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Emilia Biffi *Editor*

Microfluidic and Compartmentalized Platforms for Neurobiological Research

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Microfluidic and Compartmentalized Platforms for Neurobiological Research

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Series Preface

Experimental life sciences have two basic foundations: concepts and tools. The *Neuromethods* series focuses on the tools and techniques unique to the investigation of the nervous system and excitable cells. It will not, however, shortchange the concept side of things as care has been taken to integrate these tools within the context of the concepts and questions under investigation. In this way, the series is unique in that it not only collects protocols but also includes theoretical background information and critiques which led to the methods and their development. Thus it gives the reader a better understanding of the origin of the techniques and their potential future development. The *Neuromethods* publishing program strikes a balance between recent and exciting developments like those concerning new animal models of disease, imaging, in vivo methods, and more established techniques, including, for example, immunocytochemistry and electrophysiological technologies. New trainees in neurosciences still need a sound footing in these older methods in order to apply a critical approach to their results.

Under the guidance of its founders, Alan Boulton and Glen Baker, the *Neuromethods* series has been a success since its first volume published through Humana Press in 1985. The series continues to flourish through many changes over the years. It is now published under the umbrella of Springer Protocols. While methods involving brain research have changed a lot since the series started, the publishing environment and technology have changed even more radically. Neuromethods has the distinct layout and style of the Springer Protocols program, designed specifically for readability and ease of reference in a laboratory setting.

The careful application of methods is potentially the most important step in the process of scientific inquiry. In the past, new methodologies led the way in developing new disciplines in the biological and medical sciences. For example, Physiology emerged out of Anatomy in the nineteenth century by harnessing new methods based on the newly discovered phenomenon of electricity. Nowadays, the relationships between disciplines and methods are more complex. Methods are now widely shared between disciplines and research areas. New developments in electronic publishing make it possible for scientists that encounter new methods to quickly find sources of information electronically. The design of individual volumes and chapters in this series takes this new access technology into account. Springer Protocols makes it possible to download single protocols separately. In addition, Springer makes its print-on-demand technology available globally. A print copy can therefore be acquired quickly and for a competitive price anywhere in the world.

Wolfgang Walz

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Preface

Microfluidics is a technology which features the manipulation of small amounts of fluids in channels with dimensions of tens to hundreds of micrometers. Microfluidics takes advantage of both soft lithography and poly(dimethylsiloxane) (PDMS), a silicon-based elastomeric material which is cheap, easy to mold and with good optical properties, as well as nontoxic to cells and gas permeable, offering a suitable solution for cell and tissue culture experiments.

The design of microfluidic devices as platforms with different compartments had been inspired by the so-called Campenot chambers, developed to compartmentalize axons and cell bodies. Microfabricated versions of these devices have already been used in a wide range of biological applications thanks to their low consumption of samples and reagents, the ability to precisely control parameters within the cellular microenvironment, and the capability to perform highly concurrent and reproducible analyses. Thanks to their scale, compatible with neuron size, compartmentalized microfluidic devices are nowadays a crucial tool in the field of neuroscience. They indeed provide a powerful platform for the manipulation of subpopulation of neuronal cells and the study of complex system dynamics associated to the connectivity between two or more adjacent regions of the brain. Thanks to the feature of fluidically separated compartments, these devices are also suitable for local cell stimulation, for the creation of dynamic concentration gradients, or for high-content pharmacological screening.

In this volume, the main cutting-edge techniques to design and fabricate compartmentalized microfluidic devices are described in *Part I*. In Chap. 1, a microfluidic cell coculture platform that uses pneumatically or hydraulically controlled valves to reversibly separate cell populations is described. Chapter 2 illustrates the procedures to fabricate microfluidic devices reversibly sealed to different flat substrates through magnetic forces, which are a suitable approach for long-term cultures of neurons due to their reliable hydraulic tightness. In Chap. 3, microfluidic circuits for arraying neurons with single cell precision, enabling high-throughput experimentation, are presented as well as approaches to couple these minimalistic cocultures to open access reservoirs for electrophysiology recordings.

Part II is dedicated to the topic of axon guidance and manipulation. Chapter 4 provides details of a microfluidic chip with a modular design for highly defined isolation of axons, asymmetric genetic manipulation, and whole-cell patch-clamp recording. In Chap. 5 a unique laser cell-micropatterning system for the creation of a compartmentalized, axon-isolating, polarized neuron-growth platform at the single-cell level is described. Chapter 6 explains how Campenot cultures and microfluidics chambers can be coupled and used together for biochemical analysis and for high resolution imaging of Dorsal Root Ganglia (DRG) neuronal cell bodies and their extensive axons.

In *Part III*, compartmentalized devices for synapse manipulation are described. Chapter 7 illustrates 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 and facilitating the study of synaptic connections. In Chap. 8, the use of three-compartment microfluidic devices to model in vitro activity-dependent synaptic plasticity with dual inputs is detailed and the synaptic competition model is presented.

Part IV is dedicated to the study of how different cell populations interact in either physiological or pathological condition. Chapter 9 describes a six-compartment neuron-glia coculture microsystem platform, where interactions between the axon and glia can be studied in isolation. In Chap. 10, compartmentalized microfluidic devices to study peripheral neuro-osteogenic interactions are discussed, and qualitative and quantitative analyses for two- or three-dimensions cocultures are also presented. Chapter 11 provides details of a compartmented in vitro model of the lower motor neuron-neuromuscular junction circuit, incorporating primary spinal motor neurons, supporting glia and skeletal muscle, and spatially mimicking the unique anatomical and cellular interactions of this circuit.

In *Part V*, compartmentalized devices for pharmacological research and drug discovery are described. Chapter 12 details a powerful tool based on microfluidics used to mimic the key pathological hallmarks of Alzheimer's disease and observe the long-term disease spreading at the microscale. In Chap. 13 a device for long-term growth of twin neuronal networks and for their controlled biochemical stimulation and electrophysiological recording is described. Finally, in Chap. 14 the protocol and methodological considerations for developing synapse microarrays enabling ultrasensitive, high-throughput and quantitative screening of small molecules involved in synaptogenesis are illustrated.

The authors and I believe you will find this volume invaluable in gaining an understanding of the practical skills needed to fabricate and use microfluidics and compartmentalized platforms with cell cultures as well as the strengths of these exciting devices and their precious contribution in the field of neuroscience.

Milan, Italy ***Emilia Biffi***

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Part I

Designs and Methods

Chapter 1

The Fabrication of Microfluidic Platforms with Pneumatically/Hydraulically Controlled PDMS Valves and Their Use in Neurobiological Research

Bryson M. Brewer, Donna J. Webb, and Deyu Li

Abstract

Microfluidic technology has made a significant impact in neurobiological research. The new capabilities offered by microfluidic devices allow researchers to investigate neurobiological phenomena in ways previously unachievable using traditional cell biology techniques. Here we detail the fabrication of a microfluidic cell coculture platform that uses pneumatically or hydraulically controlled valves to reversibly separate cell populations. Using this platform, communication between cell populations in different culture chambers can be enabled or restricted as desired. This allows for both growth of different cell types in each respective optimal culture media and separate treatment of individual cell populations with transfecting agents, growth factors, and drugs, etc. At a desired time-point, cell-cell interactions can be studied by deactivating the valve. The device has been used previously to transfect neurons with different fluorescent presynaptic and postsynaptic markers and then observe in real-time the subsequent process of synapse formation. Additionally, the platform has been an effective tool for investigating the role of glia–neuron communication on synaptic formation and stability. In this chapter, the design, fabrication, and operation of the valve-enabled microfluidic cell coculture platform are clearly described so as to enable the reader to replicate the device for use in future neurobiological research.

Key words Microfluidic cell coculture, Synapse formation, Neuron–glia coculture, Pneumatic/hydraulic valve actuation, Soft lithography

1 Introduction

The ongoing development of microfluidic technology has produced tools for neurobiological researchers that provide several advantages over traditional methods and techniques [1]. The spatiotemporal control afforded by microfluidic devices makes the technology well suited for manipulating the cellular microenvironment [2]. This capability is especially useful for in vitro studies at the single-cell level [3], and as a result, an increasing number of microfluidic platforms have been successfully employed for neurobiological investigations [4]. A variety of applications have been demonstrated,

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including (1) a platform that incorporates a multi-electrode array with a microfluidic chip to monitor the electrophysiological activity of cultured neuronal networks [5], (2) a gradient-generating design that optimizes the proliferation of neural stem cells in culture [6], and (3) a device that utilizes fluidic isolation to separate neuronal cell bodies while allowing axonal growth through connecting microgrooves in order to independently probe axonal injury, regeneration, and transport [7]. These are just a few examples of many that demonstrate the potential of microfluidic technology in the field of neurobiology.

By far, the most popular material used to construct microfluidic devices is polydimethylsiloxane (PDMS) [8]. This flexible, optically transparent material is cost-effective, biocompatible, and capable of rapid prototyping via soft lithography [9–12]. Standardized procedures exist for attaching fabricated PDMS chips to different substrates (with the most popular substrate being glass) [13], as well as bonding and assembling multiple PDMS layers together, providing the capability of three-dimensional microfluidic designs [14]. In addition, the flexibility of PDMS allows pneumatically or hydraulically controlled valves to be easily integrated onto a microfluidic chip, allowing for greater control over fluid flow within the microchannels [15]. These properties of PDMS make it a suitable material for the fabrication of the pneumatically/hydraulically valve-enabled microfluidic platform described in this chapter.

The valve-enabled microfluidic device that is the focus of the following sections was developed by the authors and can be used for a variety of cell coculture applications. For example, the device has been used in the monitoring of cancer and endothelial cell cross-migration to investigate the effects of specific angiocrine factors on tumor cell motility [16]. The platform is particularly well suited for a number of neurobiology applications, being used to observe the dynamic formation of synaptic contacts between neuronal populations [17] and to investigate the effects of glia coculture on both neuronal transfection efficiency [18] and synapse formation and stability [19].

Figure 1 illustrates the key elements of the microfluidic platform. The device consists of either two or four microfluidic cell culture chambers that are connected by short, narrow barrier regions. These barrier regions either take the form of an array of microgrooves or a single continuous slit, depending on the application. A pressure control chamber attached above the barrier regions can be pressurized, forcing the flexible PDMS barriers to collapse, effectively separating the cell culture chambers. This capability allows cell populations in each chamber to be treated independently (with different culture media, transfection agents, etc…) until the pressure is removed and the barriers are released from the substrate, re-enabling communication among cells in different culture chambers. Additionally, the microfluidic device uses a passive

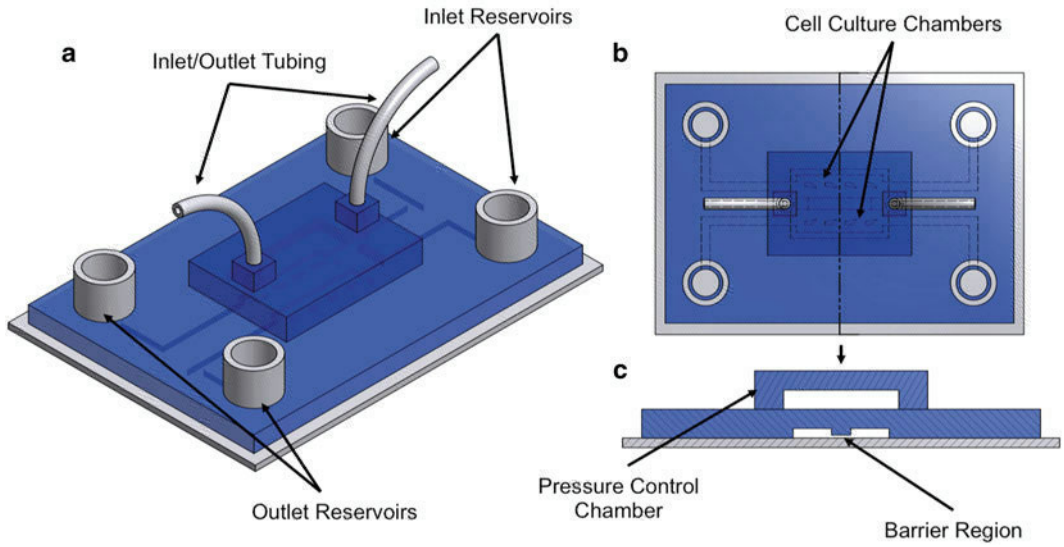

Fig. 1 Overview of the two-chamber valve-enabled microfluidic platform. (**a**) The three-dimensional schematic demonstrates the multiple layers of the device, as well as the location of the inlet/outlet reservoirs used for loading cells and media and the inlet/outlet tubing for the pressure control chamber. (**b**) An overhead view of the device clearly showcases the position of the cell culture chambers in relation to the pressure control chamber. (**c**) A cross-sectional slice identifies the pressure control chamber and barrier region connecting the two culture chambers. Note that the images here are not to scale

pumping method [20, 21] to continuously provide fresh media to cell cultures, eliminating the need for external flow control equipment such as syringe pumps. As a result, the entire platform can be contained inside a petri dish, reducing the risk of contamination during transport between culture hoods, incubators, and microscopes. Finally, the device has been successfully used with a variety of microscopy techniques, including high magnification confocal microscopy employing objectives with small working distances.

The following sections describe the fabrication and operation of the pneumatically/hydraulically valve-enabled microfluidic neurobiology platform. Three primary fabrication steps are detailed:

1. *Photolithography*—used to create an SU-8 replica mold on a silicon wafer that will serve as the "negative" pattern for the different PDMS layers. The mold can be used to make dozens of PDMS devices before needing to be replaced.
2. *Soft Lithography*—used to fabricate the PDMS layers containing the cell culture chambers/connecting barriers, the pressure control chamber, and tubing supports. This step involves pouring liquid PDMS over the SU-8 mold, curing, and removing to form the flexible, solid PDMS components of the device.
3. *Assembly*—used to combine all of the device components into an operational microfluidic platform. Plasma bonding is employed to irreversibly seal the different PDMS layers to a

glass substrate, and liquid PDMS is used to attach tubing and cylindrical reservoirs to the microchannel inlets/outlets.

Finally, operation of the device via activation/deactivation of the pneumatic/hydraulic valve barrier is discussed.

2 Materials and Instruments

2.1 Photolithography Using SU-8 Negative Photoresists

- SU-8 negative photoresist (SU-8 2005, SU-8 2050, and SU-8 2150).
- Acetone.
- Isopropyl alcohol.
- Microchem SU-8 developer.
- 3″ polished silicon wafer.
- Spin coater.
- Two hot plates.
- Photolithography mask.
- Ultraviolet exposure system.

Please *see* **Note 1** for additional information.

2.2 PDMS Based Soft-Lithography and Device Assembly

- Dow Corning Sylgard 184 Silicone Encapsulant 0.5 kg Clear Kit (PDMS).
- 2 and 5 mm biopsy punches.
- 14-gauge blunt needle.
- 0.20″ I.D/0.60″ O.D. Tygon Microbore Tubing.
- 10 mm × 10 mm pyrex cloning cylinders.
- Air/oxygen plasma cleaner.
- Vacuum pump.
- Vacuum chamber or desiccator.
- Oven.
- Deionized (DI) water.

2.3 Device Operation

- 5 mL plastic syringe.
- 23-gauge.
- Stainless steel metal pinch clamps.

3 Methods

3.1 Photolithography Using SU-8 Negative Photoresists

The first step in the fabrication of a pressure controlled valve-enabled microfluidic neurobiological coculture platform is the creation of a master mold using photolithography with the negative photoresist SU-8. Using this SU-8 master, the PDMS-based device

layers can be created. In addition, the master molds can be used numerous times (>100) before needing to be replaced. This allows for the fabrication of many microfluidic devices without spending much effort on the relatively time-consuming photolithography process. Note that all steps presented here (except the design of the photolithography mask) regarding the production of the master molds should be carried out in a clean room, preferably with a Class 1000 rating or better. This will decrease the likelihood of contamination (via dust or other airborne particles) during fabrication that could damage the small features of the mold.

3.1.1 Design of the Photolithography Mask

In order to fabricate the SU-8 mold, a photolithography mask containing the channel features must be created. SU-8 is a negative photoresist; therefore, the channel features that are desired for the mold should be transparent on the mask, and all other areas should be opaque. First, CAD software (such as AutoCAD) is used to design the desired channel features. Then, a high-resolution photomask (greater than 20,000 DPI) of the design is printed on a plastic transparency sheet (typically obtained using a CAD printing service, *see* **Note 2**).

The fabrication of a valve-enabled neurobiology microfluidic platform requires at least three photomasks, two for the cell culture chamber PDMS layer and one for the pressure chamber PDMS layer. The two masks for the cell culture chamber layer include one for the taller channel/chamber features and one for the shorter barrier features. Figure 2a, b demonstrates a CAD drawing containing the designs for both the channel and barrier layers of standard two-chamber and four-chamber SU-8 molds. To begin with, alignment marks are found on the perimeter of both mask designs to aid in the alignment process during the actual photolithography step. For the channel-chamber layer mask, the inlet and outlet microchannels are 200 μm wide (w) and 10–20 mm long (l), and the inlet and outlet ports shown are 5 mm in diameter, though the actual size of the port will depend on a later fabrication step. The cell culture chambers are 1 mm (w)×5 mm (l) and contain several semi-lunar shaped support pillars that serve to prevent the roof of the chamber in the assembled device from collapsing upon the application of pressure. The separation distance between neighboring cell culture chambers (or width of the barrier region) shown in Fig. 2 is 100 μm. In the barrier layer mask, the connecting microgrooves are 50 μm long. The width of both the connecting microgrooves and the continuous barrier is dependent upon the width of the barrier region. Generally, the width of the grooves or continuous barrier on the mask is selected to be at least three times as wide as the barrier region itself; this additional tolerance makes the alignment of the two layers during the actual mold fabrication easy to achieve. Note that the dimensions mentioned here can be slightly modified if needed, as long as several design rules are followed (*see* **Note 3**). In addition to the photolithography masks

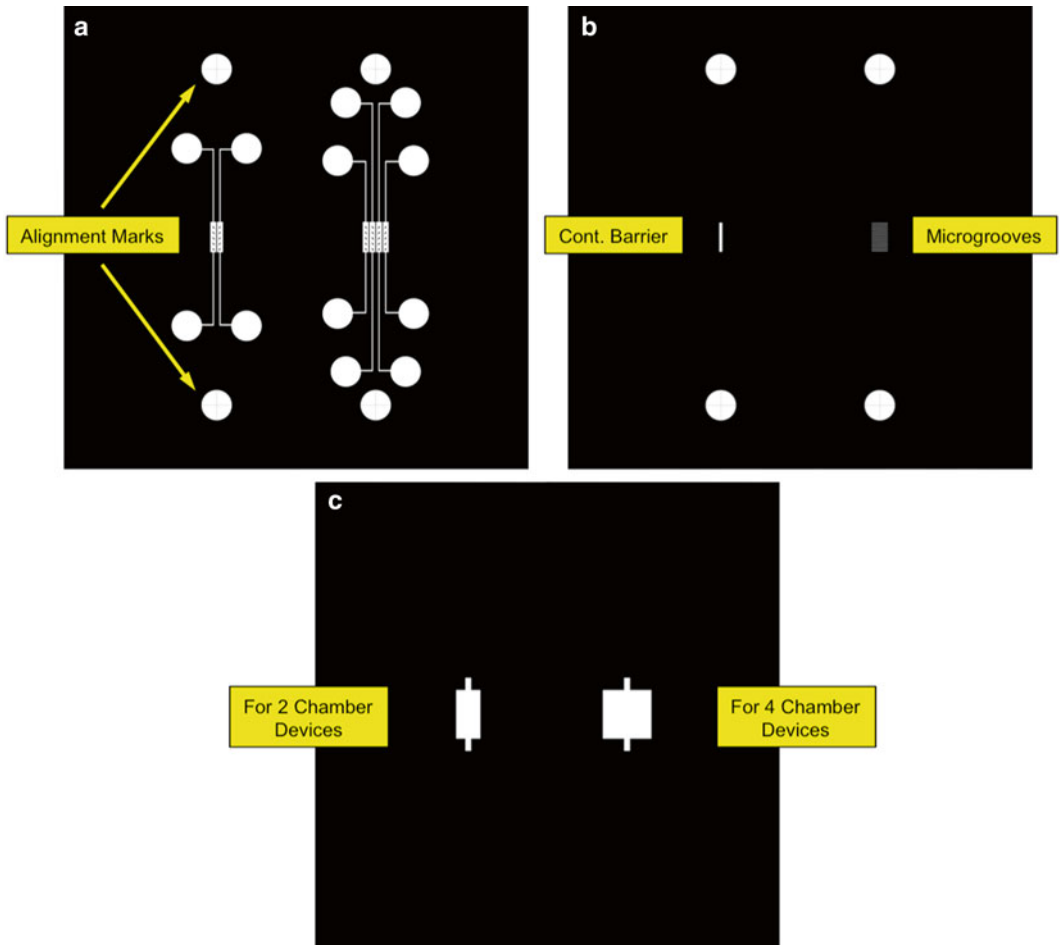

Fig. 2 Lithography photomasks for each device layer. (**a**) A CAD produced photomask containing the two- and four-chamber designs. Note the location of the alignment marks. (**b**) The mask used for making the connecting barrier features contains designs for both a continuous barrier and microgroove array. Identical marks to those in the channel design mask are used to facilitate proper alignment during fabrication. (**c**) Photomasks for the pressure control chambers corresponding to both two- and four cell culture chamber designs, which do not require alignment marks, as this mold requires only a single layer of SU-8. These designs are to scale (relevant dimensions listed in the text)

for the channel layer, a mask must also be designed for the pressure control chamber layer. The designs for both the two- and four-channel pressure control chambers are shown in Fig. 2c. For the two-channel design, the chamber is 12 mm long × 4 mm wide, while the four-channel design is 8 × 12 mm. The short inlet and outlet regions are 1 × 2 mm.

3.1.2 Fabrication of the Cell Culture Chamber Layer Masters

After obtaining a high-resolution photomask with the desired features, the SU-8 master mold can be fabricated using photolithography. The steps listed here should be carried out in a clean room and will produce a master containing barrier features that are

~5 μm tall and channel/chamber features ~100 μm tall. A summary of the protocol is presented in Fig. 3a, b.

1. Clean a 3″ silicon wafer by placing the wafer on a spin coater, washing the surface with acetone, and spinning dry at 2,000 RPM for 2 min. After drying, visually inspect the wafer for any impurities or remaining residue and re-clean if necessary before proceeding.
2. Deposit a ~1.5″ diameter drop of SU-8 2005 on the center of the wafer.
3. Program the spin coater for a two-step process. The first step is a spin at 500 RPM for 10 s at an acceleration of 100 RPM/s. This is followed by a 35 s spin at 2,000 RPM at an acceleration of 300 RPM/s. After running the program, a uniform SU-8 layer of ~5 μm tall should cover the surface of the silicon wafer. For other layer thicknesses, *see* **Note 4**.
4. Using a dry non-abrasive wipe, remove any excess SU-8 around the perimeter of the wafer by carefully placing the wipe against the bottom edge of the wafer with the vacuum on and slowly rotating the wafer one full revolution.
5. Turn off the vacuum on the spin coater and place the wafer on a hot plate set to 95 °C for 2 min.
6. Remove the wafer and allow it to cool to room temperature (usually 1–2 min).
7. Position the photomask containing the barrier features on top of the wafer with the inked side making contact with the wafer. Place a large cover glass (≥4″ diameter) on top of the mask to ensure clean contact with the wafer. Then expose the wafer using a UV source with a dose of 390 mJ/cm^2 (*see* **Note 5**).
8. Remove the large cover glass and photomask. Then place the wafer on the 95 °C hot plate for 3 min.
9. Remove the wafer, let it cool for 1–2 min, and place it back on the spin coater and turn on the vacuum.
10. Spray SU-8 developer on top of the wafer until the entire surface is covered. Wait 30–45 s, then spin the wafer at 2,000 RPM until all excess SU-8 developer has spun off of the wafer surface (*see* **Note 6**).
11. Stop the spinner and visually inspect the wafer to determine if any SU-8 remains that is not crosslinked. If any excess SU-8 remains, spray another puddle of SU-8 developer on the surface and wait 20 s before spinning at 2,000 RPM. If no excess SU-8 remains, start spinning at 2,000 RPM immediately after applying another puddle of SU-8 developer.
12. After the SU-8 developer has spun off, spray the wafer with acetone, followed by a wash with isopropyl alcohol (with the wafer still spinning at 2,000 RPM through all washing steps).

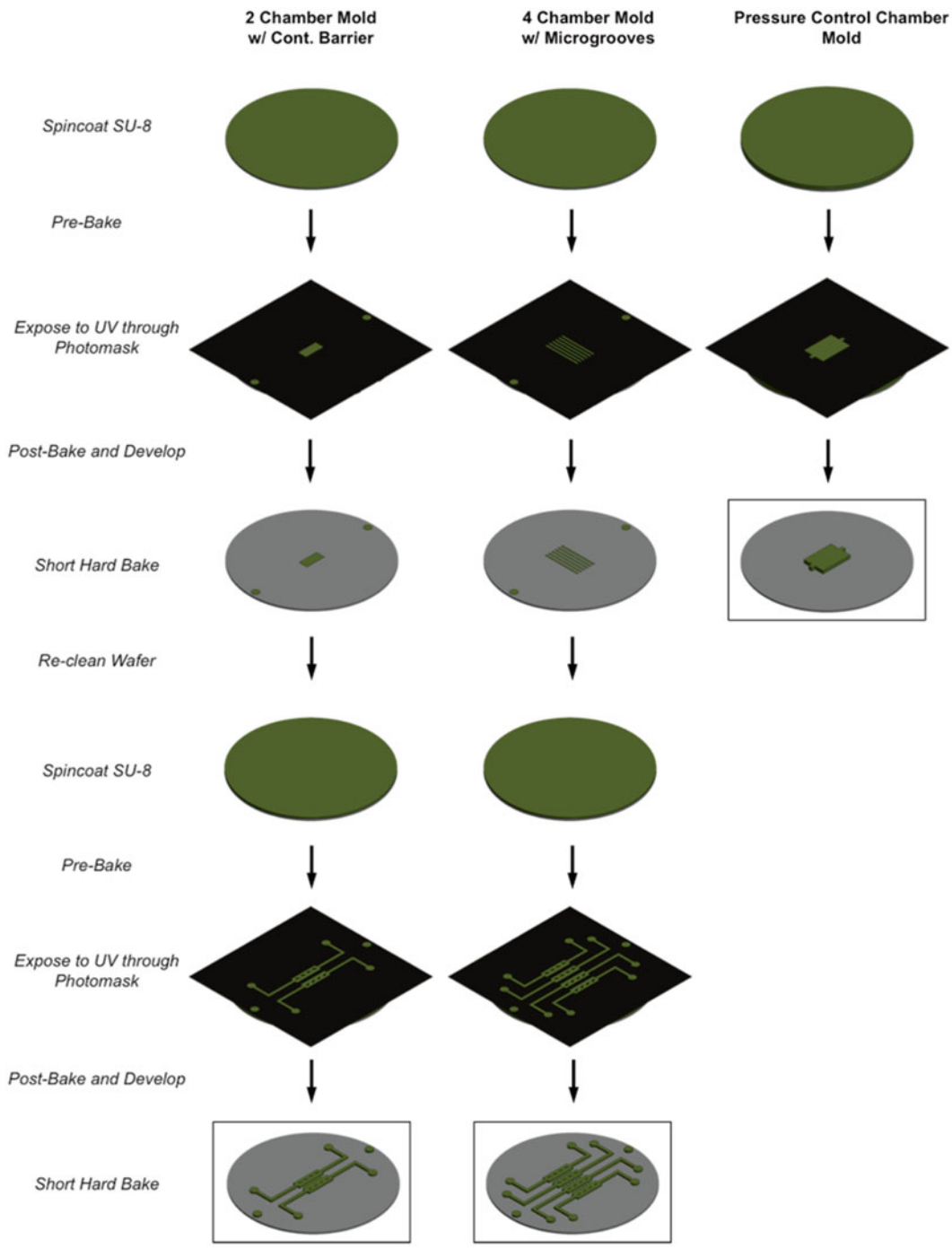

Fig. 3 Summary of fabrication of SU-8 replica molds using photolithography. This figure demonstrates the photolithography processes necessary to fabricate the SU-8 molds for the two- and four-chamber cell culture chamber layers, as well as the pressure control chamber. The cell culture chamber layer molds require multiple mask layers, with the photolithography process essentially being performed twice with proper alignment between the barrier region and channel layers. Note that microgrooves can also be used with a two-chamber design, and vice-versa

13. Remove the wafer from the spin coater and blow dry with nitrogen gas. Inspect the SU-8 features under a microscope to ensure that no defects are present, then place the wafer on a 95–150 °C hotplate for 5 min to anneal any minor surface cracks that are present. This concludes the fabrication of the 5 μm thick layer.
14. The process must now be repeated to fabricate the 100 μm channel/chamber layer on top of the 5 μm barrier layer. Again, clean the wafer as described in step 1.
15. Deposit a ~1.5″ diameter drop of the more viscous SU-8 2050 on the center of the wafer.
16. For this layer, the two-step spinning program is a spin at 500 RPM for 10 s at an acceleration of 100 RPM/s, followed by a 35 s spin at 1,650 RPM at an acceleration of 300 RPM/s. After running the program, a uniform SU-8 layer ~100 μm tall should cover the surface of the silicon wafer.
17. After removing excess SU-8 with a dry non-abrasive wipe, place the wafer on a 65 °C hot plate for 5 min, followed by 20 min on a 95 °C hot plate.
18. Remove the wafer, allow it to cool for 1–2 min, and place the wafer under the UV exposure system.
19. Place the photomask for the channel/chamber layer (either the two- or four-chamber design) on top of the wafer while carefully overlapping the alignment marks from the 5 μm SU-8 layer with the marks on the photomask (*see* **Note 7** for tips on achieving proper alignment). Expose with a dose of 390 mJ/cm^2.
20. Place the exposed wafer on a 65 °C hot plate for 1 min and a 95 °C hot plate for 10 min. Then remove the wafer and allow it to cool for 1–2 min.
21. Repeat steps 10–12 to develop the non-cross-linked SU-8. However, for the thicker 100 μm SU-8 layer, the SU-8 developer puddle should be left on the wafer for ~10 min to remove all of the excess photoresist (*see* **Note 8**).
22. Repeat step 13 to complete the fabrication of the SU-8 master mold for the two- or four-chamber cell culture chamber layer.

3.1.3 Fabrication of the Pressure Control Chamber Layer Master

The pressure control chamber master mold is fabricated in the same manner as the cell culture chamber layer master. However, the pressure control chamber mold should be ~400 μm in height. Thus, the following modifications to the above procedure can be used to fabricate a 400 μm tall SU-8 pressure control chamber master mold. Note that an alternative fabrication method can be used here that does not require the use of clean room (*see* **Note 9**). The steps are summarized in Fig. 3c.

1. Clean a 3″ silicon wafer using the previously described method.
2. Deposit a ~1.5″ diameter drop of SU-8 2150 on the center of the wafer.

3. Program the spin coater for a two-step process. The first step is a spin at 500 RPM for 10 s at an acceleration of 100 RPM/s. This is followed by a 35 s spin at 1,625 RPM at an acceleration of 300 RPM/s. After running the program, a uniform SU-8 layer ~400 μm tall should cover the surface of the silicon wafer.
4. After removing excess SU-8 from the edge and bottom of the wafer, place it on a 65 °C hot plate for 8 min, followed by 105 min on a 95 °C hot plate.
5. Once the wafer has reached room temperature, position the photomask for the pressure control chamber on top of the SU-8 as previously described and expose using a 390 mJ/cm^2 dose two separate times, waiting 1 min between doses.
6. Place the wafer on the 65 °C hotplate for 5 min, followed by 30 min on the 95 °C hotplate. Remove the wafer and allow it to cool to room temperature.
7. Development should be performed as described previously; however, for the ~400 μm layer, complete removal of the non-cross-linked photoresist will take ~20 min.
8. After placing the developed SU-8 mold on a 95–150 °C hotplate for several minutes to anneal any surface cracks, the fabrication of the pressure control chamber master mold is complete.

3.2 PDMS Based Soft-Lithography and Device Assembly

After successfully fabricating the desired SU-8 masters, the newly constructed molds can be used to make each PDMS device layer. The following protocol can be used with any of the molds fabricated in Sect. 3.2 to produce the desired PDMS device layer. Figure 4 highlights the main components of this protocol.

3.2.1 Fabrication of PDMS Device Layers

1. Mix together the two components of the Sylgard 184 silicone encapsulant kit in a 10:1 w/w ratio (base polymer–curing agent). *See* **Note 10**.
2. Place the desired master mold in a petri dish and pour the PDMS mixture over it (*see* **Note 11**).
3. Place the mold in a vacuum chamber or desiccator at −80 kPa for 45–60 min (*see* **Note 12**).
4. With all air bubbles removed, place the mold in an oven at 70 °C for ~2 h to allow the PDMS to cure (*see* **Note 13**).
5. Remove the mold from the oven and allow it to cool to room temperature.
6. Using a scalpel or razor, cut the PDMS around the perimeter of the device layer while leaving plenty of clearance around the edges of the channel features.
7. Carefully and slowly peel off the cut PDMS layer from the SU-8 mold.

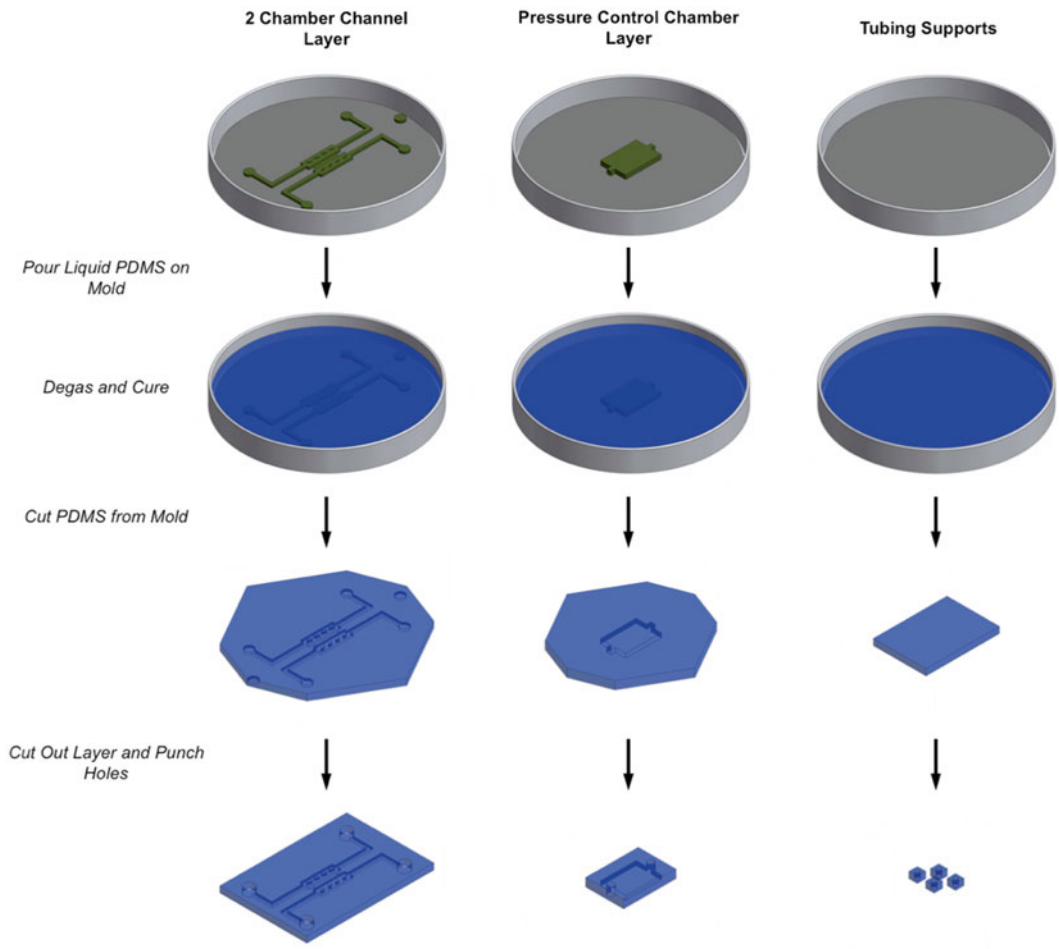

Fig. 4 Summary of soft lithography processes used to fabricate each device layer. Here the workflow required for fabrication of the PDMS cell culture chamber layer, pressure control chamber layer, and tubing supports are detailed. The schematics are not to scale, as in reality two cell culture chamber device layers and multiple pressure control chambers can be produced per 3″ silicon wafer

8. Place the PDMS on the center of a clean glass slide and trim the excess PDMS from the edges so that the entire PDMS layer fits cleanly on the slide (*see* **Note 14**).
9. Use a 5 mm biopsy punch to cut out the inlet and outlet reservoirs for all channels in the two- and four-chamber channel layers. Use a 2 mm biopsy punch to cut the holes in the inlet and outlet regions of the pressure control chamber layer.
10. Cover both the top and bottom surfaces of the PDMS layer with acrylic office tape ("Scotch Tape"). *See* **Note 15**.

In addition to the molded PDMS device layers, tubing supports must also be cut out from a plain, ~2 mm thick layer of PDMS. Each tubing support should be 3 mm (l) × 3 mm (w), and a 14 gauge needle should be used to punch out a hole in the center of each piece. Place each support on a piece of tape to maintain a clean surface (*see* **Note 16**).

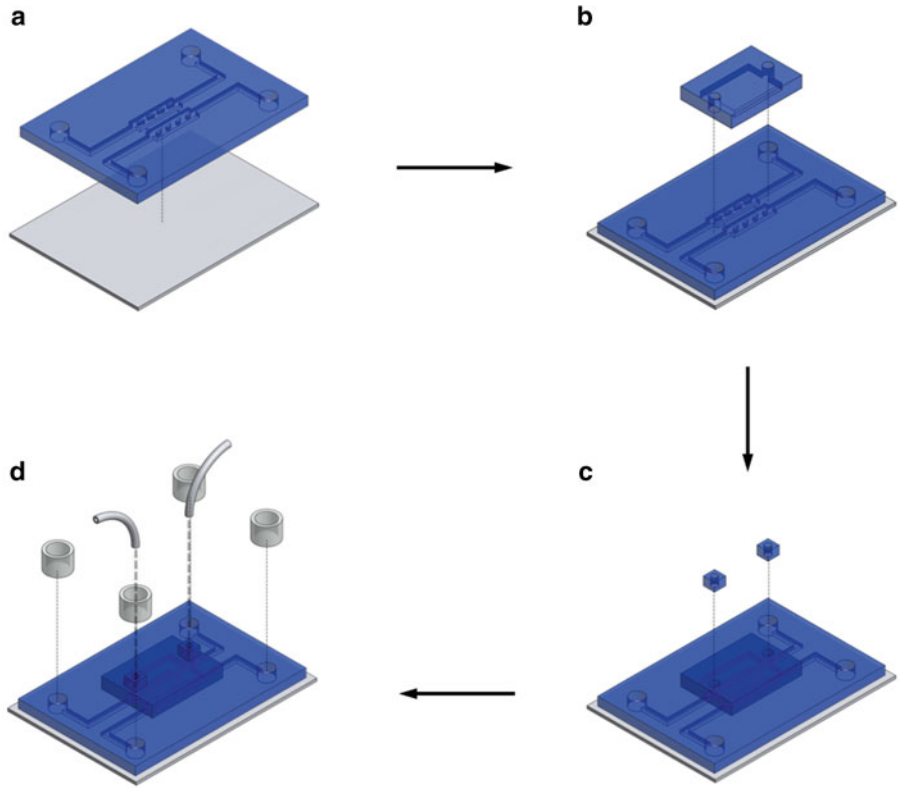

Fig. 5 Summary of the assembly of components into a final valve-enabled microfluidic device. (**a**) The first assembly step requires attaching the PDMS cell culture chamber layer to a glass coverslip via air plasma bonding. (**b**) Next, plasma bonding is used to attach the pressure control chamber to the cell culture chamber layer. (**c**) Then, tubing supports are aligned and plasma bonded to the pressure control chamber. (**d**) Finally, liquid PDMS is used as a glue to attach inlet/outlet tubing and reservoirs to the device

3.2.2 Device Assembly

The final step in fabricating the valve-enabled neurobiological platform is the assembly of all the fabricated pieces into a completed device. This involves a combination of using a plasma cleaner to activate the surfaces of both PDMS and glass to facilitate irreversible bonding, as well as using small amounts of uncured PDMS to "glue" and seal certain components (tubing and reservoirs) to the device. The following procedure outlines the details of device assembly and is summarized in Fig. 5.

1. Clean a glass coverslip or slide (*see* **Note 17**) using compressed nitrogen and place it inside the chamber of the plasma cleaner.
2. Remove the tape from the open channel side of the two- or four-chamber PDMS cell culture chamber layer and place it in the plasma cleaner with the exposed area face up (*see* **Note 18**).
3. Close the plasma cleaner chamber and expose the glass and PDMS to air plasma for 30 s by applying ~18 W to the RF coil (*see* **Note 19**).

