Chapter 7

Development of Microfluidic Devices for the Manipulation of Neuronal Synapses

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Abstract

Key determinants in the development of complex morphology and function are the cues present in a cell's environment and its response to them. Primary among these extracellular factors is the presence and influence of neighboring cells. It is crucial, therefore, in studying development to be able to replicate in vitro these network-like conditions. This is especially true of neuroscience, tissue engineering, and clinical biology, where network formation and function are critical aspects of any investigation.

Here we describe an easy and inexpensive technique based on microfluidics that provides a high degree of control in positioning and guiding cells, thereby enabling the laying down of desired cellular networks. This approach facilitates the study of synaptic connections where information is communicated between neurons. Such microscale devices are increasingly being employed for studying neurons in highly controlled environments wherein different regions of a network, or even a cell, are cultured in fluidically isolated compartments. Enhanced strategies such as highly regulated manipulations of fluid flow and physical guidance cues when combined with this compartmentalization provide an unparalleled degree of spatiotemporal control over the conformation of the neural network and the stimulation of synapses. This facilitates high-resolution investigations despite the cellular complexity. Consequently, the microfluidic culture platform presents an unparalleled context for unraveling the changes occurring at the microscale and nanoscale of synaptic connections, thereby aiding elucidation of the nuances of neuronal development, wiring, and function.

Key words Microfluidics, Microdevices, Neurons, Synapse, Cell signaling

1 Introduction

The flow of information through the intricate wiring of the nervous system relies on precise, complex interconnections between a multitude of extensions of neurons and glia with the majority of neuronal interactions occurring at synapses. Synaptic plasticity, a phenomenon that encompasses the establishment, strengthening, weakening, and elimination of synapses, is the basis of the development and modification of the neuronal network. As such, it forms the structural and functional foundation of learning and memory. Hence, not surprisingly, a large percentage of neuroscientific studies

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have focused on synapses and their functioning, and a proportionally large number of techniques have been developed to assist these investigations [1]. Some of the more common include assessing responses to stimulants and inhibitors via patching [2], use of pressure pipettes [3, 4], focal stimulation through controlled application [5, 6], or light-controlled activation (uncaging, optogenetics) [7, 8].

While these techniques provide enhanced treatment capabilities, they are more often than not based on, and limited by, the conventional paradigm of neuronal dish culture where the entire cell population exists in the same environment. This limitation can be obviated by the use of compartmentalized cultures that provide the added functionality of maintaining different cell populations, or different regions of the same cell, in separate, individually controlled domains. The original compartmentalized culture system, the Campenot chamber [9], has undergone several modifications over the years into a range of microfluidic devices uniquely suited to varied biological applications [10–14]. Neuronal cultures in these microdevices are a more faithful simulation of the complexity of the mammalian central nervous system, where distinct subregions of the neurons have distinct chemical environments [15, 16]. Today, state-of-the-art microdevices enable maximal control of the microenvironments around different subregions of a neuronal network—synapse, presynaptic and postsynaptic processes, and cell soma—allowing selective and localized stimulation of these specific regions [15, 17].

The process begins with soft lithography (Fig. 1), which can be used to fabricate devices of virtually any design using Computer-Aided Design (CAD)-generated patterns [18]. These enable incorporation of features with dimensions as low as 10 nm. The devices are fabricated using polydimethylsiloxane (PDMS)—a polymer that is inexpensive, flexible, and optically transparent down to 230 nm. It is impermeable to water, nontoxic to cells and permeable to gases, making it ideal for biological studies [19, 20]. Based on the substrate properties desired, other materials such as polyacrylamide or glass can also be used.

