

## Applications of microfluidics for neuronal studies

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Received 22 June 2006; received in revised form 1 November 2006; accepted 7 November 2006

### Abstract

Microfabrication processes have changed the technology used in consumer goods, and have now advanced into applications in biology. Microfluidic platforms are microfabricated tools that are gaining popularity for studies of molecular and cellular biology. These platforms can allow precise control of the environment surrounding individual cells and they have been used to study physiologic and pharmacologic responses at the single-cell level. This article reviews microfluidic technology with emphasis on advances that could apply to the study of the nervous system, including architecture for isolation of axons, integrated electrophysiology, patterned physical and chemical substrate cues, and devices for the precisely controlled delivery of possible therapeutic agents such as trophic factors and drugs. The potential of these chips for the study of neurological diseases is also discussed.

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**Keywords:** Microfluidic; PDMS; Single cell; Nanotechnology; Neuron; Axon

### 1. Introduction

As our ability to fabricate tools on the micro and nano-scale has progressed, we can now study cells at a scale compatible with their own size, and this is revealing new information about their responses to cues from their immediate environment, including molecules that diffuse into them and physical structures that they directly contact, such as natural or artificial support stroma and neighboring cells. Recent applications of micro- or nanotechnology to biological systems have included novel micro or nano-scaled carriers for drug delivery [1–4], micro or nano-scaled sensors like quantum dots that operate at the cellular level [5–9], and nanoelectrodes [10]. In addition, nanotechnology in the form of self-assembled monolayers and scaffolding, or car-

bon nanotubes, has been used as substrates for artificial milieu for cell culture [11–17].

In neuroscience specifically, nanotechnology has been applied to several fields of research [18,19]. For example, nanoparticles have been used for free radical scavenging in ischemic and neurodegenerative diseases [20]. Self-assembling nanofiber scaffolds are being developed for neuroregeneration [21]. Nanoparticles that can transport molecules across the blood–brain barrier are being studied [22]. High resolution studies of the surface of live neurons are being carried out by atomic force microscopy (AFM) [23,24], and single-molecule tracking using quantum dots has revealed details about membrane receptors [7,25,26]. Finally, nanotubes, nanowires, and nanoneedles are being developed for use as cellular electrodes [10,27,28]. On a slightly larger scale, microfabrication technology has been used to create microfluidic platforms that have been used for a variety of applications in the study of the nervous system, as will be discussed in this review.

The term “microfluidics” refers to microfabricated devices that can process microscopic volumes of fluids through a

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series of channels and chambers that range from one to a few hundred microns in size. These devices can be designed and constructed to function as valuable tools for studies at the single-cell level. Although they have been used for some *in vivo* applications, such as the precise delivery of drug solutions from on-chip reservoirs located on neural implants [29], their main application has been for *in vitro* studies for the delivery and processing of biochemical molecules, like those needed for DNA sequencing [30], protein analysis [31], or for many cell-based applications. More specifically, they have been used for flow cytometric cell counting, cell sorting and analysis [32]; micropatterned cell growth on substrates by regulated sequential delivery of cell adhesives and their target cells [33–37]; and creation of simulated *in vivo* tissues by progressive deposition of layers of stromal support molecules within cell culture chambers to study the effect of different 3-dimensional structures on the function of cells [12,13]. Additionally, microfluidic chambers have also been used to study the effect of forces such as laminar flow on the function of endothelial (and other) cells [38,39]; artificial circulation between various organ-simulating cell culture chambers to test the pharmacokinetics of various agents [40]; delivery of test specimens to chambers with cell-based biosensors that utilize cells as transducers to assay for the presence of suspected toxins and other agents (a.k.a. “lab-in-a-cell” technology) [41–43]; and study of basic cell physiology and cell–cell interactions on a single (or near-single) cell basis in a controlled environmental chamber [44–49].