4. Remove the glass coverslip and PDMS channel device layer from the plasma cleaner chamber. Carefully bond the open chamber side of the PDMS cell culture chamber layer to the plasma treated side of the coverslip by gently dropping the PDMS layer onto the glass (*see* **Note 20**).
5. Remove the tape from the open chamber side of the PDMS pressure control chamber layer and place it in the plasma cleaner chamber alongside the newly bonded coverslip/PDMS cell culture chamber layer. Perform the air plasma treatment as described in step 3.
6. After removing both pieces from the plasma cleaner, bond the PDMS pressure control chamber to the top of the PDMS cell culture chamber layer. Be careful to align the control chamber over the cell culture chambers.
7. Place the coverslip/cell culture chamber layer/pressure control chamber assembly back into the plasma cleaner, along with two PDMS tubing supporters. The side of the tubing supporter that was protected by tape during storage should be face up in the plasma cleaner.
8. Perform the standard plasma treatment as described in step 3. After removing from the cleaner, use forceps to place the tubing supporters on top of the pressure control chamber, aligning the holes in each component. Apply slight pressure to the top of the tubing supporter to promote a good seal.
9. Immediately fill the cell culture chamber with DI water using a pipette. Each chamber should require ~25–50 μL of water (*see* **Notes 21** and **22**). The pressure control chamber does not need to be filled with water.
10. Next, insert 0.02″ ID/0.06″ OD Tygon microbore tubing into the inlet and outlet tubing supporters of the pressure control chamber using forceps. Each piece of tubing should be ~3″ long. Mix up 1–2 g of PDMS and curing agent at a 10:1 ratio, and use the liquid mixture to seal the insertion point of the tubing into the tubing supporters (*see* **Note 23**).
11. Attach 10 × 10 mm (*diameter* × *height*) Pyrex cloning cylinders to the inlet and outlet of each cell culture chamber by applying a thin layer of liquid PDMS mixture onto the bottom of a cylinder and gently pressing onto the punched hole of each chamber inlet/outlet (*see* **Note 24**).
12. Add 200–300 μL of DI water to each cylinder and place the entire device in a 70 °C oven for 45–60 min to allow the liquid PDMS seals to cure. Be careful not to allow the reservoirs or channels to dry out during this curing process.
13. Remove the device from the oven and allow it to cool down to room temperature. Store in a petri dish and cover in Parafilm to prevent evaporation before use.

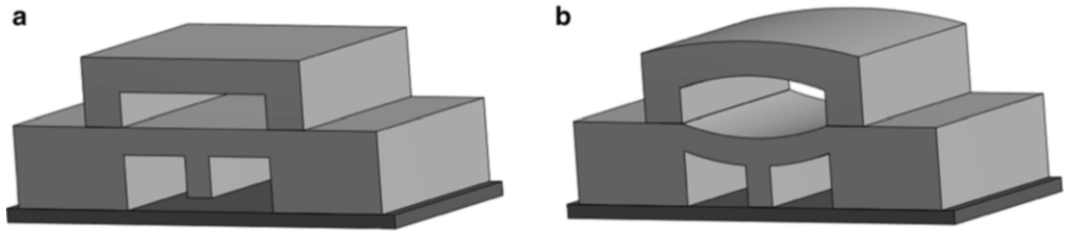

Fig. 6 Three-dimensional cross-section of a two-chamber, continuous barrier device before and after applying pressure to the control chamber. (**a**) An unpressurized device is shown with the continuous barrier elevated above the substrate. Here the two cell culture chambers are connected. (**b**) A device that has been pressurized using either pneumatic or hydraulic pressure in which the barrier is pushed down to the substrate, effectively separating the two cell culture chambers

This completes the fabrication of the pneumatic/hydraulic valve-enabled microfluidic neurobiology platform. The devices can typically be stored in Parafilm sealed petri dishes for weeks before use, as long as the channels and chambers remain filled with water.

3.3 Device Operation

After assembly, the microfluidic device is ready to be used for suitable neurobiological assays. The following steps demonstrate how to prepare the device for use and activate/deactivate the pressure valve (Fig. 6a, b). For more detailed examples of previously documented biological protocols using this device (coating of channels, cell loading, etc.), please see references [17–19].

1. First, each device should be sterilized using UV (ultra-violet) irradiation found in most cell-culture hoods. UV exposure should last for at least 1 h.
2. When ready to activate the pressure valve, a 5 mL plastic syringe with a 23-gauge needle should be filled with either air or water. Insert the syringe into the inlet tubing of the pressure control chamber.
   (a) For pneumatic pressurization, clamp the outlet tubing of the pressure control chamber closed using a metal pinch clamp. Then inject 0.2–0.3 mL of air into the pressure control chamber and clamp the inlet tubing (*see* **Note 25**).
   (b) For hydraulic pressurization, fill the entire pressure control chamber and tubing with water. Then, clamp the outlet tubing. Finally, inject an additional 0.2–0.3 mL of water into the chamber and clamp the inlet tubing (*see* **Note 26**).
3. To deactivate the valve, unclamp the inlet and outlet tubing of the pressure control chamber (*see* **Note 27**).

In order to check that your device is working properly, fluorescent dye or food coloring can be added to one of the channels after pressurization. Any leakage through the barrier/microgrooves should be apparent. Additionally, the dye should perfuse freely through the barrier/microgroove region after releasing the pressure (*see* Fig. 7).

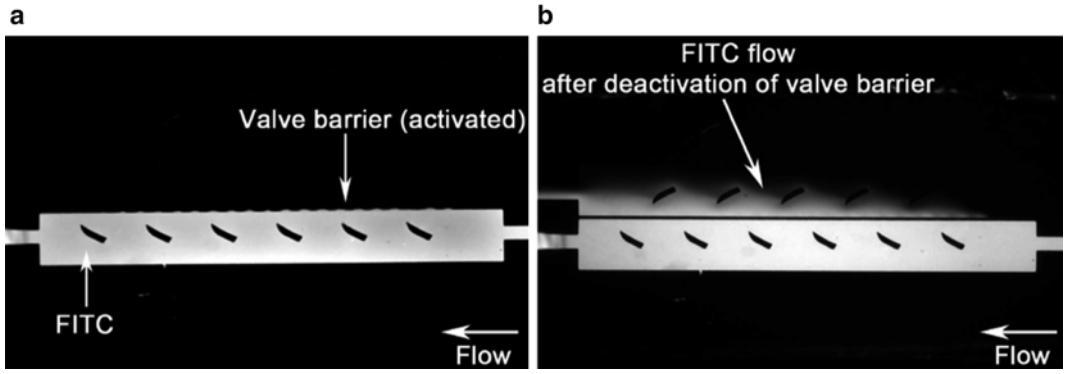

Fig. 7 Demonstration of proper valve behavior using fluorescent dye. (**a**) Fluorescein isothiocyanate (FITC) loaded into the bottom cell culture chamber is contained as long as the valve barrier is properly activated using pneumatic/hydraulic pressure. (**b**) After deactivating the barrier, FITC is free to flow across the barrier region into the top cell culture region. Figure reprinted with permission from ref. [18]

4 Notes

1. The fabrication of the molds or replica-masters to be employed later in the soft-lithography step is achieved using standard photolithography protocols. For best results, this step should be conducted in a Class 1000 clean room (or better).
2. Although there are many available options for printing photomasks, the authors have had past success using CAD/Art Services, Inc. for designs similar to those presented here.
3. The dimensions of the device features can be modified to fit a particular application. For example, the cell culture chambers could be made 1–10 mm long and/or 200–2,000 μm wide, and the width of the barrier region varied from 50 to 500 μm without sacrificing device performance. However, note that in order to better control the location where cells will be seeded within the channel, the cell culture chamber should be at least twice as wide as the inlet/outlet microchannels. The flow velocity decreases in the wider cell culture region, slowing down the cells and allowing them to settle onto the substrate. In addition, the aspect ratio of the final mold must be considered when designing a mask, as SU-8 features with a high aspect ratio may be prone to breaking during later soft-lithography steps. Concurrently, PDMS microchannels with a very low aspect ratio may collapse in the absence of support pillars. Finally, be mindful of the dimensions of the glass substrate you will use when fabricating the final device. All device features should be designed to be at least 3 mm from the edges of the glass substrate after final device assembly.

4. Data sheets containing curves relating the spin-speed to layer thickness for each SU-8 model can be found on the MicroChem website: http://microchem.com/Prod-SU8_KMPR.htm. It is generally helpful to verify the thickness of the SU-8 layer being produced after fabrication by measuring the feature height using a profilometer, as some variation from the spin-speed/thickness curve may be observed.
5. The actual required exposure dosage will vary depending on the model of SU-8 used, the thickness of the layer, wafer diameter, and the type of exposure system used. Again, the data sheets found on the MicroChem website, http://microchem.com/Prod-SU8_KMPR.htm, provide general guidelines on exposure dosage; however, it may be necessary to test a range of exposure doses to find the optimum value for a particular equipment setup. The authors typically use a Novacure 2100 Ultraviolet/Visible Spot Cure System to fabricate SU-8 master molds.
6. In order to determine when SU-8 developer, acetone, or IPA has been completely spun off of the wafer, look for a ring pattern on the wafer surface. When the ring pattern becomes visible, almost all of the excess liquid has been removed from the surface. After the rings disappear, the next washing step can be applied.
7. Often, it will be difficult to directly see the alignment marks on the 5 μm thick SU-8 layer with the photomask on top of the wafer. In this case, it is helpful to scratch the outline of the alignment marks directly into the surface of the 100 μm SU-8 layer before alignment. This is achieved by using a razor blade or scalpel to carefully score lines indicating the alignment marks visible from the 5 μm thick SU-8 layer. Then, alignment of the photomask with the new scored marks should be readily achieved.
8. For longer development times, it is useful to agitate the wafer to speed up the development process. This can be achieved by either manually rotating the spin coater back and forth through ~5 cm oscillations or by spinning the wafer at a slow spin speed (~50 RPM) during development. The slow spin speed will cause the SU-8 developer to oscillate on top of the wafer without being spun off of the surface. In addition, the 10 min development time for the 100 μm layer can be split into two 5 min developments where the SU-8 developer is spun off after the first 5 min and reapplied after stopping the spinner.
9. Unlike the cell culture chamber layer masters, the mold for the pressure control chamber layer does not contain any intricate features. Thus, a clean room is not necessary for the fabrication of the pressure control chamber mold. The following steps

should be followed to produce a glass master mold that will produce pressure control chambers of ~1 mm thickness:

(a) On a standard 1 mm thick glass slide, use a diamond-tip pen to score and cut several glass rectangles measuring either 4 mm × 12 mm (two-chamber design) or 8 mm × 12 mm (four-chamber design).

(b) Using cyanoacrylate ("Super Glue") or an equivalent low-viscosity epoxy, attach 2–3 of the cut glass rectangles to the center of another uncut glass slide (25 × 75 mm). Note that a conservative amount of glue should be used. As the cut glass rectangles are pressed against the glass slide, little to no glue should leak out around the edges. Any extra glue will mold unwanted features into the final PDMS layer, potentially causing bonding problems.

(c) Place the glass mold in a petri dish for use in the standard soft-lithography protocol.

10. In the standard 0.5 kg kit, the base polymer comes in the larger container, while the curing agent is in the smaller bottle. The two components should be mixed thoroughly for 3–5 min to ensure uniformity.

11. For the cell culture chamber layer, a layer of PDMS 1–2 mm thick should cover the top of the SU-8 mold after pouring. For the pressure control chamber layer, a 3–5 mm thick layer of PDMS should cover the top of the mold features.

12. The mixing of the two components introduces air bubbles into the PDMS. Placing the mold in a vacuum removes those air bubbles. Note that after removing the mold from the vacuum, a few bubbles may still remain on the PDMS surface. These can be removed by applying a light blast of nitrogen gas to the PDMS surface. In addition, be careful to remove any air bubbles trapped underneath the silicon wafer, as these bubbles can cause an uneven cell culture chamber layer. These trapped bubbles can usually be removed by lightly pressing on the center of the silicon wafer with forceps or a scalpel.

13. The curing process will transform the PDMS from the liquid state poured over the mold to a flexible, solid rubbery state. While the PDMS should be cured for at least 2 h, longer curing times (even overnight) should not affect device performance.

14. For the cell culture chamber layers, make sure that the edges of the PDMS are flat. Any raised portion should be trimmed so that the top of the PDMS layer is flat. Raised edges can become problematic when later attaching Pyrex reservoir cylinders.

15. The acrylic tape serves to both clean the surface of the PDMS (aiding in bonding together different layers), as well as protect

the surface from further contamination during storage and transport before device assembly is performed.

16. The tubing supports serve to aid in the device assembly phase of fabrication. Additionally, they provide extra support to the connection between the tubing and pressure control chamber, helping prevent leakage and device failure when pressurizing the pressure control chamber. Note that the holes punched in the tubing supports are slightly smaller than the tubing itself to ensure a snug fit when inserting the tubing.
17. The typical coverslip used by the authors is $25 \times 60 \times 0.13$ mm (*width* × *length* × *thickness*). Although the use of a thin coverslip can make fabrication slightly more difficult due to its fragility, a thin substrate is necessary when using high magnification microscope objectives with a short working distance (i.e., when using confocal microscopy to look at individual synapses). On the other hand, when a thin substrate is not needed, a thick (~1 mm) glass slide can be used instead in order to reduce the chance of cracking the substrate during fabrication.
18. An easy way to make sure that the tape is being removed from the correct side of the device is to inspect the tape after removal. The outline of the channels should be visible when the tape was removed from the open channel/chamber side of the device.
19. A variety of plasma cleaners exist and have been used in the fabrication of microfluidic devices. The plasma cleaner typically used by the authors is from Harrick Plasma (PDC-32G). It uses a 3″ diameter × 6.5″ length Pyrex chamber and contains three power settings. For all bonding procedures, the maximum power setting is used (~18 W applied to the RF coil). In order for the plasma to be generated, a vacuum must be achieved in the plasma cleaner chamber. Thus, a typical bonding step requires closing the chamber door, turning on a vacuum pump to evacuate the chamber, sequentially stepping the power setting from low to high (approximately 10 s each at the two lower settings), and finally removing the vacuum after completing the exposure to retrieve the samples. Often, the airflow into the plasma cleaner chamber is adjusted during operation by slightly opening or closing a needle valve connected to the chamber; this is done to maintain the intensity of the plasma in the chamber throughout the exposure. A visible purple color is emitted in the chamber during operation, and the brightness of the color can be used to gauge the plasma intensity. Finally, tuning of the exposure time and power may be required for different plasma cleaners; however, many protocols can be found in literature for guidance on the proper parameters.

20. Properly aligning the PDMS cell culture chamber layer with the glass can be tricky initially. It is best to hold the PDMS layer by its edges (being careful not to touch the plasma treated face) and line up the top of the PDMS with the top of the coverslip. Then, the PDMS layer can be gently dropped onto the glass. On a dark background, the formation of the bond between the two layers can be observed. Note that this bond is irreversible, so care must be taken during alignment.
21. After plasma treatment, the surface of the PDMS changes from its natural hydrophobic state to a hydrophilic state. However, this change is temporary, and the PDMS will become hydrophobic again if left exposed to air [22]. Hydrophobic channel walls make loading microchannels with water difficult, as pressure must be applied to force the water through, and air bubbles will often get trapped in the corners of channels and chambers. On the other hand, hydrophilic channels can be loaded with media using capillary action, and air bubbles are generally not a problem.
22. Sometimes during device assembly the roof of the microgroove or barrier region will collapse to the glass substrate. In order to check if this has occurred, one should load only one channel with DI water. Then, observe whether or not the water flows through the microgroove/barrier region to the other channel(s). If the water does not flow through, slight vacuum or pressure can be applied to neighboring channels to force the roof of the microgroove/barrier to release from the substrate, allowing the water to flow freely. Generally, this problem occurs more often in continuous barrier rather than microgroove devices but can usually be avoided by careful device assembly.
23. The liquid PDMS mixture should be "painted" on top of the tubing supporter around the tubing. This can be achieved using the tip of a scalpel to apply a small drop around the perimeter of the tubing. Be careful not to add too much, as extra liquid PDMS could run off of the tubing supporters onto the pressure control chamber and form a rounded layer over the viewing window, potentially causing imaging problems in bioassays.
24. Again, a scalpel can be used to apply the thin coating of liquid PDMS to the cylinder. Make sure the surface of the PDMS is dry around the inlet/outlet punched holes before attaching the cylinder. Any water present on the surface at the attachment point will result in a poor seal and likely lead to leakage.
25. Due to the compressibility of air, more air may need to be injected to ensure that the valve is activated. By closely watching the walls of the pressure control chamber, a slight bulging

can often be observed when sufficient pressure has been applied to activate the valve. Note that PDMS is permeable to air, so using pneumatic pressure to activate the valve will only work for short periods of time (4–6 h). Hydraulic pressure should be used when long activation time-periods are required.

26. Again, slight bulging of the pressure control chamber walls generally indicates sufficient pressure has been applied to activate the valve. Be careful not to over-pressurize using water, as the PDMS-PDMS seal is not as robust as the PDMS-glass seal. Thus, high pressures can cause delamination of the different PDMS layers.
27. Sometimes it may be necessary to only unclamp the inlet tubing first, then insert an empty syringe into the inlet tubing and pull a slight vacuum. This will promote proper barrier/microgroove release from the glass substrate and fully deactivate the valve.

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Chapter 2

A Reliable Reversible Bonding Method for Perfused Microfluidic Devices

Paola Occhetta, Emilia Biffi, and Marco Rasponi

Abstract

Microfluidic devices made of poly(dimethylsiloxane) (PDMS) are suitable for cell culture applications, mainly due to both the advantageous volume and surface properties of the material itself. Bulk properties include optical transparency, gas permeability, and ease of fabrication, to name a few. On the other hand, silanol groups (SiOH) present on the surface can be easily activated through air/oxygen plasma treatments, and used to permanently bond to other materials, like silicon, glass or PDMS. The importance of a standard sealing method with no need of additional gluing materials is crucial for microfluidic applications, where micrometer sized channels and chambers are involved. Despite the reliability of the plasma treatment to permanently seal microfluidic devices, reversible-bonding methods are sometimes desirable—e.g., high magnification microscopy, sample retrieval, and multiple usages of valuable substrates. For this purpose, common techniques rely either on weakening the plasma treatment (partial treatment, only involving one of the surfaces of interest) or on increasing the self-sealing properties of PDMS (by adjusting the ratio of pre-polymer and curing agent). However, the adhesion strength of these methods is low, thus making them suitable only for static or quasi-static conditions. Whenever there is the requirement for continuous perfusion, other techniques are needed.

Here, we describe a PDMS microfluidic device for long-term culture of cells, which can be reversibly sealed to different flat substrates. The hydraulic tightness is guaranteed through magnetic forces, being the substrate interposed between a permanent magnet and the microfluidic device, locally enriched with ferromagnetic material. In particular, neuronal networks were grown within the device, reversibly coupled to a flat Microelectrode Array (MEA). Thus, the proposed approach allows to combine the advantageous features of microfluidics and the multiple use of commercial MEA substrates. Indeed, it allows for electrophysiological investigations in highly controlled microenvironments.

Key words Microfluidics, Reversible bonding, Microelectrode arrays, Neuronal culture

1 Introduction

In the last decade, the interest in poly(dimethylsiloxane) (PDMS) microfluidic devices for chemistry and biotechnology has been exponentially increasing due to their peculiar advantages compared to traditional analytical instruments such as lower reagent consumption, reduced analysis time, lower fabrication costs and ability

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to perform highly parallelized and reproducible analyses in a high-throughput fashion. PDMS itself offers several advantageous volume and surface properties such as optical transparency, biocompatibility, gas permeability, and ease of fabrication, making microfluidic devices suitable for cell culture applications [1]. Additionally, microfluidic platforms offer the potentiality to precisely monitor parameters in time- and space-controlled manners within the cellular microenvironments, providing greater control over the cell response to different stimuli [2]. Among other research fields, microfluidic devices have been widely used in neuroscience [3–5] thanks to their size-scale, compatible with neurons own size. Particularly, the direct integration of PDMS microfluidic devices with multielectrode arrays (MEAs) allows coupling the recording of neuronal electrical activity together with the inherent high control of the cell microenvironment offered by the microfluidic approach [6–8].

Currently, most of the microfluidic devices are fabricated by bonding irreversibly a PDMS stamp containing the chambers and microchannels to a second substrate layer (glass or polymer), by activating both surfaces through air or oxygen plasma [9]. This bonding technique allows for a reliable sealing able to withstand relatively high pressures (200 kPa and above) without the addition of any gluing material [10]. However, the irreversibility of the bonding introduces several limitations in terms of possible device exploitations, including cell and material patterning, surface functionalization as well as capability of samples injection and retrieval to perform secondary analyses and direct measurements not achievable within the device. Moreover, the affordability of coupling PDMS microfluidic devices with valuable substrates, like MEAs, is subordinated to the ability of disassembling the devices and reusing the substrate upon sterilization. For these reasons, many efforts have been recently directed to the development of reversible-bonding methods, which have to attain an adhesion between layers sufficient to withstand the pressure required for the microfluidic applications, while enabling a simple access to the "experimental field" and a non-disposable use of the devices. Reversible sealing between PDMS microfluidic layer and MEAs substrates have been attempted through different techniques including self-adhesion [6] and reversible plasma bonding [11]. However, these techniques do not assure a high hydraulic tightness when relatively high pressure are applied (<35 kPa), limiting their application to static or quasi-static conditions [12].

In this chapter, we describe an inexpensive and reliable technique for reversely bonding PDMS microfluidic device to different flat substrates for long-term culture of cells [13]. The presented bonding technique is based on magnetic forces which allows to obtain devices

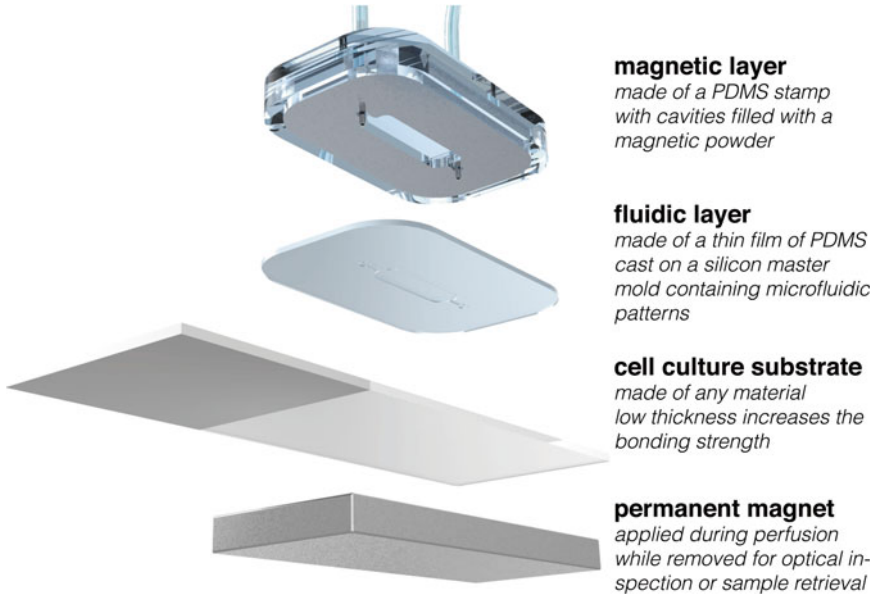

Fig. 1 Exploded view of the reversible bonding microfluidic device. The device is mainly made of PDMS and it is constituted by two layers: the bottom layer (*fluidic layer*) contains the fluidic features; the top layer (*magnetic layer*) contains a magnetic filler solution and it is optically transparent in correspondence to the fluidic regions. Once the device is assembled, channels are closed onto a cell culture substrate (e.g., histology glass slide) and maintained tight through a uniform magnetic field generated by a permanent magnet

able to withstand a high range of working pressures (up to about 150 kPa), thus suitable for cell culture applications requiring a continuous perfusion. In detail, a PDMS/iron micropowder layer is aligned onto a microfluidic layer and coupled with a flat substrate exploiting the either temporary or continuous use of a permanent magnet (Fig. 1). The introduced reversibly sealing method is characterized by compatibility with: (1) complex fluidic layer configurations, (2) micrometer size channel expanding the range of application of previously reported magnetic bonding technique, and (3) optical transparency for flow visualization and inspection over channel regions.

Specifically, neuronal networks were grown within the described device reversibly coupled to a commercial flat MEA for long-term primary neuronal cell cultures [14, 15]. However, the method offers high versatility and the design can be easily adapted to different biological applications. As an example, the same technique can be applied to precisely functionalize flat surfaces through protein perfusion or pattern either 2D cell sheets or 3D cellular structures with a microscale precision.

2 Equipment and Materials

2.1 Equipment

- Device fabrication:
  - Desktop CNC (Computer Numerical Control) milling machine (MDX40, Roland DG) with an 800 μm in diameter carbide end mill.
  - Clean room facility: mask aligner (MA56, Karl Suss)
  - Clean room facility: two flat hot plates
  - Clean room facility: fume-hood
  - Clean room facility: spin coater
  - Clean room facility: optical microscope
  - Clean room facility: nitrogen gas
  - Stereomicroscope
  - Spin coater
  - Plasma cleaner (Harrick Plasma Inc.) (*see* **Note 1**)
  - Oven
  - Vacuum chamber
- Neuronal culture and electrophysiology:
  - Humidified 5 % CO_2 incubator
  - Recording setup: pre-amplifier stage (MEA-1060-Inv-BC-Standard, gain: 55, band width: 0.02 Hz–8.5 kHz, MCS GmbH); amplification and filtering stage (FA64, gain 20, bandwidth: 10 Hz–3 kHz, MCS GmbHz); data acquisition system (USB-ME64, MCS GmbH) (*see* **Note 2**).
  - Programmable syringe pump Aladdin AL2000 (*see* **Note 3**).

2.2 Materials

2.2.1 Device Fabrication

- 4-in. polished silicon wafers
- Negative photoresist (SU-8 50, Microchem)
- SU-8 developer (Microchem)
- Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning)
- Ethanol, isopropyl alcohol (IPA), acetone, chloroform.
- Sharpened biopsy puncher (Diameter 500 μm)
- Scalpel
- Tape (Magic Tape, 3M)
- Disposable petri dish (100 and 120 mm, VWR International)
- 5 and 8 mm tick poly(methyl methacrylate) (PMMA) sheets
- Iron micropowder with grain size smaller than 212 μm (Sigma-Aldrich Corp)
- Neodymium magnet (40 × 20 × 10 mm, magnetization N42)

- Flat Microelectrode Array (60MEA200/30iR-Ti-w/o, Multi Channels System GmbH) (*see* **Note 4**)
- Tweezers for silicone wafer handling
- Stainless steel couplers (23 Gauge, 12 mm or more long)
- Blunt needles (23 Gauge)
- Tygon tubing (ID 0.02″, OD 0.06″)
- Syringes

2.2.2 Neuronal Culture

- Plating medium (before cell seeding and 4 h after): Neurobasal medium (Invitrogen), 10 % Fetal Bovine Serum (FBS; Lonza), 1 % Penicillin and Streptomycin, (Gibco)
- Culturing medium (from 4 h after the cell seeding to the end of the culture): Neurobasal medium (Invitrogen), B-27 1× (Invitrogen), GlutaMAX 1 mM (Invitrogen), 1 % Penicillin and Streptomycin (Gibco).
- Bidistilled and autoclaved water (dH_2O)

3 Methods

3.1 Device Preparation

3.1.1 Bottom Layer Mold Fabrication (Fig. 2c)

1. Design chip layout through CAD software (AutoCAD, Autodesk) (*see* **Note 5**)
2. Print out layout mask at high-res (greater than 20,000 dpi) on a transparency sheet (deep black for unexposed regions) (*see* **Note 6**)
3. Clean room operations:
   (a) Clean new wafer by rinsing with ethanol, IPA, and acetone, followed by drying with nitrogen gas.
   (b) Spin-coat SU-8 50 to the desired thickness (a thickness of 100 μm use a spin time of 30 s and a spin rate of 1,100 rpm).
   (c) Soft-bake the wafer through a two-step (two-hot plate) procedure. Move the coated wafer with tweezers on a 65 °C preheated hot plate. After 10 min, move rapidly the wafer on a 95 °C preheated hot plate. Bake it for at least 30 min and let it cool to room temperature.
   (d) Cut the mask around the designed wafer with a square shape, slightly greater than wafer diameter, and tape it upside-down to a quartz glass compatible with the mask holder of the mask aligner.
   (e) Place the wafer in the mask aligner, move it against the mask in soft-contact mode, and expose to a dose of 500 mJ/cm^2 in the i-line region (*see* **Note 7**).

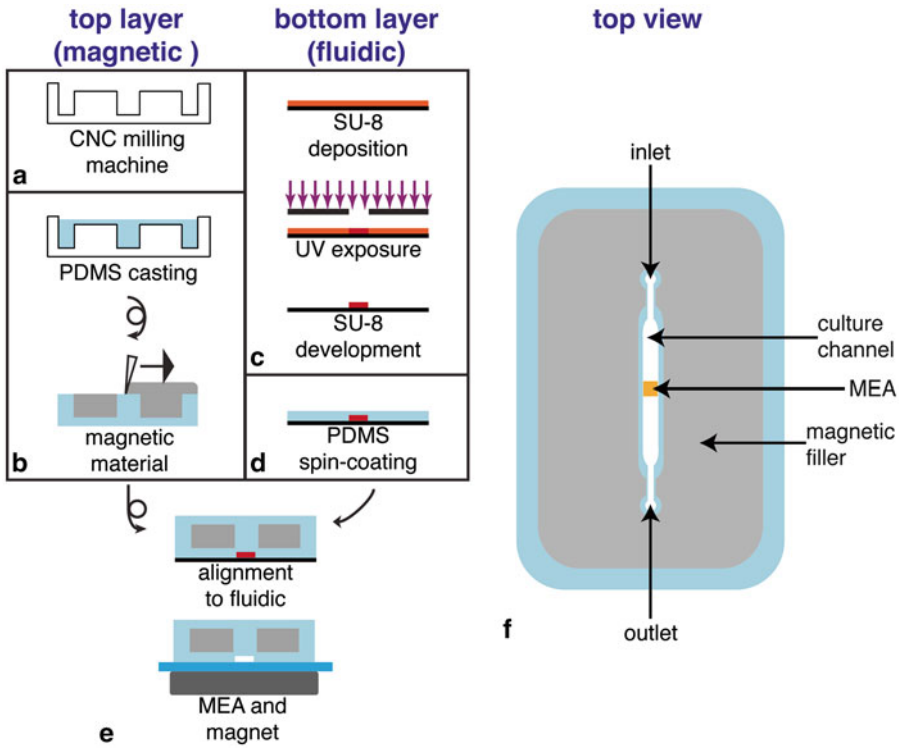

Fig. 2 Fabrication of the magnetic device. To obtain the magnetic layer a PMMA mold is realized with a CNC milling machine at a final depth of 5 mm (**a**). PDMS is cast and a ferromagnetic suspension (iron powder/PDMS mixture at ratio 4:1 as w/w) is manually plastered into specific cavities (**b**). The fluidic layer is obtained by spin-coating PDMS (thickness of 250 μm) on a silicon mold (**d**), previously realized with standard soft-lithography techniques (**c**). Finally the two layers are manually aligned under a microscope taking advantage of to the optical transparency of the top layer in the channel regions (**e**). The top view of the final assembled devices is sketched to show the culture channel, aligned to a MEA substrate, through the top layer's cavity outline (**f**)

(f) Post-bake the wafer in a similar fashion of the pre-bake: bake for 1 min at 65 °C and 10 min at 95 °C.

(g) Develop the wafer in a SU-8 developer bath for about 10 min

(h) Rinse briefly the wafer with IPA then dry it with a gentle stream of nitrogen gas

4. Place the wafer (final mold) in a petri dish and tie it with tape for further use.

3.1.2 Top Layer Mold Fabrication (Fig. 2a)

1. Decide the dimensions of the visual inspection window (*see* **Note 8**), and accordingly design the borders of the magnetic cavities.
2. Use a 3D CAD modeler to extrude the inspection window and the wall to a height of 5 mm, and export it to a CAM compatible file (e.g., iges format).

3. Machine out with the CNC milling machine the 5 mm thick PMMA sheet across its entire thickness (through hole).
4. Use a 3D CAD modeler to design an external frame for the mold (dimensions greater than the desired final device) with a thickness of 8 mm. Machine the frame out with the CNC milling machine by using the 8 mm thick PMMA sheet across its entire thickness (through hole).
5. Glue the visual inspection window PMMA piece on top of a flat 5 mm PMMA sheet (*see* **Note 9**).
6. Align the frame outside the visual inspection window and glue it to flat the 5 mm PMMA sheet.

3.1.3 Bottom Layer Fabrication (Fig. 2d)

1. Mix the PDMS-prepolymer and curing agent in ratio 10:1 (w/w) using a plastic spoon.
2. Place in the vacuum chamber and degas the mixture for at least 30 min.
3. Spin the PDMS on the mold with the spin coater up to a thickness of 500 μm.
4. Place in a vacuum chamber until all bubbles are removed.
5. Partially cure in oven at 80 °C for 15 min.

3.1.4 Top Layer Fabrication (Fig. 2b)

1. Mix the PDMS-prepolymer and curing agent in ratio 5:1 (w/w).
2. Degas the mixture for about 30 min.
3. Pour the PDMS mixture into the PMMA mold.
4. Place the mold in the vacuum chamber and degas it until no bubbles are visible.
5. Cure in oven at 80 °C for 60 min.
6. Remove the PDMS structure from the mold (*see* **Note 10**).
7. Manually fill the PDMS structure with the iron powder/PDMS suspension (*see* **Note 11**).
8. Cure in oven at 80 °C for 120 min.

*3.1.5 Magnetic Suspension Mixture (See **Note 12**)*

1. Mix the PDMS-prepolymer and curing agent in ratio 10:1 (w/w).
2. Mix the iron micropowder with PDMS in ratio 4:1 (w/w).

3.1.6 Magnetic PDMS Device Assembly (Fig. 2e)

1. Manually align the top layer (with iron powder filled openings downside) to the partially cured bottom layer under a stereomicroscope (*see* **Note 13**).
2. Bake in oven at 80 °C for 120 min.
3. Create input and output wells with the puncher.

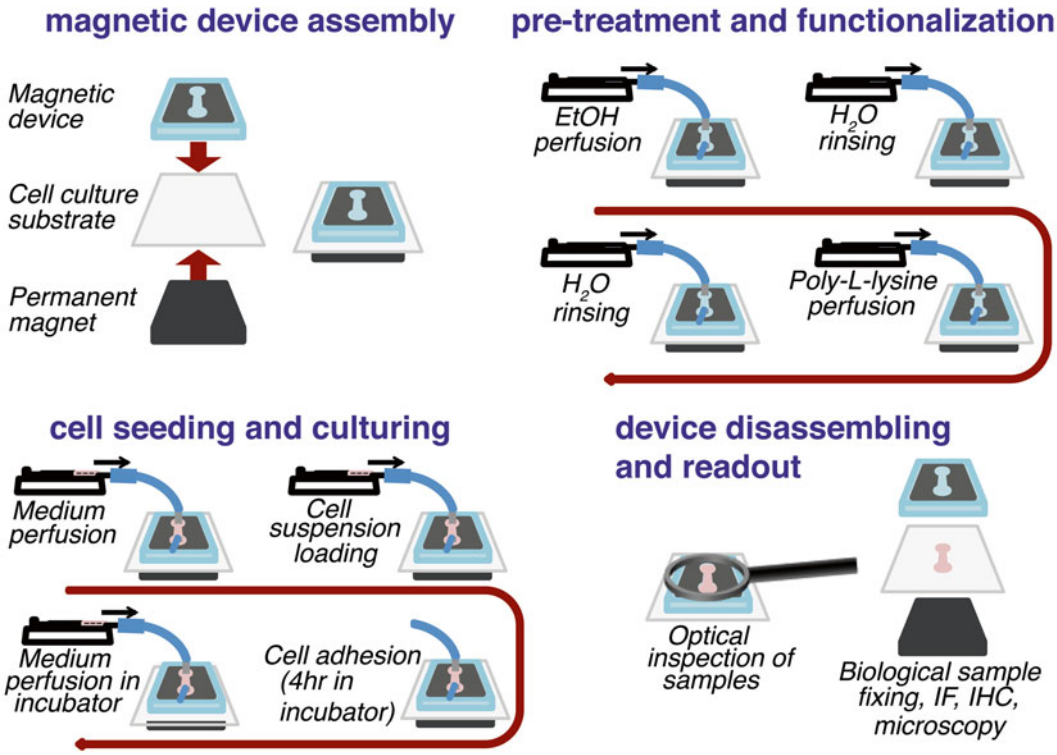

Fig. 3 Experimental setup: cell culture within a reversible assembled magnetic microdevice under continuous perfusion condition. The PDMS/iron micropowder layer is aligned onto a microfluidic layer and coupled with a flat substrate exploiting the use of a permanent magnet. Once reversibly assembled, the device is connected to a syringe pump for allowing continuous perfusion. After being washed with ethanol and rinsed with water, the fluidic channel is functionalized through Poly-L-Lysine to induce cell adhesion. The channel is then filled with medium and loaded with cells. After 4 h of static culture for allowing cell adhesion to the substrate, the device is maintained in culture under continuous perfusion condition. The transparent window in the PDMS/iron micropowder layer, aligned to the fluidic channel, allows for optical inspection of the sample while the reversibility of the bonding permits to disassemble the device, recover the substrate and perform traditional analyses on the sample

3.2 Device Assembly and Preparation for Cell Seeding (Fig. 3)

1. Place a MEA inside a sterile petri dish.
2. Place it in the air plasma cleaner.
3. Turn on the vacuum pump and wait until the pressure within the vacuum chamber has dropped below 100 mTorr (*see* **Note 14**).
4. Turn on the RF power to ignite the plasma and perfuse for 8 min to improve cell adhesion (*see* **Note 1**).
5. After the plasma treatment, take out the device inside a biosafety cabinet.
6. Lean the magnetic PDMS structure onto the MEA under a microscope, aligning it onto the MEA surface according to the final channel layout.

7. Put back the device into the petri dish.
8. Place the magnet on the opposite side (*see* **Note 15**).
9. Assemble the Tygon tubing and the stainless couplers using tweezers to preserve their sterility. Wash them by perfusing 100 % EtOH and subsequently water.
10. Connect the tubing through the couplers with the device inlets and outlets.
11. Perfuse the device with 100 % EtOH through the inlet Tygon tubing.
12. Rinse the device three times with dH_2O by perfusing through the inlet tubing.
13. Prepare 2 mg/ml poly-L-lysine (Sigma) in 100 mM Borate Buffer pH 8.5
14. Assemble a syringe filled with poly-L-lysine onto the syringe pump and connect the device through the inlet tubing (*see* **Note 3**).
15. Start perfusing the device while placing it inside a humidified 37 °C incubator overnight.
16. Wash the poly-L-lysine by perfusing water within the device.
17. Place the device, completely filled with dH_2O, in the incubator for 6 h.
18. Substitute the inlet syringe with one filled with culture medium (*see* **Note 16**).
19. The device is now ready for cell seeding.

3.3 Neuronal Culture

1. Sacrifice CD1 mice by inhalation of CO_2.
2. Dissect brains in cold HBSS (Gibco) supplemented with Glucose 0.6 % and 5 mM Hepes pH 7.4 (Sigma) and extract hippocampi (*see* **Note 17**).
3. Wash hippocampi in cold HBSS twice, with 2 min interval.
4. Treat hippocampi with Trypsin (0.25 %; Sigma), in a HBSS solution, for 10 min at 37 °C.
5. Wash again hippocampi in cold HBSS, twice.
6. Suck HBSS and add 2 ml of plating medium (*see* **Note 18**).
7. Mechanically dissociate hippocampi using pipettes until no residues of tissue are observed.
8. Count living cells filling a plastic Burker chamber with 20 μl of trypan blue and 20 μl of cell suspension.
9. Concentrate cells to 2×10^6 cells/ml by adding plating medium as necessary.
10. Withdraw 30 μl of cell suspension into a tube connected to the syringe pump.

11. Connect the loaded tube to the inlet of the device and start injecting at a flow rate of 0.5 μl/min for 30 min (*see* **Note 3**).
12. Reduce the flow rate to 1 μl/h or less (*see* **Note 19**).
13. Place the device in a humidified incubator (37 °C, 5 % CO_2) and wait for 4 h to allow cell adhesion under quasi-static condition.
14. Start perfusing the device with culture medium through the inlet port at a flow rate of 1 μl/min throughout the culture period (*see* **Note 20**).

3.4 Electrophysiological Recordings and Data Analysis

1. Carefully remove the magnet (*see* **Note 15**).
2. Place the device into the recording setup, as suggested by the manufacturer.
3. Start a recording 10 min after the movement of the device from the incubator to the recording setup to allow the stabilization of the electrical signals
4. Perform the recording continuously during the biochemical stimulation (*see* **Note 21**)
5. Use Mc_Rack software to detect spikes (*see* **Note 22**)
6. Analyze spiking behavior and bursting behavior with either a commercial or a custom software (*see* **Note 23**).

3.5 Exploitations: Selective Functionalization and Perfusion of MEAs Substrate (Fig. 4)

1. Fabricate a first magnetic device (patterning device), containing a fluidic profile replicating the pattern to be transferred on the MEAs substrate (refer to Sect. 2.1 for the fabrication procedure)
2. Fabricate a second magnetic device (culture device), containing a fluidic region compatible with (i.e. larger than) the pattern to be transferred on the MEAs substrate (refer to Sect. 2.1 for the fabrication procedure)
3. Prepare the MEAs substrate as described in Sect. 2.2 and put it into the petri dish.
4. Lean the patterning device onto the MEAs substrate and check its alignment with a microscope.
5. Place the magnet on the opposite side (*see* **Note 15**).
6. Fill the device from the inlet with 100 % EtOH for 10 min.
7. Rinse the device three times with dH_2O by adding dH_2O into the inlet and withdrawing from the outlet.
8. Fill the device from the inlet with a functionalizing protein solution (e.g., Poly-L-lysine prepared as described in Sect. 2.2) (*see* **Note 24**).

