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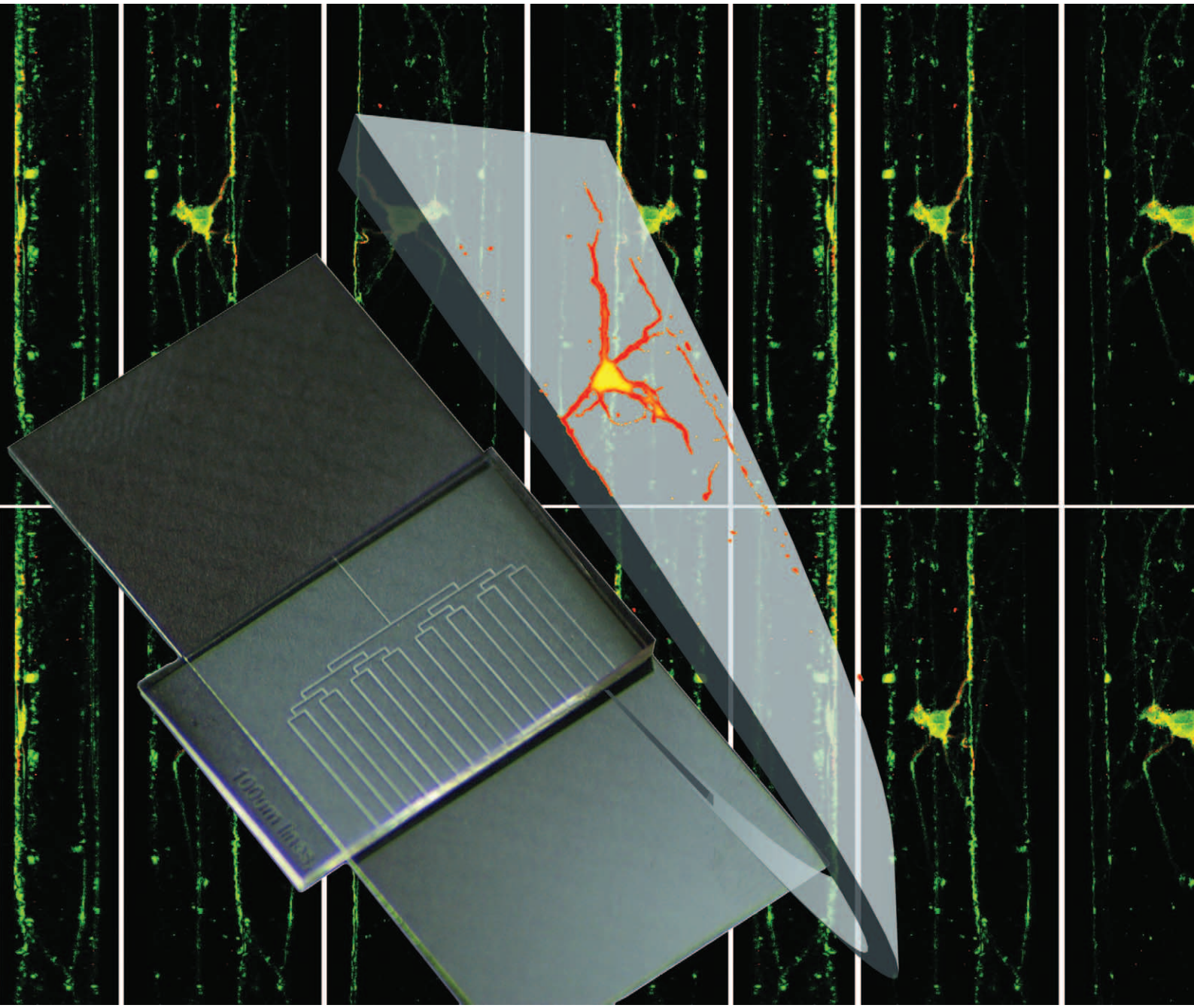

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Microfluidic devices for culturing primary mammalian neurons at low densities†

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Microfluidic devices have been used to study high-density cultures of many cell types. Because cell-to-cell signaling is local, however, there exists a need to develop culture systems that sustain small numbers of neurons and enable analyses of the microenvironments. Such cultures are hard to maintain in stable form, and it is difficult to prevent cell death when using primary mammalian neurons. We demonstrate that postnatal primary hippocampal neurons from rat can be cultured at low densities within nanoliter-volume microdevices fabricated using polydimethylsiloxane (PDMS). Doing so requires an additional fabrication step, serial extractions/washes of PDMS with several solvents, which removes uncrosslinked oligomers, solvent and residues of the platinum catalyst used to cure the polymer. We found this step improves the biocompatibility of the PDMS devices significantly. Whereas neurons survive for $\geq$ 7 days in open channel microdevices, the ability to culture neurons in closed-channel devices made of untreated, native PDMS is limited to $\leq$ 2 days. When the closed-channel PDMS devices are extracted, biocompatibility improves allowing for reliable neuron cultures at low densities for $\geq$ 7 days. Comparisons made to autoclaved PDMS and native, untreated PDMS reveal that the solvent-treated polymer is superior in sustaining low densities of primary neurons in culture. When neuronal affinity for local substrates is observed directly, we find that axons localize to channel corners and prefer PDMS surfaces to glass in hybrid devices. When perfusing the channels with media by gravity flow, cultured hippocampal neurons survive for $\geq$ 11 days. Extracting PDMS improves biocompatibility of microfluidic devices and thus enables the study of differentiation of identifiable neurons and the characterization of local extracellular signals.

