reagent channels, and the micro-channels. Following standard micromolding techniques, we pour PDMS (Sylgard 184, Dow Corning) over the master and cure it at 80 °C for at least 2 hours. The PDMS slab is then removed from the master, cored to provide inlet and outlet ports, and permanently sealed to a glass coverslip, thus completing the device.

Establishment and Quantitative Analysis of Concentration Gradients

To establish concentration gradients in the cell-culture chambers, the reagent channels are injected with a specific guidance cue at different concentrations. The soluble guidance cue in the reagent channels can quickly diffuse through the interconnecting micro-channels and establish specific and different concentration gradients in the cell-culture chambers. By slowly and continuously driving the guidance cue to flow through the reagent channels, we can maintain the concentration gradient profiles in the cell-culture chambers for as long as required, from hours to days. Figure 3 summarizes how we establish a concentration gradient in the cell-culture chamber.

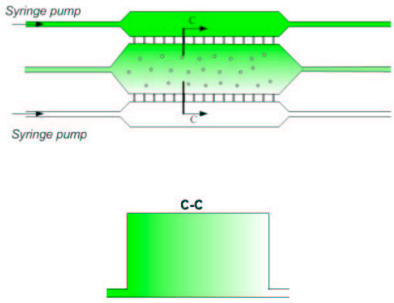

***Figure 3**: Operation of the microfluidic concentration-gradient generator. **A)** Top view of two reagent chambers and one cell-culture chamber. A syringe pump is used to drive a solution of guidance cues through the reagent chambers. **B)** Cross-section view of a cell-culture chamber where the guidance cue concentration gradient is established and maintained.*

Rapid diffusion of reagents occurs readily in microfluidic devices. The diffusion length, L, is defined as $L^2 = qDt$, where q is an integer reflecting the dimensionality of the system, D is the diffusion coefficient, and t is the diffusion time. For the cell-culture chambers in our device, we assume one-dimensional (1D) diffusion (since the chamber height is much smaller than the chamber length or width) to thus determine the concentration gradient profile:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad (1)$$

where C is the concentration of the guidance cue. Figure 4 shows the calculated concentration gradient profile of a model protein with $D = 4\text{x}10^{-7}$ cm^2/sec after 30 minutes in a cell-culture chamber [27].

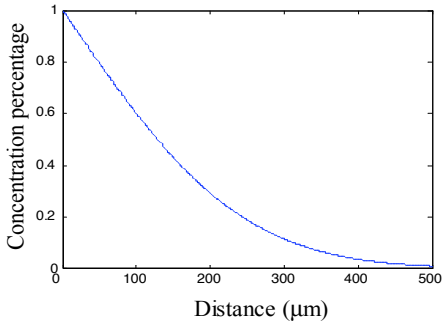

***Figure 4:** Calculated concentration gradient profile of a model protein ($D = 4\text{x}10^{-7}$ cm^2/sec) after 30 min. Eq. (1) was used.*

To image the actual concentration profile in our first-generation device, we flowed either a fluorescent cAMP analogue (100 µM cAMP-AF, Alexa Fluor 488-conjugated, Molecular Probes, D ~ 3 x 10^{-6} cm^2/sec) or fluorescent bovine serum albumin (1 mg/ml BSA, Alexa Fluor 488-conjugated, Molecular Probes, D ~ 3 x 10^{-7} cm^2/sec) into the reagent channels at a rate of 1.2 µl/min and 20 nl/min, respectively. After 30 minutes of flow, we took images of the established concentration gradient using an inverted fluorescent microscope (Figure 5). These images were digitally integrated in Matlab. As expected by Eq. (1), the established gradients result in a linear profile for the center portion of the cell-culture chambers.