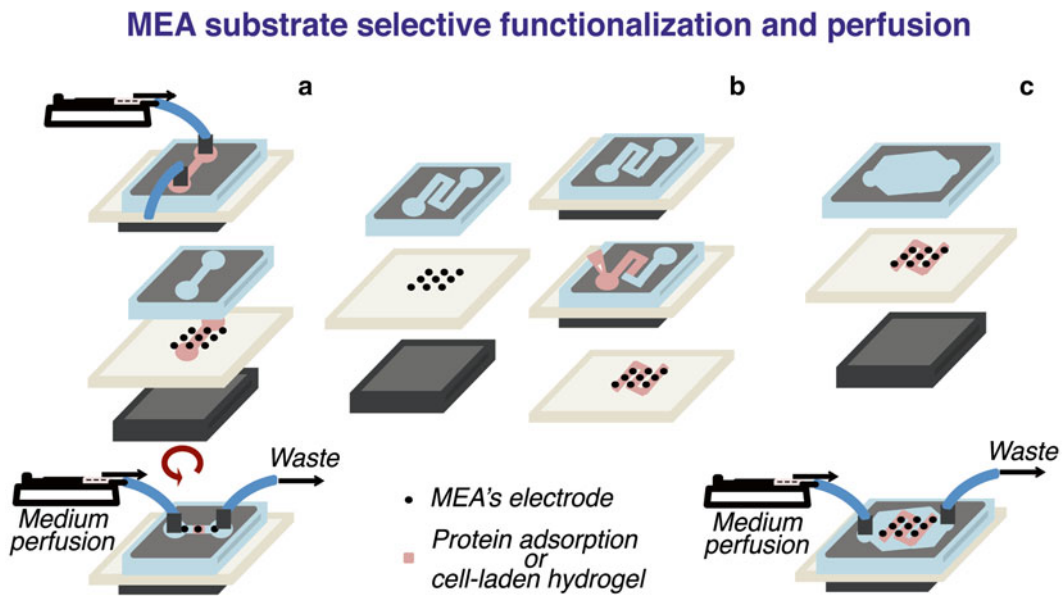

Fig. 4 The reversible magnetic bonding technique can be exploited for spatially defined 2D and 3D neuronal cell cultures on MEAs substrates. (**a**) Spatially confined 2D cell cultures are achievable by selectively functionalizing the MEAs substrate. A specific protein is perfused onto selected regions of the MEAs substrate exploiting a reversibly bonded fluidic channel, which is subsequently flipped before cell loading and culturing. (**b**) Exploiting the high hydraulic tightness of the reversible magnetic bonding, 3D cellular structures can be patterned with a microscale precision by injecting and reticulating cell/laden hydrogels within a fluidic channel reversibly bonded to the MEAs substrate [16]. Finally, (**c**) both 2D cell sheets and 3D cellular patterns can be cultured under continuous perfusion conditions by reversibly coupling the MEAs functionalized substrate with a specific shaped magnetic-fluidic layer

9. Once the functionalization reaction is over (*see* **Note 25**), wash the protein solution by adding dH_2O into the inlet and withdrawing from the outlet.
10. Disassemble the device by carefully removing the magnet and recover the MEAs selectively functionalized substrate.
11. Lean the culture device onto the MEAs substrate, aligning its channel to the transferred pattern.
12. Place the magnet on the opposite side (*see* **Note 15**).
13. Assemble the Tygon tubing and the stainless couplers using tweezers to preserve their sterility. Wash them by perfusing 100 % EtOH and subsequently dH_2O.
14. Connect the tubing through the couplers with the device inlets and outlets.
15. Assemble a syringe filled with culture medium onto the syringe pump and connect the device through the inlet tubing (*see* **Note 3**).
16. Start the perfusion (*see* **Note 26**).

4 Notes

1. Both an air or an oxygen plasma cleaner can be used. Oxygen plasma is effective in less time than air plasma treatment.
2. Any in vitro multichannel recording system can be used, provided that it can be interfaced with flat microelectrode arrays.
3. Any syringe pump is suitable, provided that it can impose infusion rates ranging from 1 μl/h to 100 μl/min.
4. Any microelectrodes array is suitable if it has a flat surface, i.e. there is not a well to contain cells and medium over the matrix of electrodes.
5. In this application a simple straight channel 100 μm high was designed, featuring two access ports (inlet and outlet) and a width compatible with MEA dimensions (channel 2 mm wide). Adding a secondary inlet, connected to the main one through a bifurcation, can minimize the risk of bubble injection into the device. The secondary inlet will be maintained clamped during the culture, while serving as outlet for possible bubbles during medium change phases.
6. Outsourcing services for mask productions are available at low cost, e.g., Micro Lithography Services Ltd.
7. To minimize the risk of cracks in the SU-8, it is advisable provide the required energy dose in three to four exposure periods rather than a single one.
8. The inspection window should have similar shape of (although be slightly bigger than) the external layout of the fluidic layer. Its dimensions should be chosen carefully; indeed, the bigger the inspection window, the more light can penetrate, thus increasing the quality of microscopy imaging, the lesser is the hydraulic tightness.
9. Gluing can be performed by slightly pressure and letting a drop of chloroform to penetrate by capillary forces between the mating surfaces.
10. Due to the high aspect ratios of the features, the PDMS stamp is fragile. To favor the detachment you can use either ethanol or water with soap.
11. Use a spatula to gently plaster the mixture until a uniform filling and leveling are achieved.
12. The magnetic mixture can be used within its gel time. At room temperature the gel time is about 12 h. In case of mass production, it is advised to prepare the required magnetic mixture and keep it at low temperatures; at –40 °C the gel time increases up to several days.
13. To keep the alignment throughout the curing process, tape can be used to tighten the top layer to the wafer underneath.

The top layer can be alternatively bound to a petri dish containing the wafer, so to reduce the risk of wafer breaking.

14. For the Harrick Plasma system, the time required to reach a pressure level compatible with plasma activation ranges from 1 to 5 min, depending on the vacuum pump extraction rate.
15. Neodymium magnets are dangerous. They are brittle, and colliding magnets could crack, with the possible consequence of catapult sharp splinters away for several meters. For this purpose, you and people around you should wear safety glasses when handling magnets. In addition, be careful not to bring close to the magnet any small magnetic items such as scissors, pincers, clamps, etc.
16. Consider imposing high flow rates for medium perfusion before culturing (tens of μl/min) to allow any potential debris or toxic residue to be removed.
17. Other cell types can be used. Outsourcing companies for embryos or brain parts sale are available (e.g., Embryotech Laboratories Inc., Neuromics Inc., AMS Biotechnology Ltd).
18. This volume is adequate when the number of hippocampi is higher than 5. If less hippocampi are available, the volume can be reduced to 500 μl.
19. It is better not to stop completely the medium perfusion in order to avoid evaporation within the microfluidic device.
20. Avoid using high flow rate to prevent bad effects on adherent cells due to high shear stresses.
21. Electrophysiological recordings can be carried out either in static conditions, to measure spontaneous electrical activity of the networks, or they can be performed during controlled perfusion conditions.
22. Any other spike detection software can be used.
23. Commercial softwares include McRack (MCS GmbH), Neuro Explorer® (Nex Technologies), Offline Sorter (Plexon Inc.). Custom algorithms are commonly developed in Matlab® (The MathWorks Inc.).
24. Cell/laden hydrogels solution can also be used instead of protein solution to directly pattern 3D cellular structures onto the MEAs substrate.
25. The time required for the functionalization reaction depends on the specific protein used.
26. The experiment can also be performed under static condition. In this case, the culture device can be manually filled with medium without using a syringe pump.

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Chapter 3

Bridging Two Cultures: Minimalistic Networks Prepared by Microfluidic Arraying, and Open Access Compartments for Electrophysiology

Jonathan West, Ngoc-Duy Dinh, Heike Hardelauf, Ya-Yu Chiang, Tracey A. Newman, Mariana Vargas-Caballero, Ayodeji A. Asuni, Katrin Deinhardt, and Martin Arundell

Abstract

Microfabrication protocols are described for two compartmentalized neuron culture platforms which extend beyond the capabilities of conventional systems. The first involves a differential flow microfluidic circuit for arraying single neurons, along with protocols for in chip biomaterial patterning and the selective treatment of somata or outgrowth compartments. These minimalistic neuronal networks are ideal for spatially resolved research using rare and precious neuronal subtypes as well as parallelization for screening biochemical libraries. The second, open-access, system solves the micro-to-macro interface challenge to enable the insertion of micromanipulators for electrophysiology studies or localized perturbation using a microinjector. This system is especially useful for the spatiotemporal investigation of mechanisms underlying disease, such as neurodegeneration and epileptic seizures. Design files along with soft lithography replication techniques are provided to facilitate the straightforward uptake of these platforms. Technology integration approaches are also presented for the alignment of arrayed neurons with individual microelectrodes for highly parallel electrophysiological and electrochemical measurements throughout nodes in the compartmentalized neuronal network.

Key words Soft lithography, User-friendly, Cell patterning, Microfluidics, Electrophysiology, Neurodegeneration, Alzheimer and Parkinson disease, Epilepsy, Long-range signaling, Blood–brain barrier

1 Introduction

Experimental methods enabling the isolation and probing of the different cellular and subcellular components of the nervous system can bring new insights. For example the membrane within the Boyden chamber allows axons to be isolated from the cell soma for the analysis of axon-specific mRNA [1]. Alternatively planar compartmentalization in the form of the Campenot chamber brings the possibility for straightforward optical and electrophysiological

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recording from living cultures. Most importantly, the Campenot and Boyden chamber systems are amendable to localized biochemical perturbations for the spatiotemporal investigation of long-range trafficking and signaling within the circuitry of the neuronal architecture [2, 3].

Taylor et al.'s refinement of the Campenot chamber in the form of microfabricated outgrowth microchannels spanning culture compartments provided the necessary precision and reliability [4, 5]. To address the growing demand, these microfluidic devices are now commercially available. A major driver behind this development was the advent of soft lithography, the straightforward replication of microstructured systems in polymers, most notably the glass-like elastomer poly(dimethyl siloxane) (PDMS) [6]. Microfluidic device replication was freed from the clean room, enabling biologists to adopt a DIY approach to produce devices in their own laboratories. Typically, the photolithographic fabrication of the molds is outsourced with these being suitably robust for repeat PDMS casting by the biologists. Soft lithography is also ideally suited for rapid prototyping and with freely available precision drawing software the biologists are now in a position to iteratively design bespoke systems to match the needs of their particular experiments.

In this chapter we describe two different systems prepared by PDMS soft lithography that add significant functionality to previous compartmentalized systems. In Sect. 4 we describe a microfluidic method for the reconstruction of neuronal circuitry with defined cell numbers [7]. This reduces local entanglement for the efficient study of inter-compartment transmission and brings the potential to isolate individual axons for visualization and analysis in a quantitative manner. Section 7 describes a method for the fabrication of an open access system to facilitate the electrophysiological analysis of distinct compartments within the network [8].

The two systems greatly extend our capabilities for investigating the brain and can be combined to leverage the benefits of both (*see* Sect. 8). Applications for these systems are numerous, including the investigation of nanoparticulate toxicity [9] and manganese propagation [10], modelling the blood–brain barrier [11] or the construction of epilepsy models that mimic the pyramidal microcircuitry in the CA1 hippocampal region. The platforms also ideally lend themselves to studying the dissemination of infectious agents such as prions [12], alpha synuclein propagation in Parkinson disease [13], amyloid beta (Aβ) transmission and tau phosphorylation cascades in Alzheimer disease [14, 15], and mechanistic responses to immunotherapy [16–18].

Another obvious merit of the microfluidic arraying method is the ability to undertake research with low abundance neuron

subpopulations rather than complex heterogeneous cocultures. For example, dopaminergic *substantia nigra* neurons suitable for Parkinson's research or peripheral neurons that can only be isolated in small numbers are still sufficient for studies using the microfluidic platform. Other examples involving rare cells are the investigation of the basis of hearing loss using an experimental arrangement of hair cells co-cultured with spiral ganglia, or studies using induced pluripotent stem cells from humans. Likewise, the low cell requirements of the system make it readily possible to undertake experiments with neurons from the miniature *Drosophila melanogaster* brain [19] and thus leverage the benefits of this powerful genetic model. The other perspective is the pragmatism of cellular economy and the small device footprint that makes high throughput research manageable. Each condition and replicate requires minimal neurons, a feature that can be used to tremendously reduce animal numbers in line with the ongoing 3Rs endeavor. For example, dose response analysis of a large panel of compounds (i.e., 1,000) can be undertaken using a single animal. Types of screen include toxicity testing and the investigation of libraries of siRNA or pharmacological agents for their effect on long-range trafficking and signal transduction in a single cell, quantitative and statistically meaningful manner.

2 Preparation of Minimalistic Neuronal Co-Cultures

This section describes how to fabricate and use microfluidic circuits for arraying neurons with single cell precision. The method enables minimalistic co-cultures to be arrayed for high throughput experimentation. For example the microfluidic circuit dispenses 100 single neurons as a spatially defined array in each compartment. With such economy, dose response screens can be undertaken using neurons from a single animal. In addition, the use of minimal neurons results in reduced levels of locally entangled outgrowths while maintaining high levels of inter-compartment connections. As such this greatly increases the efficiency of tracking interactions between the two cultures. The protocol is suitable for a standard neurobiology laboratory, requiring a microscope, hot plate, vacuum dessicator and aspiration pump. An inexpensive handheld corona discharge system is the only "exotic" instrument required. In addition to microfluidic device fabrication (Sect. 4.1), we have provided a protocol for biomaterial patterning within the microfluidic device (Sect. 4.2) and protocols for cell arraying, culture perfusion, and selective fluidic treatments (Sect. 4.3).

3 Materials 1

PDMS pre-polymer and curing agent (Sylgard 184, Dow Corning).

PDMS (Elastosil® RT 601, Wacker).

Biopsy Punches (3-mm-diameter, from Kai Medical).

Tygon® Tubing (1.65 mm ID; 3.35 mm OD) and ~170-μm-thick glass coverslips (VWR).

4-way tubing connector (Fisher or eBay).

Flow regulator (Fisher or eBay).

Poly-lysine-FITC (PL-FITC), poly-ornithine, fibronectin, and laminin (Sigma-Aldrich).

Poly-L-lysine–poly(ethylene glycol) (PLL-*g*-PEG, SuSoS, Switzerland).

4 Methods 1

SU-8 masters for PDMS device replication can be obtained by collaboration with your local microfabrication center, or fabrication can be outsourced at competitive prices from commercial services (*see* **Note 1**). High-resolution, chrome on quartz masks are used and can be purchased from a number of companies, such as JD PhotoTools (*see* **Note 2**). The 2-layer SU-8 photolithography process requires two masks. Considerable attention to detail is required to design the fluidic resistance circuits with optimal dimensions [7, 20, 21]. To bypass this inconvenience, we recommend using the 2 mask designs made freely available as supplementary information (ZIP) on the Royal Society of Chemistry website: http://pubs.rsc.org/en/content/articlelanding/2013/lc/c3lc41224e. Importantly, the first SU-8 layer should be fabricated to a depth of 2.5–3.0 μm, and the second to a depth of 25–30 μm. These are key dimensions necessary for effective neuron arraying. The 3 μm height limit is also necessary to prevent neurons being transported or migrating between the two compartments [7].

4.1 Device Fabrication and Assembly

The differential flow microfluidic circuit and 2-layer structured PDMS replicas are shown in Fig. 1. The PDMS replica molding process involves thoroughly mixing the PDMS pre-polymer with the curing agent polymer (10:1) in a plastic weighing boat or disposable plastic cup (*see* **Note 3**). The mixture is thoroughly degassed in a vacuum desiccator (typically 10–30 min) or by low *g* centrifugation in a 50 mL Falcon tube for ~5 min. Supported by laboratory tissue (*see* **Note 4**) the SU-8 wafer is placed on an 80 °C hotplate. Polymer frames, 8–10 mm thick and with an internal window of 20 × 20 mm (*see* **Note 5**), are aligned to each device on the SU-8 wafer. The PDMS mixture is poured into each frame to a depth of ≥5 mm. Thermal curing typically requires >30 min (*see* **Note 6**).

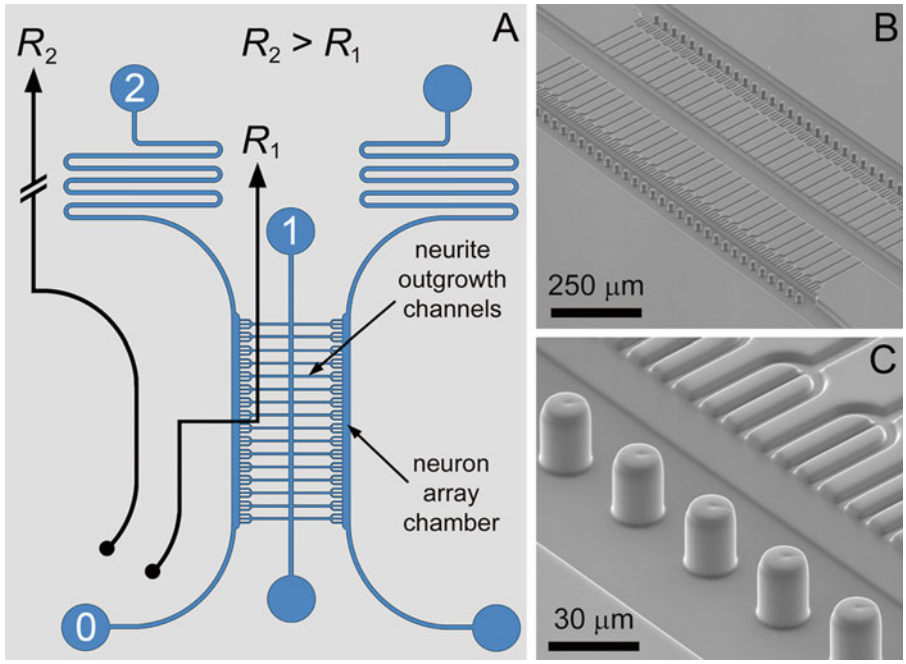

Fig. 1 The differential fluidic resistance circuit for single neuron arraying, with flanking culture chambers interconnected by neurite outgrowth channels (**a**). The path $0 \rightarrow 1$ connects the inlet channel via multiple neurite outgrowth channels to a central outlet channel. The paths have a lower fluidic resistance (R_1) than the serpentine path ($0 \rightarrow 2$, R_2) for simultaneous neuron trapping along the linear arrays of trident-shaped cell traps in both compartments. SEM images of the bilayer compartmentalized neuron co-culture array with meniscus pinning micropillars (**b**, **c**). Figure and legend reproduced with permission of the Royal Society of Chemistry (RSC) [7]

The wafer is removed from the hotplate and left to cool (*see* **Note 7**). Using a rigid scalpel, and while using protective eyewear, the frame and PDMS mold can be gently prised from the wafer by working at one corner (*see* **Note 8**). Once removed from the frame a pair of scissors can be used to tidy up the PDMS device (so-called PDMS flashing is often produced as the PDMS spreads between the polymer frame and the wafer surface). To remove remaining PDMS deposits on the wafer and protect it during storage a thin layer of PDMS can be cured on the wafer (*see* **Note 9**).

A 3-mm-diameter biopsy punch is then used to produce the 6 interface ports in the PDMS device (*see* **Notes 10** and **11**). For microchannel encapsulation, thin PDMS layers mounted on a ~170-μm-thick glass coverslip are prepared by pouring small volumes of the PDMS-curing agent mixture (10:1, *see* **Note 12**) onto the base of a hydrophobic (bacteriological grade) Petri dish and gently pressing a coverslip over the top. Thermal curing requires less than 10 min. Quickly after cooling a scalpel is used to cut and prise the PDMS-coverslip bilayer from the Petri dish (*see* **Note 13**).

The PDMS device and support PDMS layer are plasma bonded to produce a good seal. Optimum conditions are instrument specific. For example, we use a Femto plasma oven from Diener Electronic (Germany) operating at 70 W and 40 kHz in a 0.2 mbar oxygen atmosphere for 40 s (*see* **Note 14**) or a 1 min treatment with a handheld corona discharge system can be used. Once plasma-treated both surfaces are gently pressed together and left to fully bond for a few minutes.

4.2 Biomaterial Patterning by Water Masking

Immediately following plasma bonding the PDMS microfluidic channels are highly hydrophilic. Such surfaces are suitable for the adhesion and culture of cell lines such as differentiated human SH-SY5Y neuron-like cells. For the ex vivo culture of primary neurons and the culture of neuronal precursor cell lines such as the Lund human mesencephalic cell line (LUHMES) a poly-amine coating, either poly-lysine (PL) or poly-ornithine (PO), is required as an electrostatic anchor. In addition, adhesion proteins such as laminin or fibronectin are often required as the integrin-interfacing coating. However, the different material coatings readily bind neurons hindering the delivery to the arraying sites, and also imposing unwanted shear stresses on the neurons. The following protocol describes a simple procedure for in-chip biomaterial patterning to enable neuron deliver to the arraying sites and maintained registration during lengthy culture. The water masking method is documented in Fig. 2.

Shortly after plasma bonding a 0.5 μL volume of PL or PO (100 μg/mL) in 1× PBS is pipetted into the bottom central channel. By capillary action, the entire microfluidic circuit is filled, with the polyamine coating the hydrophilic PDMS microchannel surfaces. Unbound, excess polyamine molecules can be removed by aspiration-driven washing with PBS. Here, Tygon tubing, a silicone variety, is used for plug and play interconnection from the 3-mm-diameter ports to a pump.

The remaining PBS buffer is used to form the water mask. The heat from microscopy illumination causes rapid evaporation from the interconnection ports. The water mask (illustrated in Fig. 2a and documented in Fig. 2b) is established within ~5 min and can be validated by standard microscopy inspection. A video of evaporation-driven water masking is hosted as supplementary information (movie 1) on the RSC website: http://pubs.rsc.org/en/content/articlelanding/2013/lc/c3lc41224e. Stainless steel pins (*see* **Note 15**) are inserted into the flanking inlets. These couple the plasma generated by an atmospheric pressure handheld corona discharge or air plasma device (e.g., VP23, Leybold-Heraeus, USA, operating at 2 MHz, 30 kV signal) into the vacant microfluidic channels. Low light conditions are used to observe plasma patterning. Water remaining on the so-called meniscus pinning microstructures prevents plasma treatment of the neuron adhesion

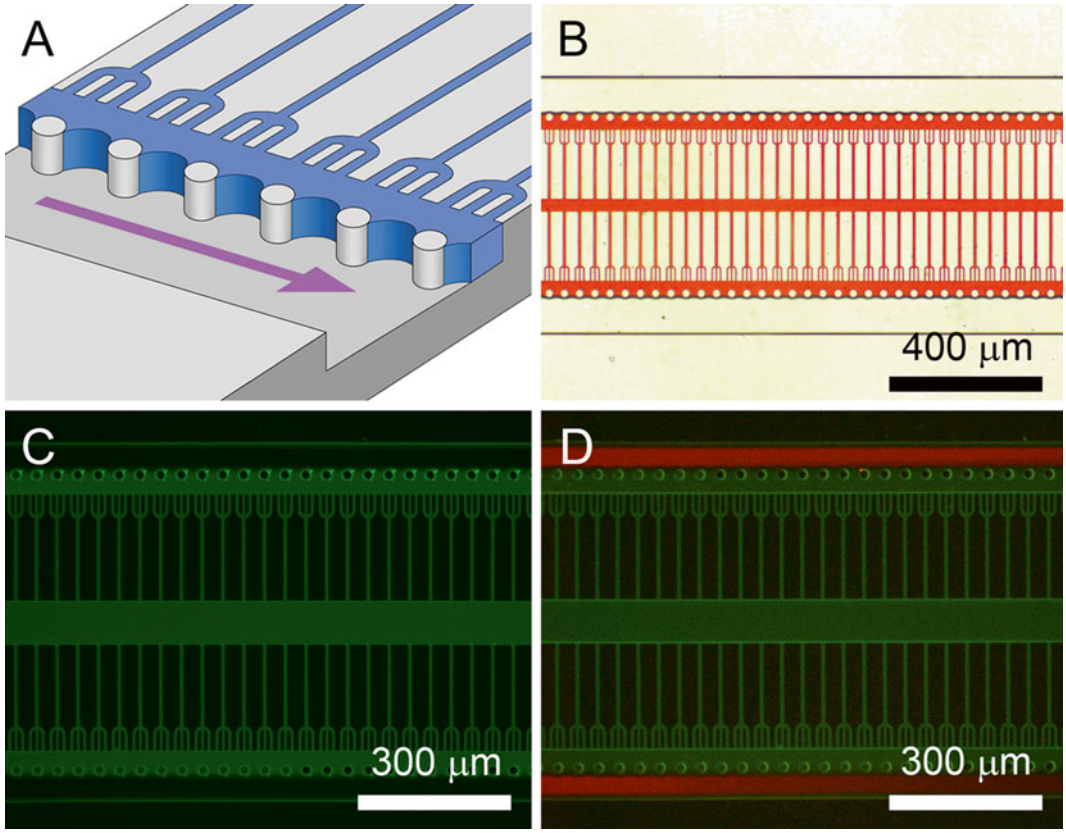

Fig. 2 Illustration of the water masking concept with the PDMS micropillars pinning the water meniscus in place for plasma stencilling (**a**). Curvature of the meniscus in the vertical plane has been neglected. Water mask visualized with a red dye and positioned, following evaporation, by meniscus pinning (**b**). The patterned PL–FITC coating imaged after plasma stencilling (**c**). Addition of protein rejecting and cell repellent PLL-*g*-PEG–TRITC to a plasma-patterned PL–FITC coating (**d**). This was used for registration of the SH-SY5Y cells and also for patterning fibronectin coatings for the LUHMES cells. Figure and legend reproduced with permission of the Royal Society of Chemistry (RSC) [7]

sites and the neurite outgrowth channels. A short, ≤1 s plasma treatment is recommended (*see* **Note 16**). The water mask is removed along with unbound polyamines by a wash step involving 20 μL volumes of 1× PBS being dispensed in the bottom three ports with parallel aspiration from the upper three ports. This is achieved using four equal lengths of Tygon tubing connected by a 4-way adaptor to an aspiration pump (e.g., N811KVP Mini Pump, KNF Neuberger, Laboport®, Germany). In this manner a PDMS-polyamine material contrast is produced (*see* Fig. 2c).

Should additional adhesion materials, such as laminin or fibronectin, be required the following steps can be used: A 10 μL volume of the graft co-polymer poly-L-lysine–poly(ethylene glycol) (PLL-*g*-PEG, 100 μg/mL, Surface Solutions, Switzerland in PBS) is pipetted into both bottom flanking channels, followed by aspiration from the

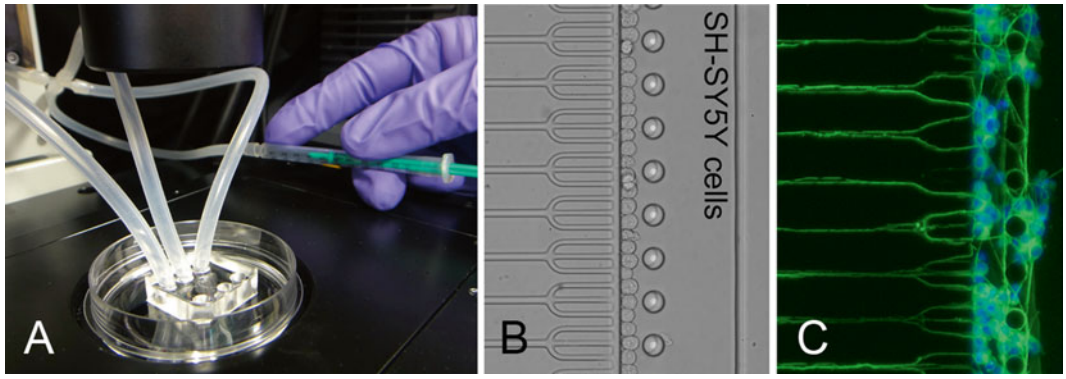

Fig. 3 Manual aspiration using a syringe is used for gentle neuron arraying. A 4-way tubing connector is used to interface the syringe with the upper three microfluidic ports for simultaneous neuron arraying in both compartments (**a**). A movie (2) of neuron arraying is hosted on the RSC website: http://pubs.rsc.org/en/content/articlelanding/2013/lc/c3lc41224e. Neuron-like human SH-SY5Y cells and other neurons are arrayed with single cell precision (one neuron per trap, **b**). Following 7 days of culture, neurite extensions interconnect the two cultures (**c**). Nuclei are stained with DAPI and actin immunostaining was used to visualize the outgrowths. Modified figure and legend reproduced with permission of the Royal Society of Chemistry (RSC) [7]

upper flanking channels for 5 min. Without interrupting the flow, excess PLL-*g*-PEG can be removed by exchange with a PBS buffer. This selectively coats the non-poly-amine-coated regions, with the PEG moiety preventing protein adsorption and cell adhesion [22, 23]. Adhesion proteins such as laminin or fibronectin can then be co-localized with the poly-amine coating. 10 μL volumes of these ECM proteins (typically 10 μg/mL, Sigma-Aldrich) are pipetted into all three, bottom ports. Aspiration from the top three ports for a few minutes produces a good quality coating. The complete biomaterial patterning protocol is illustrated at the end of Sect. 4 (Fig. 5a–d). Proteins such as N-cadherin can instead be patterned to selectively manipulate different subcellular structures (*see* **Note 17**).

4.3 Microfluidic Neuron Arraying, Culture and Fluidic Treatment

Using devices with material coatings matching the requirements of your neuron type the following describes how to array minimal numbers of neurons—i.e., ~1 neuron per trap, effectively delivering ~100 neurons to each culture compartment:

Devices are first primed with appropriate media using an aspiration pump. A 20 μL disaggregated cell suspension (1×10^6 cells/mL) is dispensed into the flanking inlet ports (0, in Fig. 1) and the upper three outlets are connected by equal-length tubing via a 4-way union to either an aspiration pump or a syringe for gentle manual aspiration as shown in Fig. 3. Neuron numbers and positions are determined by the microfluidic traps. Complete arraying typically requires less than 1 min. Excess neurons remaining in the outlet ports can be removed with a pipette for arraying in subsequent microfluidic devices. The microfluidic circuit is then disconnected from the aspiration ports and the inlet ports are filled with

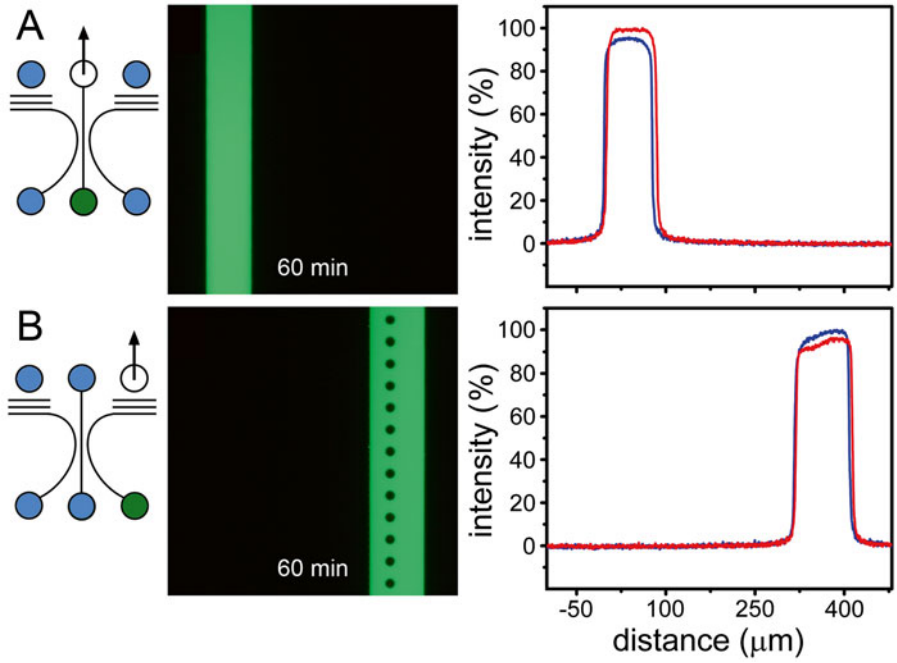

Fig. 4 Selective treatments of the central (**a**) and flanking compartments (**b**) using an aspiration pump. The *arrow* indicates the port used for aspiration. Treatments were established in 1 min (*red lines*) and were maintained for the duration of the experiment; 60 min (*blue lines*). Modified figure and legend reproduced with permission of the Royal Society of Chemistry (RSC) [7]

media and the devices are placed in the incubator for culture. Media exchange is achieved by periodic media perfusion using hydrostatic feed (e.g., using a 5 mm column height difference between ports) or by simply submerging the microfluidic devices in media. The protocols for cell loading and media perfusion are illustrated in Fig. 5e, f.

Selective fluidic treatments to either neuron culture compartment or the central neurite outgrowth compartment are achieved as follows: Dispense 20 μL of the compound in the bottom port of the channel of interest, and aspirate from the port directly above. Experiments using fluorescein-doped media show that aspiration-driven treatments are confined to either the central or flanking compartments (*see* Fig. 4a, b). The protocol for selective fluidic treatments is illustrated in Fig. 5g, h. For lengthy treatments the bottom port can be replenished with compound, or the flow rate can be greatly reduced using a flow regulator (*see* **Note 18**). An illustrated protocol describing compartment-selective fluidic treatments is shown in Fig. 5g, h.

Immunostaining protocols vary for each molecular target and associated reagents. Hydrostatic perfusion with differing column heights or an aspiration pump can be used to deliver fixative agents and other reagents for immunostaining.

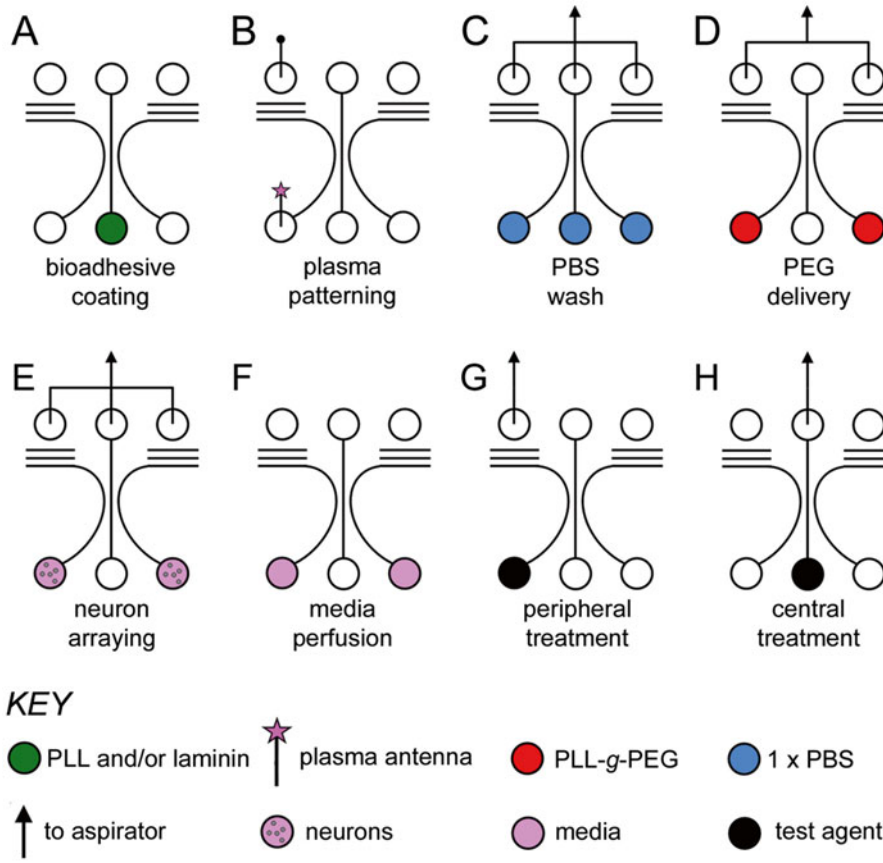

Fig. 5 Illustrated protocols for the different fluidic operations. *PL and PLL-g-PEG patterning*; addition of the cell adhesion adlayer (e.g., PL, *green*), with evaporation producing the aligned water mask (**a**), atmospheric air plasma treatment of the exposed PL (**b**), PBS (*blue*) washing by aspiration to avoid contaminating the plasma-treated region with PL (**c**), and delivery of PLL-*g*-PEG (*red*) by aspiration to coat the plasma-treated regions (**d**). *Cell arraying*; simultaneous microfluidic arraying of neurons to both flanking compartments using the aspiration pump or by gentle manual aspiration (**e**), media (*pink*) perfusion using a hydrostatic-driven flow (**f**). *Targeted fluidic treatments*; peripheral treatment of a test agent (*black*) delivered from the bottom flanking inlet by aspiration (**g**) and central treatment delivered from the bottom central inlet by aspiration (**h**). Figure and legend reproduced with permission of the Royal Society of Chemistry (RSC) [7]

5 Open Access Neuronal Cocultures

Soft lithography is ideal for the rapid replication of microfluidic structures, but these are inherently enclosed prohibiting access for micromanipulator-based electrophysiology. This section describes how to fabricate an open access device that successfully interfaces macroscopic reservoirs with microscopic outgrowth channels for electrophysiology recording of the compartmentalized cocultures. The simple fabrication protocol is suitable for undergraduate biology

and medical students to master in less than a day. The method does not rely on costly clean room facilities or specialized equipment, bringing the possibility for devices to be fabricated in a standard teaching or biology laboratory. By the use of PDMS–PDMS molding involving a detergent coating a reversible compartmentalized neuron PDMS device with a thin-walled barrier can be prepared and replicated in large numbers. Importantly, this protocol solves problems with demolding PDMS from PDMS as well as PDMS flashing (leakage of material between the molding tool and substrate). This avoids the need to cut the access holes leading to superior device quality and reproducibility. In addition the compartmentalized devices can be reused after a 70 % (v/v) ethanol–water wash. The complete fabrication process is illustrated in Fig. 6.

6 Materials 2

Microscope slides (e.g., Schott D263, Chance Glass).

Rinzl 280 μm plastic spacers (Electron Microscopy Sciences).

PDMS pre-polymer and curing agent (Sylgard 184, Dow Corning).

0.1 g/mL household detergent (Cleanline, Birmingham) in a filtered ethanol–water mixture (70 %, v/v).

10 × 10 mm aluminum posts (Wickes, UK).

Nunc 4-well non-treated rectangular culture dishes (Fisher 267061).

7 Methods 2

7.1 Positive Mold Preparation

To prepare the microfabrication master microscope slides were laser engraved by a service provider (LML, Denbighshire). These contained 20 microchannels, 3 μm in depth and 8 μm in width and with a pitch of 500 μm. For positive mold fabrication, standard microscope glass slides are placed in each of the four individual Nunc culture wells (4 × 3 cm). Glass and Nunc dishes are thoroughly cleaned with 70 % (v/v) ethanol–deionized water, dried with a nitrogen stream and placed in an oven at 50 °C. As in Sect. 4.1, the PDMS is mixed in a 10:1 ratio (w/w) and degassed under vacuum. The PDMS is then applied to all slides as a thin film using a 1.5 mL syringe. Glass master slides with the microchannels facing downwards are gently lowered on top of the planar (i.e., unstructured) glass slides with the aid of a syringe needle or small spatula (*see* **Note 19**). The glass–PDMS–glass sandwich is then placed in an oven at 50 °C for one hour for thermal curing. The master glass slide is then removed from the plain glass slide leaving a thin sheet of PDMS with 20 positive microchannels (i.e., extruding). The plain glass slide with the PDMS positive microchannels is returned to the oven for hardening for another hour.