Microfluidic-based cell studies present an advantage when compared to conventional *in vitro* techniques or mini culture systems [50] since they have the ability to precisely control the environment around individual cells. This is accomplished by using microfluidic architecture to deposit single cells in dedicated areas (thereby controlling their spatial distribution and their interactions with other cells) and by controlling the quantity of added reagents and factors distributed to these isolated cells via the microfluidic channels. Since the control offered by microfluidic platforms can avoid problems seen with standard *in vitro* techniques, such as unanticipated extraneous factors, diffusion constraints, and cell population variability, results derived from microfluidic experiments may reveal new details of cellular physiology. Cost savings are another benefit of microfluidic experiments since the volume of expensive media, hormones, and growth factors is orders of magnitude less than that used in conventional culture flasks.

Observations derived from studies of individual cells in a controlled microenvironment have the potential to reveal different facets of their physiology compared to those derived from studying the responses of populations of cells simultaneously, as is done with conventional *in vitro* studies. Many platforms have been designed around the unique anatomy of cells from the nervous system. These designs offer a novel approach to studying the cellular physiology of the nervous system and the pathophysiology of many congenital and acquired neurodegenerative diseases by allowing

isolation of cells derived from the nervous system, isolation of neurites and synapses, and analysis of single-cell responses to perturbations of their environment.

## 2. Microfluidic chamber construction

The first step in building a microfluidic chip involves drawing the design using computer software. This design is then printed out and used to create a three-dimensional mold using the same technology that is employed for constructing electronic circuitry (photolithography). Most microfluidic chambers currently used are constructed by using “hard” lithography on silicon wafers; others use soft lithography in a material called PDMS (polydimethylsiloxane) to create valves, channels, and chambers. PDMS is a silicone-type elastomer that is poured or spun onto a hard master pattern to create castings that can then be peeled off the master and placed on a rigid substrate (typically glass or silicon). It is essentially similar to clear bathroom caulk and it is marketed, for example, by Dow Corning as Sylgard 184. PDMS is optically transparent down to 280 nm, allowing both optical and fluorescent/chemiluminescent microscopy of contained cells and fluids in the visible spectrum [42]. Other major advantages of PDMS for *in vitro* cell studies are that it allows respiration of cells that are enclosed within it since it is also permeable to gases; it is nontoxic and autoclavable; and it inhibits cellular adhesion onto its surface if it is not pretreated for this purpose [51,52]. A final advantage of this material for use on microfluidic chambers is that PDMS has been shown to be a superior protective coating for onboard electronic devices (like surface-emitting lasers) since it is optically transparent yet prevents the corrosive effects of ions in the medium on these devices [53].

The process of creating a PDMS-based microfluidic system involves transferring a drawing of the desired configuration to a transparency to create a photomask. This is then placed over a substrate (silicon wafer or glass) that is precoated with a thin layer of photoresist. When exposed to UV light, the photoresist will harden only in areas patterned on the mask, and the rest will wash away with a developer, leaving a master pattern for the PDMS. The PDMS is poured onto the master, allowed to cure and then peeled off and placed on a substrate like a silicon wafer to create the final product [54]. The master can then be reused like a mold to make more PDMS castings for parallel experiments.

## 3. Microfluidic structures

Fluid flow through microfluidic channels is laminar. Therefore, diffusion is the only mechanism to mix solutes that are present at different locations in the channel cross-section. Since diffusion is a slow process, microfluidic channels can be used to create cross-sectional gradients in solute concentration to study the effect of these gradients on distinct areas of cells within this channel, or to pattern cell adhesives and repellents on the substrate prior to introduction of cells.

If desired, solutes in the chamber can be mixed by the turbulence created by deliberate oblique grooving of the floor of the channel [55–57], and cell adhesion can be influenced by the addition of nanotopographic features to the chip substrate [58]. Flow through the channels can be achieved by using gravity-driven reservoirs [59], by electrokinetic control [60], or by using PDMS valve structures that apply pneumatic pressure in a layer of air-filled channels to modify the flow in an underlying layer of fluid-filled channels [61]. Coordination and digital control of these valves can create rotary or peristaltic pumps.