Introduction

Communication among cells of the nervous system relies on chemical cues transferred locally at sites of synaptic contact within a vast network of highly branched structures. This organizational complexity has hindered efforts to analyze the precise chemical signatures that are involved at the cellular level. For mammalian neurons, the problem is compounded by both the small size of the cells and the low concentrations of the chemical species involved.[1,2] Closed-channel microfluidic devices offer the ability to define and manipulate the environment of single cells,[3] and may facilitate local chemical analysis if neurons could be cultured at low densities in such devices. At present, however, such forms of neuronal microcultures are difficult to sustain in stable form. The development of such capabilities would have an impact on research as local chemical signatures of cell–cell communication have the potential to be addressed within microfluidic devices in ways that cannot be studied using the whole brain, tissue slices, or even in dissociated cell cultures in a dish. These classical approaches for studying neurons do not provide the ability to access, maintain, and manipulate the microenvironment surrounding single neurons. Microfluidic devices provide promising platforms for controlling the microenvironment of neurons[3] and the ability to selectively probe or stimulate the somata or axons of neurons.[4–6] We address these areas using devices fabricated in polydimethylsiloxane (PDMS) with soft-lithography.[7] Devices of this type have proven to be particularly useful for biological studies due to their ease of fabrication[8] and the high gas permeability, optical transparency, and low water permeability of the structures formed from this material.[9,10] PDMS also is an inexpensive and thermally stable elastomer that allows for rapid sample replication and control over surface properties *via* simple surface modification protocols.[11]

Microfluidic devices fabricated in PDMS[12] have been characterized for many diverse biological applications,[13,14] and recently have been used as a structural component for patterning neuronal growth in culture. In one example, laminar flow gradient mixers in PDMS microdevices were used to generate defined substrate gradients for presenting developing hippocampal neurons with orienting cues for axon specification.[15] Useful protocols for proliferating and differentiating human cortical neural stem cells were developed that

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exploit stable gradients of growth factors in culture media using similar PDMS-based microfluidic gradient mixers.[16] Another approach to controlling neuronal development has been to culture cortical neurons on patterned substrates in PDMS microfluidic chambers.[17] These neurons were selectively positioned into the microdevices by incorporating an additional centrifugation step.[4] To enable studies of axon guidance in three-dimensional (3-D) defined-media conditions, open-channel PDMS microdevices have been used in conjunction with a gel matrix to investigate how topographical cues influence axon navigation.[18] Other open-channel, three-dimensional PDMS culture platforms have been selectively surface-treated to control cortical neuron attachment toward the formation of neural networks.[19,20] Similar networks have been developed on microelectrode arrays with the aid of closed microfluidic channels of PDMS that restricted growth of neuronal processes between culture wells.[21] In a set of related studies, microfabricated Campenot-like chambers made of PDMS were used to fluidically isolate neuronal cell bodies and dendrites from axons to allow for axon-specific biochemical analysis and regeneration studies.[5,6,17]

These investigations demonstrate some advantages of using microfluidic devices for studies of the nervous system by combining microfabrication and neuroscience.[22,23] There remain unaddressed issues with the approaches used in these investigations. In each of the above cited examples, neurons were used in one or more of the following ways: (i) they were cultured at high density, usually in the presence of glial cells; (ii) the cells were bathed in relatively large volumes of culture media; or (iii) the protocols included serum, a potent growth factor. It appears from most reported work that the microfabricated culture chambers used are generally large relative to the size of the neuron. In studies where 50 μm-wide closed channels are used, neuron yields are low, even in the presence of serum.[21] This low viability has been attributed to mass transfer impacts related to restricted nutrient availability and waste removal.[21,24] It also has been noted that the surface area-to-volume ratio of a device can have significant consequences for the viability of a neuronal culture, an effect that may be related to the depletion of media components and/or the leaching of agents in the microchannel material in ways that alter biocompatibility.[24] This study demonstrates improved methodologies for culturing neurons at low densities and for single-cell analysis. Developing reduced-dimension PDMS channels compatible with cell culture improves access to neurons, facilitates their chemical characterization and may ultimately help advance neuroscience through the miniaturization of experimental architectures. In this way, the context-dependent questions of cell-to-cell signaling can be addressed directly. The goal of the work reported here was to develop procedures that enable and sustain low-density cultures of primary neurons in closed-channel, microfluidic devices using serum-free media.

We describe an improvement to soft-lithographic fabrication processes that enhances the biocompatibility of PDMS. This is particularly beneficial when culturing post-natal mammalian neurons, cells that are highly sensitive to environmental conditions during development and maturation. We first cultured primary hippocampal neurons on coverslips with, and without, open-channel devices made of PDMS. We then investigated the growth of primary hippocampal neurons in static-bath, closed-channel PDMS microdevices. For neurons to remain viable and develop in closed-channels of lengths $\geq$ 4 mm (85 μm wide and 45 μm high), we found that the components of the PDMS devices must be processed through a series of organic solutions prior to their assembly. Neuronal development in these treated microchannels was characterized by direct observation. Axons were found to localize along channel corners, and in optimally treated samples to prefer attachment to PDMS over glass. We also evaluated autoclaved PDMS[25] and found it to be less effective as a material for sustaining microcultures of primary rat hippocampal neurons. Our results confirm that mass-transfer effects markedly impact the viability of the cells at low densities, as cultured neurons supported in perfused channels formed of native, autoclaved, or extracted PDMS show improvements in viability and channel-length capacity (increasing two-fold for all but native PDMS).