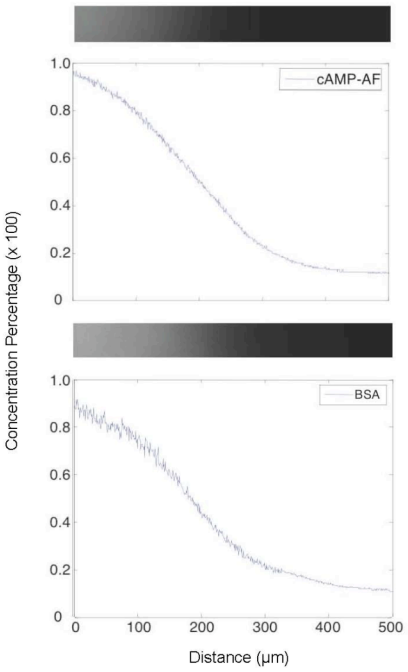

Figure 5: *Measured concentration gradients of cAMP-AF (top) and BSA (bottom).*

Neuronal Compatibility with Device Materials

As mentioned, the PDMS material we use to form the microfluidic concentration-gradient generator is biocompatible. However, to test that PDMS is compatible with primary neurons, we cultured both embryonic rat hippocampal (E18) and embryonic *Xenopus* spinal cord (Stage 20-22) neurons in the cell-culture chambers of our device under static conditions. For the hippocampal culture, we first autoclaved a sealed device and then injected Poly-*L*-lysine (PLL, 40 µg/ml, Sigma) into the device with a syringe, then incubated overnight, and finally thoroughly rinsed twice with sterilized DI water. We pre-incubated the cell-culture chamber with Neuralbasal medium (Gibco) for 30 minutes, after which we injected a re-suspended solution of dissociated neurons (500,000 cells/ml). In detail, we deposit ~3 µl of neuron solution into the inlet port of the cell-culture chambers. The neurons, through capillary action, flowed through the narrow loading channel and into the cell-culture chamber where they rapidly attached to the injected PLL-coated substrate. We next placed the devices into a 6-inch Petri dish and added sterilized DI water to the bottom of the dish in order to maintain appropriate humidity. We then incubated the device in a humidified incubator at 37 °C with a 5% CO_2 supply. For the embryonic *Xenopus* spinal cord neurons, we followed a similar procedure with the exception that we did not pre-coat the substrate with PLL. The cells of one dissociated embryonic spinal cord was re-suspended in 3 µl of freshly-prepared Modified Frog Ringer's Medium. For these neurons, we incubated the device on a bench top at room temperature.

Figure 6 is a phase-contrast photo of embryonic rat hippocampal neurons in a cell-culture chamber after 48 hrs; Figure 7 is a phase-contrast photo of embryonic *Xenopus* spinal

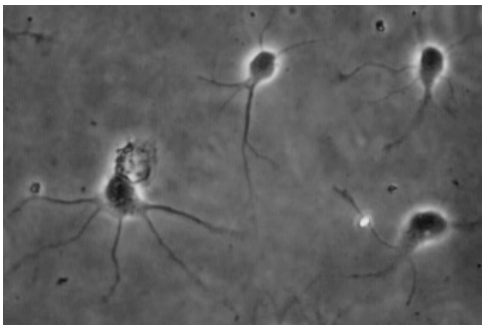

Figure 6: *Rat hippocampal neurons after 48 hr culture in a device under static condition.*

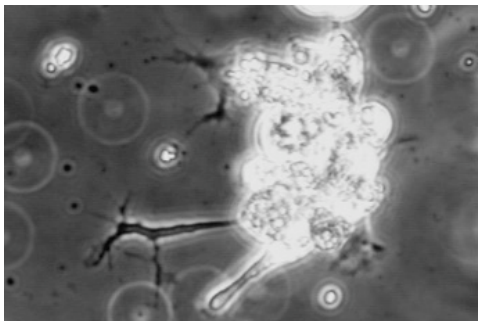

Figure 7: *Xenopus spinal cord neurons after 12 hr culture in a device under static condition*

cord neurons in a cell-culture chamber after 12 hrs. As shown, both types of neurons remain viable in our device, have undergone polarization, and are extending healthy neurite processes. The PDMS is thus non-cytotoxic to primary neuronal cultures.