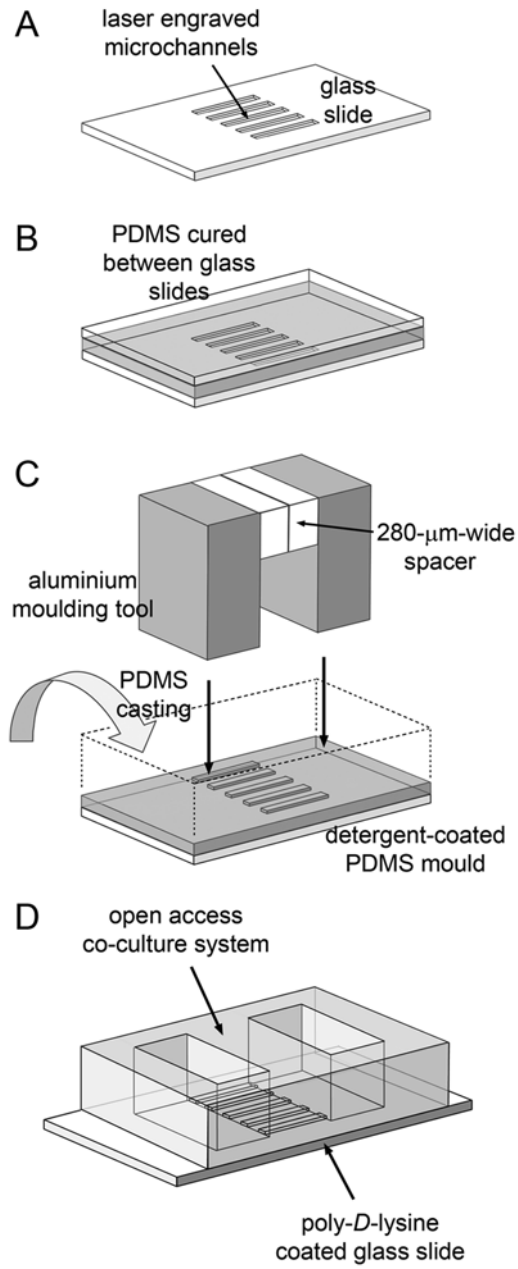

Fig. 6 Schematic of the fabrication process: A glass microscope slide with laser engraved parallel microchannels (**a**). The microchannels are molded in PDMS by sandwiching a PDMS deposit between the laser engraved glass slide and a planar glass slide (**b**). Following thermal curing, the laser engraved slide is removed and the PDMS surface is treated with detergent. An aluminum molding tool, used to define the two culture reservoirs, is aligned with the microchannels and the entire system is cast in PDMS (**c**). The PDMS replica is removed and then placed securely on a poly-D-lysine-coated microscope slide in readiness for neuron culture (**d**)

7.2 One Step Molding and Neuron Culture

The molded PDMS microchannels are treated with a small volume of 0.1 g/mL household detergent to coat the surface, then dried with nitrogen and placed in an oven at 50 °C. The molding tool for the reservoirs and thin barrier comprised two 10 × 10 mm aluminum square posts separated by two Rinzl 280-μm-thick plastic spacers (Electron Microscopy Sciences; total thickness = 560 μm) with an O-ring to hold the tool in one piece (*see* **Note 20**). The molding tool is cleaned in 70 % ethanol, dried in the oven at 50 °C, and then carefully positioned over the PDMS positive microchannels. Degassed PDMS is then poured to an approximate height of 7 mm and placed on a hotplate at 90 °C or in an oven at 60 °C for 2 h. The mold is removed and demolded with the aid of 70 % filtered ethanol–deionized water by first removing the O-ring and the aluminum molds and then removing the glass/PDMS from the Nunc dish.

Once the glass slide has been separated from the PDMS mold the thin PDMS sheet containing the extruded microchannel features can be peeled from the laser engraved glass slide (*see* **Note 21**). This will then reveal the negative microchannels (i.e., recessed) within the 560-μm-wide PDMS barrier. There is near-zero flashing (leakage of material between the molding tool and substrate). Flashing can be a problem if the molding tool is placed directly on a glass or plastic surface due to leakage of PDMS between the two surfaces. This can be difficult to remove especially around the delicate, 560-μm-thick barrier. However, when the molding tools are placed on the detergent coated microstructured PDMS there is no leakage between the two surfaces and hence no flashing after the PDMS/PDMS demolding procedure.

The PDMS compartmentalized devices can be reversibly attached to microscope slides or coverslips. The glass is initially pretreated with 100 μg/mL poly-lysine, then washed in 1× PBS and allowed to air-dry. The compartmentalized PDMS devices can then be placed on the glass substrates. To each compartment 70 % (v/v) filtered ethanol–deionized water is added to fill the microchannels and sterilize the glass and PDMS surfaces. Each compartment is then filled twice with deionized water and then filled with Neurobasal media for incubation overnight. The Neurobasal media is exchanged just prior to seeding cells in each compartment. Figure 7 shows the compartmentalized devices and neurite outgrowth between chambers.

8 Technology Integration

The benefits of the single neuron arraying microfluidic circuit can be combined with open access reservoirs for electrophysiology recordings by leveraging the detergent-based PDMS–PDMS molding strategy outlined in Sect. 7.2. Reservoirs flanking the outgrowth channels can be included using an appropriate molding tool.

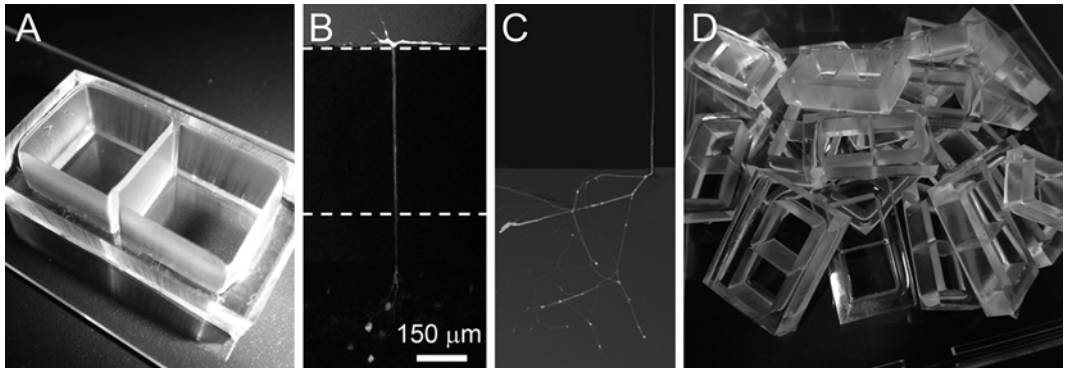

Fig. 7 Open access neuronal co-culture PDMS system (**a**). A primary cortical axon spanning the 560-μm-long microchannel (**b**, reproduced with permission of the Royal Society of Chemistry (RSC) [8]). Arborization can be observed at the exit of the microchannel (**c**). The devices can be mass produced with ease, opening the possibility for high throughput experimentation (**d**)

Importantly, the differential flow neuron arraying principle demands a fully enclosed microfluidic circuit (Sect. 4). By counter-molding the reservoirs (necessitating a detergent coating), PDMS plugs can be prepared to close the reservoirs for arraying neurons in both compartments. Once neurons are arrayed and become adherent the plugs can be removed to provide open access. The ordered linear array of neurons is ideal for high throughput patch clamping, involving periodic translation to immediately locate the next neuron. The transparent quality of PDMS also brings the possibility for live or post hoc biochemical imaging. Beyond analysis, localized and cell-type specific stimulation can be achieved using optogenetic methods [24, 25].

To make significantly larger gains in recording throughput the micromanipulator-based patch clamp can be replaced with microelectrode arrays [26, 27] arranged to interface with the array of neurons. A fully enclosed system can be used with microfluidic arraying to register individual neurons on patterned electrodes. To retain the neurons directly on the electrodes during lengthy culture the electrodes can, for example, be coated by self-assembly with thiolated adhesion materials. The challenge with this approach is the alignment of the microelectrodes with the microfluidic structures. Soft lithography is no longer suitable and instead classical photolithography processes for polymer (e.g., SU-8) and metal patterning is recommended. The additional fabrication costs are justified by the returns in the scale and quality of the data.

In addition to electrophysiological methods, the microelectrodes can be reconfigured for localized electrochemical sensing of the secretions from single cells or small groups of cells. Here, the charge transfer signatures of neurotransmitters such as serotonin and dopamine [28, 29], as well as electroactive nitric oxide and

oxygen species can be quantitatively measured in real time using amperometry [30, 31]. Importantly, electrophysiological and electrochemical measurements can be undertaken using the same electrode for recording both electrical and chemical signals from the same target [32].

9 Concluding Remarks

Cooperation at the interface between engineering and neuroscience has led to the development of the minimalistic neuronal network and open access electrophysiology platforms. These platforms extend the scientific reach of the neuroscientists by making compartmentalized electrophysiological and electrochemical measurements feasible, by enabling single cell assay precision and by providing a high throughput perspective with the possibility to generate big data sets from a single animal. Ongoing engagement will ensure that technical developments continue, arming the biologists with the custom-designed systems necessary to understand the workings of the brain and aid the discovery of therapeutic agents that prevent the intra- and inter-neuronal spread of disease.

10 Notes

1. GeSiM (http://www.gesim.de/) is one of many companies that offer low volume SU-8 wafer fabrication. Other companies can be located via the FluidicMEMS website: http://fluidicmems.com/list-of-microfluidics-lab-on-a-chip-and-biomems-companies/.
2. High resolution photolithography masks can be purchased from a number of companies, such as JD Photo-Tools (http://www.jdphoto.co.uk/).
3. The uncured PDMS pre-polymer has a honey-like viscosity often leading to minor spills that are difficult to remove from surfaces. Without care PDMS can easily spread to contaminate the laboratory surfaces and equipment. We recommend having tissue wipes on-hand throughout the PDMS preparation process to immediately remove these spills.
4. The use of laboratory tissue prevents particulates being directly sandwiched between the silicon wafer and hotplate which can pose a wafer fracture risk.
5. Polymer frames can be mechanically or laser machined in the workshops of most institutes. High melting temperature polymer (e.g., Delrin) frames are recommended. A maximum of four framed devices can be molded with the current 4″ wafer

design. Alternatively the entire wafer can be molded using a frame fashioned from aluminum foil.

6. A plastic pipette tip can be used to test the cure quality in the corner of the device (i.e., away from areas requiring imaging).
7. It is more difficult to remove hot/warm PDMS molds from the SU-8 wafer. Again this poses the risk of wafer breakage.
8. Take care to use the scalpel away from the brittle microstructured SU-8 regions.
9. Coating the entire wafer with a film of PDMS is useful for protecting the SU-8 features during storage and also for removing any particulates and PDMS deposits remaining on the wafer surface from prior use.
10. Biopsy punches can be used repeatedly to prepare inlet/outlets with minimal particulates. However, after repeated use they become worn and should be replaced to prevent the production of excessive particles.
11. To aid alignment of the ports with the microfluidic features it is recommended to work on a dark surface in good lighting. Alignment is also aided by punching from the microstructured side.
12. PDMS available from Wacker (Elastosil® RT 601) is significantly easier to detach than Sylgard PDMS. In addition, hydrophobic bacteriological grade Petri dishes which are more flexible than cell culture Petri dishes should be used. Both features significantly reduce the risk of coverslip fracture during removal.
13. When using a scalpel to remove the coverslips coated with the thin sheet of PDMS always wear eye protection as there is a risk of either the coverslip or the blade breaking.
14. Handheld plasma/corona discharge devices are inexpensive (a few hundred euros). These should be operated in a fume hood to remove ozone and other atmospheric radicals.
15. Suitable stainless steel pins are available from all good needlework departments.
16. Excessive plasma treatment evaporates the water mask producing unwanted polyamine loss. Insufficient plasma treatment can be identified using fluorescently labelled polyamines available from Sigma-Aldrich.
17. The in situ biomaterial patterning technique can be used to pattern other adhesion molecules such as N-cadherin [33] for the realization of "axon-free" dendrite zones and the investigation of dendritic responses to distal stimulation [34–36].
18. Inexpensive flow regulators can be purchased from eBay and other suppliers.

19. Care must be taken to avoid trapping air bubbles between the two glass slides. This issue means that it is especially important to thoroughly degas the PDMS before molding.
20. Suitable O-rings are available in most hardware stores.
21. When peeling away the positive PDMS mold care is required to avoid damaging the delicate, 560-μm-wide barrier.

Acknowledgements

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Part II

Axonal Guidance and Manipulation

Chapter 4

Asymmetric Genetic Manipulation and Patch Clamp Recording of Neurons in a Microfluidic Chip

Prasanna Sakha, Cecilia Brunello, Joonas Heikkinen, Ville Jokinen, and Henri J. Huttunen

Abstract

Studying the formation and function of neuronal circuitry is complicated by the heterogeneity and high density of neuronal processes and synapses. Compartmentalized cell culture systems offer a simple yet powerful solution for isolation of axons from dendrites and cell somas. This chapter describes how to manufacture and use a microfluidic chip with a modular design for highly defined isolation of axons, asymmetric genetic manipulation, and whole-cell patch clamp recording. The microfluidic chip consists of detachable and resealable layers that allow multiple modes of operation during cell culture, fluidic isolation for limited transfection, and recording with low-angle electrode access. This versatile technique is useful for functional studies that require specific expression of for example optogenetic tools in presynaptic neurons or for studying the entry of pathogenic particles, such as viruses or oligomers of misfolded proteins, into presynaptic structures.

Key words Compartmentalization, Electrophysiology, Microfluidics, Axons, Axonal isolation, PDMS, Patch clamp, Virus, Neuronal circuitry, Optogenetics

1 Introduction

Rapid development of compartmentalized culture systems has allowed highly refined control of neuronal connectivity in neuronal cultures. The most common way to achieve microscale compartmentalization is by using microfluidic chips that allow axons but not neuronal cell somas to pass along microgrooves (typically in the range of 3×7 μm). This allows isolation of cell compartment-specific materials (e.g., axon-specific mRNA and protein species) [1, 2] and dynamic cell biological studies (e.g., kinetic studies of axonal transport of vesicular and organelle cargo) [3–7]. Recently, also electrophysiological recordings have been incorporated into the compartmentalized culture platforms [8, 9]. The most common way to record from neurons utilizes an upright patch clamp setup, which sets strict spatial requirements for

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neuronal cell culture systems. Moreover, asymmetric genetic manipulation of presynaptic and postsynaptic neurons would allow sophisticated studies of synaptic function, and uptake and propagation of materials. We have recently developed a microfluidic culture system that is compatible with standard patch clamp setups and allows for example specific presynaptic expression of optogenetic genetic tools [10]. This chapter will provide detailed instructions on (1) how to manufacture a microfluidic chip for isolation of two populations of neurons while allowing their axonal connectivity and access of electrodes, (2) how to utilize fluidic isolation for asymmetric genetic manipulation of the two interconnected but isolated populations of neurons, and (3) how to perform patch clamp recording of neurons grown in this microfluidic device.

2 Materials

2.1 Master Fabrication

Making of a SU-8 epoxy photoresist master for the polydimethylsiloxane (PDMS) chips requires the use of a clean room. The required clean-room equipment includes a spin coater, a hot plate, a mask aligner, and a plasma phase deposition tool for an antiadhesion coating. Chemicals required are SU-8 5 and SU-8 100 photoresists and developer (Microchem), isopropanol, and a photomask (Compugraphics).

2.2 PDMS Microchip Fabrication

Materials required for fabricating microfluidic chips include the master mold, which contains the negative of the desired chip, and the PDMS elastomer kit. The most commonly used PDMS kit is the Dow Corning Sylgard 184 kit, which contains both the PDMS prepolymer and the cross-linking agent (*see* **Note 1**). In addition to these, a vacuum desiccator makes the process faster but is not necessary.

2.3 PDMS Microchip Cleaning

PDMS microchip cleaning requires an ultrasonic bath and isopropanol (*see* **Note 2**).

2.4 Neuronal Culture

Poly-L-lysine (PLL) solution (Sigma) provided as 0.01 % solution in water is used undiluted for coating glass coverslips. Full Neurobasal media is prepared from plain Neurobasal (Invitrogen) supplemented with 2 % B27 supplement (Invitrogen), 0.5 mM L-glutamine, and 1 % (v/v) penicillin and streptomycin (Lonza Biologics).

2.5 Electrophysiology

Artificial cerebrospinal fluid (ACSF) should contain (mM) 110 NaCl, 5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgSO_4$ prepared in Milli-Q water (pH 7.4 using 5 NaOH, 250 mOsm) (*see* **Note 3**). For rat hippocampal neurons (RHN) grown in full Neurobasal media (NB), osmolarity of culture media could range between 250 and 254 mOsm. Molarity and osmolarity of ACSF and neuronal culture media should be closely comparable.

Potassium based intracellular filling solution (ICS) for glass electrodes should contain (mM) 115 KCl, 10 HEPES, 5 EGTA, 5 MgATP prepared in Milli-Q water (pH 7.2 with KOH, 240 Osm). Cesium based ICS should contain (mM) 115 Cs-methanesulfonate, 10 HEPES, 5 EGTA, 5 MgATP, 5 QX314 (Cl Salt) prepared in Milli-Q water (pH 7.2 with 1 CsOH, 240 Osm) (*see* **Note 4**).

Electrophysiological setup consists of recording Chamber (Luigs and Neumann, slice mini chamber I) mounted with Olympus BX51 fluorescence microscope and connected to multi-Clamp700B amplifier (Molecular Devices). OptoLED light source (Cairn Research Ltd., UK) is custom-fitted to fluorescence microscope for blue light stimulation (470 nm).

Ag/AgCl wire is used as an electrode.

3 Methods

3.1 Master Fabrication

The SU-8 master is fabricated through a two-layer UV-lithography process shown in Fig. 1. The basic fabrication steps are given below and more details can be found from ref. [10].

1. Spinning SU-8 5 for 30 s at 5,000 rpm for 3 μm axonal isolation channel layer.
2. Soft bake on a hot plate, 5 min at 95 °C.
3. Exposure of the first layer using the channel mask.
4. Post-exposure bake on a hot plate, 5 min at 95 °C.
5. Development in SU-8 developer under soft agitation for ≈3 min. Rinsing with isopropanol.

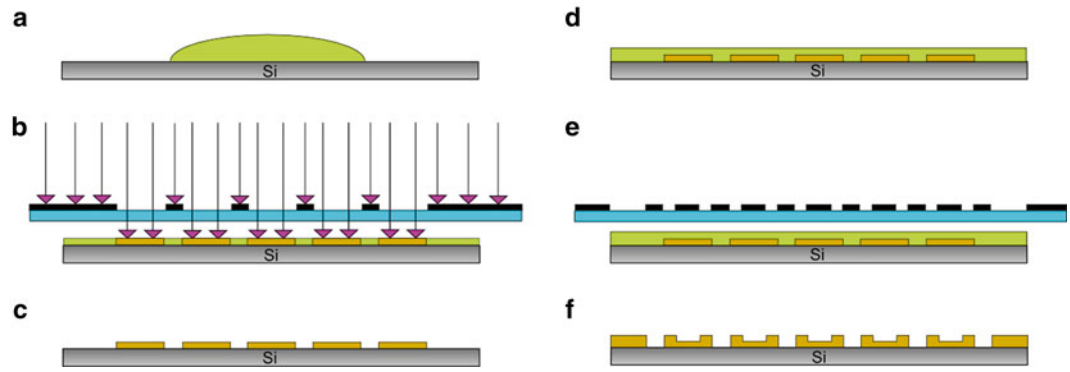

Fig. 1 Fabrication steps for SU-8 master on top of silicon. (**a**) The first layer of SU-8 is spun and soft baked. (**b**) Photoresist exposure with UV light through channel mask. (**c**) Structures on wafer after development and post-exposure baking. (**d**) New layer of photoresist is spun on top of the wafer in order to get structures for reservoirs. Ramped soft bake is needed in order to avoid cracking or buckling of resist. (**e**) Mask for reservoirs must be aligned. (**f**) After final post-exposure bake, development and application of an antiadhesion coating, the silicon master is ready for use

6. Spinning SU-8 100 for 30 s at 800 rpm for a 500 μm reservoir layer.
7. Slowly ramped soft bake: 25 min at 65 °C followed by 150 min at 95 °C.
8. Aligned exposure of the second layer using a reservoir layer mask (*see* **Note 5**).
9. Slowly ramped post-exposure bake on a hot plate, 40 min at 95 °C (*see* **Note 6**).
10. Development for ≈30 min. Rinsing with isopropanol.
11. Application of an antiadhesion coating (*see* **Note 7**).

3.2 PDMS Microchip Fabrication

The procedure for fabricating microfluidic chips out of PDMS through replication molding is presented in Fig. 2. The molding process can be done in many ways, but the main idea stays the same: PDMS is cured on top of master mold, and therefore, the patterns of master mold are transferred into structures of PDMS.

The process starts from preparing the master for molding by attaching it to a flat surface. Next, PDMS prepolymer and cross-linking agent mixture is poured on top of the master, degassed, and allowed to level off before curing. Curing the PDMS solution

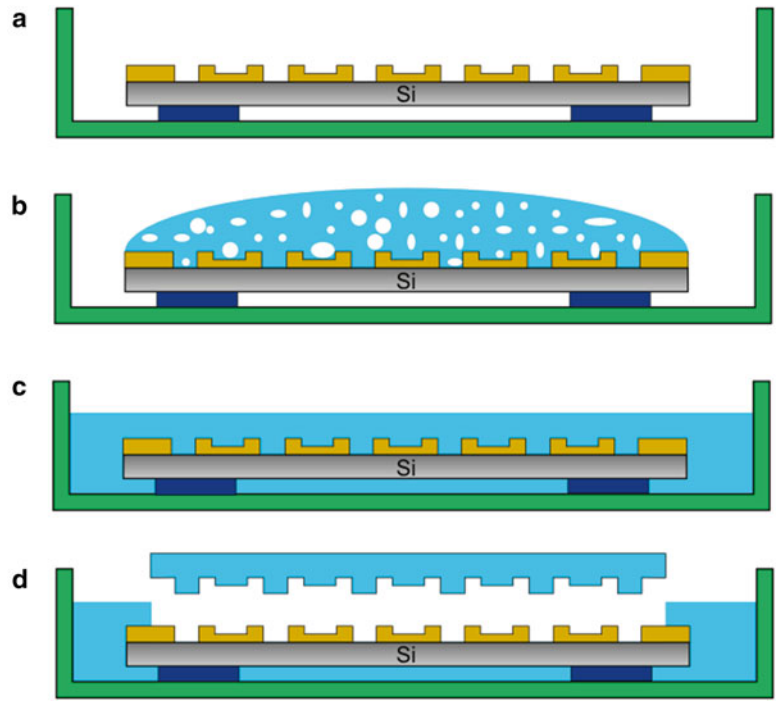

Fig. 2 Process for master molding of PDMS. (**a**) The master is attached to petri dish with tape. (**b**) PDMS prepolymer is poured on the master without degassing. (**c**) Planarized and degassed PDMS ready for curing. (**d**) Cured PDMS is cut and peeled from master. Extra PDMS is left on the dish to fill unusable space

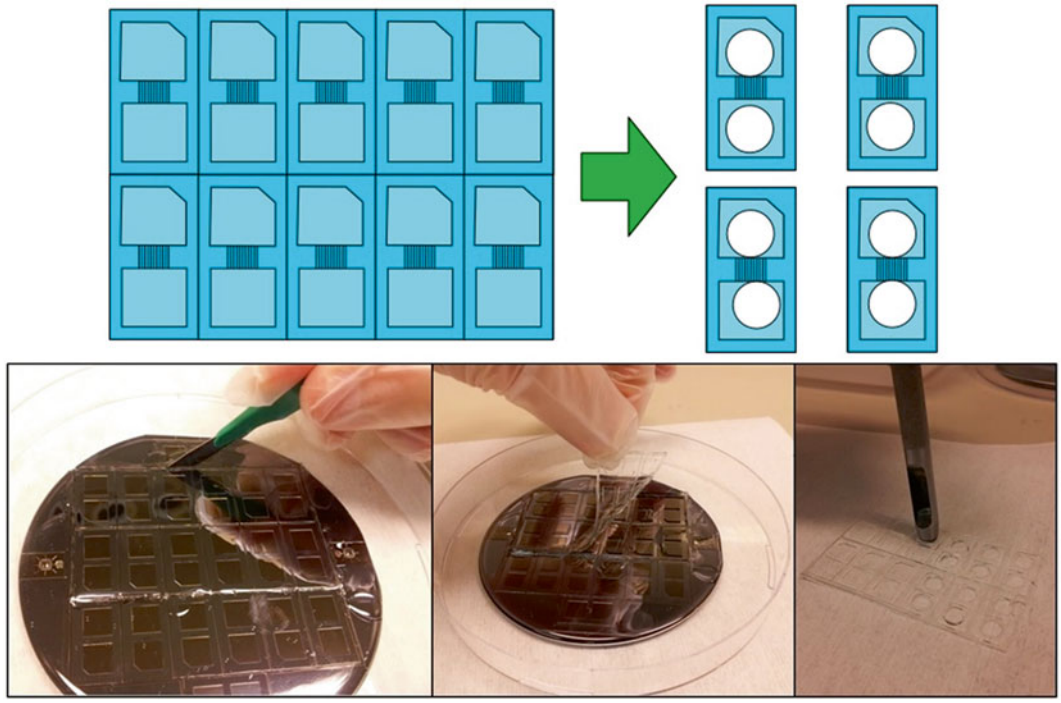

Fig. 3 Processing cured and solid PDMS. *Upper picture* shows schematic what should be done after curing. *Lower picture* shows steps of cutting PDMS from master, peeling it off, and punching holes for reservoirs

polymerizes the mixture by cross-linking and therefore the PDMS solidifies. Curing is usually done in an elevated temperature in an oven placed in a room with proper ventilation. After curing solid PDMS can be cut, peeled and diced, as shown in Fig. 3. As a step-by-step guide the whole process goes as follows:

1. Attach the master mold to flat surface of a container like petri dish (*see* **Note 8**). Light pressure is enough when attaching, because silicon wafers tend to break easily.
2. Sylgard 184 Silicon elastomer kit contains two components for making PDMS, prepolymer and curing agent. Mix the prepolymer and curing agent with ratio of 10:1 (w/w) (*see* **Note 9**).
3. Degas the mixture with vacuum desiccator for 80 min (*see* **Note 10**).
4. Cast the degassed mixture on top of the master mold. Care must be taken when pouring PDMS not to create any new air bubbles at this point. If bubbles are formed, they can be removed with a pipette or further degassing in a vacuum (*see* **Note 11**).
5. Cure PDMS in oven at 50 °C for 2 h (*see* **Note 12**). The curing stage of PDMS can be tested by touching it with for example a scalpel. When properly cured, PDMS can be sticky, but it should not be liquid-like.

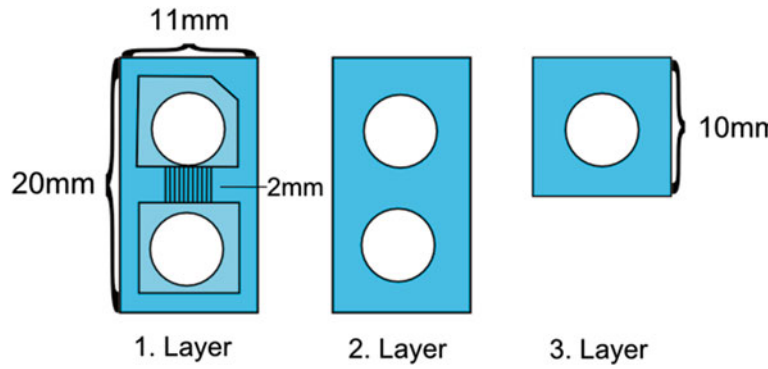

Fig. 4 The different layers of the microfluidic chip. Layer Thicknesses: (*1*) Layer: 1 mm, (*2*) Layer: 5–7 mm, and (*3*) Layer: 2 mm. The axon isolation channels on the first layer number 34 individual channels (height 3 μm, width 7.5 μm, length 2 mm)

6. Cut the desired part of PDMS with a scalpel or a knife (*see* **Note 13**). This is done by cutting all of the chips at the same time (*see* Fig. 3).
7. Carefully peel the cut piece of PDMS from the master. PDMS between the chips is quite thin, so great care must be taken not to tear the PDMS "carpet".
8. Cut the PDMS piece into microfluidic chips as shown in Fig. 3. This should be done on a clean surface and preferably channel side up.
9. Punch the holes for reservoirs (*see* **Note 14**). Cutting and punching creates particles on top of the PDMS surface and in the channels, therefore cleaning (*see* next section) should follow these steps.

The final modular PDMS chip will contain three layers. The first layer is the one with channels and therefore needs the master molding technique described above. The second and third layers have no micro feature, and they are fabricated simply by punching and cutting from plain PDMS. The thicknesses for the layers are ≈1 mm for the first layer, ≈5 mm for the second layer and ≈2 mm for the third layer. Dimensions of the layers can be seen in Fig. 4. Holes for reservoirs should be located approximately same place in every layer. This way when assembling the chips, one can avoid leakage (*see* **Note 15**).

3.3 PDMS Microchip Cleaning

After the fabrication process, the PDMS chips are often covered with PDMS debris and other contaminating particles. In order for the waterproof sealing property of PDMS to work properly, the chips need to be cleaned. Figure 5 shows examples of a properly and improperly cleaned microfluidic PDMS chips placed on top of a

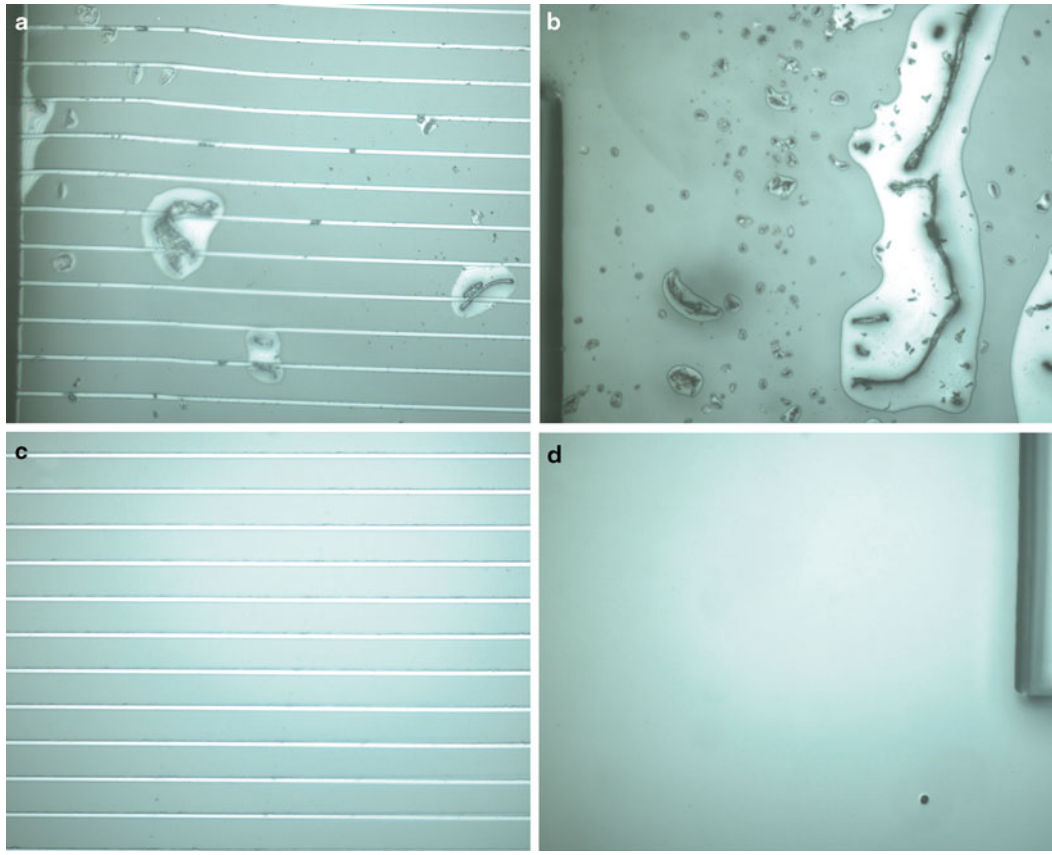

Fig. 5 Comparison between dirty and cleaned PDMS–glass interface. (**a**) Dirty channels with particles. *White areas* show lack of adhesion. (**b**) Particles outside channel area. (**c**) Channels after isopropanol cleaning. (**d**) Clean PDMS–glass interface outside channel area

glass slide. The preferred method for cleaning PDMS chips is ultrasonication in isopropanol as explained below (also *see* **Note 16**).

1. Immerse the PDMS chip in isopropanol in a container and ultrasonicate (*see* **Note 17**). The required time depends on the ultrasonicator type, but long baths (1–2 h) are preferred.
2. Dry the chips after ultrasonication by baking the chips in an oven at 50 °C or higher overnight (*see* **Note 18**).

3.4 Chip Pretreatment and Assembly

3.4.1 Cleaning and Recycling of the Chips

On the day before plating the neurons (Day 1), wash the chips in fresh 96 % ethanol. The chips can be placed in a 50 ml Falcon tube on a Gyro rocker at medium/high speed overnight. During the washes the liquid has to be clear and free of debris. PDMS microfluidic chambers can be reused up to five times, or until the sticking properties of PDMS are maintained. Once the chips are disassembled, all of the PDMS layers should be stored in 96 % ethanol and washed at least three times on a Gyro rocker before

reusing them. If the chambers have been used several times already, several washes are recommended, discarding the old ethanol and replacing it with fresh one every time.

3.4.2 Coverslip Coating (Day 1)

1. Sterilize glass coverslips in fresh 96 % ethanol for at least 30 min. For the size of chips described in Sect. 3.2, the most suitable size for square coverslips is 22 × 22 mm (*see* **Note 19**). Following this step, coverslips should be handled in a laminar flow hood in order to ensure sterile conditions.
2. Put the coverslips in a 10 cm cell culture petri dish and rinse with Milli-Q water three times to remove all ethanol. Gently shake the plate in order to allow proper distribution of the liquid. Add poly-L-lysine (PLL) 0.01 % (w/v) on the same plate in the amount necessary to cover all the coverslips, after removal of the last washing Milli-Q step. Next, place the plate in a cell culture incubator (5 % CO_2, 37 °C) for at least 6 h. PLL can be recollected and reused for up to ten times, if properly stored in +4 °C according to manufacturer's instructions, and in sterile conditions.
3. After coating, wash the coverslips three times with Milli-Q water, shaking the plate each time (*see* **Note 20**). It is important to let the coverslips to dry perfectly before assembling the chips. The easiest way to do this is to let them dry on a sterile surface (e.g., leaning vertically on the cell culture plate wall; *see* **Note 21**).

3.4.3 Assembly of the Chips for Cell Culture (Day 2)

On the day of plating, after overnight washes of the PDMS chips on the rocker, the microfluidic culture chips are ready to be dried and assembled. The chips should always be manipulated with sterile forceps and the following ethanol washes should be handled in a laminar hood.

1. The drying process takes about 1.5 h: place the chambers on a sterile surface leaning against the wall of the cell culture dish and flip them over after 30–45 min to ensure proper drying of both sides. It is very important that both glass coverslips and PDMS chambers are perfectly dry at the moment of assembly, so that the sticking properties are not compromised.
2. The thinner and lowermost layer of the chip is the one containing the tunnels, which have to be in contact with the glass (be careful to place the chips the channel sides down, meaning in direct contact with the coverslip). Make sure that the PDMS sticks to the coverslip by gently pressing with tweezers and that there are no air bubbles within the interface.
3. Continue assembling the second and the third layer as shown in Fig. 6b, c, slightly pressing the corners to ensure proper sealing of PDMS, avoiding touching the central part in order not to

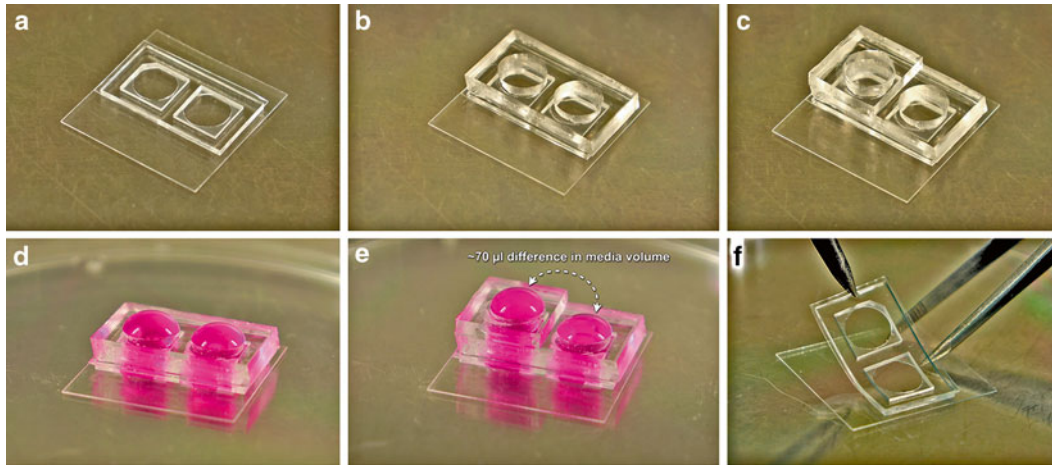

Fig. 6 Assembly of the chip. Correct assembly of the first (**a**), second (**b**), and third (**c**) PDMS layers on the coverslip. Chip in culturing conditions without (**d**) and with (**e**) the third layer. Demonstration of how to remove the first layer by peeling off with two pairs of forceps (**f**)

compress the tunnels. Marking one of the sides of the coverslips with permanent pen might help as future reference to identify one of the two somatic reservoirs. Each assembled chip on a coverslip occupies one well of a standard 6-well cell culture plate. When dealing with low volumes of media, it is advisable to leave 1–2 of the wells (of the 6-well plate) to be filled with water to limit excessive evaporation and to place the plate on the bottom shelf of the incubator, if it is provided with a fan.

4. Fill one of the reservoirs with 200 µl of full Neurobasal media, and let the media diffuse into the tunnels for about 5–10 min. Fill then the second reservoir as well (200 µl).
5. Incubate the chips for at least 30 min in a cell culture incubator before plating the neurons. During this time, it is also possible to assess whether the chips have been assembled properly and that media is not leaking out of the assembled chips. If so, fluidic isolation is not ensured and further manipulations are not advisable.

3.5 Neuronal Culture on Chip

3.5.1 Plating of Neurons (Day 2)

Up to 25,000 hippocampal or cortical neurons can be plated per reservoir: cell suspension should be prepared so that the final volume does not exceed 30 µl.

1. Remove 150 µl of pre-incubated media from chips and add cell suspension, carefully pipetting up and down to allow homogeneous distribution of the neurons within the reservoir. Repeat the same procedure for the second reservoir, if the experiment requires neurons to be present on both sides of the chip (*see* **Note 22**).

2. At this point, it is important to let the neurons to acclimatize in the incubator for a maximum of 10 min before adding the rest of the media.
3. +37 °C full Neurobasal media can be then added 150 μl per reservoir. Asymmetrical volume difference that ensures fluidic isolation of one of the two reservoirs is reached by adding extra 70 μl on the side where the third PDMS layer has been placed (Fig. 6e; *see* also Sect. 3.4.3, *see* **Note 23**).

3.5.2 Neuronal Culturing (Day 6 Onward)

Neuronal culture on a microfluidic chip is not much different compared to conventional technique of dispersed neuron cultures. Nevertheless, a few considerations have to be taken into account.

First of all, the evaporation rate can affect the culturing conditions since the media volumes are quite small. As a general guideline, media should be changed every 3 days (DIV3, DIV6, DIV9, DIV12, etc.) by removing two thirds of the total volume and replacing with a bit extra volume to compensate evaporation. This means that 130 μl out of 200 μl are taken away and 160 μl of fresh warm full Neurobasal are replaced into each reservoir.

In order to keep osmolarity as stable as possible during culturing, it is recommended to remove part of the old media any time the media has to be changed. This recommendation stands even if the evaporation rate has been higher than normal after the 3 day cycle so that the volume left is significantly decreased compared to the typical case.

Moreover, for DIV15 (and older) cultures, less media should be removed when changing the media, since mature neurons get more stressed with high frequency media changing and large volumes of fresh Neurobasal. Full Neurobasal can be stored in +4 °C with the cap properly closed to ensure sterility up to 1 week, while freezing is not recommended.

During culturing, it is important to monitor if chambers are leaking. Media leakage can occur both between the glass and the first PDMS layer and between the first and the second PDMS layers. In the first case, media can flow outside the chip in the rest of the well or, if the tunnels are not perfectly sealed to the glass, neurons can grow outside the area of the reservoir. In the second case the media can either flow out of the chip or interfere with the fluidic isolation. The latter case can be easily identified during media change, whenever a very fast passage of media added to a reservoir to the other one occurs.

3.5.3 Asymmetrical Culture

Introduction of media volume difference between two reservoirs of this chip can create a characteristic variation in the Laplace [11] and the hydrostatic pressures [12] of the reserves. The hydrostatic pressure is always higher on the side with more media while the Laplace pressure given the volumes of the reservoirs and the

recommended amounts of liquid on each side is also higher (or the same) on the side with more media. Thus, in this chip containing two separate cell culture reservoirs connected by 2 mm length of 34 separate tunnels, the balance of these two forces creates an inimitable environment for specific asymmetric genetic (or pharmaceutical) manipulation [10].

1. Maintain media difference as early as DIV3 in the cultures. Excess media of 70 μl in the reservoir with the third PDMS layer should be sufficient to create asymmetric fluid isolation (Fig. 7a) (*see* **Note 24**).
2. To check the specificity of viral transduction, infect RHN cultured on both reservoirs with lentiviruses expressing fluorescent proteins, such as GFP or DsRed. For example, viral transduction is done at DIV3 with pLenSyn1/GFP and pLenSyn1/DsRED viruses (GFP/DsRed expression limited to neurons only by the Synapsin-1 promoter) in the reservoirs containing lower and excess volume of media, respectively. Fixation and analysis at DIV 15 shows that GFP-infected reservoir has few neurons that have been transduced with the DsRed virus, while the DsRed-infected side does not have any GFP-expressing neurons (Fig. 7b). These cross-infections are mostly seen at the vicinity of axonal tunnel openings (*see* **Note 25**).
3. In order to obtain specific genetic manipulation using lentiviruses, it is encouraged to only infect the reservoir containing lower media volume (Fig. 7c). In case a virus vector without a fluorescent marker needs to be used, optimized immunostaining protocol should be used for visualization of the virally expressed proteins in the fixed samples.
4. Maintaining the media volume difference is essential throughout the culture period. The third PDMS layer also reduces the risk of media cross-spillage between the two cell reservoirs. Final volume of media between two reservoirs should have asymmetrical volume difference even after each routine change of media (*see* **Note 26**).
5. Avoid media leakage from the chip during these experiments. The occurrence of leaky chambers can be minimized by assembling the chambers well enough to form a proper seal between the stacked PDMS layers.
6. Check for internal media leakage between the reservoirs every time when changing the media. First, remove media from both reservoirs, then add a required volume of media in one of the reservoirs (preferably to the side containing lower media volume) then wait for 10–30 s. Then, add a required volume of media to the other reservoir before placing the plate back to the incubator (*see* **Note 27**).