Materials and methods

Microdevice fabrication

Open-channel microdevice fabrication. Open-channel microdevices for cell culture were fabricated using Selective Pattern Release (SPaR), a form of Decal Transfer Microlithography (Fig. 1A).[26–28] Masters were fabricated by photolithography using a transparency mask defined by ink-jet printing and SU-8(50) photoresist. The masters consisted of an array of parallel lines that converge to a single inlet and outlet. The width and height of the photoresist features on the masters used for open channel device fabrication were $\sim$33 and 46 μm, respectively. The masters for closed channel devices were similar, but the photoresist lines were $\sim$88 μm wide. The PDMS prepolymer (Sylgard 184, Dow Corning) was cast below the resist features on a master using a photoresist spinner (Fig. 1Ai). After spin-casting, the thin PDMS layer on the master was cured at 70 °C for 30 min, then exposed to UVO for 90 s, and placed in a closed, dry container along with an open vial of (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane for 20 min to generate a release layer.[8,29] The master with cured decal was then covered with a 0.5–1 cm backing layer of PDMS prepolymer, and cured at 70 °C for 2 h (Fig. 1Aii). The multi-layer PDMS stamp was removed from the master and 2 mm channel access holes were made using a biopsy punch. The PDMS surfaces were rinsed with ethanol and DI water (MilliQ), and dried under a stream of nitrogen. Glass coverslip substrates (No. 1½, Corning) were rinsed with 2-propanol, dried with nitrogen, cleaned in a piranha solution (3 : 1 H_2SO_4 : 30% hydrogen peroxide, Fisher) for 30 min, rinsed thoroughly with DI water, and dried under a stream of nitrogen. (**Caution!** The piranha solution is extremely corrosive and reactive with organic matter.) The decal was exposed to UVO for 150 s and placed in contact with a clean glass coverslip (Fig. 1Aiii).[26] This assembly was heated at 70 °C for 20 min to facilitate bonding between the coverslip and PDMS stamp. The channels were then filled with a 100 μg ml^{-1} solution of poly-D-lysine (PDL, Sigma) for 1 h[30] and rinsed (3×) with

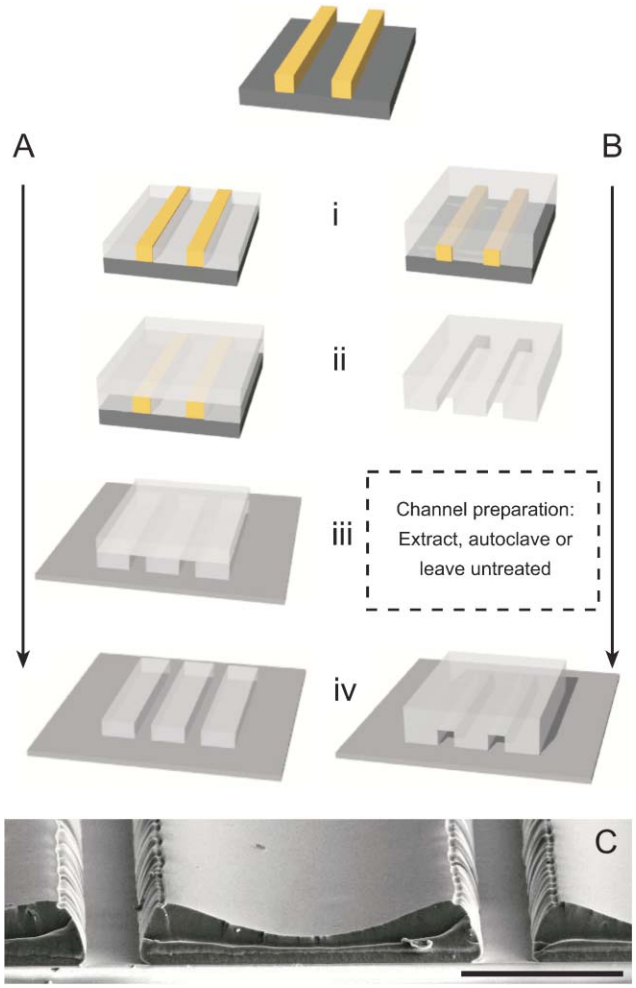

Fig. 1 Masters were made by patterning SU-8 (50) photoresist on silicon, and treating with a "no stick" layer to allow PDMS release. (A) Open channel devices: PDMS was spin-cast onto the master and cured, the PDMS was fluorinated and covered with bulk PDMS to provide support for decal transfer. The multilayer stamp was removed, oxidized and covalently attached to a clean coverslip, and bulk PDMS removed. (B) Closed channel devices: bulk PDMS was poured onto the masters, cured, cleaned, prepared (no treatment, autoclaved, or extracted) and assembled onto clean coverslips. Channels were coated with 100 μg mL^{-1} PDL, and media-rinsed prior to cell culture. (C) Scanning electron micrograph of open channel microdevice. Scale bar = 100 μm.

phosphate buffered saline (PBS) using the channel outgas technique (COT).[31] In the final step, the thick backing layer of PDMS was removed with forceps to complete the decal transfer process (Fig. 1Aiv). Electron micrographs of PDMS open-channels on glass were obtained using a JEOL JSM-6060LV scanning electron microscope (SEM) and are presented in Fig. 1C. The 'sag' observed in the PDMS structures results from a meniscus due to the wetting of the liquid prepolymer to the sides of the photoresist when the prepolymer was spin-cast below the features of the photoresist (Fig. 1Ai).[26]

Culture medium was placed on the open-channel PDMS devices and housed overnight in a humidified cell culture incubator at 37 °C with 5% CO_2. This removed air that became trapped in the open-channels upon initial media contact, making devices ready for cell plating.