We confirmed that the hippocampal neurons cultured in our devices undergo polarization by specifically immunolabeling axonal processes. At various time points, a single device was removed from the syringe pump and quickly injected with 4% paraformaldehyde in phosphate-buffered saline (PBS) to fixate the neurons. Similar to standard immunostaining protocols, the cells were then permeabilized with 0.1% Triton X-100 in PBS (PBST) and immunoblocked with 10% normal goat serum in PBST overnight at 4 C. Cells were incubated with primary antibodies to label specifically neurons (anti-β-tubulin, 1:1000 dilution, Covance) and axonal processes (SMI-312 pan-axonal neurofilament marker, 1:500 dilution, Covance). Fixed samples were then incubated with the appropriate fluorescence-conjugated secondary antibodies for visualization (Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 anti-mouse, Molecular Probes). Because the our device is optically clear and mounted directly on a coverslip, all immunostaining and visualization procedures can be performed directly on the device without disassembling the cell-culture chambers. We used a Leica DM IRBE confocal microscope equipped with Helium-Neon, Krypton, and Argon lasers to image. Figure 8 shows confocal images of two hippocampal neurons cultured within the device for 24 and 48 hours, respectively. The neurons display a typical neuronal morphology with multiple dendrites extending from the cell body (green channel) and a single, long axonal process (red channel). Therefore, neurons cultured within the PDMS device undergo normal polarization.

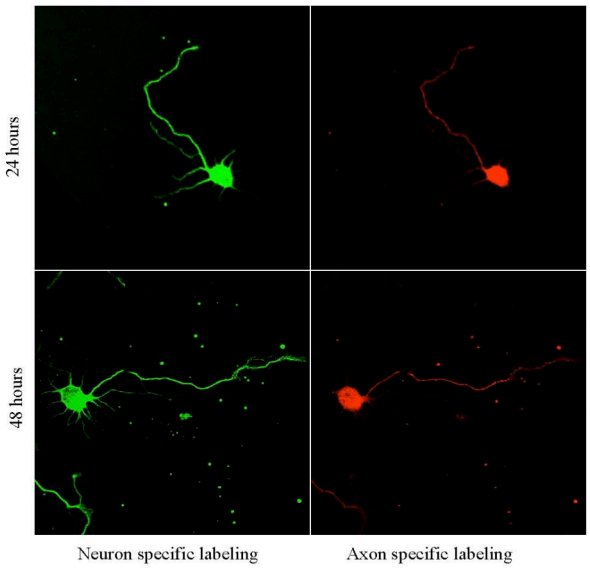

Figure 8: *Confocal images of rat hippocampal neurons after 24 hrs and 48 hrs cultures in devices under static condition.*

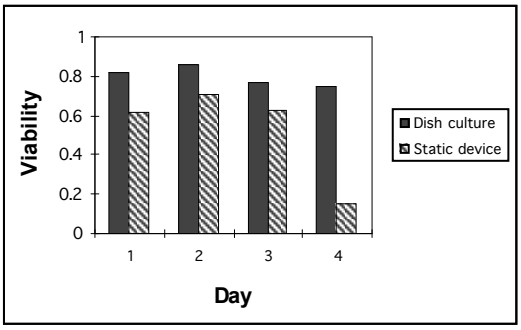

Figure 9*: Neuron viability data of dish culture versus static device culture. Each bar represents the average viability of three trials with at least 150 cells/trial.*

To further characterize viability of embryonic rat hippocampal neurons, we assessed the viability of these neurons in our device using a 0.1% Trypan blue solution (Gibco) that specifically labels membrane-compromised cells. Figure 9 shows that the neurons survive well in our device up to 3 days. The dramatic decrease in viability on Day 4 is thought to be a result of starvation, as these are static devices and the media is not being replenished. This is in contrast to the dish culture, which is also static but does have a greater volume of media.