In order to trap and isolate cells for on-chip analysis, sieves [62], dams [63], and docks [44,46–48] have been designed that non-traumatically immobilize individual cells. Holding areas with an adjustable size have been used for isolating functional groups of cells like pancreatic islets [49], and special designs that can trap pairs of cells have been used in order to study cell–cell communication via gap junctions [45]. Large arrays of micro-culture chambers connected to input and output channels have also been created in microfluidic chips. Since repeated growth/passage cycles (using proteolytic enzymes such as trypsin) could be performed on-chip by microfluidic control, these arrays could be very useful for long-term experiments [64].

There are several different design considerations that must be addressed before planning a microfluidic chamber for cell studies. These include ensuring an adequate chamber height to allow for convective movement of cell-expressed inhibitory factors away from cells [65]. In addition, the flow rate must be optimized to provide nutrients and remove wastes, without producing excessive shear stress that can change morphology of the cells or even detach them [62,66]. Finally, pretreatment of the PDMS to change its hydrophobicity, or to apply an extracellular matrix, has been shown to alter both the quality of the subsequent cell culture and actual cell morphology [42,62].

#### 4. Microfabricated devices useful for neuronal studies

Complex pyramidal networks of serpentine interconnected microchannels have been designed so that the concentration of special factors delivered to a cell culture chamber can be precisely controlled over specific areas of the chamber. These networks can create a spatial concentration gradient of the factors so that the factors' exact effect on the behavior of individual cells can be determined (see Fig. 1) [67]. The final gradient spatial range can span several microns to hundreds of microns, and is oriented perpendicular to the flow direction. Once established, the gradient is maintained by laminar flow, but the gradient can be varied in a dynamic fashion by changing the relative input flows. This technique can also be used to establish asymmetric or shaped gradients (e.g. smooth, step, or multiple peaks) [33,67,68]. This allows the quantity of factors to be precisely controlled to match the location of a cell, or to vary over the width of an individual cell, in order to study the effect of the factor on just part of a

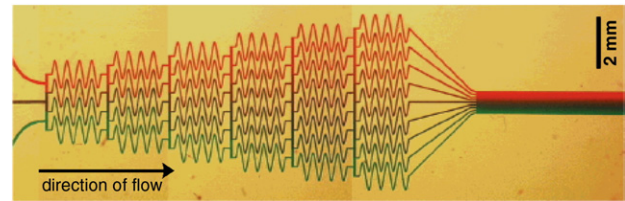


Fig. 1. A gradient-generating microfluidic device. Photograph showing gradient-generating microfluidic device with three inputs (left), mixing red and green dyes in solution into one channel (right). (Reprinted with permission from Analytical Chemistry. Dertinger SKW, Chiu DT, Jeon NL, Whitesides GM. Generation of gradients having complex shapes using microfluidic networks. 2001; 73 (6): 1240–1246. Copyright 2001 American Chemical Society.)

cell. These devices have proven useful in studying the effects of agents such as IL-8 (CXCL8) or EGF (epidermal growth factor) on the chemotaxis of neutrophils [69] or cancer cells [70], as well as the effects of various growth factors on neural stem cells [71] (see later section on growth factor studies for further discussion).

Gradient-generating devices are very useful for *in vitro* studies of neuronal cells since maintenance of precise spatial control of growth and inhibitory factors is imperative for experiments that probe the complex interactions that occur among neural lineage cells. Gradient devices are also useful in the creation of gradually varying surface topology by etching reagents, or the creation of gradients of adhesives, self-assembled monolayers (SAMs), and dyes [68]. In addition, they have been used to perform large parallel tests of the effects of various dilutions of agents on cells in large on-chip culture chamber arrays [64].