Closed-channel microdevice fabrication

Closed-channel microdevices for cell culture were fabricated using standard soft lithography methods (Fig. 1B).[8] The PDMS prepolymer was prepared as described above and poured to a thickness of ~2 mm over a master, degassed, and cured overnight at 70 °C (Fig. 1Bi). Cured PDMS devices were removed from the masters (Fig. 1Bii), randomly divided into three groups for treatment and cell culturing: (a) untreated (native PDMS); (b) autoclaved PDMS; and (c) extracted PDMS devices (Fig. 1Biii). Native PDMS samples were rinsed briefly with 95% ethyl alcohol USP (EtOH, AAPER) and dried at 70 °C overnight. Autoclaved devices were first cleaned by rinsing with 95% EtOH, then autoclaved at 121 °C and 110 kPa for 20 min with a 20 min drying time (81 °C to 91 °C). For the extracted samples, PDMS devices measuring approximately 22 mm × 22 mm × 2 mm were treated sequentially in 100 mL of the following solvents with continuous stirring (repeated solvents indicate the disposal of used solvent and the introduction of fresh solvent): pentane (Sigma) for 36 h; pentane 7 h; xylene isomers plus ethylbenzene 98.5%+ (Sigma) 1–2 h; xylenes 16 h; xylenes 7 h; 200 proof EtOH USP (AAPER) 1–2 h, EtOH again for 16 h, and finally EtOH for 7 h. After extraction with the organic solvents, the devices were submerged in 1 L of sterile DI water overnight. The extracted devices were then dried at 70 °C overnight. These three processes sterilize the PDMS, which is necessary for cell culture. Our extraction procedure was based on a previously published protocol.[32] While it may be possible to simplify the extraction procedure by using shorter extraction times or fewer solvents, we were not able to identify alternate conditions that provided comparable beneficial impacts on the health and survival of the cells. Final cross-sectional channel dimensions measured 85 μm × 45 μm in width and height, respectively. The catalytic metal content of native, autoclaved, and extracted PDMS was analyzed at the Illinois Waste Management and Research Center. Samples were analyzed specifically for platinum content by inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Elemental PQ ExCell).

For static bath closed-channel cultures, PDMS channels were cut from the treated stamps and placed in conformal contact on dry (acid-cleaned) glass coverslips (Fig. 1Biv). Solutions of PDL were centrifuged at 1400 rpm for 5 min prior to use to remove undissolved debris; the supernatant was applied to coverslips and aspirated into the device channels. Aspiration was performed using a Pasteur pipette connected to a vacuum line. In this approach, liquid was removed from the outlet to induce flow from the inlet towards the outlet. Samples were housed in a humidified incubator at 37 °C for 1–2 h until cell plating whereupon PDL was replaced with cell-containing media. Closed-channel devices for gravity-induced flow were 9 mm long prior to converging to a single channel. These samples were prepared as described with an additional overnight media rinse between PDL coating and cell plating.

Cell culture

Animal procedures were conducted in accordance with PHS guidelines for the humane treatment of animals under approved protocols established through the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee under the Vice Chancellor for Research. Primary hippocampal neurons from P1-P2 Long-Evans BluGill rats from our inbred colony were cultured following a protocol modified from Brewer.[33] Hibernate-A (Brain Bits, Springfield, IL) and Neurobasal-A (Invitrogen) media without phenol red were supplemented with 0.5 mM L-glutamine (Invitrogen), B-27 (Invitrogen), 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin (Sigma). Briefly, subjects were rapidly decapitated, the brain removed and hippocampi dissected in ice-cold Hibernate. Four hippocampi were pooled and then treated with activated papain (25.5 U mL^{-1}, Worthington) in Hibernate for 30 min at 37 °C. Following enzymatic treatment, the papain-containing solution was aspirated and hippocampi were rinsed once with 1 mL of enzyme-free Hibernate. Cells were then mechanically dissociated through trituration in 2 mL Hibernate using a fire-polished Pasteur pipette. After undissociated tissue settled, the supernatant was transferred to a new 15 mL vial and the process repeated. The resulting supernatant was combined and centrifuged at 1400 rpm for 5 min. Cells were resuspended, counted, diluted in Neurobasal media, and plated at 100 cells mm^{-2} for control cultures. For plating in closed channels, the culture media in the channels from the post-PDL rinse was exchanged with cell-containing media *via* aspiration-induced displacement. This provided a convenient cell-loading process by preventing channels from drying out. Dissociated cells were loaded into native, autoclaved and extracted closed channels between $1.0 \times 10^6 - 1.5 \times 10^6$ cells mL^{-1} and permitted to attach for 5–10 min. The media levels on the inlet and outlet sides of the device were balanced to provide a static bath. For channel perfusion, media levels were offset by adding more fluid to the inlet than to the outlet. This height difference resulted in a gravity-induced flow through the channels from the inlet to the outlet. Every 48 h, the media levels were re-established with fresh media to the initial plating levels (450 μL initial reservoir and 50 μL final reservoir) to permit continuous gravity-induced flow. All cultures were housed in a humidified incubator at 37 °C with 5% CO_2 and fed 2–3 times per week.