Culturing Neurons in the Presence of Flow in the Reagent Channels

Because the embryonic rat hippocampal neurons are very shear sensitive, we need to ensure that the cell-culture chambers remain static even while flowing media through the reagent channels. Thus, we add "plug pins" at the inlet and outlet holes of the cell-culture chambers to maintain static conditions in these chambers. To test that we indeed have static conditions, we prepared devices as described above and measured cell viability over time while flowing normal media (without guidance cues) through the reagent channels. We incubated embryonic rat hippocampal neurons for four hours under static conditions. Then, we inserted Tygon tubing (Thickness: 0.020 in.; I.D.: 0.020 in.) into the inlet ports of the reagent channels and connected to 50 μl gas-tight glass syringes (Hamilton) mounted onto a syringe pump (KDS, Model 230). The syringe pump is placed into the humidified incubator (37 °C, 5% CO_2) with the device and is turned on with a flow speed of 1 nl/min. As a negative control, we used 5 μl of media to induce hydrostatic pressure (~5 mm water) to one device in order to induce low-shear stress in the cell-culture chambers of that device.

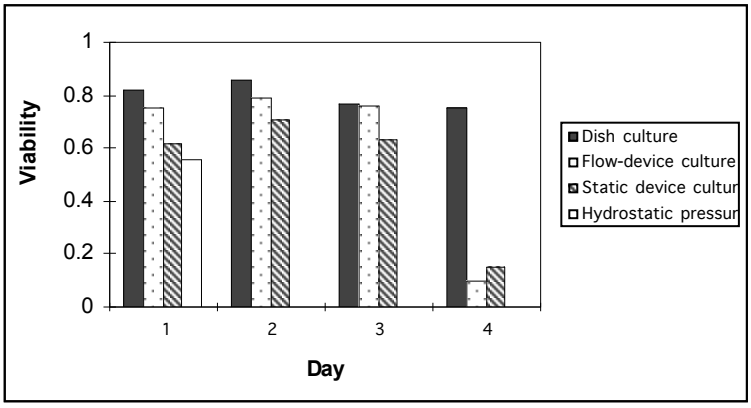

Figure 10: *Neuron viability data of cells cultured under various conditions. Each bar represents the average viability of three trials with at least 150 cells/trial.*

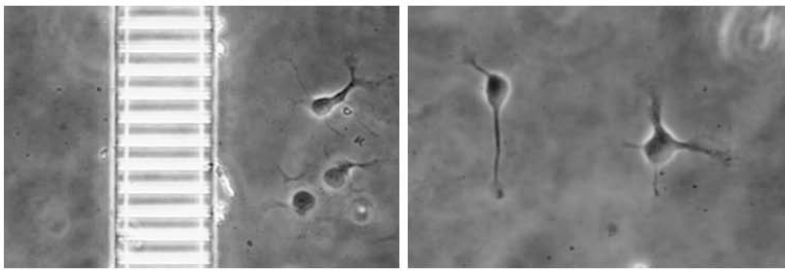

Figure 11: *Phase-contrast image of hippocampal culture after 48 hrs in the cell-culture chamber with continuous media flow through the reagent channels.*

Figure 10 shows the extreme sensitivity of hippocampal neurons to shear stress, as the cells cultured under hydrostatic pressure were completely dead by Day 2. In contrast, neurons cultured in our devices with continuous media flowing through the reagent channels maintained viability through Day 3. Similar to our previous experiments, we observed a dramatic drop-off in viability on Day 4, which we attribute to the depletion of fresh media. We also performed phase-contrast microscopy on the hippocampal cultures 48 hours after incubation, Figure 11. The cells exhibit a normal neuronal morphology and have extended neurites.