A typical device for synaptic investigations (Fig. 2) has three parallel primary channels about 100–200 μm wide and 50 μm high. These are connected through narrow cross-communicating channels about 7 μm (W)×7 μm (H)×50–800 μm (L), termed interconnects, that run perpendicular to the primary channels. Each primary channel has its own inlet and outlet ports to allow independent control of fluids such as media and stimulants. Neurons are seeded into the two outer channels and, guided by the physical cues of the interconnects, they send axons and dendrites into the central channel where synapse formation occurs. The interconnects permit the neuronal processes to grow through while their small size prevents the soma and whole cells from entering, thereby keeping the cell bodies confined within the primary

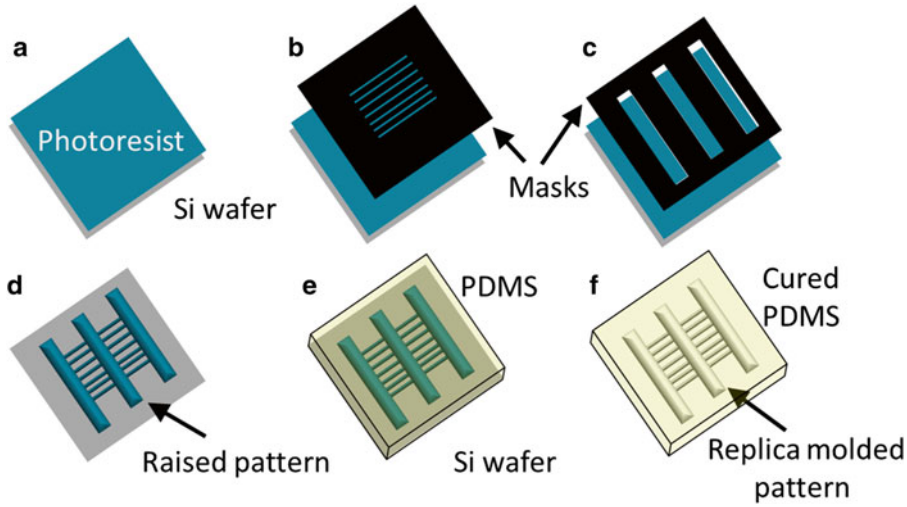

Fig. 1 Workflow for device fabrication. Fabrication begins with photolithography (**a**–**c**) that creates raised patterns on silicon masters (**d**) which can then be used and reused to create PDMS devices through soft lithography (**e**, **f**). To create low-height interconnects in addition to the taller channels, two sequential photolithographic processes are employed. The higher resolution interconnects are created first by exposure through a high-resolution quartz/chromium mask (**b**), followed by the three larger parallel channels created by exposure through a transparency mask (**c**)

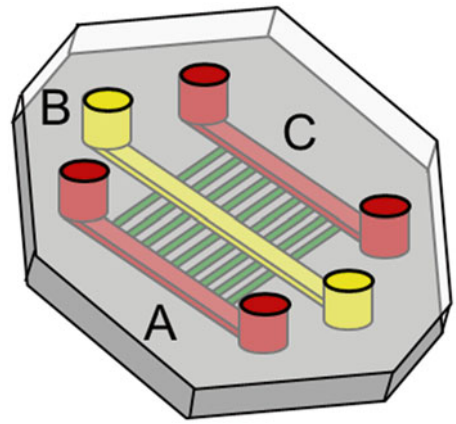

Fig. 2 Microfluidic device for synaptic manipulation. Devices consist of three channels A, B, and C communicating through narrow interconnects (about 7 μm × 7 μm × 300 μm long). Neurons seeded into the outer channels, A and C (*red*), send neurites through the interconnects (*green*), into the central synaptic channel B (*yellow*). Test reagents can then be introduced into this central channel. Widths of cell channels A and C, and synaptic channel B can be varied to obtain the desired device and synaptic configurations

channels. Once the network is established, reagents can be introduced into the central channel for controlled stimulation (or inhibition) of the synapse. Treatments are restricted to the central channel through controlled manipulation of hydrostatic pressure between the channels. Keeping the level of the media higher at the inlets and outlets of the outer channels as compared to the central channel ensures an inward pressure that prevents any fluid leakage into the outer cell compartments—providing a fluidically isolated central synaptic compartment.

Further control of the network can be achieved through additional modifications, such as the use of low-density networks [21], guidance of neurites through fluidic or surface-bound gradients of permissive and/or repulsive cues [22–24], or controlled positioning of the cells through the external application of physical forces [25].

2 Materials

2.1 Device Fabrication and Assembly

Silicon wafer; quartz/chromium mask; SU-8 photoresist; (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane; PDMS pre-polymer (Sylgard 184, Dow Corning); HPLC-grade pentane (Fisher Scientific); xylene isomers plus ethylbenzene 98.5+% (xylenes) (Sigma); 200-proof ethanol (EtOH); sterile DI water; 22×22 mm square No. 1 cover glass (acid-cleaned, Corning); 35 mm petri dish.