Immunochemistry

Neurons cultured in microdevices were characterized using basic immunocytochemistry.[30] After fixation, the cells were rinsed with PBS between each step, except between blocking and primary antibody incubation. Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.25% Triton in PBS for 10 min, and blocked for 30 min at room temperature with 10% BSA in PBS. Primary antibodies used include: (a) mouse monoclonal anti-MAP2 (1 : 1000, Chemicon) for labeling dendrites; (b) sheep polyclonal anti-tau (1 : 100, Pierce Endogen) for axon identification; (c) rabbit polyclonal anti-neurofilament M (1 : 1000, Chemicon) for marking neuron-specific intermediate filaments; and (d) mouse monoclonal anti-GFAP (1 : 1000, Chemicon) to identify glial cells. Secondary antibodies were purchased from Molecular Probes and used in a 1 : 1000 dilution at room temperature for 1–2 h. These included: (a) Alexa Fluor 488 goat anti-mouse; (b) Alexa Fluor 488 goat anti-rabbit; (c) Alexa Fluor 568 goat anti-rabbit; and (d) Alexa Fluor 633 goat anti-sheep. For labeling actin filaments (f-actin), rhodamine-conjugated phalloidin (1 : 1000, Molecular Probes) was incubated for 20 min at room temperature followed by three PBS rinses over a 20 min period. Samples were briefly rinsed with DI water, dried, and mounted with Prolong Gold anti-fade reagent (Molecular Probes).

Microscopy

Labeled neurons in microchannels were imaged within as little as 24 h immediately after preparation due to the tendency of the antifade reagent to begin clouding while inside the PDMS channels, resulting in poor image quality. To evaluate neuronal development and axonal localization within the microdevice, confocal images were acquired on a laser-equipped Zeiss LSM-510 and processed with the Zeiss image acquisition software to render compressed two-dimensional images from the 3-D Z-stack data.

Results and discussion

Neuronal development in open-channel microdevices

The compatibility of primary mammalian neurons with the microdevice materials was investigated by plating neurons on the open-channel microdevices, as described above. These open-channel devices have large diffusion volumes where the material surfaces and neurons have free access to bulk media. Under these conditions, direct substrate-dependent neuronal development can be studied apart from conditions influenced by the closed-channel environment. While neuronal growth has been characterized on open-channels fabricated entirely from PDMS,[18] our fabrication method produces open-channels that present the cell with two interfacial materials—the PDL-coated coverslip and PDMS. As shown in Fig. 1C, we employed open-channels that had ~33 μm-wide glass bottoms and ~39 μm-tall PDMS walls. Neurons in open-channels were maintained for 7–8 days *in vitro* (DIV) in a static bath culture and immunocytochemically processed to label the dendritic microtubule associated protein (MAP2) and the neuron-specific intermediate filaments (neurofilament-M) (Fig 2). These results show characteristic neuron polarization and development marked by long, branching dendrites. Neuronal processes frequently were retained inside the channel and localized to channel corners; single neurites occasionally were observed to migrate from the channel, across the PDMS surface, and into an adjacent channel (Fig. S1).†

Others also have observed the localization of cellular processes to channel corners. For example, an adrenal gland cell line (PC12) was stimulated with nerve growth factor to induce neuron-like morphology in microchannels of different sizes.[34] In this work, open-channel structures were fabricated with polyimide on coverslips and coated with collagen wherein they demonstrated that properties of process outgrowth

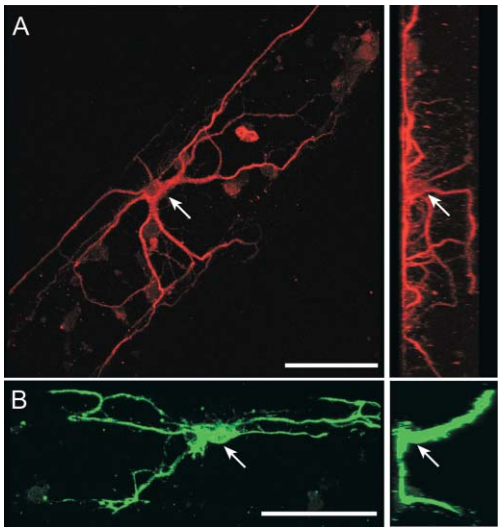

Fig. 2 Morphology of primary rat hippocampal neurons developing in open-channel microdevices. Neuronal somata (arrows) and processes labeled with antibodies that recognize neuronal markers, (A) MAP2 and (B) neurofilament-M. Cultures were collected for immunochemistry at 8 DIV. Images show a top-down view looking into the open-channel devices (left) and side views rotated 90-degrees for respective images (right). Scale bars = 50 μm.

(length, number, and angle of emergence from soma) were dependent on the width of the channel.[34] Despite the material and biological differences between these systems, we see a similar behavior of neuronal processes exhibiting a preference for channel corners. Notable differences can be attributed to the topographical scales, materials, substrates, cell types and location where the cell develops in the channel (in the center or corners). Specifically, neuronal cell bodies and processes in our system were able to attach to PDMS walls and neurites to navigate up sidewalls where they may exit the channels due to channel topography (Fig. 2). Here we emphasize an important variable for consideration in these kinds of studies: substrate-dependent cell behavior. For example, when the channels were coated with laminin, we observed neurons aggregating inside the channels to form clusters of cell bodies; however, channels coated with PDL prevented this aggregation.

Neuronal development in closed-channel microdevices

We next investigated the growth of dissociated neurons in closed-channel PDMS microdevices. For our initial studies, we used native PDMS microfluidic channels that were ~4–5 mm long, 85 μm wide, and 45 μm high, which then converged to a single channel outlet. Most cells died prior to the first media change at 48 h in these native PDMS closed-channel devices (on occasion, a single live cell with poor development was observed, data not shown). Many process variations for the cell plating and incubation were examined to improve neuron viability in closed-channel native PDMS devices, but none were successful.