III. Future Work

The results outlined in the previous section demonstrate our ability to fabricate and employ a microfluidic-based concentration-gradient device for the systematic and quantitative study of axonal chemotaxis. We are currently on the "cusp" of performing our very first experiments using BDNF. As well, we are in the process of writing a manuscript that we intend to submit to *Nature Biotech*. or *Nature Methods.* Further, we have been honored to present a Platform Talk on this work at the 51st Biophysical Society Meeting in Baltimore, MD in early

March 2007. Finally, we will be presenting an oral presentation at the Materials Research Society Spring Meeting in San Francisco, CA in April 2007.

An outgrowth of this work is that we are also using our microfluidic device to determine the intrinsic and extrinsic factors that influence how myoblasts and monocytes respond to external cues from their local environment thus leading to cell migration, orientation, and fusion. We are currently establishing concentration gradients of different chemokines, such as interleukin 4 (IL-4), growth hormone, insulin growth factors I and II, SDF-1/CXCR4, and vascular endothelial growth factor (VEGF), in order to determine how these chemokines gradients influence the migration, orientation, and fusion activities of muscle cells and monocytes/macrophages. Our understanding of the way myoblasts and monocytes assemble in response to local microenvironmental cues is critical for establishing new systems that enable the design of new diagnostic and therapeutic tools. In the future, this work may lead to novel substrates, new technologies, and designer biomaterials that could be used in artificial limbs or to promote skeletal muscle growth after injury.

Overall, the potential impact and significance of our microfluidic concentration-gradient generator are far reaching, since our device can be used as a quantitative platform technology for regenerative neurobiology. Below are four high-impact areas for our device.

1. Our work will be the first quantitative study of neuronal polarization and axon pathfinding of mammalian neurons in response to environmental microgradients. The results obtained with the microfluidic concentration-gradient generator will provide insight for future *in vivo* studies and regenerative strategies by quantifying the effects of neuronal guidance cues on a cell type (embryonic rat hippocampal neurons) that more closely resemble human neurons.

2. With our device, we can measure the minimum concentration gradient required to guide the pathfinding of the axon in response to known guidance cues. Another feature of the device is the ability to test multiple environmental cues simultaneously to discern potential signaling crosstalk. In future studies, we could evaluate opposing and reinforcing gradients of guidance cues in order to identify combinations that optimize the distance over which the axon can be guided or regenerated. Beyond having an immediate impact on our understanding of developmental neurobiology, our long-term goal is to translate this information to the clinical regeneration and guidance of injured axons.

3. Our optimized microfluidic concentration-gradient generator could prove useful in identifying novel guidance cues that regulate neuronal polarization and guide axonal movement. Because the system allows monitoring of multiple cell populations in real time on one device, screening of potential therapeutic agents for axon guidance and regeneration can be quickly achieved.

4. Our device design utilizes a novel approach to culturing shear-sensitive cells within a microfluidic concentration-gradient generator device and would therefore be ideally suited for culture of other "difficult" cell types, such as stem cells, epithelial cell, and endothelial cells, in defined environmental niches.

IV. Publications & Technical Results

a) Papers published in peer-reviewed journals: N.A.
b) Papers published in non-peer-reviewed journals or in conference proceedings: N.A.
c) Papers presented at meetings, but not

•A. Thupil, N. Ma, M-m. Poo, and L. L. Sohn, *Axonal Chemotaxis in a Microfluidic Gradient Generator*, Platform Talk, 51st Annual Biophysical Society Meeting, March 2007.

•A. Thupil, M-m. Poo, and L. L. Sohn, *A Controllable Microfluidic Gradient Device for Studying Neuronal Polarization*, Oral Presentation, Materials Research Society, April 2007.

d) Manuscripts submitted but not published: N.A.
e) Technical reports submitted to ARO: N.A.

V. List of all participating scientific personnel showing any advanced degrees earned by them while employed on the project

N.A.

VI. Report of Inventions

N.A.

VII. Bibliography

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