2.2 Cell Harvest and Seeding

Source animal—Long-Evans BluGill rats; phosphate buffered saline (PBS); 100 mg/ml solution of poly-D-lysine (PDL, Sigma); Hibernate-A (Brain Bits, Springfield, IL) and Neurobasal-A (Invitrogen) media without phenol red, supplemented with 0.5 mM L-glutamine (Invitrogen), B-27 (Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma); papain (25 U/ml, Worthington) in Hibernate. PDL, media, and the enzyme to be stored at 4 °C.

2.3 Synapse Stimulation and Analyses

Stimulant such as L-glutamate (100 μM); paraformaldehyde (4 %); synaptic markers—anti-synapsin 1 antibody (1:100, Millipore), anti-synaptophysin antibody (1:100, Abcam), anti-PSD-95 antibody (1:100, NeuroMab), Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies (1:1,000, Molecular Probes); Normal Goat Serum; Triton X-100 (0.25 % in PBS); FM 4-64 (10 μM in Hibernate, Life Technologies); all stored at 4 °C.

3 Methods

The procedure detailed here begins with device fabrication [22]. To save time and effort, commercially available microdevices may also be used, taking special care to ensure the selection of suitable device design. Once the device is ready, subsequent steps involve

cell seeding and network formation [26, 27], followed by synapse stimulation and analysis. After use, the PDMS device can be discarded since PDMS is relatively inexpensive and identical new devices can be created easily using the silicon master.

3.1 Device Fabrication and Assembly

1. Device fabrication begins with two sequential photolithographic processes to make silicon masters with multi-height photoresist features based on the desired device design (**Notes 1–3**). Create the low-height interconnects first by spinning SU-8 in a thin layer (~7 mm) onto the master and then exposing through a high-resolution quartz/chromium mask (defined by electron beam lithography). Next create the three larger parallel channels by spinning a thicker layer (~50 mm) of SU-8 onto the master and exposing through a transparency mask (defined by ink-jet printing) aligned with the interconnects. Resist spinning, exposure, and baking are performed as directed by the manufacturer's specifications.
2. To ensure easy release of devices, treat the masters with (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane. At this stage the Si-master is ready for use.
3. Pour the PDMS pre-polymer, a 10:1 mix of base and curing agent, onto the Si-master to a thickness of 2–5 mm. Place it in a vacuum desiccator to facilitate the removal of air bubbles. Allow it to cure at 70 °C for 2 h.
4. Once cooled, peel off the PDMS from the Si-master and create inlets and outlets in it using a 4 mm biopsy punch so as to create the desired design (Fig. 2).
5. Subject the device to sequential solvent extraction to remove impurities and free oligomers (*see* **Note 4**). Submerge the devices in (1) pentane for 16 h, (2) xylene for 1–2 h, (3) xylene for 2–4 h, (4) ethanol for 1–2 h, and, finally, (5) sterile DI water overnight. Allow 1 day for drying.
6. Bond the device to the coverslip using oxygen plasma. Any available oxygen plasma generator may be used. Place the device bottom-side up and the cover glass in the generator for a plasma exposure of 40 s to 1 min. Upon removal from the generator, immediately flip the device onto the cover glass, pressing gently to ensure good conformal contact.
7. As soon as possible, fill the channels of the assembled device with sterile water by placing droplets at the inlets (*see* **Note 5**).
8. At this stage, the device may be tested for fluidic isolation by flowing media containing a fluorophore, such as fluorescein-isothiocyanate (FITC), through each of the channels individually and in different combinations. As long as a positive hydrostatic pressure is maintained, there should be no leakage of fluorophore. Time-lapse imaging for 1 h with 1 ms intervals

for the first 5 min, followed by 1 min intervals for the next 50 min, will enable detection of fast, as well as slow, diffusion, if any.

Unless specified otherwise, all subsequent steps involving the device are to be carried out in a laminar flow hood to avoid contamination.