Despite their advantages, closed-channel systems can impede the growth and survival of cells for a variety of reasons, including reduced nutrient supply, waste accretion, and the leaching of cytotoxins from the device materials.[24] It has been reported that up to 5% (w/w) of bulk PDMS is un-crosslinked oligomers, which can be removed by extraction through soaking/swelling PDMS in a series of organic solvents,[32] a treatment procedure, among others, that has been shown to influence the attachment and proliferation of cell-lines and human endothelial cells.[13] PDMS also has been shown to absorb small molecules, thereby modifying local media composition in low-volume device contexts.[35]

We hypothesized that extracting PDMS prior to microdevice assembly and cell culture would improve the survival of primary rat hippocampal neurons by removing leachable materials such as un-crosslinked, low molecular weight oligomers. To test this hypothesis, we extracted PDMS using the protocol described above prior to preparing closed microdevices for neuronal culture. In addition to extraction, autoclaving also alters PDMS,[25] by driving the polymerization reaction to higher degrees of conversion, thus reducing uncrosslinked oligomers, and by out-gassing volatile impurities, such as solvents and low molecular weight components. These changes could improve the viability of low density cellular cultures, therefore we included this treatment process for experimental comparison. The effectiveness of the extraction[32] and autoclaving processes for removing platinum from PDMS was measured by ICP-MS. The platinum content of the samples decreased in the order of native $>$ autoclaved $>$ extracted PDMS. Details of the extraction process and platinum content of the samples are provided in the ESI.†

The effects of the PDMS treatment conditions on neuron viability were studied by culturing hippocampal neurons in microdevices made of native, autoclaved and extracted PDMS. Low-density cultures were sustained for 7 DIV in 4–5 mm-long channels. These were replicated until a total of 70 microchannels for each PDMS-type had been examined. We found a total of 3 neurons in the native PDMS channels, 28 neurons in the autoclaved channels, and 51 neurons in the extracted PDMS channels. Apart from the effects of the PDMS-types, Neurobasal media supplemented with B-27 is unfavorable for the growth of glia at low densities which serves to enrich neuronal content.[36] Glial cells were rarely observed in any of the microchannel cultures.

We observed that neurons cultured in microdevices of extracted PDMS exhibited characteristic development to produce a ramified axon and branching dendrites (Fig. 3).[37] On autoclaved substrates, neuronal processes differentiated to produce polarized neurons having an axon and dendrites; however, the processes often appeared finer (having no taper) with fewer branch points than with extracted substrates. Neuronal processes also showed a strong preference for channel corners in these closed-channel devices, as was previously seen for the open channel cultures.

Through immunocytochemistry, neurons in closed channels were labeled with antibodies against MAP2 and the axon-specific marker, tau, both of which are microtubule-associated proteins (Fig. 3). More axons were found to localize to the PDMS surface as compared to the coverslip (Fig. 3A–C). This could be due to: (1) the increased gas availability at the PDMS–media interface being preferred to that of the coverslip–media interface; (2) the physical surface properties of PDMS being more favorable than the PDL-coated coverslip;

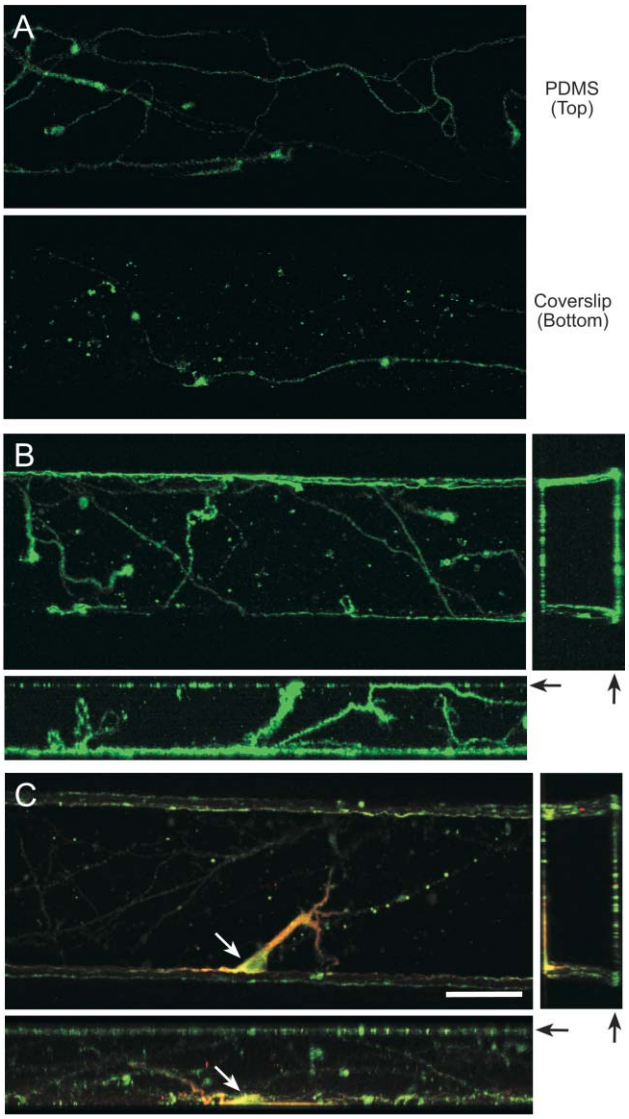

Fig. 3 Neurite localization within closed-channel devices. (A) Axons preferentially localize to extracted PDMS surfaces as demonstrated by two separate images from a single Z-stack; the top image shows processes on the PDMS surface, which is parallel to the channel bottom, the coverslip. (B) In a separate channel, axons at the inlet localize to channel corners and extracted PDMS surfaces in closed-channel microdevices. (C) An individual neuron is identified (cell body, white arrow) with axons localizing to corners and walls. Cells in channels are labeled with antibodies against tau protein (pseudocolored green), MAP2 (red) and GFAP (not shown as no glia were present). All cultures at 7 DIV. Black arrows (right) mark the top of the channel for compressed side view images. Scale bar = 50 μm.