There are many other innovative techniques and devices that employ microfluidic channels that have potential application in the study of neural lineage cells. For example, rapid, highly sensitive determination of transmembrane potential has been accomplished in microfluidic devices with the use of charged membrane-permeable, potential-sensitive dyes, with minimal consumption of reagents [72]. Similarly, the relative acidity of fluids in microfluidic channels and chambers has been determined with pH-sensitive fluorescent-tagged monolayers (SAMs) that are attached to the substrate of a microfluidic platform [73] or with a cytosensor microphysiometer that uses microliter flow chambers to measure pH changes down to  $0.5 \times 10^{-3}$  pH units [74]. The oxygen content in microscale cell cultures has also been determined by using on-chip oxygen sensors based on fluorescent quenching of ruthenium dye particles encapsulated in the PDMS of microfluidic devices [40].

NMR (nuclear magnetic resonance) microcoils have been used to study single non-perfused neurons [75], and planar NMR probes have been microfabricated on the glass substrate of microfluidic platforms and tested with sucrose solutions, but these have not yet been tested on cells [76].

Finally, combined microfluidic/micro-electroporation chips have been used to genetically manipulate cells [77,78] and this could represent an important tool in the investigation

of genetic diseases like Huntington's Disease, the numerous hereditary ataxias that have genetic defects identified, autosomal dominant Parkinson's Disease and Alzheimer's, Familial ALS, and Charcot–Marie–Tooth Disease. These chips use microfluidics to deliver and immobilize a single cell over integral electroporation electrodes. Other molecular biology processes have been incorporated into microfluidic platforms capable of single-cell genetic studies. For example, these chips can isolate and lyse single mammalian cells and then purify and recover their mRNA [79]. Using a different design, investigators have detected RNA levels as low as 34 RNA templates with chips capable of performing 72 parallel, 450 picoliter reverse-transcriptase PCR reactions [80]. This capability could potentially allow detection of genes that are only expressed at very low levels. Microfluidics have also been used to synthesize cDNA from subpicogram mRNA templates isolated from single cells [81]. In addition to these “diagnostic” procedures, genetic manipulation at the micro-scale has been performed by successfully transforming plasmid DNA into competent cells using microfluidics [82]. All of these chips that are dedicated for molecular biology procedures incorporate large arrays of micro-scale channels, holding areas, pumps, valves, electrodes, and heaters, and they represent some of the more complex architectures found in microfluidic chips.

## 5. Integrated electrophysiology

Many microfluidic studies on neurons have monitored electrical characteristics of these cells, typically by using microelectrode arrays (MEAs) [35,36,43,83–89] (see Fig. 2).

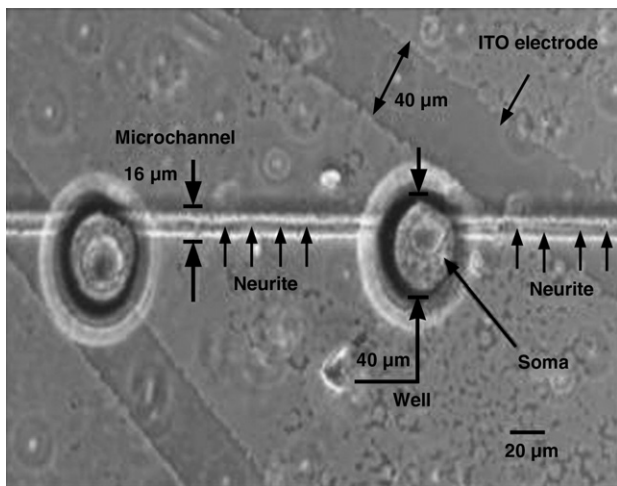


Fig. 2. Neurites in microchannel crossing ITO multielectrode array. A neuron (25  $\mu\text{m}$  in soma diameter) sprouting two microchannel-guided neurites (170 and 130  $\mu\text{m}$  long respectively) over an ITO electrode (right-hand side neurite). Picture taken after 4 days in culture. (Reprinted with permission from Journal of Neural Engineering. Claverol-Tinture E, Ghirardi M, Fiumara F, Rosell X, Cabestany J. Multielectrode arrays with elastomeric microstructured overlays for extracellular recordings from patterned neurons. 2005; 2 (2): L1–7. IOP Publishing Limited.)