3.2 Cell Harvest and Seeding

Described here is a procedure for harvesting neurons from rat hippocampi. The device design also permits the simultaneous study of neurons from different brain regions (**Note 2**), or even of non-neuronal cells (**Note 3**). Different harvest procedures would need to be employed in those cases.

1. Empty the inlet ports and add poly-D-lysine (PDL). Apply suction at the outlets to ensure the PDL enters the channels. Leave it to coat for a minimum of 2 h. Rinse out with Neurobasal-A media prior to cell loading. Meanwhile, proceed with cell harvesting.
2. Decapitate 1- to 2-day-old rat pups and dissect out the hippocampi from the brains in ice-cold Hibernate-A.
3. Pool the tissue and place in activated papain (25 U/ml) for 15 min at 37 °C.
4. In a laminar hood, aspirate out the enzyme, rinse the tissue with 1 ml Hibernate-A at 37 °C.
5. Add 2 ml fresh Hibernate-A at 37 °C and triturate with a fire-polished Pasteur pipette to free the dissociated cells. Place the supernatant in a 15 ml vial, and repeat trituration with 1 ml fresh Hibernate-A at 37 °C, pooling the resultant supernatant with the earlier.
6. Add fresh papain (25 U/ml) to the remaining tissue and repeat steps 3–5.
7. Pool the supernatant and centrifuge at 14,000 rpm for 5 min.
8. Resuspend the resulting cell pellet in 500 μl Neurobasal-A at 37 °C.
9. Calculate cell density of the suspension using a hemocytometer and, if required, dilute to a density of 1×10^{-6} cells/ml using Neurobasal-A at 37 °C. Cells are now ready for loading into the device.
10. Empty the inlets of the outer channels of the Neurobasal-A-rinsed device (step 1) and fill them with the cell suspension.
11. Apply suction at the outlets to facilitate cell entry into the channels. View under a microscope and once a sufficient number of cells are seen to have entered the channels, stop flow by adding media to the outlet. Replace the cell suspension at the inlets with fresh Neurobasal-A at 37 °C and check again under the microscope to ensure there is no flow that might dislodge the cells.

12. Leave the device undisturbed in the incubator for 15–20 min.
13. Once the cells have adhered, return the device to the hood. Empty out the outlets and inlets, and refill the inlets with fresh media to actuate gravity-induced flow before returning the dish to the incubator (*see* **Note 6**).
14. Empty the inlets and outlets and refill the inlets with fresh media twice daily, until cells are required for experimentation (*see* **Note 6**).

3.3 Synapse Stimulation and Analyses

Neurites will begin entering the interconnects within a day and, depending on the device design, traverse the interconnects within a few days to start forming synapses in the central channel. Once a network of desired elaboration is formed, the synapses can be stimulated by introducing stimulants into the central channel. Described here is an example of stimulation with glutamate, with synaptic activity visualized using FM 4-64, a lipophilic dye used as a reporter of vacuolar dynamics. Following observations on live neurons, the culture can be fixed and immunocytochemistry performed to visualize cytoskeletal proteins and synaptic markers.

1. When ready to begin stimulation, fill the device with fresh Hibernate-A at 37 °C.
2. In all subsequent steps, care should be taken to ensure that the media level at the inlets and the outlets of the outer channels remains higher than that for the central channel. This prevents potential backflow of markers and stimulants from the synapse chamber to the cell chambers.
3. Empty the inlet of the central channel. Add into this port FM 4-64 at 10 μM. Empty the outlet to facilitate flow. Allow flow for 30 s to 1 min.
4. Empty the central inlet again and rinse with fresh media. Allow media to flow in and wash out unbound FM 4-64 for a minimum of 3 min.
5. Place the device on the microscope stage and focus in on a suitable region of the synapse chamber at 40×.
6. Set up the imaging software for time-lapse capture with no cycle delay and begin imaging.
7. Taking care not to touch the cover glass, aspirate out the media from the central inlet and introduce the glutamate solution.
8. After 10–20 s of introducing glutamate, aspirate it out from the inlet. Rinse and refill the port with fresh Hibernate-A (*see* **Note 7**).
9. As the introduced glutamate flows down the channel, it will induce signal transmission at the functional synapses. Postsynaptic sites will then appear in the imaging as FM 4-64 positive, thus helping identify all active functional synapses.