(3) media components adsorbed by the PDMS producing a higher local concentration of constituents more favorable for axon elongation; and (4) a combination of these and/or other factors. Our observations suggest that factors 2 and 3 have considerable importance. Notably, we found that neurons growing near channel inlets (where the cell body is not in a closed channel) have axons that preferentially migrate into the first 300 μm segment of the closed-channel, and that these axons show a preference for guidance along the PDMS surfaces (Fig. 3B). Fig. 3C shows that dendrites, like axons, localize to channel corners, an observation explained by and supporting topological models for the influences that surface feature geometries have on neurite outgrowth.[38] Surface corrugations present the neuron with a greater environmental complexity, a factor that may promote neuron development above that available in simpler culture environments, (*e.g.*, coverslips without channels). Localization of neuronal processes at the channel corners may also be attributable to physicochemical properties of PDMS (such as high gas solubility) that are not yet completely understood.

We also explored the impact of channel length on cell viability in closed-channel static-bath cultures. If the channels were 3 mm long, neurons grew equally well in all devices regardless of the PDMS type, with cells showing growth similar to that observed in control cultures on coverslips without channels (Fig. 4). When neuron survival was monitored in 6 mm-long closed channels, no viable cells were found in devices of any PDMS type. We attribute these results to the difference in the diffusion-length scales inside the channels relative to the bulk media outside the closed-channel device. Shorter channels provide better access to bulk media conditions, whereas longer channels require longer diffusion times, thus reducing the cell's access to bulk media. Therefore, in long closed channels, diffusion-limited replenishment of extinguished antioxidants and waste accretion can negatively impact cell growth under static-bath conditions. These issues with longer closed channels can be addressed by inducing laminar flow to increase the supply of fresh media to the cell's local environment.

Neuronal development in closed-channel microdevices with laminar flow

To improve fluidic control over the microenvironment of individual neurons, we designed and fabricated a channel array to sustain continuous gravity-induced flow for 48 h between media changes. Specifically, the closed-channel PDMS device consisted of sixteen parallel microfluidic channels (l = 18 mm, w = 85 μm, h = 45 μm) that converged to a single channel outlet (Fig. 5A). This design enables control over flow rate by trimming the lengths of the inlets and/or the outlet to change flow resistance and ultimately flow rate. Flow rates were determined by perfusing channels with culture media containing 2 μm polystyrene monodisperse microspheres (Polysciences). For this study, the inlets and outlet channels were 9 and 4 mm long, respectively. Under these conditions, offsetting the fluid heights at the inlets and outlet reservoirs induces gravity-based flow that is sustained for 48 h, at which time it is reinitiated. Microsphere linear flow rates were measured between 7–14 μm s^{-1}. After 24 h, the flow rates were reduced to 1.6–3.2 μm s^{-1}, and, after 48 h, the flow was insufficient to continue moving most of the microspheres down the channels. In this sample setup, the volume difference between the initial and final reservoirs is 400 μL. Based on a 48 h period for reservoir equilibration by 200 μL, we estimate the total fluid displacement per channel at 13 μL for the 48 h period.

When we evaluated neuronal survival and differentiation in 9 mm-long channels under this gravity-driven flow field, we

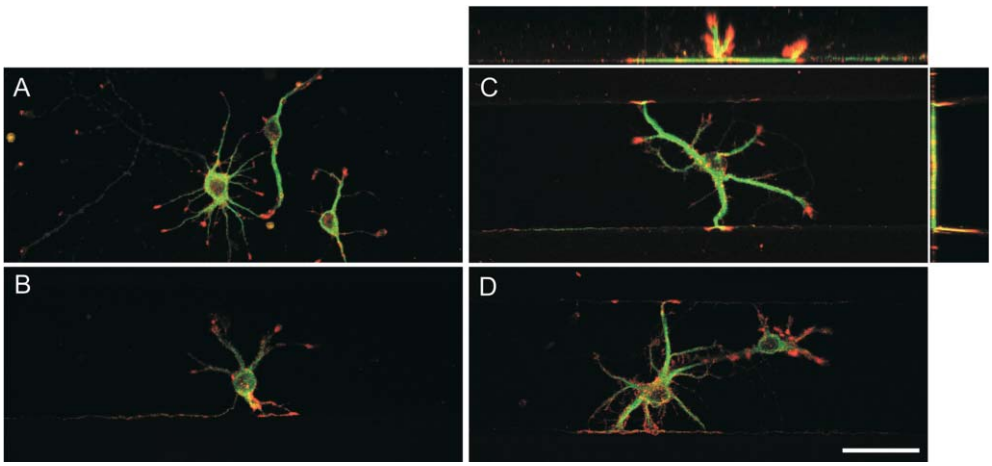

Fig. 4 Neuronal development in control cultures (coverslip only) and 3 mm static-bath, closed-channel microdevices. (A) Control culture of rat postnatal neurons. Neurons develop characteristic morphologies in short (3 mm) channels in (B) native, (C) extracted and (D) autoclaved PDMS. Neuronal processes are identified by MAP2 (green) and rhodamine phalloidin (red) staining at 7 DIV in all cultures; (C) these processes are observed migrating up the channel sidewalls, as indicated. Scale bar = 50 μm.