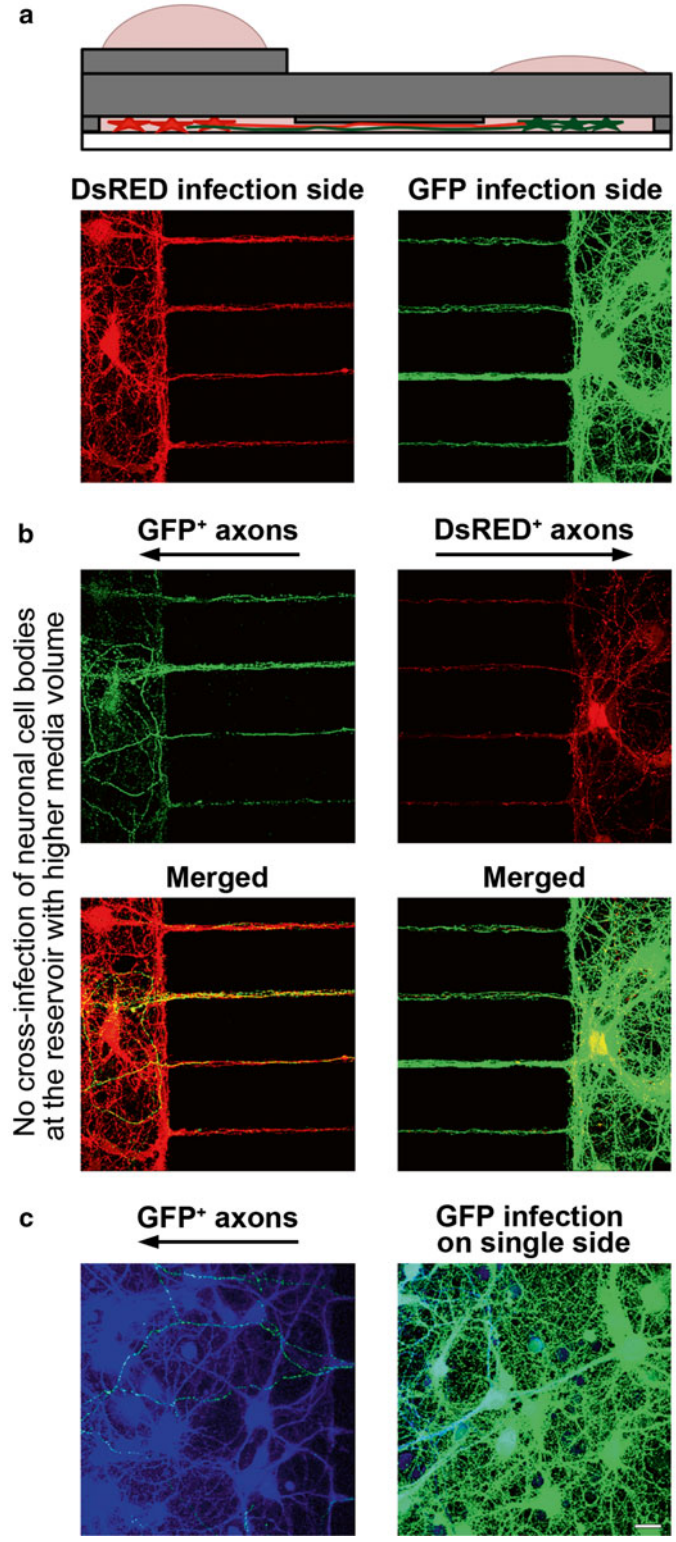

Fig. 7 (**a**) Fluidic isolation by asymmetric media volume between the two reservoirs. (**b**) Rat hippocampal neurons transduced with DsRed and GFP lentiviruses in the reservoir containing excess (*left*) and low (*right*) media volume, respectively. DsRed- and GFP-expressing axons grow via microgrooves to the neighbor

3.6 Cell Fixation and Chip Disassembly

3.6.1 Fixation with 4 % Paraformaldehyde

Optimal timing for fixing the cells is at around DIV15–DIV16. Axons are fully grown in the tunnels already at around DIV10 (with chips using 2 mm tunnel length). Also, neuronal cultures on microfluidic chips do not last in general as long as normal dispersed cultures. The multilayered chambers can be disassembled either before or after fixation, but doing it before fixation can cause damage to the neurons, and to the axons in particular, which can be easily disturbed by even small horizontal movement of the tunnel walls at the interface of PDMS and glass. Fixation is typically done with 4 % paraformaldehyde (PFA) in PBS, but other methods may also be used. For PFA fixation, proceed as follows. Samples do not have to be handled in a laminar hood anymore at this stage but for safety reasons, step 1 can be done in a fume hood. The fixation protocol in 4 % paraformaldehyde is:

1. Remove most of the media from both reservoirs, so that one-third of the media is left on the neurons to protect them from drying and damage caused by pipetting. Add 1 ml of 4 % PFA in PBS or more to the chambers, without worrying about the overflow.
2. Incubate the neurons for 30 min at room temperature.
3. Following removal of the PFA solution, carefully rinse the chambers a couple of times with PBS before chip disassembly.

3.6.2 Chips Disassembly

Two pairs of forceps should be used to disassemble the microfluidic chips (Fig. 6f). First, remove the top and the middle PDMS layers, being careful not to move the bottom layer. Particular attention should be paid when removing the bottom layer, as it is very easy to damage the axons. With one pair of forceps, hold the coverslip trying to keep it as steady as possible, while using the other pair of forceps to peel one of the short sides of the PDMS layer. It is important to slow upward movement in a longitudinal direction for detachment of the PDMS layers, as shown in Fig. 6f.

3.6.3 Processing of the Fixed Samples

The coverslips can be then processed as any other fixed sample, after three 15 min washes with PBS. A bit more attention should be paid during PBS washes, as it should not be added directly on the cells or from close proximity. In the same way, if using Gyro rocker, the speed should not exceed 15 rpm, as even fixed axons can easily detach.

Fig. 7 (continued) reservoir (growth direction indicated by *arrows*). Cross-infection of neuronal soma with DsRed virus in reservoir containing low media volume (*right*), while reservoir with excess media volume does not contain GFP infected soma (*left*). (**c**) Unidirectional transduction of GFP virus in a reservoir with low media volume. Only GFP-expressing axons enter to uninfected reservoir (containing excess media), without cross-infecting neurons on the uninfected side. Uninfected neurons (*left*) were immunostained with microtubule-associated protein-2 (MAP2) with Alexa Fluor 405 conjugated secondary antibody. Scale (*white bar*): 25 μm

3.7 On-Chip Electrophysiology

This protocol is applicable for a standard commercially available electrophysiological setup with an upright microscope for visual guidance and custom-fitted for blue light transmission (470 nm) from OptoLED device (Cairn Research Ltd) for optogenetic experiments. However, the general conceptions of this protocol can be applied with any electrophysiological setup. In addition, different laboratories may have varying procedures for gaining whole-cell patch clamp access, but the specific details described here can be beneficial for anyone using this chip for electrophysiological recordings.

3.7.1 Prerecording Preparations

1. Electrophysiological recording from cultured neurons can be done between week 2 and 3 after a functional network of neuronal connections has been established. Plate RHN on both reservoirs of the fully assembled chip (one reservoir having three PDMS layers; Fig. 6e). For optogenetic EPSC (excitatory postsynaptic current) recordings, asymmetric transduction with lentivirus coding for YFP-tagged channel rhodopsin (ChR2; with H134R mutation) [13] is required. In practice, this means that only one of the reservoirs (containing lesser media volume) is transduced with the lentivirus. This system allows studying the functional connectivity of light-activatable axons that enter the reservoir containing the uninfected neurons.
2. Recording chamber of the electrophysiology setup should be pre-warmed and perfused with warm ACSF before the cultured neurons are placed into it. It is recommended to maintain recording temperature lower than + 37 °C to minimize excessive spontaneous activity of neurons. Setting up desired temperature for neuronal culture recording however varies with the objective of the experiment.

3.7.2 Chip Transformation from Neuronal Culture to Electrophysiology Mode

1. Remove approximately ¾ of culture media from both reservoirs and gently detach the top two PDMS layers with forceps. The bottom PDMS layer containing the axonal tunnels should remain attached to the coverslip with approximately 30–40 µl of culture media to prevent immediate drying of cultured neurons (*see* **Note 28**). Store the top two PDMS layer in 96 % ethanol (*see* Sect. 3.4.1).
2. Handling the coverslip (with the cultured neurons and the remaining bottom PDMS layer) requires precision since the glass coverslip tends to stick to plastic surface (of the 6-well plate) (*see* **Note 29**).
3. Neuronal samples should be transferred into the ACSF-perfused recording chamber as quickly as possible. This entire transfer time should not exceed 3–4 min.
4. Visual assessment of neurons and their processes before any recording session is essential. Excessive blebbing of axons is an

indication that cultures are not healthy. Presence of excessive debris or extensive detachment of cell somas and neurites is another indication that cell viability is low, and that the particular chip should be discarded (*see* **Notes 30** and **31**).

3.7.3 Whole-Cell Patch Clamp

After placing the sample into recording chamber of the electrophysiology setup, procedure for whole-cell access includes: applying pressure to the pipette and offsetting the electrode (Ag/AgCl electrode) once it touches ACSF in recording chamber, checking electrode resistance, directing the electrode to cell membrane of a neuron, offsetting the electrode once again at the level of cell surface, touching the cell membrane, releasing pipette pressure when resistance change is seen, holding the cell at −70 mV, gigaseal formation, capacitance compensation, and breaking the seal to gain whole-cell access.

Cell Identification and Gigaseal Formation

In dispersed hippocampal cultures, pyramidal cells can be visually defined based on their shape and membrane properties. Neurons might also be clumped together, thus appearing rounder (*see* **Note 32**). Both glutamatergic and GABAergic activity can be observed during spontaneous activity recording from cultured neurons in this chip [10], so it is essential to distinguish pyramidal from non-pyramidal cells.

Membrane properties of RHN (resting membrane potential, input resistance, etc.) can vary in dispersed cultures depending on the age of the culture and the state of cell during whole-cell access. For neurons cultured in this chip, typical resting membrane potential can vary between −20 and −55 mV (Recommended further reading: [14–17]).

Suitable electrode resistance is essential for patching cells correctly. For example, if electrode resistance is low (3–4 MΩ), risk of electrode tip suctioning the cell completely from coverslip might increase, and if it is high (7–9 MΩ), the sharp tip of the electrode might penetrate the cell and suction the nucleus. For the latter reason, try to find pyramidal cells with clear cytoplasmic demarcation between cell membrane and nucleus. In order to maintain membrane properties (e.g., access resistance) relatively constant, it is advisable to use electrodes with the similar resistance range for a set of experiments. Electrode resistance of 5–8 MΩ should be adequate for patching RHN cultured in the chips described in this chapter.

Orientation of cell surface and the angle of the glass electrode touching the cell can be a crucial factor for getting a good gigaseal. The tip of the glass electrode and surface curvature of the neuron should be on a same plane, and importantly, the electrode should touch the cell surface very gently without causing excessive deformation of cell membrane.

Finally, release the pipette pressure and apply gentle suction simultaneously while touching the cell membrane. This should be adequate to gain a gigaseal. However, if gigaseal does not form immediately, wait for at least 30 s to check the state of seal formation and then apply another very gentle suction and hold into it by closing the pipette valve. Once the gigaseal is formed, open the valve to release the pressure.

Whole-Cell Access and Electrophysiological Recording

1. After formation of stable gigaseal, apply a gentle quick suction, wait for 3–10 s, and apply another quick suction but with slightly greater force than the previous one. Usually, whole-cell access can be gained within the first few trials. Check the membrane properties of the neuron after gaining whole-cell access.
2. To observe action potentials (AP), obtain a stable current clamp recording at –70 mV (±3 mV) by applying constant current between 20 and 100 pA (without exceeding ±200 pA, otherwise patched neuron would become leaky and responses would be compromised). Step depolarization of 20–100 pA with 10–20 pA interval, 800 ms duration and 5 s sweep interval can be applied to evoke a train of AP. Frequency of AP firing should increase incrementally with injected current during step depolarization and traces should show characteristic spike adaptation pattern. For example, RHN grown in this chamber showed AP frequency of 16.66 ± 1.13 Hz at 60 pA depolarization [10].
3. Spontaneous activity can be also recorded from the same cell in voltage clamp mode at –70 mV together with any pharmacological studies (*see* **Note 33**).

Optogenetic Stimulation for EPSC Recording

For optogenetic experiments, it is essential to first determine the transduction efficiency of ChR2 viruses, as using excess amount of virus may cause neuronal toxicity. Getting a 100 % transduction would assure that light-activatable channels are expressed in every neuron and also throughout the neuronal processes. Determination of viral titter can be carried out since different batches of virus may have different transducing units (*see* **Note 34**). A convenient way to determine transduction efficiency is to check the rate of transduction (of ChR2-YFP lentivirus) from a fixed, dispersed neuronal culture under a fluorescent microscope. In addition, depolarization responses evoked by blue light in virus-infected neurons should be tested. In voltage clamp mode, using Cs-based electrode-filling solution, the blue light-induced current responses can vary from 50 pA to over 400 pA. A step-by-step guide for the optogenetic stimulation and EPSC recording is presented below.

1. Patch a pyramidal neuron from the uninfected reservoir and obtain stable whole-cell access in voltage clamp mode (–70 mV). Channel rhodopsins are light sensitive, so infected

samples should be exposed to minimum amount of light. However, for visual guided patch system certain degree of light exposure is inevitable. It is advisable to turn off all light sources after getting the gigaseal, and to wait for 30 s to stabilize the cell in dark before breaking it open.

2. Excessive spontaneous firing of the cell might be reduced if recording is done at +30 °C and age of the culture does not exceed 3 weeks. Cs-based ICS should be used as filling solution in the glass electrode.
3. Duration of blue light pulse for optogenetic stimulation can be between 2 and 10 ms (optimal 5 ms). However, sweep interval should be over 10 s.
4. As fluorescent light cannot be used to detect axons expressing ChR2-YFP, finding light-responsive EPSCs from patched neurons can be labor-intensive. Thus, in the uninfected reservoir it is advisable to patch cells that are quite close to the opening of axonal tunnels (no further than 1 mm) to increase the chance of finding light-responsive, uninfected neurons, which have made functional connections with the entering axons that express ChR2.
5. In addition, it is possible to visually track the direction of infected axons entering the uninfected reservoir. Axons do grow along almost straight paths inside the tunnels; this directionality is however not maintained once infected axons reach out for somatodendritic contacts in the uninfected reservoir. There should be a slight contrast difference along those axons entering uninfected part of the chip as it grows over the cell monolayer, which can be checked by fine-tuning the focus of light microscope. Keeping this is mind it is possible to select pyramidal cells for recording, which have axons crossing its somatodendritic surface. However, not all infected axons entering to uninfected side would have made functional postsynaptic contacts and might not respond to blue light sweeps. In that case, patch another cell to check if the next one is responding (*see* **Note 35**).
6. It is essential to check if a patched neuron fires prominent EPSCs to blue light stimulation with at least ten sweeps before patching another one. Temporal resolution of EPSC is essential to determine if responses are the right ones. Blue light-induced EPSCs should be initiating roughly at the same time point.
7. Do not exceed 200 light stimulations/sweeps for any sample. It is not rare to spend time patching many neurons from the same chip just to find a single light responding cell, but excessive stimulation of neuronal network with blue light leads to hyperactivation of neuronal circuits significantly adding noise to the recording (*see* **Note 36**).

8. Positioning of cell to light stimulation area is important. OptoLED setup connected to the microscope lens for blue light stimulation has a narrow central focus area in the recording chamber. Use an X–Y stage manipulator to place the neuron of interest to the central field of the microscope (visualized through the live monitor) before patching.

4 Notes

1. Sylgard 184 should be stored at below 25 °C.
2. Isopropanol is an organic solvent and is thus easily flammable, but does not otherwise have any special storage considerations.
3. Measure and balance the pH and then check osmolarity of ACSF. In order to decrease the osmolarity add Milli-Q water and use 1 M sucrose to increase it. Osmolarity over ±4 mOsm for ACSF should still be acceptable for hippocampal neuron recording. ACSF can be stored at +4 °C for a week and should not contain any precipitation of salts.
4. Accurate measurements of salts have to be done as content of ICS is crucial for proper responses from neurons. After dissolving salts, balance the final volume with MQ water. Adjust pH and then measure the osmolarity. Do not add too much of water or sucrose to balance osmolarity (for example, at the most 1.5 ml of MQ water can be added for 25 ml of ICS). Aliquot ICS into smaller volume (300–900 μl) and store at −80 °C (can be stored for up to 6 months).
5. The visibility of the alignment marks on the first SU-8 layer through the thick second SU-8 layer might be poor, depending on the microscopes on the mask aligner. In case the marks are not visible, separate processing step can be done at the start to fabricate for example aluminum alignment marks on the silicon substrate.
6. Very thick SU-8 layers sometimes have adhesion loss during post-exposure baking. This adhesion loss can manifest itself as either buckling of structures or full detaching of structures. Slower temperature ramping or better dehydration before spin coating can help. If the problems persist, it might help to make the post-exposure bake in lowered temperature, such as 4 h in 65 °C.
7. Many different methods can be used to fabricate the antiadhesion coating. We have used a plasma phase deposition of a fluoropolymer, but liquid phase depositions or aerosol depositions are also possible.
8. Master mold can be attached with double sided adhesive tape, but also rolled one sided tape works. Cured PDMS between the master and flat surface will glue them together.

9. The mixture should turn milky white at this point from all the air bubbles. The elasticity of the PDMS can be controlled by the ratio of the prepolymer and the curing agent. Ratio 10:1 forms the most commonly used elastic PDMS, but if even more elastic PDMS is needed, ratio can be increased to 20:1 or even more. A ratio of 5:1 produces less elastic PDMS.
10. If vacuum desiccator is not available, the mixture can be degassed in a refrigerator (e.g., overnight).
11. Alternatively, the degassing and casting can also be done in the other way around by first casting the mixture on top of the master and then degassing.
12. PDMS can also be cured at 80 °C for 30 min [18]. Higher temperatures allow faster curing but make sure that the container can tolerate the higher temperature. If an oven is not available, PDMS can be cured by leaving it at room temperature for 2 days. With very thick PDMS layers (>1 cm) more time might be needed for curing. If a completely planar PDMS layer is required, leveling of the vacuum desiccator and the curing oven is required.
13. While cutting the PDMS, one should avoid scratching the master. The PDMS outside of the master can be leaved in the petri dish as walls for next use. This way, next time less PDMS is needed for molding.
14. Cutting and punching is suggested to be done channel side up. Also, the punched holes are preferred to be as close to the channel part of the chip as possible, as this makes the cell culture process easier.
15. Stacking the PDMS layers tightly with each other forms waterproof seal between layers. If permanent and irreversible bonding is needed, PDMS pieces can be treated with oxygen plasma. This changes the surface chemistry of PDMS and allows permanent bonding.
16. As an alternative process, PDMS can also be cleaned using adhesive tape. In this method, a clean tape is attached on the PDMS surface and pulled off. The process is repeated as many times as necessary for obtaining a clean seal as shown in Fig. 5c.
17. PDMS chips tend to float in isopropanol but they can be attached to the bottom of the container by gently pressing. The chips should be so that the microfluidic channel side is facing the solvent (and not the container).
18. As an alternative to step 2, the residual isopropanol can also be extracted by soaking the PDMS chips overnight in water and then dried by baking for 2 h at 50 °C PDMS is somewhat permeable to small solvent molecules and absorbs them [19], which is why the drying steps are necessary. The PDMS might

appear milky white instead of transparent after the isopropanol bath or the overnight water extraction of the alternative presented in this note. However, in both cases the PDMS will return to transparent after the oven-baking step.

19. Round coverslips are less handy during further manipulation of fixed samples.
20. Washes at this stage should not be done with PBS, which reduces sticking of PDMS to glass.
21. The purpose of setting the chips and coverslips in a vertical leaning position against the cell culture plate walls is to allow proper air-drying and to prevent the coverslips from sticking to the surface of the culture plate.
22. Cell density can very according to individual experiments. If the amount of neurons is lower than 20,000, make sure to pipette the cell suspension close to the entry of the tunnels.
23. Since the size of the chips (in particular the thickness of the third and second PDMS layer of the manually generated chips) can vary from one to another, quantities indicated above can be changed according to individual chips.
24. 70 μl is only a practical volume that can be accommodated in the third layer; excess of this might cause media spillage.
25. Transduction efficiency for both viruses has to be determined prior to asymmetric infection. Almost 100 % transduction efficiency is expected for individual viruses.
26. Rate of media evaporation might vary even in the same chip during prolonged culture, so check the volume of media remaining in each reservoir before calculating the replaceable volume and maintain excess of 70 μl media while replacing it.
27. Apart from the usual rate of media evaporation, there should not be any drastic changes in the volume difference maintained for asymmetric infection. In addition, if media cross-spillage or overflow is seen between the two reservoirs as a result of handling, the chambers should be discarded.
28. Use only those chambers that are not leaking during the culturing period. Excessive leakage of media might cause viral cross-contamination if one of the chambers has been asymmetrically infected.
29. Use 6-well plates for holding the assembled chambers during cell culture. This helps to create a microenvironment for cultured neurons by limiting excessive evaporation of cell culture media. Also, one of the wells should be filled with 8–12 ml of sterilized Milli-Q water for the same reason. In case the coverslip gets stuck to the bottom of the 6-well plate, simply pipette 600–800 μl of warm MQ water around the coverslip and gently lift its edges. While doing so, avoid using excessive

force as it might break the coverslip and damage the neuronal networks. Moreover, using thicker glass coverslip (1 mm thickness) can reduce the risk of coverslip breakage during this process.

30. Neuronal cell culture requires extra care throughout the study period. If one is absolutely unsure on the state of cultured neurons based on visual observation, it is advised to stain the cultures with dyes such as propidium iodide, neutral red or other commercial kits to check the viability of neurons.
31. Sterility of cell culture incubator should be checked before culturing neurons. In addition always make sure that a water-saturated incubator has a sufficient amount of sterile water (preferably slightly above the marked level).
32. Plate 20,000–25,000 RHN in each reservoir for an electrophysiological experiment. RHN grown above or below this density may result in nonuniform distribution of neuronal cell monolayer. Preferably, neurons should neither be too clumped nor extremely isolated and should have homogenous monolayer formation while culturing them. In the chip described in this chapter, with 7×7 mm cell culture reservoirs, it is typical that the edges have lower density of cells than near the tunnels or in the central part of the reservoir.
33. Rate of ACSF perfusion can be as low as one drop per 4 s (around 400 μl per minute). However, for pharmacological treatments, perfusion rate should be increased to one drop per second (dilute the drug in ASCF to its final concentration). Time for drug diffusion to saturate bottom PDMS layer might be longer, so it is possible to increase the perfusion time by twofold to threefold. For example, if time for complete perfusion of any particular drug is about 10 min in acute slice or in a normal dispersed culture then perfusion time can be increased to 25–30 min in these chambers.
34. Producing virus in bulk quantity is highly encouraged. Virus should be stored at −80 °C freezer and can be used for over 3 years. Once thawed, it should be either used within a week (storing at +4 °C) or refrozen (−80 °C). Repeated freeze-thawing of virus should be avoided. It is suggested to aliquot viruses into desired volume before freezing it.
35. Pull at least 7–10 pairs of electrodes at a time. This will be handy during optogenetic recordings where extensive numbers of cell need to be patched. On the same note, it is not always essential to fill the glass electrode completely with ICS. Filling about ½ its length should be sufficient. Avoid air bubbles inside the electrode capillary. If present, gently flick or tap with your fingertips by holding the electrode in a slanting position. Using excessive force to do so might break the tip of your electrode.

36. Do not spend over 2 h trying to find light responding uninfected cells since by then one would have stimulated plenty of blue light pulse to hyperactivate neuronal network. Whole-cell-accessed, uninfected neuron might not respond to blue light pulses because of poor synaptic connection formed by ChR2-expressing axon. Excessive light stimulation would however activate any existing ChR2 expressing axons around the vicinity of patched cell.

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Chapter 5

Development of a Compartmentalized Biochip for Axonal Isolation and Neuronal-Circuit Formation at the Single-Cell Level

Ting Huang, Russell K. Pirlo, Wan Qin, Yongliang Lin, Lina Wei, Lucas Schmidt, Nick Erdman, Tingfei Xi, Mauris N. DeSilva, and Bruce Z. Gao

Abstract

In vitro neuronal networks in cell cultures have tremendous potential for the investigation of synapse formation, development, and function, especially with the development of microelectrode arrays. Most current techniques used to form a defined neuronal network are based on microcontact-printing, but the intercellular connections in the patterned low-density network are formed randomly, systematic study of a specific network is not possible. For such study, a practical tool for creating defined neuronal networks in which each intercellular connection can be formed according to a predetermined pattern is critical. In addition, because glia—particularly astrocytes—play an important role in neuronal network processing, a precise platform to study glia–neuron interaction at the single-cell level is necessary. In this chapter we describe a biochip-microfabrication technique and a unique laser cell-micropatterning system for creation of a compartmentalized, axon-isolating, polarized neuron-growth platform at the single-cell level.

Key words Microfabrication, Laser cell-micropatterning, Axon isolation, Single cell-resolution circuits, Polarize

1 Introduction

In vitro neuronal networks in cell cultures have tremendous potential for the investigation of synapse formation, development, and function, especially with the development of microelectrode arrays (MEAs). Utilizing MEAs allows noninvasive, long-term, simultaneous recording of electrical activity from multiple sites of a neuronal network [1–3], which is critical to the investigation of neuronal networks. In conventional MEA-based studies, a large number of cells are randomly placed in a culture, and thus neuronal networks are formed arbitrarily. Because the MEA has a fixed pattern of electrodes, the arbitrary cell arrangement may cause

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either simultaneous recording of the electrophysiological activities of multiple neurons that belong to different units of the network or no recording. Thus, an MEA cannot be used for a systematic examination of the network. To construct a defined neuronal network in vitro, controlling the process of axonal pathfinding for network formation regarding the pattern of the electrodes is essential.

It has been suggested that in vivo axonal pathfinding is guided both by chemical signals acting as attractants and/or repellents and by physical constraints encountered in the extracellular environment [4, 5]. Microcontact printing techniques were introduced to simultaneously mimic chemical guidance and physical constraints to facilitate creation of a network with a defined circuit that matches the pattern of the electrodes [6–10]. Network patterning is achieved by directly depositing chemicals, either with or without a gradient, to form an axon-extension path. However, in microcontact printing, a particular circuit of the network is formed by multiple cells that are randomly placed on the printed path so that each cell is exposed to a mixture of proteins, ions, and neurotransmitters released from neighboring cells. Although these multiple cell-cell interactions represent conditions of an in vivo network, inductive research methodology requires a single-cell-level in vitro neuronal network in which each intercellular connection can be individually controlled [11, 12]. In addition, study of an in vivo single-cell event, such as pioneer neuron pathfinding (a critical process in formation of the initial neuronal network during development), requires creation of a single-cell-level neuronal network [13].

Replication of in vivo neural tissue composition (and thus function) also requires the ability to recruit multiple cell types during network formation. It has been suggested that glia, an important component of the brain tissue, have an active role in brain function and particularly in information processing during both development and adulthood [14]. MEA testing has shown that adding astroglia to the neuronal culture promotes greater synapse density and higher activity in neuronal networks [15]. Therefore, to obtain more in vivo-relevant data, it is necessary to include glia (i.e., astrocytes) when a single-cell-level neuronal network is constructed.

Because it is quite difficult to create a defined chemical gradient in a system with very high spatial resolution, microcontact printing cannot easily be used to control a network's circuit polarity. The geometry of microtopology and 3D physical constraints have been employed to guide axonal polarity during pathfinding [16–19]. For example, it has been shown that the direction of axonal guidance can be regulated by varying the angle of turns in microchannels [20]. In our design, microtunnels constructed in different 3D geometrical shapes, like tapered, snag, and hook, have been used to guide axons to build a polarity circuit [21], as shown in Fig. 1.

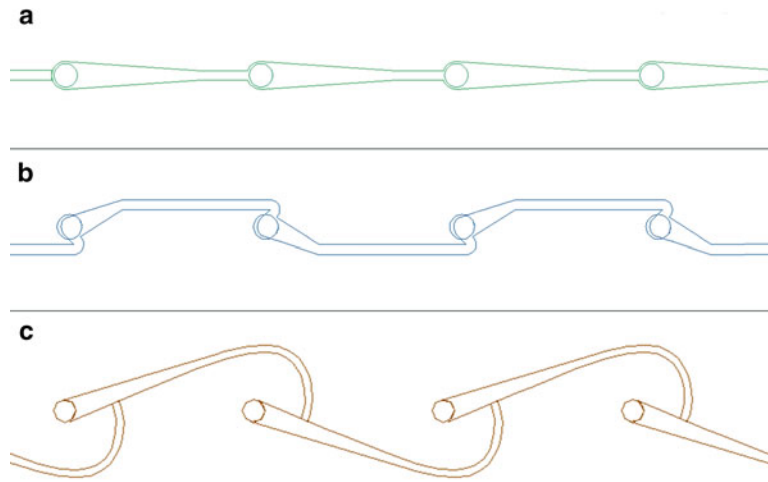

Fig. 1 The design of polarized microtunnels (**a**) Linear design: An axon will be guided to extend in the tapered microtunnel from the wider side to the narrower side to achieve polarized extension. (**b**) Snag design: An axon will be compelled to extend into the microtunnel from the straight other than from the angled entrance to achieve polarized extension. (**c**) Hook design: An axon will extend in the microtunnel initially along only one direction and then remains in the same direction without changing into the other direction because of the 900 turning design, and thus achieve polarized extension

Once the network has been formed, neurons are highly polarized: their axons extend from the cell body (soma) over considerable distances through varying extracellular microenvironments. In such a network, the somas and the axons experience different local environments and respond to them independently. To systematically study a network's properties and make full use (such as in drug testing) of the unique response of different components of the network, it is crucial to be able to separately control the microenvironments of the somas and the axons. In 1977, the Campenot chamber, a type of compartmentalization, was introduced as an in vitro peripheral-nervous system model that separates axons from somas [22]. As microfluidic techniques that control fluid dynamics in microchannels were being developed, Jeon's group introduced several microfluidic biochips to facilitate compartmentalized axon-soma isolation in in vitro study of the central nervous system [23, 24]. Microtunnels with a height of less than 3 μm were designed as axonal compartments; they sufficiently constrained somas and allowed only axons to pass through. These microfluidic biochips are mainly microfabricated using optically transparent polydimethylsiloxane (PDMS), which has been proven to be biocompatible and reusable. When used with MEAs, PDMS increases the signal-to-noise ratio of electrodes [25]. Microfluidic compartmentalized biochips are widely used in axon-isolation

studies and coculture studies, such as glia–neuron interaction and heterotypic neuronal networking [26, 27]. However, none of the microfluidic biochips, compartmentalized or noncompartmentalized, can be used to construct neuronal networks at the single-cell level due to lack of a proper single-cell deposition technique.

To address this issue, we combined the laser cell-micropatterning and microfluidic techniques to construct single-cell-level neuronal circuits. The concept is to microfabricate PDMS membranes with microholes and microchannels. When the membrane is placed on top of the MEA, microwells and microtunnels form the microholes and microchannels, respectively. The microwells (30 μm in diameter, 35 μm in height [the thickness of the membrane]) were used as soma compartments to prevent migration of neurons that were laser-patterned on the substrate (e.g., an MEA). Two types of microtunnels were designed. One type (10 μm wide on average and 2 μm high) was used to connect the microwells and to provide axon-extension paths to guide axonal pathfinding for formation of the designated neuronal network. The other type (approximately 100 μm wide and 80 μm high) was used as a microfluidic channel to deliver multiple cell types to the exit section of the microfluidic channel for laser-guidance. The fabrication process included standard multilayer photolithography and soft lithography techniques to separate the network-patterning region and the cell-delivering region in space both horizontally and vertically.

Compared to conventional methods of single-cell manipulation, such as those that use micropipettes or micromanipulators, the laser cell-micropatterning system developed in our lab has much higher spatiotemporal resolution and causes less cell damage [21, 28, 29]. The entire laser cell-micropatterning system is shown in Fig. 2. The working principle of our system is based on the optical forces that arise from a focused Gaussian laser beam incident on a dielectric particle, such as a cell, with a different refractive index from that of the surrounding medium. According to Maxwell's theory, light carries momentum with a magnitude proportional to its energy and in a direction along its propagation. When a propagating laser beam interacts with a cell, some of the light scatters and causes a change of the light's momentum in the direction along its propagation. As a result of the law of momentum conservation, the cell undergoes an equal and opposite change in momentum; this gives rise to optical force. The force in a weakly focused beam has two components: radial force acting from the outside to the center of the beam along the radii and axial force acting along the direction of the beam's propagation [30]. During the patterning process, a cell in the suspension is delivered by the microfluidic channel. This channel leads to the cell-deposition chamber at the bottom of which the network patterning region has been fabricated. At the exit section of the microfluidic channel, the delivered cell is trapped and guided by the laser beam as the chamber with

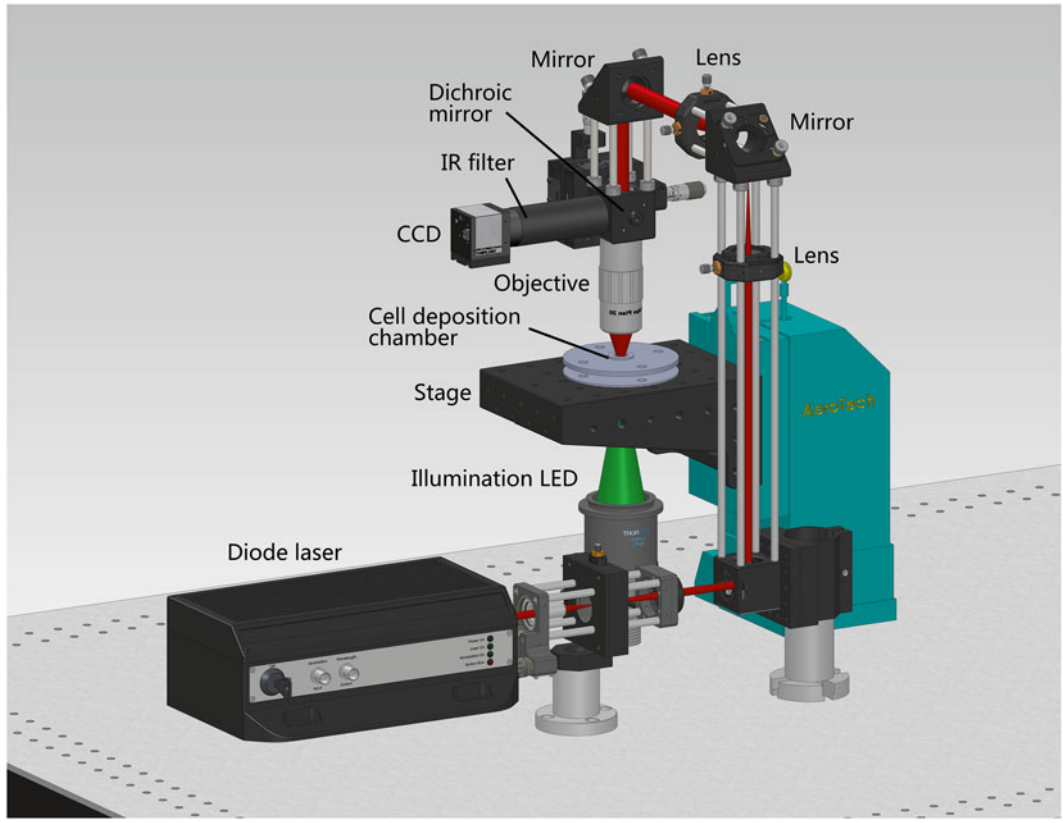

Fig. 2 Schematic of the laser-guided cell micropatterning system

the attached biochip is moved by the motorized stage to bring the cell towards the desired point on the substrate (i.e., the microwell in the biochip). The cell is pushed down to the substrate by the axial force in the beam's propagation direction. The laser cell-micropatterning system achieves high-resolution placement of an individual cell to establish a single-cell-level circuit in vitro, not only for homotypic neurons but also for heterotypic neurons and glia pairs.

2 Equipment and Materials

This section outlines all equipment and materials used in biochip fabrication, laser cell micropatterning, and cell dissection. The preparation of a special culture media for chick-forebrain neurons and astrocytes is also included.

2.1 Biochip Microfabrication and Preparation

1. Spin coater (Laurell WS-400B-6NPP/LITE).
2. Microaligner with 200 W lamphouse (Suss MJB-3).
3. High-vacuum pump (BOC Edwards RV3 Rotary Vane).

4. Vacuum oven (Isotemp, Model 285A).
5. Vacuum desiccator.
6. Two planar hot plates.
7. Nitrogen (Airgas).
8. Plasma cleaner/sterilizer (Harrick PDC-32G).
9. Microelectrode arrays (MEAs, MultiChannelSystems).
10. Polished silicon wafers (University Wafer, 50 mm in diameter).
11. SU-8 (2002, 2050) negative photoresist (MicroChem).
12. SU-8 developer (MicroChem).
13. Photomask (CAD/Art Services).
14. Isopropyl alcohol (Fisher).
15. Chlorotrimethylsilane (TMCS, Sigma).
16. Sylgard™ 184 Silicone Elastomer Kit (PDMS, Dow Corning).
17. Xylene (Fisher).
18. Extracting solvents: triethylamine, ethyl acetate, acetone (Fisher).
19. Polyethylenimine (PEI, Sigma).
20. Biopsy punch (Harris Uni-cores, 5 mm).
21. Corona treater (Electro-Technic Products, Inc., BD-20).

2.2 Laser Cell-Micropatterning System

1. Optical Configuration: The laser source is a single-transverse-mode diode laser (200 mW, 830 nm, continuous wave (CW), S6020–200, Intense Inc.) attached to a diode-laser mount (TCLDM9, Thorlabs Inc.), which provides current and temperature control. An aspheric collimating lens (f = 4.51 mm, NA = 0.55, C230TME-B, Thorlabs Inc.) is immediately outside the laser diode chip, and this is followed by an anamorphic prism pair (NT47–274, Edmund Optics) used to transform the elliptical beam into a circular one. Then the beam is expanded and steered using a biconvex lens pair (f = 15 mm, D = 0.5 in. and f = 20 mm, D = 0.5 in., LB1092-B, LB1450-B, Thorlabs, Inc.). The second lens is mounted on a motorized translational stage (PT1-Z8, Thorlabs, Inc.) to steer the beam's focal point so that the guidance region of the beam coincides with the object plane of the imaging system. The beam then passes through a 45° dichroic mirror (DMLP567, Thorlabs Inc.) that reflects the visible image to the CCD camera while allowing passage of the 830 nm beam. Next, the beam is focused on the cell-deposition chamber using a long-working-distance objective (20×, Mitutoyo Plan Apo Infinity-corrected, NT46-145, Edmund Optics) with NA = 0.42 and f = 10 mm. The illumination source is a green (530 nm) 200 mW LED (M530L2, Thorlabs Inc.). The illumination beam is reflected upward by another dichroic mirror and enters the chamber

through the bottom cover glass, which serves both as the optical window and the cell-culture substrate. The imaging beam passes through the 20× objective and is reflected to the camera by the dichroic mirror. Immediately after the dichroic mirror, a tube lens (f = 160 mm, not shown in Fig. 2) is used to form the image on the CCD camera (XC-ST50 Sony). In front of the CCD camera, several IR filters are used to remove artifacts from the guidance beam. The CCD camera is mounted on a 3D translational stage to allow alignment of the center of the CCD with the laser-guidance region.

2. Cell-Deposition Chamber: The components of the chamber include a top plate with a transparent-glass window and a bottom plate, which has a hole with mounting steps that hold both a standard MEA chip and a glass-bottom 35 mm petri dish. To seal the MEA chip, the top and bottom plates are clamped with a built-in ring or the petri dish. The whole chamber is stabilized on a three-axis motorized stage (Aerotech FA90-25-25-25) driven by three Aerotech N-drive units with an IEEE1394 interface.
3. System control software: The control system is run on an Intel Core 2 Quad computer with 4 GB of RAM and Microsoft Windows® XP. The control software is written in LABVIEW8.6. The Aerotech stage uses real-time executive (RTX) to communicate with the computer through an IEEE1394 port. The laser-intensity adjustment and the opening/closing of the laser shutter are controlled via serial port/RS232 access through VISA in LABVIEW. Cell manipulation and navigation are primarily controlled by an Xbox 360 controller. The program consists of four primary timed loops respectively to (1) handle user input from the front panel, keyboard, and Xbox 360 controller; (2) capture, process, and display the patterning video with navigational overlays; (3) read motion-control data from the analog sticks and compute the movement vectors; and (4) issue motion commands to each of the three axes. The application, which provides 3D-position memory marking and recalls functions, makes the patterning process more effective with an intuitive user interface and control system for navigating cells from the microfluidic channel to the deposition point on the substrate.