Frequently, MEAs can be pretreated by using microcontact printing to deposit patterned cell-adhesive proteins (attached by physisorption or covalent alkanethiol monolayers). This patterned neuronal deposition can result in higher cell density at the electrodes and subsequent increased apparent electrical activity of the culture [35,36]. Patch clamp systems have also been implemented on microfluidic chips, but they have not been tested on neurons yet [90–93]. Other complementary nanotechnology devices have potential application for electrophysiology studies of neurons cultured on these chips. For example, nanoelectrodes can be integrated onto the chip substrate to monitor action potentials [10,28] and quantum dots can be used to stimulate action potentials [5,6]. This ability to monitor characteristics of spontaneous and induced action potentials of isolated neurons could provide a convenient model to study the non-structural influences of drugs and toxins on axonal physiology.

## 6. Physical and chemical guidance cues

Physical and chemical cues play a significant role in guiding cell attachment and neurite growth. It is therefore helpful to understand how different cells of the nervous system respond to these cues when planning regeneration and development studies, especially when performing single-cell studies. In regard to physical cues, research has shown that certain cells prefer certain nanotopographies. For example, astroglia preferentially attach to pillars over wells, and they respond to the topography by changing their expression of cytoskeletal proteins like actin and vinculin [94]. Neurons also respond to topography, and it has been shown that neurons confined in narrow square-bottomed channels elaborated fewer neurites, and each neurite was longer and more likely to be oriented parallel to the channel wall, presumably due to inflexibility of the cytoskeleton [95]. Although a two-dimensional system will never truly reflect the *in vivo* milieu, these factors must be taken into account if the system is going to be used to study disease or to screen the effects of extrinsic molecules.

Studies on independent static chemical cues have isolated certain short peptides that preferentially promote neural adhesion (amino acid sequence IKVAV) over fibroblast and glial cell adhesion (amino acid sequence RGD) when adsorbed onto the substrate [96]. When collagen or poly-L-lysine was patterned in parallel stripes on flat substrates, rather than left as uniform layers, these protein patterns caused shorter, thicker, less branched, and slower growing neurites, and caused changes in either the depolarization or repolarization of the action potential [37]. Laminin gradients generated with microfluidic designs also directly influenced neurite extension by specifying which early neurite would become the axon, although the gradient did not guide the direction of axonal growth [33]. It is of particular importance to understand the effects of laminin since it has been shown to play a critical role in axonal pathfinding in the embryonic CNS *in vivo* [97].



Prior to assembly of a microfluidic chip on a substrate, a PDMS stencil or microstamp can be used to apply proteins and other agents onto the platform substrate to guide future cell attachment and differentiation, and to create cell co-cultures in controlled geometric patterns [98–100]. Stenciling or microcontact printing of factors can help control the exact position of neurons on a substrate, and the resultant controlled neuronal patterns are very helpful in studying neural networks and interactions occurring within synapses [35,36,99,100].

Other work has combined topographic and static chemical cues. For example, substrate grooves treated with adsorbed laminin have allowed selective orientation of astrocytes in order to provide a permissive environment for some neural studies [101]. Similarly, the response of chick spinal neurons to poly-lysine-coated “V”-shaped channels and pits showed that neurites stayed within the untreated channels, but could grow out of the channels when the entire surface was pretreated with poly-lysine, demonstrating that chemical guidance cues may supersede the influence of topography [34].

Recently, synergistic effects of topography, chemical, and biological cues have been noted to directly influence the differentiation of adult rat hippocampal progenitor cells *in vitro* [102]. When these cells were co-cultured with astrocytes aligned on laminin-coated substrate grooves, there was enhanced neuronal differentiation and alignment of neurites parallel to the astrocyte processes and substrate grooves. It is presumed that the astrocytes first align on the grooves, and then secrete soluble factors that are concentrated locally by the topography, resulting in facilitated neuronal differentiation of the progenitor cells.