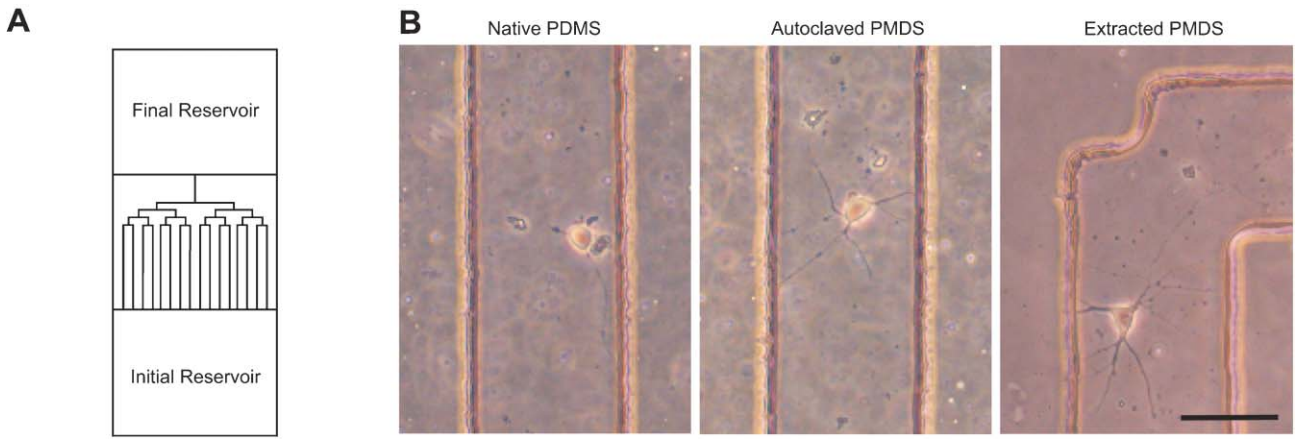

Fig. 5 Neurons cultured in treated and untreated closed-channel PDMS microfluidic devices at 7 DIV with continuous, gravity-induced flow. (A) Schematic of channels fed by gravity flow. Reservoirs were re-established every 48 h with fresh media to maintain continuous perfusion. (B) Cell viability and morphology improved with autoclaved PDMS and even more with extracting PDMS. Viability trends are similar to static bath cultures in channels perfused with culture medium. Total numbers of paired or isolated neurons in a 16-channel array per PDMS-type were 1 (native), 57 (autoclaved), and 139 (extracted). Scale bar = 50 μm.

found that cells survived and developed better in extracted PDMS microdevices compared to autoclaved and native PDMS devices. Isolated or paired living intact cells with phase-bright neuron morphology were counted for each sample; for comparison, neurons were analyzed at 7 DIV although they lived $\geq$ 11 DIV. In the 16-channel arrays, 1 poorly developed neuron was observed in a native PDMS sample, 57 neurons were found in the autoclaved PDMS channels, and 139 neurons were observed in the extracted PDMS channels. Neurons in channels of the different PDMS-types exhibited a characteristic, treatment-dependent morphology, with elaboration of processes enhanced in parallel with neuronal survival (Fig. 5B). Compared to native and autoclaved PDMS, extracted channels were more permissive for cell survival. A consequence was that neuronal aggregates formed inside the channels near the inlets. These occasional aggregates were outside the confines of the counting criteria and were not included in the above data. Overall, we found that inducing flow in extracted and autoclaved PDMS channels allowed for the survival of neurons in channels 9–10 mm long. This is an improvement, considering that neurons in static bath cultures could not be sustained in closed channels $>$ 5 mm long. Taken together, these results demonstrate that: (1) low-rate perfusion does not improve the neuron sustainability of native PDMS; and (2) inducing media flow further enhances the ability to culture neurons at low densities in autoclaved and extracted PDMS, when compared to static-bath cultures using the same material treatments.

Channels continuously supplied with fresh media show improved neuronal viability, indicating that neurons are sensitive to different local environments. PDMS has been shown to collect and remove media components (*e.g.*, proteins and amino acids) after media incubation.[25] Low-rate perfusion should continue supplying these compounds to developing neurons and, thus, improve viability. These results underscore the importance of understanding how material properties

influence the local environments of the microchannels and ultimately the development of the cell. While PDMS has many beneficial properties, there does appear to exist some need to improve its biocompatibility so that it can support low-density cultures of sensitive cells such as the primary mammalian neurons examined here. Ongoing work in our labs is focused on using microfluidic devices to analyze the chemical cues released from neurons. Neurobiological signals might be collected by immobilizing capture agents, such as hydrophobic self-assembled monolayers (SAMs), in the microchannels and then analyzing the collected chemical cues by matrix-assisted laser desorption ionization (MALDI).

Conclusions

We have developed microfluidic device treatments that enable the survival and differentiation of primary hippocampal neurons at low densities under serum-free conditions. Both open- and closed-channel architectures formed of native, autoclaved and extracted PDMS were evaluated. Although neuron viability was improved over native PDMS by autoclaving, exhaustive solvent-based extraction of the PDMS provided superior results, with a nearly two-fold increase in neuron viability and optimal cell development. Axons localized to channel corners in both open- and closed-channel microdevices, and were more abundant on PDL-coated PDMS than on PDL-coated coverslips. Perfusion by gravity flow more than doubled the allowable channel length for sustained neuronal cultures. With this advance in defining material and flow conditions for PDMS-based microdevices, we believe it is now possible for small numbers of postnatal mammalian neurons to be probed to analyze local cell-to-cell signals in ways not previously possible.

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