2.3 Cell Dissection and Cell Culture Medium

1. Dissection:
   - Day 7 or Day 14 embryonic chick eggs
   - Zeiss dissection microscope (Stemi 2000)
   - Dissection tools: one blunt forceps, one curved forceps, two #5 biology forceps, one serrated scissor
   - Nylon mesh (73 μm pore diameter)

- Ice-cold HBSS with 1 % antibiotic/antimycotic (Gibco)
- 0.25 % Trypsin-EDTA (Sigma)

2. Serum-free, glia-conditioned media:
   - Neurobasal without L-glutamine or phenol-red (Gibco)
   - 0.5 % antibiotic/antimycotic (Gibco)
   - 0.5 % gentamicin (Gibco)
   - 0.5 % (1×) GlutaMAX (L-glutamine substitute) (Gibco)
   - 2 % B27 neuronal supplement (Gibco)
   - 100 ng/mL NGF (Gibco)
3. Glial media:
   - Media 199 (with L-glutamine) (Sigma)
   - 10 % fetal bovine serum (Gibco)
   - 1 % antibiotic/antimycotic (Gibco)
   - 50 μg/ml gentamicin (Gibco)
   - 2.5 μg/ml amphotericin (Gibco)

3 Methods

3.1 Microfabrication and Biochip Preparation

The biochip comprises two major parts. The first is the network-formation chip, which is produced (as briefly described in the introduction) by placing a PDMS membrane with particular features on top of an MEA or a petri dish to form compartmental neuron-culture microwells and microtunnels. The second is the microfluidic cell-delivery chip, which is mounted in the same chamber, near the network-formation chip. The PDMS-based soft photolithography technique, including mask design, mold etching, and PDMS-membrane formation, is used to manufacture the two chips (network-formation chip and cell-delivery chip). The manufacturing process is as follows.

3.1.1 Network-Formation-Chip Fabrication

The microwells and the microtunnels on the network-formation chip are fabricated in a two-layer photolithographic process: one layer is etched using the mask with the features of the microtunnels, and the other layer is etched using the mask with the features of the microwells.

Photolithography-Mask Design

The two masks are designed using AutoCAD™ and laser photoplotted on a transparency with a resolution of 2 μm (CAD/Art Services Inc). Outside the feature region, at the exactly same locations on both masks, at least three markers with a typical dimension of several millimeters are designed (as shown in Fig. 3). These markers are used to align the two layers during fabrication (*see* **Note 1**).

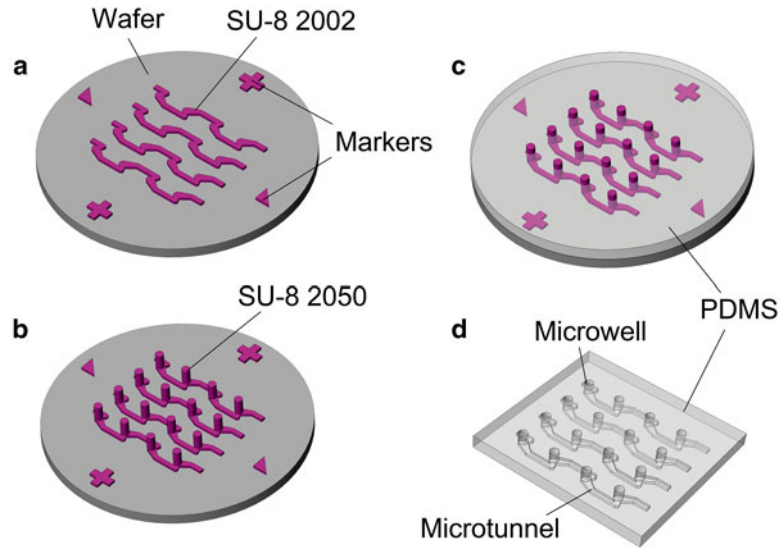

Fig. 3 The procedure for network-formation chip fabrication (**a**) First-layer photolithography. (**b**) Second-layer photolithography. (**c**) Soft lithography. (**d**) The pattern of a network-formation chip with microwells and microtunnels

First-Layer Photolithography

A standard photolithographic process is employed to manufacture the first layer to create the mold for the 2 μm thick microchannels and, simultaneously, the markers, *see* Fig. 3a.

1. Layer spinning: The spinning program is sequentially set as 500 rpm/10 s, 1,500 rpm/15 s, 3,000 rpm/45 s, and 500 rpm/10 s. Before placing a clean silicon wafer on the spinner, the nitrogen vacuum valves must be opened. Then the vacuum button is pressed to ensure that the wafer is securely mounted to the holder. Next, SU-8 2002 is poured cautiously (to prevent air bubbles, *see* **Note 2**) to cover the entire wafer. The spinner lid is then closed, and the preset program is run, after which the wafer is covered uniformly with a 2 μm thick negative resist (SU-8).
2. Prebake: After being released from the spinner, the wafer is baked on a 65 °C hot plate for 2 min, followed by a 95 °C hot plate for 2 min (*see* **Note 3**).
3. UV exposure (photo etching): The UV lamp of the MJB3 aligner is turned on to allow the output of the lamp to reach a constant intensity of 196 mW/cm^2. During the warm-up period, the mask is cut and taped to the mask holder, which is then loaded onto the aligner. Then the wafer covered with the photoresist is loaded onto the vacuum chunk. After the wafer is placed precisely under the mask, it is brought into contact with the mask. The *Z*-axis control of the aligner may be adjusted to make the contact as tight as possible (*see* **Note 2**),

which will be automatically evaluated by the aligner. After the aligner sends out a contact-approval signal, the exposure shutter can be turned on, and exposure begins. The exposure time is preset as 24 s (*see* **Note 3**).

4. Postbake: After being exposed, the wafer is baked at 95 °C hot plate for 2 min.
5. Development: The exposed wafer is sprayed and washed with SU-8 developer for approximately 2 min until all the uncrosslinked residue is washed off, and the mold is visibly formed (*see* **Note 4**). This is followed by a second wash with isopropyl alcohol for 10 s. Then the wafer is dried with pressurized nitrogen.
6. Hard bake: Wafers with the formed molds are hard-baked at 137 °C for at least 2 h to anneal any surface cracks.

Second-Layer Photolithography

The photolithographic procedure for the second layer has minor changes from that for the first-layer. The second layer is created on the wafer that has the mold of the first layer. Alignment must be perfect; this is achieved by aligning the markers on the second mask with the markers on the wafer that the first mask created, *see* Fig. 3b. The steps are briefly described here.

1. Layer spinning: Before spinning, markers that were created during fabrication of the first layer on the wafer should be covered by small strips of tape (*see* **Note 5**). SU-8 2050 is spread on the wafer followed by the spin with the sequential speed of 500 rpm/15 s, 1,700 rpm/45 s, and 500 rpm/10 s to achieve an even 100 μm thick layer. The edge is scratched off with a blade, and the tape that covers the markers is peeled off before the wafer is released from the spinner. Care must be taken to keep the markers on the wafer.
2. Prebake: The wafer is baked on a 65 °C hot plate for 10 min and then on a 95 °C hot plate for 20 min.
3. Alignment and exposure: After the wafer is placed on the chuck and moved beneath the second mask, the viewing field of the microscope is changed to one of the markers by adjusting the position of the motorized stage. A rough alignment should be made before raising the chuck (without letting it come into contact with the mask) to make sure the markers on both the mask and the wafer are visible under the microscope. The X, Y, and Theta micrometer controls are adjusted to align the wafer to the mask—at least two markers must be perfectly aligned under the 5× objective. Once alignment is achieved, the chuck is carefully raised to bring it into full contact with the mask. The exposure time is set to 65 s.
4. Postbake: The wafer is baked on a 65 °C hot plate for 5 min and then on a 95 °C hot plate for 10 min.

5. Development: The wafer is immersed in SU-8 developer with light agitation for about 7 min, until all uncrosslinked photoresist is washed away. Then, the wafer is sprayed and washed separately with fresh developer and isopropyl alcohol for 10 s and dried with compressed nitrogen.
6. Hard bake: Molds are hard-baked at 137 °C for at least 2 h.

Soft Lithography

Soft lithography refers to processes that use lithography via the elastomeric (soft) transfer of a pattern. Typically, PDMS is used as the elastomer, *see* Fig. 3c, d. The procedure used for creating the soft lithographic PDMS membrane is summarized as follows.

1. Silanization: To facilitate removal of PDMS membranes from the mold created on the silicon wafer, the surface of the wafer is first silanized. Hard-baked molds are placed face-upwards in a vacuum desiccator. A petri dish with three-to-seven drops of chlorotrimethylsilane (TMCS) is also placed in the desiccator. Once a maximum vacuum level is attained, the desiccator valve is shut, and the molds are evaporation-coated for 30 min. TMCS is toxic, so all of these steps should be done in a chemical hood.
2. Polymer mixing: The base and the curing agent of PDMS are mixed at the ratio of 9:1; 10 % xylene is added to decrease viscosity (*see* **Note 6**). All of the components are mixed and vacuumed for about 1 h, until no bubbles exist.
3. Spinning and baking: The uncured PDMS solution is spin-coated onto the silicon wafer with the created mold at sequential speeds of 500 rpm/10 s, 2,000 rpm/10 s, 4,000 rpm/40 s, and 500 rpm/10 s to achieve a thickness of approximately 35 μm. The PDMS is then cured by baking the wafer on a hot plate at 125 °C for 1–3 min until the membrane is cured.
4. Re-spinning and re-baking: PDMS solution is deposited annularly around the feature area and then spun at a rate of 800 rpm/60 s to achieve a 100 μm annular thickness around the feature area (*see* **Note 7**). Then, the wafer is baked at 125 °C for 1–3 min.
5. Peeling and shaping: After the PDMS membrane is cooled, it can be peeled off the mold from edge to center. Care must be taken not to tear any holes through the membrane. Then the network-formation membrane is cut into a 10 mm × 10 mm square piece with the micro features in the center, serving as the network-formation chip. A single silicon mold may be used to produce over 20 elastomeric membranes before losing integrity (due to features breaking off).
6. Postbaking: A collection of membranes is baked in a vacuum oven at 137 °C for at least 2 h to ensure maximum crosslinking.

3.1.2 Microfluidic Cell-Delivery Chip Fabrication

Photolithographic-Mask Design

The procedure for mask design is the same as that described in "Photolithography-Mask Design" under Sect. 3.1.1. Only one layer of microfluidic channel is required. The microfluidic channel for cell delivery is 200 μm wide and 100 μm deep. The length can be several millimeters, depending on the desired cell-delivery rate.

Photolithography

SU-8 2050 is used to create a 100 μm-high mold for the 100 μm-deep channel. The photolithography procedure and parameters are the same as those described in section "Second-layer photolithography" except that photoresist is spread on a new, clean silicon wafer, and no additional alignment is required because there is only one layer.

Soft Lithography

1. Silanization: The procedure is the same as that described in section "Soft Lithography" under the Sect. 3.1.1.
2. Polymer mixing and vacuum: The base and the curing agent of PDMS are mixed at the ratio of 10:1 without addition of xylene. The silicon wafer with the mold, made as described in section "Photolithography", is put in a 60 mm petri dish, and then the mixture is poured on the wafer to a height of approximately 2 mm. Simultaneously, the mixture is poured on two additional petri dishes to fabricate flat membranes with a height of 1 mm and 3 mm, respectively. The mixture is vacuumed until no bubbles are visible.
3. Baking: The dishes with the clear mixtures are baked on a flat hot plate at 80 °C for approximately 1 h, until the mixtures are cured.
4. Peeling and shaping: After the PDMS is cool, it can be peeled off the mold from edge to center using a surgical blade. As shown in Fig. 4, the 2 mm PDMS membrane with the grooves serves as the channel layer. The region on this channel layer that starts at the end of the microfluidic groove is cut to form a square hole (e.g., 15 mm × 15 mm). The actual size of this hole will be determined by the size of the patterning zone (e.g., the network-formation chip). After the square hole is cut, the middle of the side wall of the channel layer that is opposite the inlet reservoir is cut open (typically 1–2 mm wide) to make the channel layer a horseshoe shape. The 1 mm PDMS membrane that serves as the top layer is cut into a rectangular shape with a size that can cover the entire channel layer. The 3 mm PDMS membrane, which serves as the cell-suspension reservoir (inlet) is cut into a 6 mm × 6 mm square. Next, a 5 mm diameter hole is punched in each of the three membranes using a biopsy punch. The holes should be aligned with the inlet microfabricated on the 2 mm PDMS membrane.

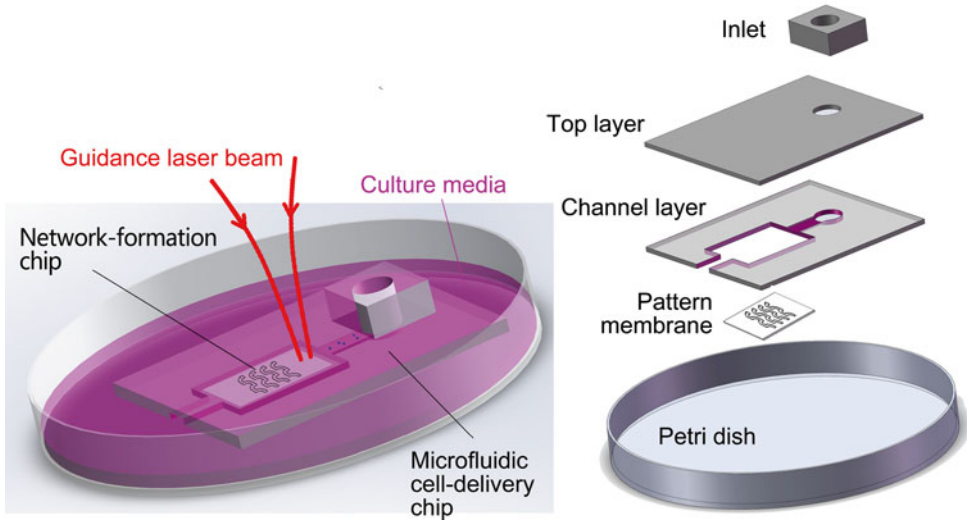

Fig. 4 Assembly of the biochip with both network-formation and cell-delivery chips

3.1.3 Biochip Preparation

The PDMS membranes must be treated to become bioactive. Typical steps include oligomer extraction, surface modification, and cell-adhesive-agent coating as described in the following procedures. Then various parts of the biochip are assembled to form a functional biochip.

1. Oligomer extraction: To further reduce the toxicity of PDMS membranes, uncrossed oligomers must be extracted using the process described in Millet's work [31]. The PDMS membranes, including both the network-formation chip and the microfluidic cell-delivery chip, are immersed in the following three solvents: triethylamine, ethylacetate, and acetone, each for at least 2 h. The membranes are then vacuum-baked at 137 °C for at least 2 h to remove the solvents. After extraction, the PDMS membranes are soaked in 70 % ethanol for at least 1 h for cleaning and sterilization.
2. Assembly of network-formation chip: The network-formation membrane is placed directly on the glass bottom of a petri dish to form the network formation chip. If the membrane is used to form a chip with an MEA, alignment of the network-formation membrane with the MEA must be performed under a dissection microscope. Before the membrane is spread on the MEA surface, several drops of 70 % ethanol are used as a lubricant to aid in sliding the membrane (using two forceps) to ensure all microholes are aligned with the electrodes. It is important not to create any air bubbles during alignment. Once proper alignment is achieved, the assembled chip is allowed to dry in the

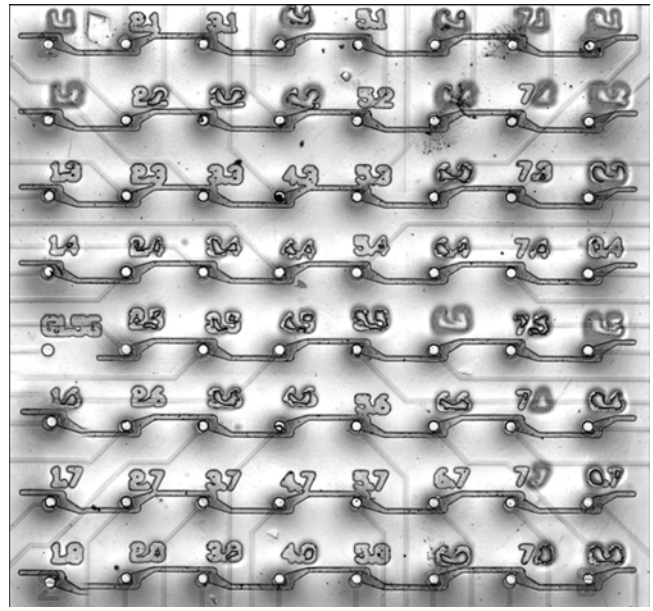

Fig. 5 A microscopic image of a typical assembled PDMS membrane on an MEA for the study of single cell-based neuronal network

hood and then heated at 50 °C for 2 h, creating a stronger bond. A picture of a typical assembled PDMS membrane on an MEA is shown in Fig. 5.

3. Surface modification and coating: After the PDMS membranes are assembled on the MEA (or a petri dish), the formed network-formation chip is treated with oxygen plasma (Harrick PDC-32G) for 5 min at 200 mTorr to increase surface hydrophilicity (*see* **Note 8**). Immediately following the plasma treatment, the chip is coated with a cationic polymer polyethylimine (PEI) diluted to 0.05 % w/v in 8.5 pH borate buffer for at least 0.5 h. A vacuum process may be used to facilitate flowing the PEI into the microtunnels. The chip is then washed three times with sterilized MilliQ water and air-dried in the hood.
4. Microfluidic cell-delivery chip assembly (*see* Fig. 4): After 30 s plasma treatment with corona treater, the channel layer and the top layer are permanently bonded together by placing the top layer in contact with the surface of the channel layer so that the microfabricated microfluidic grooves form the microfluidic tunnel. The 5 mm hole on the top layer must be aligned with the 5 mm hole punched at the microfluidic channel's inlet on the channel layer. Next, the inlet PDMS membrane that functions as a cell-suspension reservoir is assembled after plasma treatment to the other side of the top layer, with the punched 5 mm holes being aligned with each other. Irreversible bonding is achieved after baking the three stacked membranes for

1 h at 135 °C. Then the formed microfluidic chip is sterilized by UV light for 30 min and is ready for use.

5. Biochip assembly (*see* Fig. 4): The microfluidic cell-deliver chip is attached to the activated network-formation chip with the patterning zone in the middle of the square hole and the exit of the microfluidic channel next to desired deposition positions. Consequently, the top layer, the channel layer, and the patterning substrate form a cell-deposition chamber. Now the biochip is ready for the laser cell micropatterning process.

3.2 Cell Dissection and Preparation

3.2.1 Neuron Dissection and Culture

Embryonic chick forebrain dissection is based on Heidemann's protocol [32], by which telencephalon tissue is harvested, but not the entire forebrain.

1. Day 7 eggs are cleaned with 70 % ethanol wipes, the top of each egg is removed with blunt forceps, and the chick embryo is removed with curved forceps.
2. The embryo is decapitated and the head is placed in a 35 mm dish with ice cold HBSS solutions.
3. The head of the embryo is flipped upright; a #5 forceps in one hand is used to hold the head by pressing it to the bottom of the dish. A similar forceps in the other hand is used to pinch-clip the skin around the forehead and then peel and scoop the two frontal lobes.
4. The lobes are then moved to a separate dish where the meninges and the posterior diencephalon are removed to get pure, white telencephalon tissue.
5. The telencephalon is removed and placed in a 1.5 ml tube filled with 0.25 % Trypsin-EDTA.
6. After incubation at 37 °C for 5 min, the trypsin is removed and replaced by 1 ml glial medium containing 10 % serum.
7. The tissue is then triturated up to ten times with a 1 ml pipette tip before it is centrifuged at 1,000 rpm for 5 min.
8. The supernatant is discarded and replaced by glia-conditioned medium. Cells are resuspended by trituration before being counted with a hematocytometer.
9. The concentration of the cell suspension is adjusted to 1×10^6 cells/ml and is now ready for the laser cell micropatterning process.

3.2.2 Astrocyte Dissection and Culture

The protocol for Embryonic Chick Cerebral Hemisphere astrocyte cultures presented here is based upon Kentroti [33].

1. Day 14 Eggs are wiped down with 70 % ethanol, and the top is cracked and removed. The embryo is removed from the egg and the head is decapitated.

2. The skull is opened with serrated scissors, and the cerebral hemispheres are moved to a new dish. The meninges are removed from around the hemispheres.
3. The cerebral hemispheres from up to three chicks are broken into fine pieces with a pair of forceps.
4. These pieces are mechanically dissociated by sieving them through a nylon mesh (73 μm pore diameter) into glial medium.
5. The cells are suspended and plated in a T150 flask and incubated at 37 °C and 5 % CO_2.
6. After 24 h only a small fraction of the cells (<10 %) have survived and attached to the plastic substratum, and the glia medium may be replaced.
7. Cultures reach confluence after 1–2 weeks, and then the cells are passaged by dissociating with 0.25 % Trypsin-EDTA for 5 min and neutralizing with normal glial medium. After centrifuging at 1,000 rpm for 5 min, the supernatant is removed and the cells are resuspended in 1 ml of glial medium. A fraction of these cells is replated in T150 flasks.
8. Cells are passaged three times according to the above step to get high-purity astrocyte cultures. Then they are ready to create glia-conditioned medium or for the laser cell micropatterning process.
9. Making glia conditioned medium: After the pure astrocyte cultures reach confluence, they can be used for 3 days to create glia-conditioned medium. 20 ml serum-free preconditioned medium is added, and the conditioned medium is collected 24 h later. 60 ml conditioned medium in total can be obtained per flask for 3 days. The conditioned medium may be frozen after being collected. When needed, it is thawed and filtered with a 0.22 μm filter, and 2 % B27 and 100 ng/ml NGF 7 s are added to create the a finished neuron-culture medium. In our experience, glia-conditioned culture medium enabled higher viability of forebrain neuron cells than typical culture medium (*see* **Note 9**).

3.3 Laser Cell Micropatterning Process

The detailed laser cell-patterning processes include cell loading, prepatterning, patterning, and postpatterning as follows.

1. Cell loading: First, a 1 ml syringe loaded with cell-culturing medium is used to inject medium into both the patterning zone and the microfluidic channels. The volume of the medium should be controlled so as to wet and fill the entire pattern zone, but the liquid level should be slightly lower than the top surface of the top layer to prevent the optical window formed by this surface from being occluded by the media. A pressure pulse may be applied to the microfluidic channel network via the cell reservoir to expel any trapped air. Once the microfluidic

chip is primed, gravity serves as the driving force for the microfluidics. After stable microfluidic flow has formed in the channel, a 60 μl cell suspension of about 1×10^6 cells/ml density is loaded into the inlet reservoir (*see* **Note 10**). Next, the entire chip is placed into the chamber bottom. Finally, the chamber cover is installed over the substrate, and screws are inserted and tightened until the clamp is lightly secured.

2. Prepatterning: The cell-deposition chamber is placed into the motorized pneumatic stage. Then, the nitrogen supply is turned on to the pneumatic stage. Next, the laser-source power is turned on with the laser output set to 200 mW. The stage with the cell-deposition chamber is raised so that the substrate comes into focus on the imaging screen. The laser-patterning control software is initialized and the Xbox 360 controller must be recognized. The patterning speeds are set to 150 μm/s horizontally and 25 μm/s vertically (*see* **Note 11**). Then patterning may begin.
3. Patterning: The entire patterning process should be completed in less than 30 min to maintain high cell viability (*see* **Note 12**). The patterning process is carried out by exactly using the following instructions.
   (a) Use the X-Box controller thumb-sticks to navigate through the chamber and locate the area of network-formation chip where the cells should be deposited. Save this coordinate point as the first deposition point.
   (b) Use the X-Box controller thumb-sticks to navigate the edges of the chamber to locate the exit of the microfluidic channel. Save this coordinate point as the injection point.
   (c) Identify a single cell that looks typical based on one's experience in the area of the exit of the microfluidic channel, position the predetermined radial trap cross hairs on top of the cell of interest, and pull the left trigger to open the laser shutter to capture the cell.
   (d) With the cell inside of the radial trap, use the controller thumb-sticks to guide the stage in the direction of the onscreen navigation arrow to the deposition point. The laser may be harmful to some sensitive chip components, so automatic intensity reduction may be used (*see* **Note 13**).
   (e) Push the cell to the surface until it will not slide. The cell has been patterned.
   (f) Release the laser trigger. Find the next deposition point and mark it.
   (g) Repeat Step 3—the chamber will automatically reposition the radial trap and field of view to the exit of the microfluidic channel.
   (h) When all cells are patterned, press the STOP button.

4. Postpatterning: When patterning is complete, the chamber exterior is sprayed with 70 % ethanol or Enivrocide™ and wiped down. The biochip is immediately removed from the chamber and placed into a cell-culture hood. The remaining medium in the cell reservoir is removed via a pipette and the microfluidic cell-delivery chip is peeled off the substrate using sterilized forceps. The network-formation chip with patterned cells is then incubated at 37 °C and 5 % CO_2.
5. Heterotypic cell patterning: If glial cells need to be patterned to the same culture 24 h after the neurons are patterned, the cell-delivery chip should not be peeled. Instead, it should be rinsed by flowing cell culture medium until there are no visible cells in the microfluidic channels. The procedure of patterning glial cells is the same as that used to patterning neurons described above. If many cell types need to be patterning simultaneously, multiple cell-delivery channels can be microfabricated in the microfluidic cell-delivery chip.

3.4 Data Analysis

1. Analysis of geometric guided polarity: We use live-cell phase microscopy to observe the time history of axonal growth before the axons fully connect to neighboring cells. The observed neurite outgrowth is classified as one of the four following scenarios: extension of a neurite in the direction guided by the microtunnel geometry (+); in the opposite guidance direction (−); into both directions (both); not in any microtunnel (neither). Neurons that did not extend neurites at all are not included in the analysis.
2. Analysis of heterotypic patterning: In our experiments, heterogeneous cell patterns are formed by first depositing neurons in one laser cell-patterning session and then depositing glial cells beside those neurons in a second session 24 h later. In the set of heterotypic patterning experiments, contrary to the behavior of neurons, glial cells rarely stay within the microwells; they prefer to migrate into the short and narrow channels. Onstage time-lapse video is used to analyze contact guidance and barrier activity between neurons and glial cells.

4 Notes

1. From our experience, the features that are meant to match the MEA can be enlarged by 5 % in the AutoCAD™ drawing to account for shrinkage of the PDMS molds during curing. Further, at least 5 μm of overlap between the first and second layer features is desirable. The markers for alignment may be added on the edge of the masks to make covering with tape easier in the subsequent steps.

2. Air bubbles, dust, scratches, or an "edge-bead" (an artifact of the spinning process) during spinning and baking processes should be avoided; these could cause surface inconsistencies and hinder full contact of the mask, resulting in poor resolution or poor alignment, respectively. Good contact, sufficient to cause good resolution, is affirmed by a vacuum reading of at least 4.5 mTorr before exposure.
3. In our experiments, the prebake and postbake time is two times longer than the manufacturer's suggestion so as to make the solvent of SU-8 thoroughly resist volatility and the exposed feature stick more securely to the substrate. An overexposure of 10–20 % is typically used because overexposure ensures full crosslinking and penetration to the bottom of the resist; this may reduce resolution.
4. During development, care should be taken not to spray the center features directly because even the force from this rinsing could damage the features. Especially for the small features on the first layer, holding and lightly swaying the wafer with forceps is recommended. When rinsing with isopropanol, if a milky residue appears on the wafer, then the wafer requires further development.
5. Because the first layer on the silicon wafer contains features only 2–3 μm high, it is nearly impossible to discern these features with the Karl Suss MJB3 aligner through a second coat of photoresist of more than 10 μm thickness. We experimented with creating multiple-layer alignment with increased thickness, but the overwriting critically reduced resolution. Using tape to protect the marker from second-layer spinning greatly increases the clarity and the accuracy of alignment. After the first-layer mold has been hard-baked, the marks will not be torn by taping.
6. During the fabrication of a network-formation chip, the PDMS base is mixed with the curing agent in a ratio of 9:1 (rather than the manufacturer's suggestion of 10:1) because a higher curing-agent content has been shown to be more biocompatible with in vitro cell cultures [25]. To this mixture we added 10 % xylene to decrease viscosity, allowing greater uniformity of the hole-forming pillars. Several journal publications plot PDMS spin speed versus PDMS film thickness. While these charts did not account for PDMS mixed with xylene, we chose to use the chart published by Zhang [34], which served as a good guide while we experimentally determined the appropriate speed to spin PDMS.
7. A PDMS membrane of 35 μm thickness is too thin to manipulate because of it will stick to itself. Therefore, additional annular layers of about 100 μm are spun on top the 35 μm layer, excepting above the central features, which facilitates peeling the PDMS membrane off the mold and aligning it to the MEA.

8. The surface modification techniques that are used to transform the PDMS membrane into a hydrophilic surface are necessary to promote neuron attachment and neurite outgrowth. Oxygen-plasma treatment can burn off organic residues, and it gives the surface a negative charge by adding O- and OH-groups. However, longer time and higher power render the PDMS surface too glassy and lead to cracking and greater shrinkage; oxygen plasma with a high setting should be applied no longer than 10 min. A cationic polymer coating should be done within 10 min after plasma treatment, or the surface hydrophilicity will fade away.
9. Heidemann and colleagues found that little-to-no neurite outgrowth from chick forebrain neurons plated below 5×10^3 cells/cm^2 and glia-conditioned medium substantially inhibited neuron development and hastened cell death, during even the first day of culture [32]. However, in our experiments, the astrocyte-conditioned medium critically improved neuron survival at low densities (as low as 10 cells/cm^2), and 85 % of the neurons that were laser-patterned into microwells in our biochips survived.
10. The microfluidic cell-delivery chip was created to substitute for the microinjection system we previously used because it is more easily manipulated and thus enables contamination to be avoided. It is driven by hydrostatic-pressure difference between the inlet and outlet reservoir. A single-cell streamline can be achieved in the microfluidic channel; it can keep running for at least 2 h, which in our experience is long enough for patterning [35]. Before cell loading, medium is pumped by a syringe to wet the internal surface of the channel, which is essential to keeping cells in solution. If the deposition area is large, more than one outlet can be designed, according to the requirement. The flow speed can be easily adjusted according to the patterning speed by changing the fluid-level difference between inlet and outlet.
11. Controlling the speed of the laser-pattering process is one of the key points for success. The ability to manipulate cells with the laser-patterning system is dependent on the optical force. This force was able to support only a certain acceleration of the cells without "dropping" the cell, which was left behind as the stage moved on. Furthermore, the fluid-filled chamber caused a drag force which limited the maximum speed a cell could be pulled by the optical force without dropping the cell. While these values depend on the size and type of cell, they are typically on the order of 150 μm/s on the X and Y axes. In the Z axis, the speed is based not on pulling but on keeping up with the forward motion of the cell caused by the axial force. This downward velocity is typically 50 μm/s.

12. Single, round, dark, healthy-looking cells should be selected from microfluidic channel outlet during patterning to increase the viability of cells in the microwells. After repeated practice, guiding each cell to a point on a substrate takes between 10 and 15 s. With cell selection added to the overall patterning time, the overall average time to pattern a cell is closer to 30 s.
13. The electrodes of a standard MEA are made of Indium tin-oxide (ITO) coated with platinum. Even without the platinum coating, the ITO electrodes absorb significantly more of the 800 nm laser radiation than the glass substrate or silicon nitride insulation. This absorbed radiation creates sufficient heat to boil the medium overlying the electrode, thus fouling or damaging it. Additionally, this amount of heat is likely to critically damage a cell as well as forcefully expelling it from the electrode as a bubble is formed. To avoid such heating, the laser intensity can be reduced from 100 to 15 mW. If automatic intensity reduction is desired, check the box of the control panel displayed on the screen for auto reduction and input the desired values for X/Y closeness and for Z distance above. This will reduce the laser intensity to a set level when the stage/laser/cell is within a set distance from the marked deposition point in the horizontal plane (i.e., 50 μm) and a set distance above (i.e., 150 μm).

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Chapter 6

Campenot Cultures and Microfluidics Provide Complementary Platforms for Spatial Study of Dorsal Root Ganglia Neurons

Sara J. Fenstermacher, Maria F. Pazyra-Murphy, and Rosalind A. Segal

Abstract

Dorsal root ganglia (DRG) neurons are a functionally diverse population of sensory neurons with specialized morphology to respond to external stimuli. These pseudo-unipolar neurons extend a single axon that bifurcates to innervate the periphery and spinal cord, allowing sensory information from the environment to be transferred rapidly to the central nervous system. During development, these DRG neurons rely on peripheral target-derived neurotrophins for survival. Due to their unique morphology, DRG neurons exhibit spatially complex signaling and regulated gene expression that are challenging to study in vivo or in conventional cultures. The development of compartmented culture systems has been invaluable to the study of neurotrophin signaling, mRNA transport and localization, and local protein synthesis in axons. Here we describe the setup and maintenance of rat DRG neurons in two different compartmented culture platforms: Campenot cultures and microfluidics chambers. These systems are highly complementary and so together can be used for biochemical analysis and for high resolution imaging of neuronal cell bodies and their extensive axons.

Key words Neuroscience, Dorsal root ganglia (DRG) neurons, Campenot cultures, Microfluidics, Compartmented culture, Axonal RNA, Local protein synthesis, Neurotrophins

1 Introduction

Sensory neurons of the dorsal root ganglia (DRG) convey information about the environment to the central nervous system including pain, touch, temperature, and body position. To perform these specialized functions, DRG neurons are a heterogeneous population of neurons with diverse molecular and physiological properties. During development, DRG neurons extend long axons towards the periphery to innervate skin, muscle, and other target tissues. These primary sensory neurons rely upon target-derived neurotrophins for their survival [1, 2]. Neurotrophins, including Nerve Growth Factor (NGF) and Brain-derived Neurotrophic Factor (BDNF), bind receptor tyrosine

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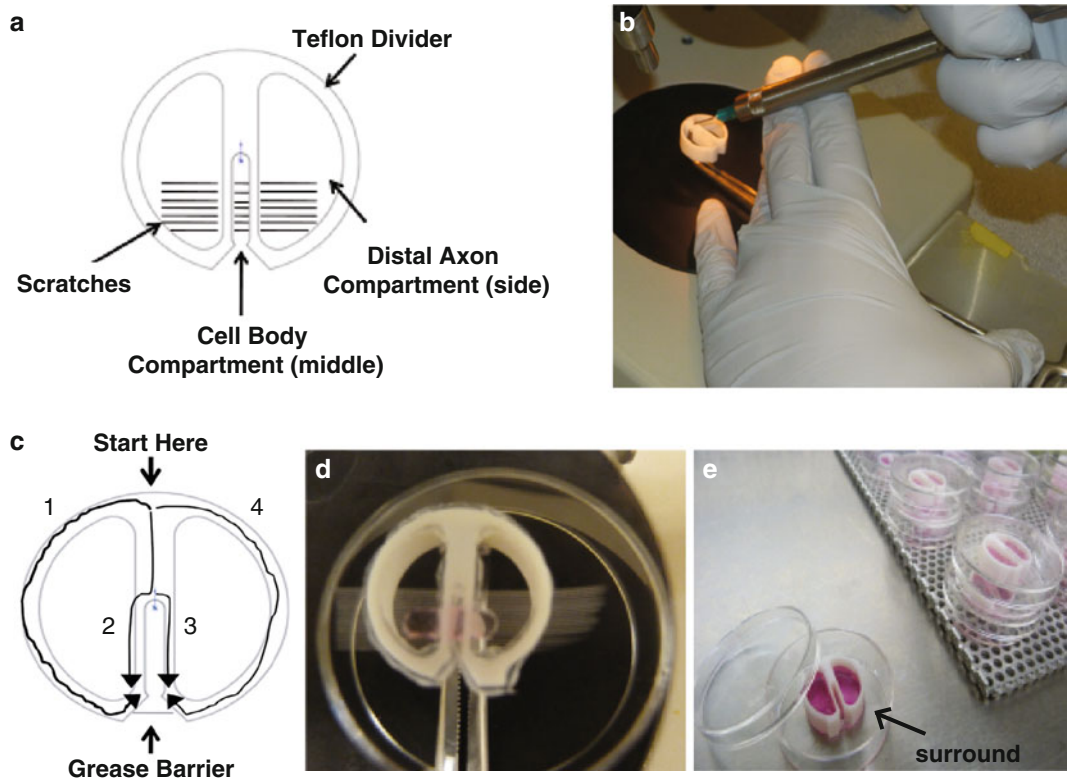

Fig. 1 Assembling Campenot cultures. (**a**) Labeled schematic of Campenot culture. (**b**) Image of hemostat with attached divider and hand placement for adding grease to the divider. (**c**) Schematic of grease application. (**d**) Image of greased divider attached to p35 dish. (**e**) Image of testing assembled cultures for leaks before use

kinases (Trk receptors) at the DRG axon terminal and initiate a retrograde signal to activate a prosurvival response within the neuronal cell body. Thus, DRG neurons that properly innervate their target and receive neurotrophins survive and maintain appropriate connections within this peripheral sensory circuit. In order to study this highly spatial neurotrophin-dependent signaling in neurons, researchers use unique in vitro culture systems to recapitulate the in vivo spatial separation of cell bodies and axons of these long and polarized cells.

In 1977, Robert Campenot introduced a compartmented culture known today as the "Campenot chamber" [3]. This three-chambered culture system allows neurons to be plated within a center compartment and neurites to extend outward into the two surrounding axonal compartments (Fig. 1a), thus retaining the in vivo spatial configuration of neurons. In addition, these compartments are fluidically isolated, allowing for local neurotrophin and/or selective experimental treatment of cell bodies or axons. Finally, cellular material from the individual compartments of

Campenot cultures can be collected separately, allowing for analysis of RNA and protein from neuronal cell bodies and distal axons independently. Initially, Campenot cultures were used with sympathetic neurons to study axonal outgrowth and lipid metabolism and transport [3–8]. Since their development, Campenot cultures have been used extensively by many investigators to examine the unique nature of retrograde neurotrophin signaling in both sympathetic and DRG neurons, providing a powerful in vitro tool to examine these challenging spatial questions [9–18].

While Campenot cultures are ideal for RNA isolation and protein biochemistry from subcellular neuronal compartments, they are not well-suited for high-resolution microscopy or for live imaging, since the neurons are grown on plastic tissue cultures dishes. In contrast, microfluidics chambers are superior for imaging as neurons are grown on glass coverslips. Microfluidics are newer compartmented culture platforms which are commercially available or can be fabricated within your lab [19]. DRG neurons are plated in one side of these two-chambered devices and axons grow through a set of microgrooves to extend outward into a distal axon compartment (Fig. 3a, b). Like Campenot cultures, they provide physical separation between the neuronal cell bodies and distal axons and are fluidically isolated to allow differential treatment of cell bodies and distal axons (Fig. 3c). Microfluidic cultures are an ideal system for following RNA and protein live within the distinct cellular compartments, especially within axons and growth cones, and in response to localized extracellular cues. In addition, cultured neurons can be fixed and used for fluorescence in situ hybridization (FISH) and immunofluorescence to identify subcellular localization of RNA and protein, respectively, within DRG neurons.

Since the original development and use of compartmented cultures for studying axonal growth and retrograde neurotrophin signaling, they have more recently become an essential tool for studying RNA localization and local protein synthesis within axons [20]. Hundreds of RNAs have been identified in axons of DRG and other neurons using RNA isolated from compartmented cultures [21–24] and the cultures provide a way to selectively label and identify locally synthesized proteins by incorporation of non-canonical amino acids [25–27]. In addition, local stimulation within compartmented cultures allows for investigation of cue-induced mRNA localization and translation [21, 28, 29]. Novel methods for labeling and following RNA and newly synthesized protein are being developed regularly and compartmented cultures are powerful tools to use in these experiments and for the continued investigation of spatial regulation of gene expression in neurons [30–33].

Here we describe how to set up and maintain DRG neurons in both Campenot and microfluidic cultures and demonstrate the clean spatial separation of cell bodies and distal axons achieved in

both culture platforms. In addition, we describe how these complementary culture systems can be used experimentally to explore neurotrophin regulated signaling and gene expression with both neurotrophin stimulation and deprivation paradigms. Together these unique cultures platforms provide a specialized and functional system for investigating spatially localized events within DRG neurons.

2 Campenot Cultures

2.1 Reagents and Materials

Collagen I (BD Biosciences, Cat.# 354249)

P35 tissue culture dish (Fisher Scientific, Cat.# 150318)

Methylcellulose (Xenex, Cat.# E4M)

Trypsin (Worthington, Cat.# LS004452)

Teflon dividers (Tyler Research, CAMP10) (*see* **Note 1**)

Pin rake (Tyler Research, CAMP-PR)

Grease loader (Tyler Research, Camp-GLSS)

DMEM (Life Technologies, Cat.# 11965118)

NGF (PeproTech Inc., Cat.# 450-01)

BDNF (PeproTech Inc., Cat.# 450-02)

High-vacuum grease (Fisher Scientific, Cat.# 146355D)

Cytosine ß-D-Arabino Furanoside (AraC) (Sigma-Aldrich, Cat.# C-1768)

23 gauge tubing adapter (Fisher Scientific, Cat.# 427565)

90° angle hemostats (Roboz Surgical Instruments Co., Cat.# RS-7035)

Cell culture incubator at 37 °C with 7.5 % CO_2

2.2 Methods

Preparation of reagents:

1. Collagen Coating: Make up collagen to a final concentration of 0.71 mg/mL in 0.001 N HCl. Coat p35 tissue culture dish by adding 500 µL to each plate and place in a 37 °C oven for 2 days, or until completely dry.
2. Grease Loaders: Fill grease loaders with vacuum grease, wrap with foil, and autoclave.
3. Teflon Dividers: Teflon dividers can be reused following each experiment, but must first be properly cleaned. To clean dividers, wipe off all remaining grease and place in sulfuric acid for 2 days. Remove the dividers from the acid and rinse 3× with water. Boil dividers in water for 20 min, let dry, place in a glass p100 petri dish, and autoclave.
4. Methylcellulose: Put 1.5 g of methylcellulose into a 500 mL bottle. Add a stir bar and autoclave for 20 min on dry (from

this point all work must be sterile). Next, add 500 mL of plain DMEM, and stir in a cold room until dissolved. Aliquot into 50 mL conicals and store at −20 °C. For working stock, aliquot one of the 50 mL conicals into 1.5 mL tubes and store at −20 °C.