## 7. Growth factor studies

As previously discussed, microfluidic channels can be arranged to create dynamic concentration gradients of special factors in order to determine their effects on neural cells. These experiments have shown, for example, that neurite growth favored media containing neurotrophin-3 [103]. The effect of various concentrations of combined growth factors (epidermal growth factor, fibroblast growth factor 2, and platelet-derived growth factor) on neural stem cell proliferation and differentiation has been studied using microfluidic chambers that were precoated with poly-L-lysine and laminin. During the study, the cells were exposed to constant low flow (0.1  $\mu\text{l}/\text{min}$ ) of media to minimize autocrine and paracrine effects of secreted factors that would be difficult to regulate in a standard tissue culture system [71]. The stem cells proliferated in direct proportion to the growth factor concentration, differentiated into astrocytes in inverse proportion to the growth factor concentration, and migrated towards areas with higher concentrations. Further microfluidic studies on the effects of growth factors may be helpful in understanding their influence on stem cells that have been transplanted into the CNS.

## 8. Studies to isolate axons

Individual cells from neural lineage have been studied within microfluidic chambers in the past, often with the aid of integrated analytical devices, but now, chip design has progressed to allow studies of isolated axonal segments. Previously, this was only achievable with Campenot chambers that use neurotrophic factors to “artificially” stimulate axonal growth from macro-scaled cultures across grease layers [104]; however, the microfluidic platform designed by Jeon’s group [105] represents a much more sophisticated device for axonal isolation. It incorporates tiny grooves of adequate size and length to allow isolation of axons from the regular cell culture chamber, so that their physiology can be studied independently. These grooves in the PDMS create a high resistance to fluid transport and allow temporary fluidic isolation of the somal compartment from the axonal compartment by using different medium volumes in each chamber resulting in a hydrostatic pressure gradient. The grooves also prevent the passage of cell bodies and guide neurite growth into the axonal isolation compartment where chemical manipulation of the axon can be accomplished without directly exposing the cell’s soma (see Fig. 3) [105–107].

Jeon’s group was able to use their isolated compartments to detect axonal mRNA and changes in somal gene expression in response to axonal chemical and physical manipulation, and vice versa. Since there are no ribosomes in the axon, the source for the axonal mRNA would be mitochondrial DNA. They also co-cultured oligodendrocytes with the axons and demonstrated early *in vitro* myelination to prove that this platform held promise for studies on myelination, axonal injury, and regeneration.

More specifically, Jeon’s chip could be used to study aspects of neuronal pathophysiology in acquired axonopathies (i.e. neurodegenerative diseases including Multiple Sclerosis, Alzheimer’s, traumatic CNS damage), in acquired neuropathies (Guillain–Barré), and in congenital hereditary neuropathies (Charcot–Marie–Tooth and other primary inherited neuropathies, hereditary sensory autonomic neuropathies, giant axonal neuropathy, etc.). Since single axons can be isolated, the effect of transection can be assessed directly on the axon and its associated soma. Axoplasmic flow can be evaluated with dyes, and the local vs. global effects of extrinsic proteolytic enzymes like matrix metalloproteinases (MMPs) or proteins like  $\beta$ -amyloid applied to one compartment can be studied. In addition, cells that model genetic diseases can be cultured in the chips and the effects of axonal stresses on these cells can be compared to effects seen when identical stresses are applied to normal cells.

## 9. Conclusion and potential applications

By providing the ability to isolate individual cells and precisely control their milieu while minimizing nonspecific