10. Control experiments may be performed with introduction of glutamate-free media.
11. After the desired experiments have been performed, it is advisable to check for expression of synaptic proteins through immunocytochemistry. Introduce the following into all three channels in the given order: 4 % paraformaldehyde or glutaraldehyde in PBS for 30 min, fresh PBS for 5 min for rinse, 0.25 % Triton in PBS for permeabilization, fresh PBS for 5 min for rinse, 5 % normal goat serum (NGS) in PBS for blocking and fresh PBS for 5 min to rinse. Introduce the relevant primary antibodies, such as the microtubule-associated proteins (MAPs)—tau, which labels axons, and MAP2, which labels dendrites, and established synaptic markers synapsin1, synaptophysin, or PSD-95. Each primary antibody should be followed by incubation with secondary antibodies to the host-species for the primary antibody with 2.5 % NGS in PBS. After the final incubation, rinse with fresh PBS for 10 min before imaging.

4 Notes

1. In the described procedure, axons and dendrites from both the outer channels (A and C) meet and connect in the central chamber. Another possible configuration is to have one population, population A, be selectively presynaptic and provide only axons, and no dendrites, which then synapse with dendrites from the other population, population C, which becomes exclusively postsynaptic. This is achieved by making the interconnects towards population A (between channels A and B) longer (>700 μm), which is greater than the expected dendrite length [11]. Since only axons achieve the lengths necessary to traverse these longer interconnects, only they reach the synaptic chamber, resulting in a device that selects against dendrite entry into the central synapse chamber.
2. The 'adjacent-stripe' architecture of these networks also makes them ideal for studies of layered structures in the brain and elsewhere [28]. For instance, neurons from different cortical or hippocampal layers can be laid out side-by-side to approximately recapitulate the in vivo structures, thereby enabling studies of inherent as well as emergent properties of these networks.
3. The methodology described can be used with minor modifications to study other neuronal interactions, as well. These include neuromuscular junctions [29] and neuron–glia interactions [30, 31]. A two-channel device is usually employed for such investigations, with one of the channels seeded with neurons and the other with the non-neuronal cell type.

The maintenance of fluidic isolation ensures no inter-mixing of media or added growth factors between the different cell populations. Further design modifications can be incorporated for more specialized experiments [8].

4. A few studies have reported adverse effects of PDMS on cultured cells, for instance, differential gene expression in the cultured PC12 cell line when cultured on PDMS vs. polystyrene [32]. These use native-PDMS that has not been subjected to solvent extraction, a process that has been shown to leach out and wash away unpolymerized oligomer and heavy metal contaminants [21]. They also may use PDMS as a substrate upon which cells are cultured, leading to significant differences in substrate stiffness, which itself has profound effects on the differentiation of cells [33, 34].
5. Plasma treatment makes the PDMS surface hydrophilic. If left in contact with air, over time this reverts to hydrophobicity. Filling the channels with water upon assembly ensures the maintenance of hydrophilicity at the surface.
6. Maintaining a healthy neuronal culture in a microdevice requires the maintenance of a gentle flow of media to replenish nutrients and wash away toxins and cell metabolites. Due to very limited volumes within chambers of microdevice cultures, diffusion cannot be relied upon for this transfer as it is in dish cultures. However, high flow rates can result in shear stress, which is detrimental to cell health. Flow rates in the range of 10–20 μm/s are ideal. Maintaining a fluid differential between the inlet and the outlet with regular monitoring is usually sufficient to achieve these rates through gravity induced flow, though syringe pumps can be used for higher precision.
7. The duration of stimulant exposure in the given protocol is 10–20 s. This can be increased to as long as desired with the device being returned to the incubator until needed for further experiments. With a few modifications in device design, the exposure can be decreased as well to achieve pulse stimulation [35].

5 Conclusions

This convergence of synaptic investigations and the technology for engineering microenvironments, when coupled with high-resolution imaging and sophisticated software for data analysis, has the potential to decipher the guidance cues that direct the development of neuronal networks and thence the nervous system. Findings will fill gaps in understanding fundamental principles that wire the brain and be relevant to treatment of brain dysfunctions believed to be due to connectivity errors and loss.

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