5. Media: For culturing DRG neurons in compartmented cultures use DMEM with 5 % heat-inactivated horse serum and 1 % penicillin–streptomycin (*see* **Note 2**). To make up media with neurotrophins dilute nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) from 1 mg/mL stock concentration into the media (*see* **Note 3**). Thus, to make 100 ng/mL NGF/BDNF media, dilute NGF and BDNF 1:10,000 into media. To make 10 ng/mL NGF/BDNF media, dilute NGF and BDNF 1:100,000 into media, or dilute the 100 ng/mL NGF/BDNF media (1:10) with media. In addition, Cytosine ß-D-Arabino Furanoside (AraC) should be added to media for a final concentration of 0.3 μM, to limit the growth of Schwann cells and other glia.

Setup of the compartmented chambers (start this process 1–2 days before the dissection):

1. Using the pin rake, scratch across the middle of a collagen-coated p35 dish, from one edge to the opposite edge. Pipette 20 μL of methylcellulose into the middle of the scratch.
2. To prepare the divider, first attach a 23-gauge Luer stub adapter to the grease loader. Next, use a pair of 90° angle hemostats to grip the Teflon divider by the thick middle portion and lay flat with the divider facing up under a dissecting microscope (Fig. 1b). Apply the grease by coating the surface of the divider following the directions within Fig. 1c. Make sure to insert the adapter directly into the grease at each new starting point, creating a continuous line of grease to seal the divider to the culture dish (*see* **Note 4**). Next, turn one of the prepared p35 dishes upside down and place onto greased-divider with the methylcellulose across the middle of the divider. Use tweezers to press the dish onto the grease making a complete seal (Fig. 1d). It is important to create a full seal with the dish, but if too much pressure is used, the axons will not cross into the side compartments (*see* **Note 5**). Lastly, pick up and turn over the hemostats and unclamp the divider. Under the microscope, make a small grease barrier (0.25 cm) at the front of the middle compartment. The barrier is added to ensure that the cells do not leak out of the middle compartment at the time of cell plating.
3. Add DMEM into each of the side compartments and place in incubator in which the cells will be maintained. Allow the cultures to sit for several hours and then check for leakage into either the middle compartment or surrounding area (Fig. 1e).

Leaky cultures should not be used for plating cells. This is a critical step in the procedure, as it tests each culture for fluidic isolation before use.

DRG dissection:

Compartmented cultures can be used to culture embryonic or adult DRG neurons [34] from rat and mouse, as well as iPS-derived neurons. To culture embryonic rat neurons, Sprague–Dawley rats from embryonic day 15 (E15) are used for the DRG dissection. Each dissected spinal cord yields approximately 500,000 cells. Thus, a dissection of 10 rat embryos yield about five million cells, providing enough cells to plate about 50 cultures, with 100,000 cells plated per culture. To culture embryonic mouse DRG neurons, mice from E13 are used and each animal generates approximately 200,000 cells. Thus, a dissection of 10 mouse embryos should provide enough cells to plate 20 cultures. Although DRGs contain a heterogeneous population of DRG sensory neurons, DRGs are collected from the entire length of the spinal cord and pooled for trypsinization and plating (*see* **Note 3**).

1. Remove rat (E15) embryos from the timed pregnant female animal (as seen in ref. [35]). In a sterile dissection hood, use sharp forceps to tear open and remove embryos from the amniotic sac. To properly euthanize embryos, use scissors to sever each head from the embryo. Transfer each embryo body to a 50 mL conical filled with plain DMEM. Rinse embryos in DMEM and then transfer to a plastic dissecting dish with fresh DMEM. The next steps should be performed using a dissecting microscope and lamp in a sterile environment.
2. Lay embryo ventral side down, limbs pointing outward. The whitish spinal cord should be visible in this orientation.
3. While holding the embryo still with forceps with one hand, use bent forceps (Roboz, Cat.# RS-5005) to pull away the top layer of skin from atop the spinal cord. Do this by sliding one edge of the bent forceps down from the anterior opening between the skin and spinal cord, and then pull back with pressed forceps to remove skin. This step should be performed several times along the length of the embryo to expose the whole spinal cord and all neighboring DRGs. The DRGs should now be visible as two rows of small spheres ventral and lateral to the spinal cord.
4. Use microdissecting spring scissors (Roboz, Cat.# RS-5600) to cut directly below the DRGs, therefore cutting the DRGs away from the underlying tissue while they remain attached to the spinal cord. Cut along both sides of the spinal cord, such

that the spinal cord can be pulled from the embryo with all of the DRGs attached. Place the spinal cords with DRGs in fresh DMEM on ice. Dissect and collect all spinal cords with DRGs in DMEM before proceeding.

5. To remove DRGs from the spinal cord, take each spinal cord with DRGs and place in a p35 dish with 2 mL Hank's Buffered Salt Solution (HBSS). Use fine-tipped forceps to gently pluck each DRG off of the spinal cord and leave in surrounding HBSS. It is best to grab the DRG by the base where it attached to the spinal cord and pull, rather than squeezing the forceps directly around the DRG. After DRGs have been removed from the spinal cord, discard the spinal cord and move on to the next.
6. Add 1 mL trypsin (1 mg/mL made up in HBSS) to plucked DRGs, swirl gently, and incubate at 37 °C for 50 min.
7. Add 3 mL DRG media to trypsinized DRGs and spin cells down in centrifuge at 80 rcf (636 RPM) for 5 min at 4 °C. It is important to use the serum-containing DRG media here, as the serum functions to inactivate the trypsin.
8. Carefully aspirate media off pellet and resuspend cells in 100 ng/mL DRG media + AraC for plating.

Plating and maintaining DRG neurons in compartmented cultures:

1. Before plating DRG neurons, replace DMEM in side compartments with 100 ng/mL NGF/BDNF media + AraC. Perform DRG neuron dissection and plate 100,000 cells per compartmented culture into the middle compartment, in a volume of 25–40 μL (cell density is 2.5–4 million cells per mL media). Be sure to plate cells in 100 ng/mL NGF/BDNF media + AraC. *See* Table 1 for media conditions.
2. After incubating cells overnight, add 10 ng/mL NGF/BDNF media + AraC to the area surrounding divider until media crosses over grease barrier and exchanges with media in middle compartment.
3. Media should be changed on subsequent days (Table 1) to slowly reduce NGF and BDNF levels over the week in culture. In addition, we find that pulsing application of AraC substantially limits the number of glial cells. To change media, first aspirate all media from surround and side compartments. Second, add media to side compartments. Third, add media to surround until it exchanges with the middle compartment. One can drip a few drops over the grease barrier to ensure exchange with the middle compartment. When removing media, it is important to aspirate from the very top of each side compartment

Table 1
Media conditions for DRGs in Campenot cultures

DIV (Days in vitro)	Middle compartment	Side compartments
Plating cells	100 ng/mL NGF/BDNF + AraC	100 ng/mL NGF/BDNF + AraC
1 DIV	10 ng/mL NGF/BDNF + AraC	Leave as is
2 DIV	10 ng/mL NGF/BDNF	100 ng/mL NGF/BDNF
5 DIV	0 ng/mL NGF/BDNF + AraC	1 ng/mL NGF/BDNF + AraC
8 DIV	Use for experiment	

so as to avoid the axons. Lastly, do not remove media directly from the middle compartment as that can disturb the cell bodies, instead only remove media from the surround.

4. Axons will grow into the side compartments by around 5 days in vitro (DIV) and should have extended well across the side compartment by 8 DIV, when ready for experimental use.

Collecting RNA or protein from compartmented cultures:
Since DRG middle and side compartments are fluidically isolated, they can be used to collect cell body and distal axon fractions separately for biochemical analysis (*see* **Note 6**).

1. To collect RNA from Campenot cultures, carefully remove all media from surrounding the divider, and from both the side and middle compartments. Place cultures on ice and add 10 μL TRIzol to each compartment. Take a pipette tip in hand and scratch several times perpendicular to the scratches in the side compartments. Use a narrow gel-loading tip to gently scratch the middle compartment. Be careful not to bump or move the divider so as to maintain fluidic isolation and keep cell fractions separate. Collect RNA from side and middle compartments. Pooled RNA from at least 6 compartmented cultures should be used for each experimental condition. Following RNA extraction using TRIzol, RNA can be analyzed by qRT-PCR (Fig. 2a).
2. To collect protein from the cultures, repeat the procedure for collecting RNA but instead add 5–10 μL lysis buffer to each compartment. Lysates pooled from 8 to 12 compartmented cultures should be used for each experimental condition to optimize detection and reproducibility. Lysates can be analyzed by western blot (Fig. 2b).

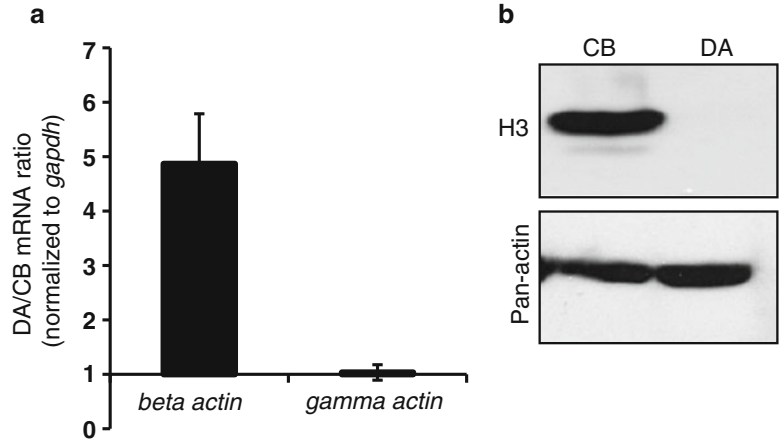

Fig. 2 RNA and protein markers demonstrate fluidic isolation of cell bodies and distal axons in Campenot cultures. (**a**) mRNA ratio of distal axons to cell bodies (DA:CB) isolated from E15 DRG neurons grown in Campenot cultures, normalized to *gapdh* and analyzed by qRT-PCR (Reproduced from ref. [28]). *Beta-actin* is relatively enriched in DA, while *gamma-actin* is not. Beta-actin mRNA localizes to axons, while gamma-actin mRNA is restricted to CB, indicating neuronal cell bodies are restricted to the CB compartment and the Campenot cultures are fluidically isolated during RNA extraction. (**b**) Protein collected from CB and DA analyzed by western blot shows that histone (H3) is only present in CB, while the loading control, pan-actin, is present in both CB and DA. Thus, cell bodies are restricted to the CB compartment and cultures are fluidically isolated during protein collection

3 Microfluidics

3.1 Reagents and Materials

Poly-D-lysine (Sigma, Cat.# P7280)

Laminin (Life Technologies, Cat.# 23017015)

Coverslips, No. 1.5, Size: 40×24 mm (Fisher Scientific, Cat.# 12544C)

Hank's Balanced Salt Solution (HBSS) (Invitrogen, Cat.# 14175103)

BD Falcon petri dishes 150×15 mm (Fisher Scientific, Cat.# 08-757-148)

Microfluidics (Xona Microfluidics, Cat.# SND450, *see* **Notes 7** and **8**)

BD Falcon petri dishes 60×15 mm (Fisher Scientific, Cat.# 08772B)

Neurobasal (Life Technologies, Cat.# 21103049)

B-27 Supplement (Life Technologies, Cat.# 17504044)

NGF (PeproTech Inc., Cat.# 450-01)

BDNF (PeproTech Inc., Cat.# 450-02)

Cytosine ß-D-Arabino Furanoside (AraC) (Sigma-Aldrich, Cat.# C-1768)

Cell culture incubator at 37 °C with 7.5 % CO_2

3.2 Methods

Preparation of Coverslips: Perform 1 day before cell plating (all in sterile hood).

1. Sterilize Coverslips: Place coverslips in a p150 petri dish and rinse with 70 % EtOH. Let coverslips dry by leaning along edge of plate. Place coverslips in a glass p100 petri dish and autoclave.
2. Coverslip coating: Cut piece of Parafilm to fit in p150 petri dish, wipe Parafilm with EtOH and press into bottom of petri dish. Place sterilized coverslips on Parafilm. Coat coverslips with 0.2 mg/mL PDL and leave overnight at room temperature.

Media:
For culturing DRG neurons in microfluidics use Neurobasal, 2 % B-27, 1 % GlutaMAX, 1 % penicillin–streptomycin, 0.08 % Glucose (1:250 dilution of 20 % glucose stock solution) (*see* **Note 2**). To make up media with neurotrophins dilute nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) from 1 mg/mL stock concentration into media (Table 2) (*see* **Note 3**). Thus, to make 100 ng/mL NGF/BDNF media, dilute NGF and BDNF 1:10,000 into media. To make 50 ng/mL NGF/BDNF media, dilute 100 ng/mL media (1:1) in media. Lastly, AraC should be added to media for a final concentration of 0.3 μM, to limit the growth of Schwann cells. We find that addition of AraC for at least the first 3 days in culture is effective at reducing Schwann cells in microfluidics.

Table 2
Media conditions for DRGs in microfluidic cultures

DIV	Cell body compartment	Distal axon compartment
Plating cells	50 ng/mL NGF/BDNF + AraC	100 ng/mL NGF/BDNF + AraC
1 DIV	10 ng/mL NGF/BDNF + AraC	100 ng/mL NGF/BDNF + AraC
2 DIV	1 ng/mL NGF/BDNF + AraC	10 ng/mL NGF/BDNF + AraC
3 DIV	1 ng/mL NGF/BDNF	10 ng/mL NGF/BDNF
4 DIV and longer	1 ng/mL NGF/BDNF or use for experiment	10 ng/mL NGF/BDNF

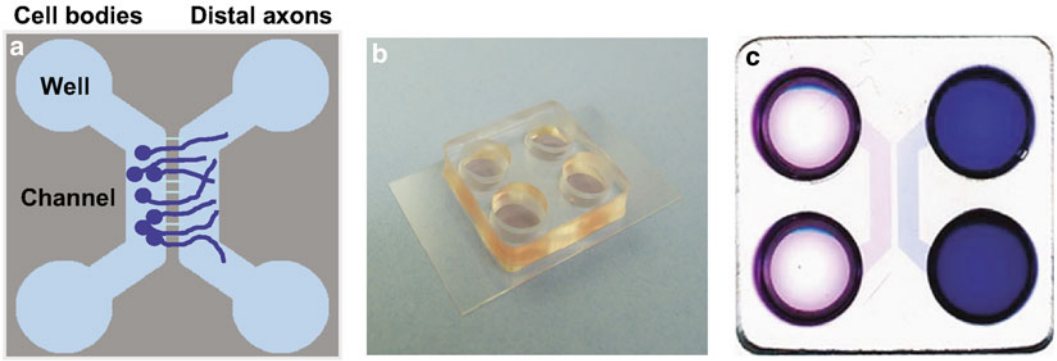

Fig. 3 Microfluidic cultures. (**a**) Labeled schematic of microfluidic culture. (**b**) Assembled microfluidic on glass coverslip. (**c**) Microfluidic filled with media on left side and trypan blue in right side is fluidically isolated for 24 h. Media volume in wells is two times the volume of trypan blue to create isolation by hydrostatic pressure. *Blue* remains restricted to the right side of the culture after 24 h

Culture Assembly: Day of DRG dissection (at least 4 h prior to cell plating).

1. Wash Coverslips: Aspirate off PDL and wash coverslips 3× with sterile water. Following last wash, remove water and let coverslips dry in hood.
2. Sterilize Microfluidics: Place microfluidics (*see* **Note 9**) in a p150 petri dish (grooves facing up) and wash with 70 % EtOH. Let dry in hood.
3. Culture Assembly: Place a dry, PDL-coated coverslip face up into a p60 petri dish. Use forceps to place microfluidic chamber (groves down) onto coverslip (Fig. 3b). To reinforce seal with coverslip, use back end of forceps to gently press down on chamber in center, as well as the four edges and corners (*see* **Note 10**).
4. Laminin Coating: Dilute 50 μL laminin in 5 mL HBSS (1:100 dilution) to a final concentration of 10 μg/mL. Add laminin to each channel by pipetting 50 μL into top two wells with pipette tip pointing directly towards channel. Be sure that laminin flows down to coat the entire channel surface on which cells will grow (culture can be tipped up to encourage flow-through). Add 100 μL of laminin to each well. Place cultures in a 37 °C incubator for 3 h.

Plating cells in microfluidics:

1. Following dissection, trypsinization, and cell counting, spin down 500,000 cells at 80 rcf (636 RPM) for 5 min at 4 °C.
2. Carefully aspirate off media, leaving as little media as possible. Resuspend cells in 50 μL media (50 ng/mL NGF/

BDNF + AraC). This step is important for obtaining the proper cell density within the microfluidic.

3. Remove laminin from microfluidics by aspirating off all four wells and placing tip directly towards channel from top and/or bottom to remove all laminin from the channels (*see* **Note 11**).
4. Plate 40,000 cells in each microfluidic by adding 4 μL cells to the top well of cell body side. Pipette tip should point directly into the channel and watch that cells flow down, reaching the base of the channel. If cells do not flow down, apply a slight downward force by aspirating briefly from the bottom of the channel using a 200 μL pipette tip.
5. Immediately put cultures into the 37 °C incubator to let cells attach to substrate for 5–10 min.
6. Add media (100 ng/mL NGF/BDNF + AraC) to distal axon compartment channel by pipetting 40 μL directly into channel in top right well, and subsequently add media to all four wells: 100 μL media (50 ng/mL NGF/BDNF + AraC) to both cell body wells and 100 μL media (100 ng/mL NGF/BDNF + AraC) to both distal axon wells. Return cells to incubator.

Maintenance of microfluidics cultures:
Due to the small volume of media that can be added to microfluidics cultures, media should be changed every day or every other day to minimize effect of evaporation, and ensure optimal health and axon growth. One can adjust the cell feeding schedule depending on the humidification of your incubator, based on changes in the media volumes following feeding. Importantly, media must be changed without disturbing cells. Therefore, it is important that you not remove media directly from the channels themselves (which contain approximately 4 μL media). *See* Table 2 for schedule of media conditions when feeding cells.

1. When changing media, first aspirate media from all four wells. Keep pipette tip away from entrance to the channel containing the cells.
2. Add 40 μL of specified media to top well of each culture. Do not point tip into channel, just add to the well.
3. Tip cultures up to allow media to flow through the channel and into the bottom well. Be sure to see that media begins to fill the bottom well.
4. Add 100 μL specified media to each well and return cultures to incubator (*see* **Note 12**).
5. Axons will grow across microgrooves and enter the distal axon compartment by 1–2 DIV and extend across the compartment by 4–7 DIV. Continue to change media daily or every other day until used for an experiment (Table 2) (*see* **Note 13**). Keeping cells in AraC for first 72 h will significantly limit glia

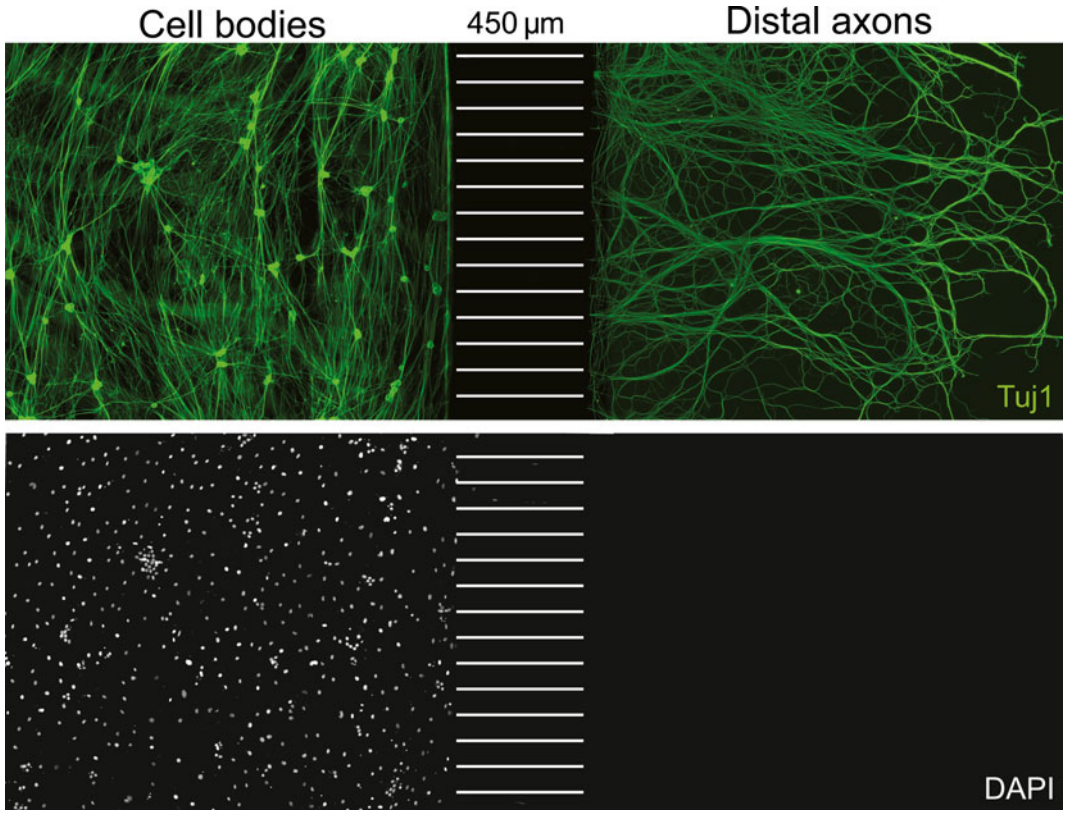

Fig. 4 Immunostaining in microfluidic cultures. Tuj1 immunostaining of E15 DRG neurons grown in microfluidic culture shows growth of axons into distal axon compartment. DAPI staining demonstrates that cell bodies are restricted to the cell body compartment

within the cell body compartment and no glia should be present in distal axon compartment.

Fixing microfluidics:
While microfluidics can be used for live imaging of DRG neurons, the cultures can also be fixed for immunofluorescence (IF) or fluorescent in situ hybridization (FISH) (Fig. 4). Since axons are physically separate from the DRG cell bodies, this allows for high resolution imaging of individual axons and growth cones. This is far superior to imaging in mass cultures where most fields of view contain a mix of cell bodies and axons, making it difficult to select the appropriate exposure time and focal plane for optimizing detection of axonal signal. If using cultures for RNA analysis by FISH, be sure to use RNase-free fixatives.

1. To fix microfluidics, first remove half of the media from each well.
2. Perform a gentle fix by adding 4 % PFA to the media in each well (final ratio of media to PFA is 1:1) and leave on cells for 10 min at room temperature (*see* **Note 14**).

3. Remove all media/PFA from each well and add 50 μL fresh 4 % PFA to each top well and let flow through to bottom wells. Add additional 4 % PFA to each well and fix for 10 min.
4. Wash three times with 1× PBS (5 min each): Wash microfluidics by adding 50 μL 1× PBS to both top wells and tip to allow PBS to flow down through both channels. Then add 100 μL to all four wells and wait 5 min. Aspirate PBS from all four wells and repeat twice more.
5. Fixed cells can be stored in 1× PBS at 4 °C for immunostaining or FISH.
6. Immunostaining and FISH can be performed with the microfluidic chamber still attached by adding reagents just as described previously for media and PBS (Fig. 4). Mount attached microfluidics by adding 30 μL of Fluoromount G to each top well. This is sufficient for imaging cell bodies and distal axons. Alternatively, to image axons within the grooves, one can remove the microfluidic chamber for staining. To do this, outline attached microfluidic chamber with a hydrophobic barrier pen following fixation, remove the microfluidics and proceed with staining, and mount the coverslip onto a slide for imaging.

4 Neurotrophin Stimulation and Deprivation Experiments

Compartmented cultures can be used to study various aspects of neuronal development, function, and regeneration. Here are two experimental paradigms which can be used to study neurotrophin responses within DRG cell bodies and axons. First, neurotrophin stimulation experiments can be performed alongside a control, vehicle-stimulated condition, to determine how gene expression is regulated both temporally and spatially in response to neurotrophins. Alternatively, these systems can be used to study changes observed when neurotrophins are removed. These experiments can be performed in either compartment culture system, depending on the analysis method required by your experiment. Lastly, these experiments can be combined with a number of other manipulations, such as knockdown by shRNA, transgene overexpression, or acute injury to investigate the direct role of specific molecules in DRG neuron function and survival (*see* **Notes 15** and **16**).

4.1 Neurotrophin Stimulation Protocol

1. To perform experiments with neurotrophin stimulation, first remove all media from the cultures. In Campenot cultures this requires aspirating off all of the media from side compartments and the surround. In the microfluidics, this requires aspirating media from the four wells.
2. Add plain media (either DMEM or Neurobasal) to the cultures for 2 h. This brief treatment allows signaling pathways to reset to baseline following growth in neurotrophins.

Additionally, blocking antibodies to NGF and/or BDNF can be used during the starvation to rigorously eliminate the effect of any residual neurotrophin.

3. Stimulate the distal axons with 100 ng/mL NGF/BDNF or vehicle control (100 ng/mL BSA) (*see* **Note 17**). In the Campenot's this can be done by making a 1:100 dilution of NGF and BDNF in plain DMEM and adding 5 μL of this to each side compartment (assuming side compartments contain about 500 μL media). In the microfluidics, plain media with neurotrophins or BSA should be made in advance and added as previously described. Cell bodies are left in plain media during stimulation. This selective stimulation of distal axons recapitulates the way in which DRG neurons receive neurotrophins from the periphery in vivo. To study the differences in the spatially selective response to neurotrophins, one can also compare neurotrophin stimulation of the distal axons with stimulation of the cell bodies, or with combined stimulation of cell bodies and axons (global stimulation similar to conventional mass cultures).
4. The length of neurotrophin stimulation can vary from minutes to hours, or even days, depending on the timing of the biological response. Ideally, one should perform a time course to determine the optimal stimulation time for experimentation and to investigate the temporal properties of the biological response.
5. Following neurotrophin stimulation, collect (Campenot's) or fix (microfluidics) cells for subsequent analysis (Fig. 5a).

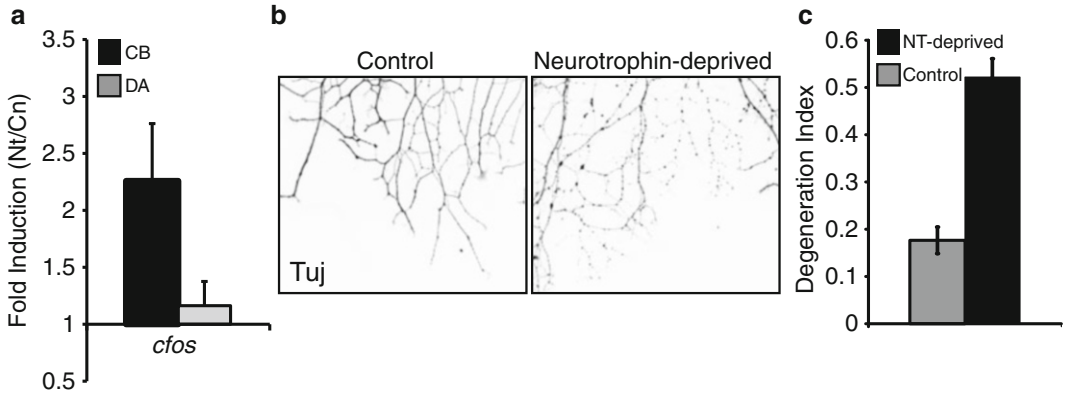

Fig. 5 Neurotrophin stimulation and deprivation experimental data. (**a**) Campenot cultures were stimulated with neurotrophins at distal axons (DA) for 2 h (Reproduced from ref. [28]). Fold induction is compared with control, vehicle-stimulated, neurons. The transcription factor *c-fos*, an immediate early gene, is upregulated in CB, but not DA. (**b**) Distal axons (DA) in microfluidic cultures were deprived of neurotrophins for 24 h, fixed and stained for Tuj1, and compared with control (plus neurotrophin). (**c**) Images of DA were binarized and axon fragments were defined by the Analyze Particle (0–200) function in National Institute of Health ImageJ software. Quantified images show that NT-deprived DA exhibit an increase in axon fragmentation by this degeneration index (ratio of fragmented axons to total axon area)

4.2 Neurotrophin Deprivation Protocol

1. To selectively deprive distal axons, change axonal compartments to plain DMEM or Neurobasal media and put cell bodies in low neurotrophins (10 ng/mL NGF/BDNF) for 24 h.
2. Following deprivation, cultures can be fixed and stained for Tuj1 to perform a degeneration assay (Fig. 5b, c).

5 Notes

1. There are many other dividers available from Tyler Research. This protocol is designed especially for the Teflon dividers with one small middle compartment and two larger side compartments (CAMP10–20 mm OD), but the compartment setup protocol would be the same with many other shaped dividers.
2. This serum containing media has been used successfully for many years to grow DRGs in compartmented Campenot cultures. As a serum-free alternative, Neurobasal with B-27 can also be used with these cultures. In contrast, we find that growth of DRG neurons in microfluidic chambers is far superior with Neurobasal and B-27. The use of different media for each culture system is based on empirical evidence that the specified media results in optimal growth of DRG neurons in each culture. The difference in effectiveness may be a result of the media interaction with different substrates.
3. DRG neurons are a heterogeneous group of neurons that express different Trk receptors and, in turn, rely on different neurotrophins for their survival during development. These protocols use both NGF and BDNF for culturing DRG neurons, thus supporting the survival and maintenance of TrkA and TrkB expressing neurons which account for the vast majority of embryonic DRG neurons. The TrkC expressing neurons, which are a very small percentage of total DRG neurons, are not maintained in these cultures. Alternatively, the neurons can be grown in NGF alone, yet this will alter the complement of neurons that survive in the cultures.
4. Observing assembly of the cultures can be extremely helpful, especially for proper grease application. To watch this process, see the referenced JoVE video [11].
5. The amount of pressure used to seal the p35 dish to the greased Teflon divider will greatly influence the culture outcome. The ideal amount of pressure will seal the compartments and create fluidic isolation, as well as allow the axons to grow through the grease and into the side compartments. In the beginning, it is inevitable that several cultures will be leaky, so be sure to set up more than needed for an experiment.

6. It is possible to collect RNA or protein from microfluidics [19], although not described here. However, the amount of material is more limited than from the Campenot cultures.
7. This protocol was established using the 450 μm width microgroove microfluidics, which seem optimal for DRG neurons. Similar protocols can work for shorter and longer microgrooves. If using shorter microgrooves, there is a greater possibility that fluidic isolation may be compromised.
8. Instead of purchasing premade microfluidics, one can also fabricate the chambers using poly dimethylsiloxane (PDMS) [19]. This requires an initial investment to manufacture the master mold.
9. The microfluidic chambers are not readily reusable as are the Campenot dividers, which can be washed and reused for years. Some success with detergent-based cleaners has allowed the microfluidics chambers to be reused, but optimal growth and fluidic isolation is often not achieved in many of the washed cultures.
10. Following assembly of the culture, the coverslip often sticks to the tissue culture plate. After pressing the chamber onto the coverslip, use the forceps to gently loosen the coverslip away from the plate.
11. If bubbles form within the channels when aspirating laminin or plating cells, aspirate off cells, add 50 μL media to the channel, and aspirate again to clear the channel. Then replate cells into the channel. It is important to examine the channels carefully before adding cells to the culture.
12. While cells are growing, we find it optimal to maintain equal volumes of media on each side of the microfluidics cultures. During an experiment when fluidic isolation is desired, volumes should be kept unequal to create a small hydrostatic pressure difference and ensure that there is no mixing of media between compartments (Fig. 3c). We have verified that small molecules (>750 Da) in the axonal compartment are not detectable in the cell body compartment if we add 60 μL media to the axonal wells, with 200 μL media in each cell body well (filled to top). This volume difference maintains fluidic isolation overnight and up to 24 h. Figure 3c demonstrates that the hydrostatic pressure difference can retain trypan blue to one side of the culture for at least 24 h.
13. When performing experiments with changes in media that will add or remove various treatments (i.e., small molecules, plain media starvation), do two washes through each microfluidic channel with 40 μL media and aspirate from wells following

each wash, before subsequently adding additional media to the wells. This ensures that cells are cleaned of any prior treatment and are successfully transferred into media of the desired concentration.

14. If you find that axonal morphology is not maintained during PFA fixation, try a methanol pretreatment to stabilize axonal microtubules. Add 50 μL ice-cold methanol (kept at −20 °C) to the top two wells of each channel and let flow through to bottom wells. This should be done quickly and only left on cells for 1 min. Following this, remove methanol from each of the wells and perform a 4 % PFA fix for 15 min.
15. For transgene overexpression in microfluidics, DRG neurons can be transfected using Amaxa Rat Neuron Nucleofector Kit (Lonza) prior to plating cells. Following transfection of two million cells, spin cells down for 3 min, resuspend in 50 μL media (without Pen-Strep or AraC), and plate 4 μL into cell body channel. Variability in cell death during transfection leads to differences in cell number; therefore, recount cells following transfection or adjust media volume slightly after plating first culture to desired density.
16. Recombinant protein can be selectively introduced to a single compartment of the Campenot cultures using the Chariot protein transfection system (Active Motif) to locally overexpress protein in cell bodies or axons [28].
17. It is important to use a vehicle control (i.e., BSA) alongside the neurotrophin-stimulated cultures for the period of treatment. This controls for the response to new media following starvation with plain media, as well as the mechanical stimulation during the media change.

Acknowledgements

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Part III

Manipulation of Synapses

Chapter 7

Development of Microfluidic Devices for the Manipulation of Neuronal Synapses

Anika Jain and Martha U. Gillette

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

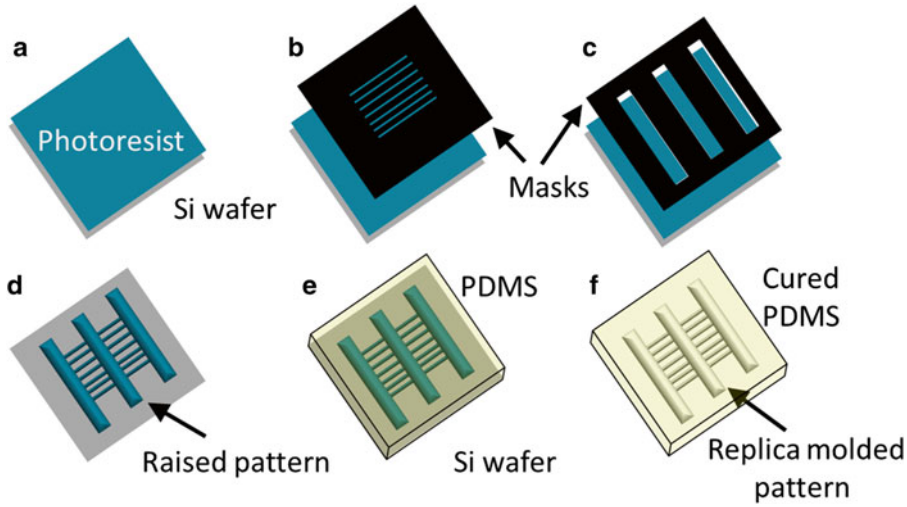

Fig. 1 Workflow for device fabrication. Fabrication begins with photolithography (**a**–**c**) that creates raised patterns on silicon masters (**d**) which can then be used and reused to create PDMS devices through soft lithography (**e**, **f**). To create low-height interconnects in addition to the 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**)

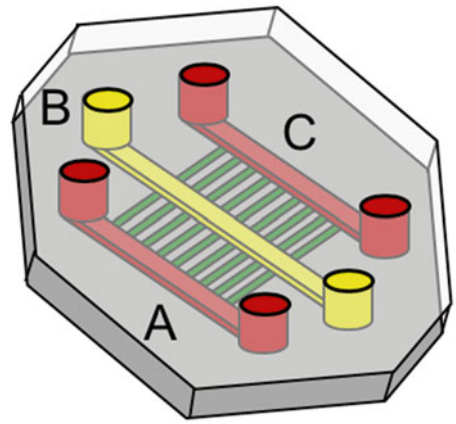

Fig. 2 Microfluidic device for synaptic manipulation. Devices consist of three channels A, B, and C communicating through narrow interconnects (about 7 μm × 7 μm × 300 μm long). Neurons seeded into the outer channels, A and C (*red*), send neurites through the interconnects (*green*), into the central 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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Chapter 8

Use of a 3-Compartment Microfluidic Device to Study Activity Dependent Synaptic Competition

Ainsley Coquinco and Max Cynader

Abstract

The brain undergoes rapid changes throughout development. Balancing the formation and elimination of synapses is essential for proper neural circuit formation. Many of these processes are driven by activity the brain receives during development. Therefore, a significant amount of time and effort has been given to understanding how activity shapes the brain. Current in vitro and in vivo methods have provided us with many insights. However, with in vivo methods, it is difficult to observe the changes that occur at the molecular level. Current in vitro methods allow us to see those molecular changes but regional influences of activity is difficult to control. We begin bridge the gap between the two modes of analysis by using microfluidics. Through the use of 3-compartment microfluidic chambers, neuronal cell bodies are both physically and environmentally isolated while their axons are free to interact between groups of cells. Here we outline the steps to establish such a system.

Key words Synaptic competition, Synaptic plasticity, 3-Compartment microfluidic chambers

1 Introduction

In the developing brain, neurons undergo a substantial number of morphological and synaptic changes [1, 2]. A constant balance of synapse formation, stabilization, and elimination is required for the proper development of the neural circuit [2]. A major contribution to these mechanisms is driven by the experience/activity input that the brain receives during development [3]. The most classic and extensively studied example of this phenomenon occurs in the development of the visual cortex. When one eye is closed during development, electrophysiological observations show increases in activity and territory in favor of the open eye in the visual cortex [4, 5]. This is also clearly observable structurally with anatomical techniques in the formation and maintenance of ocular dominance

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columns [6, 7]. The mechanism by which neural activity shapes neural circuitry during development remains a key question to be resolved in neuroscience research.

While visual cortex plasticity is one of the more robust and well-studied models of neural development, it is not without its own difficulties. Because of the complexity of the mammalian brain, it becomes technically difficult or expensive to observe changes at the cellular and molecular levels. Furthermore, there is the additional difficulty of genetic manipulations either yielding extremely low gene expression efficiency or requiring the use of transgenic mice. To work around these issues, in this and many other systems, many labs have chosen to *use* in vitro models. The use of primary neuronal cultures allows for easier observation of neurons and delivery of specific genes via transfection [8–10]. These reduced models make it easier for the researcher to genetically manipulate and/or visualize individual neurons.

An important advantage of in vitro models is the ease of visualizing and genetically manipulating neurons. However, changing the activity of neurons in culture is currently limited to non selective chemical treatment of the entire culture or genetic modification of a small population of neurons within the culture [11–16]. Most models that look to change the activity of a subset of neurons in culture require the use of genetic modifications. This enables the user to examine the effects of manipulating a particular neuron's activity relative to its unaltered surrounding environment. The disadvantage is that it is difficult to identify a specific target region and control the proximity of altered and unaltered neurons.

Microfluidic technology can be used to address these issues. Microfluidics takes advantage of the properties of laminar flow to manipulate minute amounts of fluids creating chemically isolated environments [17]. Park et al. [18] developed two and three compartment microfluidic devices that could create distinct chemical environments by varying the relative volumes in each compartments. Furthermore, microgrooves between compartments still allowed for interaction between compartmentalized neuronal groups while remaining environmentally isolated.

Using the three compartment microfluidic device, we developed a new model to study in vitro activity dependent synaptic plasticity with dual inputs. Using this three compartment device, we designated one of the center compartments (center) as the target while the other two lateral compartments act as inputs. Utilizing the microfluidic properties, we can then selectively alter the activity of the desired neuronal population pharmacologically.

Here we outline the steps needed to establish the culture system. This protocol has the flexibility to be utilized with a variety of cells and genes of interest.

2 Materials

- Three compartment microfluidic chamber available (Millipore, AX50010) (Fig. 1a).