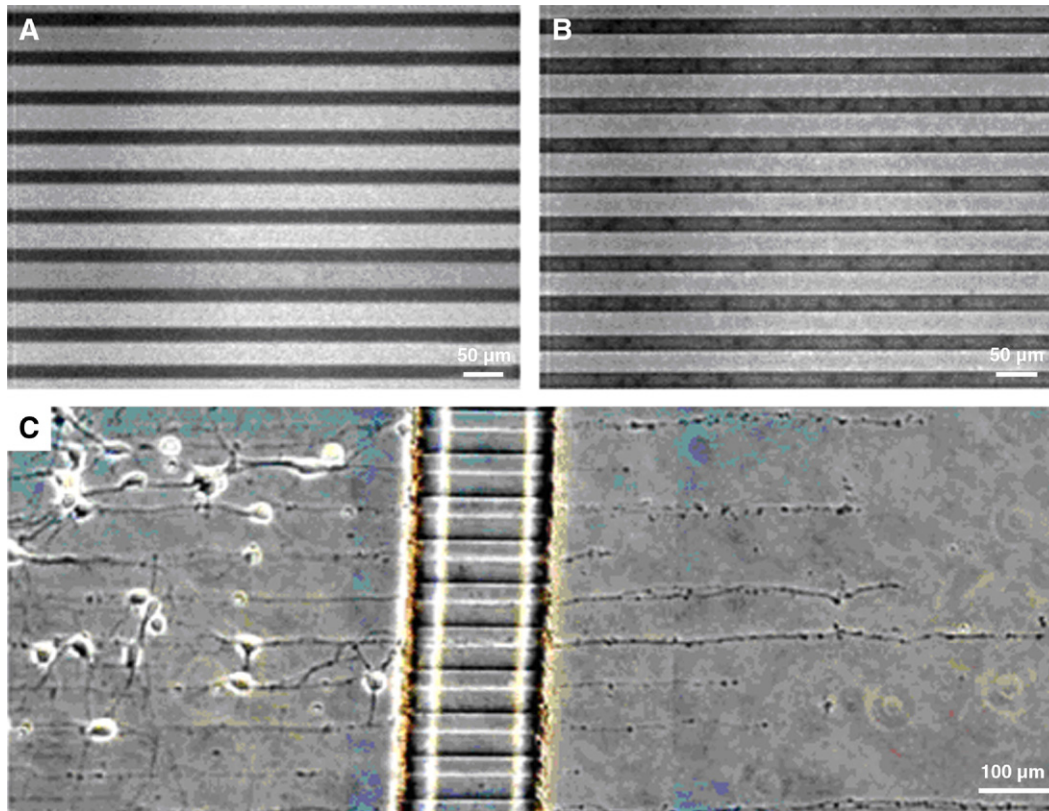


Fig. 3. (A) Fluorescence micrograph of FITC-labeled polylysine patterned with micromolding in capillaries (MIMIC). (B) Fluorescence micrograph of FITC-labeled polylysine patterned with microcontact printing. (C) Phase micrograph showing neurites crossing the microgroove barrier and following the polylysine patterns. (Reprinted with permission from Langmuir. Taylor AM, Rhee SW, Tu CH, Cribbs DH, Cotman CW, Jeon NL. Microfluidic multicompartiment device for neuroscience research. 2003; 19 (5): 1551–1556. Copyright 2003 American Chemical Society.)

interactions, microfluidics can enable a “ground-up” approach to studying the interactions of different physiologic agents (growth factors, cytokines, chemokines, etc.) on individual neurons and glia, as well as allow high throughput screening of pharmacologic agents. These platforms can also facilitate study of localized physiology in isolated cellular projections (axons or dendrites) and in individual cell–cell interactions, such as that which occurs in synapses, and in the process of myelination. Incorporation of other micro- and nanotechnology tools and on-chip analytical devices into these platforms such as multielectrode arrays, pH and ionic current monitors, NMR coils, and molecular biology devices should additionally help to elucidate more details of neuronal physiology.

Potential applications of this technology could involve single-cell morphologic and physiologic studies of the effects of pharmacologic agents, developmental factors, inflammatory molecules, toxic environmental agents, and neurotransmitters. Combining these platforms with single-molecule evaluations (carried out electronically, optically, or by AFM force spectroscopy techniques) could provide valuable data on the response of receptors and ion channels to these agents. In summary, studies of individual neuronal cells within microfluidic platforms represent a new approach to experimental investigation and may reveal critical

new information about cellular physiology in health and in disease.

#### Acknowledgement

This work has been supported by The Nancy Davis Foundation — The Race to Erase Centers Without Walls and the Dan Murphy Foundation. We thank W. French Anderson, MD for reviewing and editing the manuscript.

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