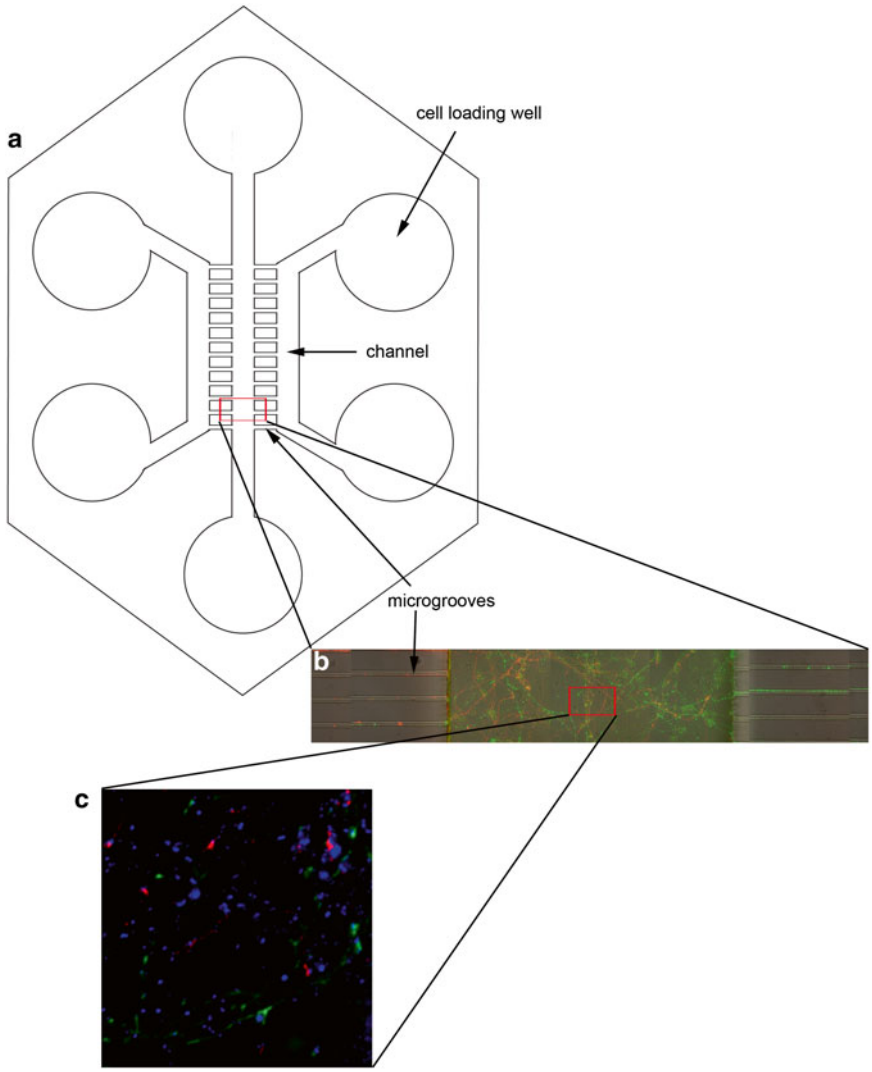

Fig. 1 Illustration of a 3-compartment microfluidic device. (**a**) Each compartment consists of two cell loading wells connected together by a channel. The middle channel is 500 μm wide, and the lateral channels are 1,000 μm wide. When dissociated neurons are placed into a cell loading well, they flow into and through the channel; many will eventually settle inside the channels and begin to grow. All observations and measurements are performed on neurons located inside the channels. For the purposes of this protocol all observations and measurements are performed using the middle compartment's channel. (**b**) compartments are separated by microgrooves (500 μm long, 10 μm wide) that allow axons from each compartment to interact with the other compartmentalized neurons. (**c**) axons from the two lateral compartments are differentially labeled with fluorescent protein tagged synaptophysin (transfection) and puncta from those axons that colocalize with PSD-95 (*blue*, immunocytochemistry, viewed from the center compartment) are counted as synapses

- Sylgard 184 elastomer kit (Dow Corning).
- #1.5 (0.17 mm) cover glass: Glass is first treated in a 50 % hydrochloric acid (HCl) solution overnight. After HCl treatment, glass is then rinsed with distilled water and sonicated for 30 min. After sonication, glass is then rinsed with 100 % ethanol followed by subsequent washes with 75 % ethanol. Glass is then stored in 75 % ethanol and ready to be used.
- Poly-D-Lysine in borate buffer: 0.1 M borate buffer solution (1.24 g boric acid, 1.9 g sodium tetraborate, 400 mL water, pH 8.5).
- Rat primary cortical neurons (will also work with mouse).
- Rat Nucleofector kit (Lonza, VAPG-1003).
- Dulbecco's Modified Eagle Medium with 10 % fetal bovine serum.
- Neurobasal (Invitrogen, 12348-017) with B27 supplement (Invitrogen, 17504-044), GlutaMAX (Invitrogen, 35050-061) and penicillin–streptomycin.
- YFP or mcherry tagged synaptophysin was used to label axons coming from the outer compartments and allowed us to differentiate between the two. Plasmids were on the pLL 3.7 backbone with a synapsin promoter.

2.1 Equipment

- Nucleofector™ 2b Device (Lonza, AAB-1001).
- Fluorescent microscope.

3 Procedure

All experiments are performed in accordance with the guidelines and regulations specified by relevant authorities.

3.1 Assembly of Microfluidics Chambers

- Chambers are sterilized with a quick wash of 70 % ethanol.
- Chambers are then incubated in PBS (pH 7.4) overnight at 37 °C and 5.5 % CO_2.
- Chambers are rinsed with distilled water and dried thoroughly at room temperature. Cover glass should also be dried thoroughly (H_2O rinse is not required for glass) (**Note 1**).
- Additional sterilization step (optional): chambers and glass are UV sterilized for 30 min prior to assembly.
- Once completely dry chambers are pressed firmly onto treated No. 1.5 cover glass. Make sure no air bubbles form in between the glass and chamber (**Note 2**).
- Poly-D-lysine is then put into half of the wells allowing the coating solution to pass through the channel into the other half of the wells (**Note 3**).

- Chambers are then incubated at 37 °C for 1–2 days.
- After incubation with poly-D-lysine, the chambers are then washed with double distilled H_2O (ddH_2O). Remove PDL but without drying the channel and then place sterile ddH_2O in the top half of the wells allowing the water to flow through rinsing the inner part of the chamber. This step is repeated three times (**Note 4**).
- After washing, fill all wells with sterile ddH_2O and incubate overnight at 37 °C and 5.5 % CO_2.
- Remove H_2O and replace with Neurobasal with B27 supplement and GlutaMAX. Allow media to flow through similar to wash steps. Incubate for at least 1 h. Chambers are now ready for plating of neurons. Ideally chambers should be used the same day upon completion but can be used up to 1 week later. (However, ensure the wells and channels remain filled with liquid).

3.2 Cell Culture, Nucleofection, and Plating of Rat Primary Cortical Neurons

- Rat cortical neurons are prepared from embryonic day E18 rats and are dissociated into a cell suspension to be used for nucleofection.
- For nucleofection: a suspension density of four million cells per mL is used. The cell suspension is aliquoted to four million cells and spun down in a centrifuge at 600 rpm for 30 s.
- 110 μL of electroporation buffer is mixed with 2 μg of FP-tagged (mcherry or YFP) synaptophysin contained within a pLL3.7 plasmid backbone with synapsin promoter and chilled for about 10 min on ice (**Note 5**).
- Media is then removed and cells are resuspended in the electroporation buffer.
- Cells are then electroporated at setting O-003 using the Amaxa Nucleofector.
- 1 mL of DMEM with FBS is added to the cells. Mix well.
- 10 μL of the cell suspension is placed on the top half of each compartment and allowed to flow through the channel to the other end.
- Another 10 μL of the cell suspension is then placed at the bottom of the channel.
- Allow cells to settle for at least 10 min then top up each well with 200 μL of Neurobasal (Invitrogen) with B27 supplement (Invitrogen) and GlutaMAX (Invitrogen) (**Note 6**).

3.3 Troubleshooting

- Cell density inside the channel is too low. After plating examine the flow through of the cells through the channel. If cell flow through is slow tilt chamber to increase flow force. If more force is required, a quick vacuum suction on one end of the

channel can be performed. Wait for cells to settle and determine if that is the desired density.

- Cell growth appears to be slower than cultures in classical dishes. If this occurs make sure all surfaces that neurons are exposed to are coated with poly-D-lysine.
- Axons do not stay within the micro-channels. If both chamber and cover glass are not completely dry this will occur. Any moisture prior to assembly will affect the hydrophobic bond formed between the glass and Sylgard.
- Leaking of chemical into the other chambers is very high. This problem will occur if assembly is performed when components are not completely dry or if there are unnoticed air pockets between contacting surfaces of the chamber and glass.

4 Procedure for In Vitro Synaptic Competition Model

The following steps are where we begin to look at synaptic competition. By completing the previous steps, there is now a system in which three groups of neurons are in three chemically and physically separate environments with the exception that their axons can interact among them. We are then able to change the activity of one group of neurons (in this case one of the lateral groups) and observe its ability to form synapses in the center compartment compared to the other lateral group. The lateral groups should have been differentially marked via transfection (in this protocol we used YFP and RFP tagged synaptophysin).

5 Treatment of Neurons

- Treatment is performed by removing the media from one compartment only.
- Then new media with the desired activity enhancer/inhibitor (e.g., tetrodotoxin and high potassium, chloride concentration) is added to one half of the compartment only.
- Wait for about 10–30 s for the new media to flow through the channel, removing the excess untreated media from the channel. (You should see the other half of the compartment begin to fill).
- Remove the media again and then add 50 μL of treated media to each well. Adjust the total volumes of each compartment. Typically, we maintain total volume difference between the treated and untreated channels of at least 150 μL.

6 Immunocytochemistry

- After the desired treatment ends, cells are fixed with 1 % paraformaldehyde in PBS (Phosphate Buffered Saline pH 7.4, GibCo) for 4 min followed by cold methanol (−20 °C) fixation for 6 min (**Note 7**).
- Cells are then washed 3× with PBS.
- Cells in the central compartment (target/postsynaptic cells) are immunostained for PSD-95 using PSD-95 antibody (monoclonal mouse, 1:1,000 dilution from Affinity Bioreagents) in 3 % BSA (bovine serum albumin, Sigma) in PBS overnight at 4 °C. Cells are then washed with PBS again then incubated for 1 h at room temperature with Alexa 647 anti mouse secondary antibody (1:100 dilution, Invitrogen). This acts a post synaptic marker for all neurons in the compartment (**Note 8**).
- Neurons are then imaged under a fluorescent microscope at 63× (**Note 9**).
- The number of synapses formed for each lateral group within the central compartment can then be quantified and compared by counting the number of colocalized synaptophysin and PSD-95 puncta (Fig. 1b, c).

7 Notes

1. To speed up the drying of the chambers use a vacuum to remove any excess water seen on the chamber.
2. Check for air bubbles again several minutes after placing the chamber and glass cover together. Sometimes air bubbles will appear later.
3. If flow of poly-D-lysine is slow or not occurring use a vacuum to create a brief suction on the other end.
4. Make sure the water is able to flow through the channel also when washing. Do not just wash the well. Alternate filling connected wells to be more thorough.
5. Transfection steps can be done with any plasmid. Also, if the transfection is preferred to be performed when the cells are older, methods such as the use of Lipofectamine can be done. Simply follow the transfection protocol with the treatment protocol described here in mind.
6. After plating of neurons, it is advisable to surround the completed chambers with additional media to minimize evaporation of media from the cell culture.

7. Fixation and washes are performed with the chamber and cover glass attached together.
8. After fixation and washes, the chamber can be removed from the cover glass prior to staining if desired.
9. If cells are desired to be viewed at a later date (or the microscope used is upright), remove chamber from coverslip and mount the coverslip on a microscope slide.

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Part IV

Interaction of Different Cell Populations

Chapter 9

Multi-compartment Neuron–Glia Coculture Microsystem

Jaewon Park, Sunja Kim, Jianrong Li, and Arum Han

Abstract

Conventional cell culture plate based neuron–glia coculture methods have limitations in conducting localized physical and biochemical manipulations of cells that allow detailed investigation into axon–glia interactions and spatial regulation of functions. Here, we describe a multi-compartment neuron–glia coculture microsystem platform where axons can be fluidically and physically isolated from neuronal somata and dendrites so that interactions between the axon and glia can be studied in isolation. The multi-compartment configuration having six axon/glia compartments allows multiple experimental conditions to be conducted on a single microsystem.

Key words Neuron culture microsystem, Compartmentalized culture, Axon isolation, Localized biomolecular treatment, Axon–glia interaction, Coculture microsystem

1 Introduction

Oligodendrocytes (OLs) are post-mitotic cells that arise from oligodendrocyte progenitor cells (OPCs) that progressively mature into pre-myelinating oligodendrocytes before finally differentiating into myelinating cells in the white matter of the central nervous system (CNS) [1, 2]. Regulation of OL development and differentiation has been studied extensively and the signaling molecule that affects OPC proliferation, survival as well as differentiation has been well identified [3–5]. However, the molecular basis of axon–glia signaling that leads to the formation of CNS myelin still remains largely unknown. This is due to the complexity of the myelination processes as well as lack of suitable in vitro myelination models that are easily accessible for experimental manipulations.

Conventional cell culture methods, where neurons and glial cells are cocultured in randomly mixed form, fail to provide means to locally manipulate the physical and biochemical cellular environments and make it difficult to investigate localized interaction of axons and glia in the absence of neuronal somata for detailed mechanistic studies. In order to overcome the limitations of

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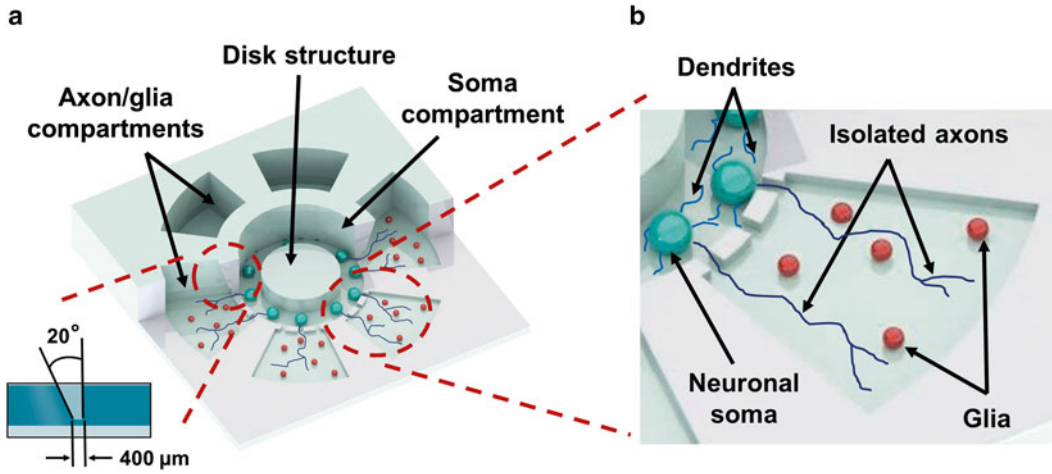

Fig. 1 (**a**) Illustration of the multi-compartment neuron–glia coculture microsystem capable of studying localized axon–glia interaction under six different experimental conditions on a single device. *Inset*: Cross-sectional view of the PDMS wall separating the soma and the axon compartment that is thicker at the top (*see* **Note 5**). (**b**) Illustration showing the isolation of axons from neuronal somata for localized axon–glia interaction studies [8]

conventional cell culture methods, various microfluidic neural culture platforms have been recently developed [6–10]. In this chapter, we describe fabrication steps for a multi-compartment neuron–glia coculture microsystem and its usage where axons can be fluidically and physically isolated from neuronal somata and dendrites into six axon/glia compartments for studying localized axon–glia interaction under multiple experimental conditions in a single microsystem [8].

The coculture microsystem is composed of one soma compartment and six satellite axon/glia compartments connected by an array of microchannels (width: 20 μm, height: 3 μm, length: 400 μm) (Fig. 1a). Shallow height of the microchannels works as a physical barrier to keep neuronal somata in the soma compartment, while allowing axons to grow into the neighboring axon/glia compartments for isolation (Fig. 1b). The length of microchannels (400 μm long) restricts the growth of dendrites into the axon/glia compartment, thus only axons are present in the axon/glia compartment. The multi-compartment configuration where the soma compartment and the axon compartments are only 400 μm apart can be achieved by a micro–macro hybrid soft-lithography master fabrication (MMHSM) technique [11], and enables multiple experimental conditions of axon/glia interaction studies to be performed in parallel on a single device. This fabrication technique also allows the microsystems to be prepared with reduced fabrication time and improved reproducibility with minimum device-to-device variations by eliminating the manual reservoir punching process.

The coculture microsystem introduced in this chapter can not only be used for studying interaction between neurons and glia cells but also can be used for various other applications such as studying migration/response of cocultured microglia, axon transection and regeneration, or axonal transport of target molecules. The design can be easily modified to have increased number of satellite compartments for achieving higher-throughput culture platform if needed.

2 Equipment and Materials

2.1 Equipment

- Imprint master fabrication: Mask aligner, spin-coater, hot plate, reactive ion etcher with CF_4 gas.
- PDMS device fabrication: Digital balance, laboratory oven, vacuum connected desiccator, oxygen plasma cleaner, CNC milling machine, temperature-controlled hydraulic press, autoclave.
- Neuron culture: Humidified 5 % CO_2 incubator.

2.2 Materials

2.2.1 Microsystem Preparation

- 3 inch silicon wafer (SQI Inc.)
- SU-8™ 2015 photoresist (Microchem).
- SU-8™ developer, (propylene glycol methyl ether acetate (PGMEA), Microchem).
- Transparency film mask (CAD/Art service).
- Isopropyl alcohol (IPA) (EMD Millipore).
- Sulfuric acid (EMD Millipore).
- Hydrogen peroxide (BDH Chemicals).
- (tridecafluoro-1,1,2,2-tetrahydrooctyl)-Trichlorosilane (United Chemical Technologies, Inc.).
- Poly(methyl methacrylate) (PMMA) block (McMaster-Carr).
- Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning).
- Sharpened stainless steel punch (Diameter: 10 mm, Technical Innovations, LLC).
- Round glass coverslip (Diameter: 25 mm, Electron Microscopy Sciences).
- Poly-D-lysine (PDL) (Sigma Aldrich).
- Weighing boat (VWR International).
- Disposable stir rod (e.g., plastic fork).
- Razor blades (VWR International).
- Parafilm (VWR International).
- Disposable petri dish (100 mm, VWR International).

2.2.2 Neuron Culture

- Six-well culture plate.
- NBB27 with glutamate (DIV 0–DIV 4): Neurobasal medium (100 ml, Invitrogen), B27 supplement (2 ml, Invitrogen), L-glutamine (2 mM, Sigma-Aldrich), L-glutamic acid (25 μM, Sigma-Aldrich), *N*-acetyl cysteine (63 ng/ml, Sigma Aldrich), penicillin/streptomycin (2 ml, Invitrogen).
- NBB27 (from DIV 4): Neurobasal medium (100 ml, Invitrogen), B27 supplement (2 ml, Invitrogen), GluMAX (0.5 mM, Invitrogen), *N*-acetyl cysteine (63 ng/ml, Sigma-Aldrich), penicillin/streptomycin (2 ml, Invitrogen).
- DMEM/NBB27: Neurobasal medium (100 ml, Invitrogen), B27 supplement (2 ml, Invitrogen), GluMAX (0.75 mM, Invitrogen), DMEM (100 ml, Hyclone), sodium pyruvate (1 mM, Sigma-Aldrich), 100× SATO (2 ml), D-biotin (10 nM, Sigma-Aldrich), penicillin/streptomycin (2 ml, Invitrogen), *N*-acetyl cysteine (63 ng/ml, Sigma-Aldrich), insulin (5 μg/ml, only during the first week of coculture, Sigma-Aldrich).
- 100× SATO: Transferrin (100 μg/ml, Sigma Aldrich), Bovine serum albumin (BSA) (100 μg/ml, Sigma Aldrich), Progesterone (60 ng/ml, Sigma Aldrich), Putrescine (16 μg/ml, Sigma-Aldrich), sodium selenite (5.2 ng/ml, Sigma Aldrich).

3 Methods

3.1 Microsystem Preparation

3.1.1 Imprint Mold Fabrication (Fig. 2) (See **Note 1**)

1. Apply SU-8™ 2015 photoresist on a cleaned silicon wafer and spin-coat at 3,500 rpm for 30 s.
2. Soft-bake the wafer for 4 min at 95 °C on a leveled hot plate.
3. Expose the wafer through a transparency film mask (20,000 dpi) containing radial array of microchannel patterns and alignment marks with the dosage of 170 mJ/cm^2.
4. Post-exposure bake the wafer for 4 min at 95 °C on a leveled hot plate.
5. Develop with SU-8™ developer (PGMEA) for 45–60 s.
6. Rinse with IPA (*see* **Note 2**).
7. Dry the SU-8™ patterned wafer with pressurized nitrogen gas.
8. Dry etch the SU-8™ patterned silicon wafer using CF_4 gas (etch depth: 3 μm).
9. Remove the remaining SU-8™ etch mask from the wafer with Piranha solution (H_2O_2–H_2SO_4 = 1:3) at 180 °C for 20 min followed by thorough rinse with DI water (*see* **Note 3**).
10. Vapor-coat the patterned silicon wafer with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-trichlorosilane for 20 min inside a vacuum chamber, followed by rinsing with IPA (*see* **Note 4**).

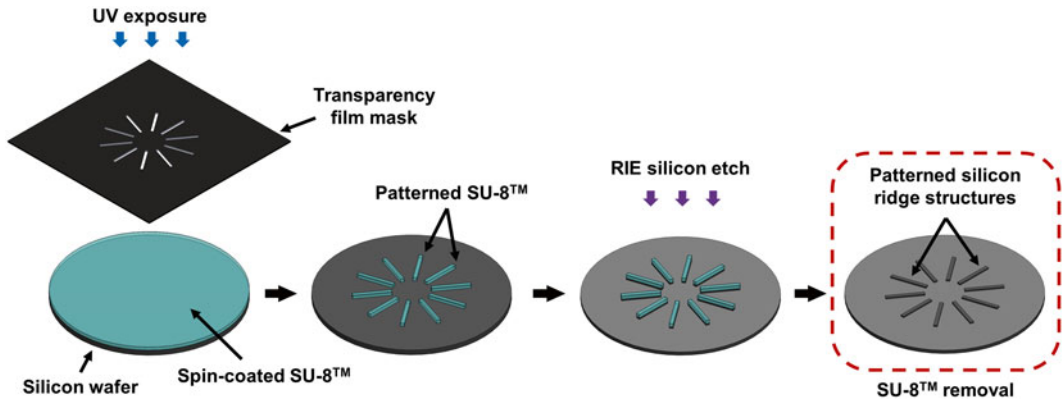

Fig. 2 Imprint mold fabrication process using photolithography followed by dry etch of the silicon wafer

3.1.2 PMMA Master Mold Fabrication (Fig. 3a)

1. Design one soma compartment and six surrounding axon/glia compartments that are 400 μm apart from the soma compartment using any computer-aided design (CAD) program (*see* **Note 5**).
2. Prepare a poly(methyl methacrylate) (PMMA) block (50×50×12.5 mm^3) that will be used as a PMMA master mold.
3. Pattern compartment structures on the PMMA block using a computer numerically controlled (CNC) milling machine.
4. Rinse the PMMA block with IPA three times.
5. Align imprint mold (prepared in Sect. 3.1.1) with compartments-engraved PMMA block using the alignment marks.
6. Hot emboss imprint mold on the PMMA block at 115 °C with 1.1 MPa of pressure for 5 min using a temperature-controlled hydraulic press.

3.1.3 PDMS Master Mold Fabrication (Fig. 3b)

1. Thoroughly mix PDMS prepolymer with curing agent (10:1 by weight) in a weighing boat using a plastic fork (*see* **Note 6**).
2. Place the PMMA master mold in a disposable container and slowly pour the PDMS mixture until the PDMS reaches 3–5 mm above the surface of the PMMA master.
3. Place in a vacuum chamber for 10–15 min to remove excessive bubbles.
4. Place the PDMS-poured PMMA master mold inside a leveled drying oven and cure for at least 3 h at 85 °C.
5. After PDMS has been fully polymerized, detach the PDMS master mold from the PMMA master mold (*see* **Note 7**).
6. Vapor-coat the PDMS master mold with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-trichlorosilane for 20 min inside a vacuum chamber, followed by a brief rinsing with IPA.

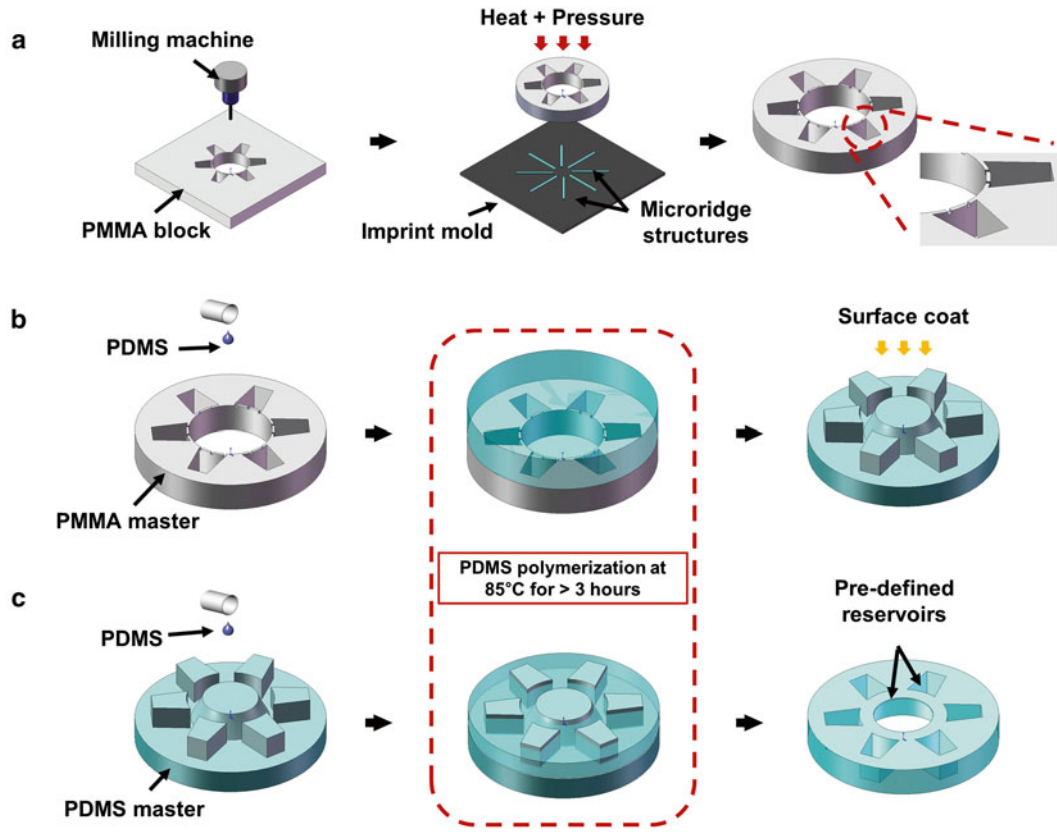

Fig. 3 MMHSM fabrication process of multi-compartment neuron–glia coculture microsystem. (**a**) PMMA master mold, (**b**) PDMS master mold, and (**c**) multi-compartment PDMS device

3.1.4 Multi-compartment PDMS Device Fabrication (Fig. 3c)

1. Thoroughly mix PDMS prepolymer with curing agent (10:1 by weight) in a weighing boat using a plastic fork.
2. Place the PDMS mixture in a vacuum chamber for 10–15 min to remove excessive bubbles (*see* **Note 8**).
3. Place the PDMS master mold in a disposable container and slowly pour the PDMS mixture until the PDMS reaches 2–3 mm below the top surface.
4. Place in a vacuum chamber for 10–15 min to remove excessive bubbles from the PDMS mixture.
5. Place the PDMS-poured PDMS master mold inside a leveled drying oven and cure for at least 3 h at 85 °C.
6. Detach the final PDMS device from the PDMS master mold and cut out rough boundaries with a razor blade so that the bottom surface of the device is perfectly flat.

3.1.5 PDMS Disk Fabrication

1. Thoroughly mix PDMS prepolymer with curing agent (10:1 by weight) in a weighing boat using a plastic fork.

2. Pour approximately 15 ml of PDMS mixture to a petri dish (100 mm) for obtaining an approximately 2 mm thick PDMS slab.
3. Place the PDMS mixture in a vacuum chamber for 10–15 min to remove excessive bubbles.
4. Place the petri dish inside a leveled drying oven and cure for at least 3 h at 85 °C.
5. Cut out the PDMS disk using a round-hole punch.

3.2 Device Assembly and Neuron Culture Preparation Steps (Fig. 4)

1. Place a clean glass coverslip, a PDMS disk, and a PDMS device (channel patterned surface of the device facing up) inside the oxygen plasma cleaner.
2. Turn on the vacuum pump and wait for 2 min to reach vacuum pressure sufficient to generate plasma.
3. Turn on the RF power to ignite the plasma and perfuse oxygen gas for 2 min.
4. After the plasma treatment, gently assemble the PDMS disk and the PDMS device onto the glass coverslip (*see* **Note 9**).
5. Immerse the assembled device in a beaker with DI water.
6. Place the beaker inside a vacuum chamber for 5–10 min to remove any air bubbles trapped inside the microchannels.
7. After vacuum, autoclave the device in water-immersed state without drying procedure for sterilization.
8. Take out the device inside a biosafety cabinet and place it inside a six-well culture plate for easy handling.
9. Aspirate excessive water in compartments and apply PDL solution to the soma compartment (400 µl) and axon/glia compartments (100 µl) (*see* **Note 10**).

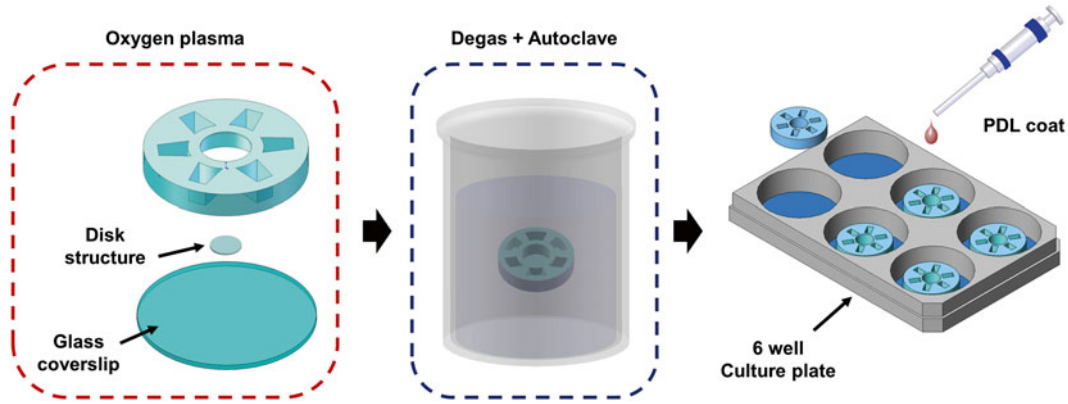

Fig. 4 Oxygen plasma enhanced device assembly process and neuron culture preparation steps

10. Place the device inside a humidified 37 °C incubator overnight.
11. Aspirate PDL solution and thoroughly rinse with sterile DI water three to five times with 5-min intervals (*see* **Note 11**).
12. Fill the device with culture medium and incubate inside a humidified 37 °C incubator overnight before use (*see* **Note 12**). The device is now ready for cell seeding.

3.3 Microsystem Neuron Culture

1. Aspirate culture medium from the soma compartments.
2. Dilute neurons in 100 μl of NBB27 culture medium with glutamate (cell concentration to match 1,000 cells/mm^2 to the cell culture substrate area) and apply to the soma compartment (*see* **Note 13**).
3. Incubate the PDMS device with neurons inside a humidified 5 % CO_2 incubator for 30 min for neurons to attach to the bottom substrate.
4. Aspirate culture medium from the six axon/glia compartments.
5. Apply additional 300 μl of culture medium to the soma compartment and add 200 μl of culture medium to each of the six axon/glia compartment.
6. Exchange medium every 72–96 h by gently aspirating half of culture medium inside all compartments and adding fresh culture medium (*see* **Note 15**).
7. At DIV 4, exchange NBB27 with glutamate to NBB27 without glutamate.

3.4 Microsystem Neuron–Glia Coculture

1. Culture neurons inside the PDMS coculture platform for 7–10 days for axons to grow across the microchannels and into the axon/glia compartments to establish isolated axonal layer (*see* **Note 14**).
2. Aspirate culture medium from the axon/glia compartments (*see* **Note 15**).
3. Dilute glia cells in 200 μl of DMEM/NBB27 culture medium (at desired areal cell density) and apply to axon/glia compartments.
4. Exchange the soma compartment culture medium to DMEM/NBB27.
5. Replace half of culture medium every 72–96 h.

3.5 Localized Biomolecular Treatment

Fluidic isolation between the soma compartment and the axon compartment can be achieved by generating fluidic level difference between the compartments (Fig. 5). Minute but sustained flow from the higher fluidic level to the lower fluidic level counters diffusion and enables localized biomolecular treatments to either the somata only or to isolated axons only depending on which compartment has higher fluidic level [6–8].

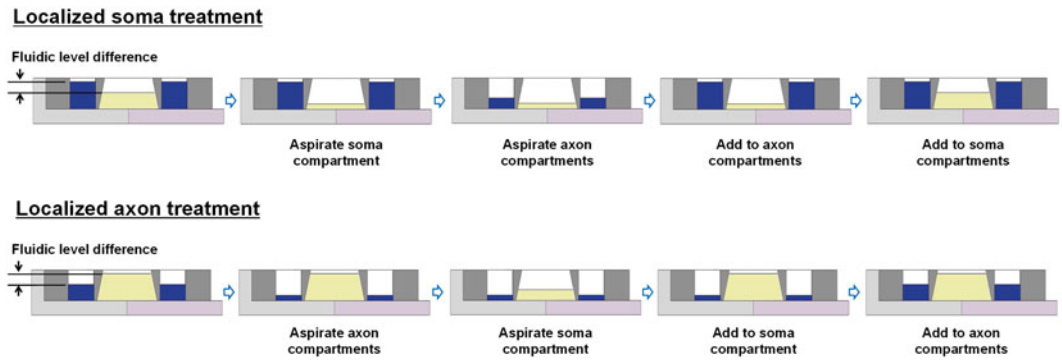

Fig. 5 Illustrations showing culture medium exchange process during the localized biomolecular treatments

3.5.1 Localized Soma Treatment

1. Aspirate culture medium from the soma compartment (*see* **Note 16**).
2. Dilute biomolecule to be treated in 200 µl of culture medium and add to the soma compartment (*see* **Note 17**).
3. Keep culture medium in the axon compartments to be 200 µl for keeping fluidic level higher than that of the soma compartment (*see* **Note 18**).

3.5.2 Localized Axon Treatment

1. Aspirate culture medium from the axon compartment (*see* **Note 16**).
2. Dilute biomolecule to be treated in 80 µl of culture medium and add to the axon compartment (*see* **Note 17**).
3. Keep culture medium in the soma compartments to be 400 µl for keeping fluidic level higher than that of the axon compartment (*see* **Note 18**).

4 Notes

1. SU-8™ is used as an etch mask for silicon RIE etching. Any other available material such as a patterned metal (e.g., aluminum) layer or a patterned SiO_2 layer can be used as an etch mask.
2. White residue can be observed when uncross-linked SU-8™ is not sufficiently removed during the development process. Completely dry the sample with pressurized nitrogen gas and further develop in SU-8™ developer (PGMEA).
3. Piranha procedure must be performed inside a fume hood.
4. Coating of the sample with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-trichlorosilane facilitates release of PDMS layer after replication.
5. When designing the PMMA master mold, side wall of the axon/glia compartments interfacing the soma compartment

should be tilted outward to have thicker wall on top (Fig. 1a inset). This prevents PDMS wall from being easily torn during the final device replication process (Fig. 3c). Alignment marks should be added to the design as appropriate.

6. Improper mixing of polymer base and curing agent can result in unevenly cured polymer.
7. When handling polymerized PDMS, be careful not to contaminate the surface with any particles or dust. Wrapping the polymerized PDMS objects with Parafilm helps to keep it from dust.
8. Excessive bubbles can be generated during the degassing process when using a PDMS structure as a replication master mold. Additional degassing of PDMS mixture prior to pouring it onto the replication master mold is recommended.
9. Do not apply excessive pressure when bonding the compartment layer since it can result in collapse of the microchannels.
10. Generating fluidic level difference between the soma compartment and the axon/glia compartments enable PDL solutions to easily flow into the microchannels for proper coating.
11. Generate fluidic level difference between the soma compartment and the axon/glia compartments for the first two washes to properly rinse PDL inside the microchannels.
12. Incubation of the device inside a 37 °C incubator overnight allow any potential debris or toxic residue to be removed.
13. Cell numbers can be adjusted according to the experimental design (500–2,000 cells/mm^2 is recommended).
14. Starting time point of the coculture can be adjusted by the experimental design.
15. Aspirate the culture medium from the outer end of the axon/glia compartment so that the isolated axons are not damaged.
16. Be careful not to damage the cells on the bottom of the substrate.
17. Fluidic level difference is determined by the size of the soma compartment and the axon compartment as well as the culture medium added to each compartment. Therefore, volume of the culture medium for fluidic isolation can be adjusted depending on the size of the compartments.
18. Fluidic level difference should be maintained even during the culture medium exchange process while performing localized treatments. Aspirate half of the culture medium inside the compartment with localized treatment first, followed by other compartment. For adding the fresh culture medium, the compartment without biomolecular treatment should be filled first (Fig. 5).

Acknowledgement

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Chapter 10

Compartmentalized Microfluidic Platforms as Tool of Choice to Study the Interaction Between Neurons and Osteoblasts

Estrela Neto, Diogo Paramos-de-Carvalho, Ana Henriques Lourenço, Paulo Aguiar, and Meriem Lamghari

Abstract

Microfluidic platforms have greatly evolved in the last few years. This technology is nowadays transversely spread all over the most abroad fields of research. Compartmentalized microfluidic devices, initially applied in the neuroscience field for the simplest biochemical tests, is presently used at the most complex assays such as cocultures for developmental and regeneration studies. Excitingly, these devices have emerged as a potential tool to study, not only the interaction within central nervous system cells but also the innervation of peripheral tissues. This latest issue have led us to select the compartmentalized microfluidic devices to conduct the studies of peripheral neuro-osteogenic interactions. In this chapter we describe and standardize the major proceedings to ensure the success of the coculture model for neurons and osteoblasts and further recommend the qualitative and quantitative analysis for two- or three-dimensional cocultures.

Key words Microfluidic, Coculture, Innervation, Bone, Neurons, Osteoblasts

1 Introduction

Compartmentalized microfluidic devices are platforms with different compartments which allow the separation of distinct cell components. The compartmentalization of these devices relies on micro-sized channels through which axons and dendrites are able to extend, but not the cell soma [1–3], and through the micro-grooves, it is possible to create either dynamic flow between channels or fluidic isolation. Studies on isolated cell soma and axons resulting in biochemical analyses of pure axonal fractions and precise physical and chemical treatments can be easily carried out using these platforms. If firstly these devices were mainly used for neuroscience research, to examine a wide range of neural functions as axonal transport or injury and regeneration, due to their unique ability to allow high-resolution live imaging, nowadays it is

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transversely spread all over other fields. This occurs due to the low costs in manufacturing, reduced consumption of reagents, increased separation efficiency and reproducibility, leading to high-throughput analysis. These features have, in time, allowed the integration of more complex cell cultures and, from intra-system cocultures (e.g., coculture of neuronal system cells: neurons and oligodendrocytes [4, 5], or neurons and Schwann cells [5]) researchers ended up managing to have inter-systems cocultures (e.g., nervous and skeletal systems: neurons and osteoblasts [3] or nervous and muscular systems: neurons and myocytes [6]).

Taking this into account, the compartmentalized microfluidic platforms have emerged as a potential tool to study, not only the interaction within central nervous system cells but also the innervation of peripheral tissues.

In skeletal biology, there is a strong interest in the role of peripheral nervous system in bone homeostasis during developmental stages. Therefore, we provide a protocol to perform both cocultures with embryonic dissociated dorsal root ganglion (DRG) or entire adult DRG since they offer different biological insights. On one hand, the coculture using rat embryonic dissociated DRG and osteoblasts can be useful for studies of the role of peripheral nervous system in bone homeostasis during developmental stages. On the other hand, the coculture of entire adult DRG and osteoblasts allows the analysis of these interactions between the two systems in mature or challenged scenario. It is also possible to improve the microenvironment of these cultures by including 3D cultures within compartmentalized microfluidic devices to mimic the spatial properties of bone tissue extracellular matrices, which provide mechanical and biochemical signalling factors.

In this chapter we address the application of the compartmentalized microfluidic platforms as a tool of choice to understand the cross talk in the peripheral neuro-osteogenic path. We describe the key elements to perform and analyze cocultures of neuronal and non-neuronal cells, namely, osteoblasts. The major proceedings to ensure the success of this protocol are the appropriated coating of the glass substrate, the correct assembling of the microfluidic devices, the maintenance of the culture of both neurons and osteoblasts and finally a proper analysis and quantification of the obtained results.

2 Materials

2.1 Coating of Glass Coverslips

1. Glass coverslips (24 × 24 mm, Microscopic Glass Factory).
2. Nitric acid 65 % (v/v) (cat. No. 1004411000Merck). Danger, corrosive reagent; requires proper disposal.
3. Milli-Q (mQ) water (sterile).

4. Ethanol 70 %.
5. Plastic petri dishes (sterile).
6. Forceps (sterile).
7. Poly-D-lysine (PDL, cat. No P7280, Sigma-Aldrich) 0.1 mg.ml^{-1}.

2.2 Assembling of Microfluidic Devices

1. Microfluidic chambers, AXIS™ Axon Isolation Device, 450 μm (cat. No AX45005, Millipore).
2. Forceps (sterile).
3. Scotch tape (3 M Schotch 471).
4. Biopsy punch (2.0 mm, cat. No BP-20 F, Kai Medical).
5. Ethanol 70 %.
6. Plastic petri dish (sterile).
7. 6-well plates (sterile).
8. Coated glass coverslips.
9. Laminin (cat. No L2020, Sigma-Aldrich) 5 μg.ml^{-1}.
10. Neurobasal medium (cat. No 21103-049, Invitrogen).
11. Vacuum system.

2.3 Cell Culture

2.3.1 Sensory Neurons Culture

1. Laboratory animals (rat embryos E18 or 6–8 weeks-old mice).
2. Stereoscope.
3. Dissecting tools (scalpel, thin scissors, and forceps).
4. Flamed Pasteur pipettes.
5. Hank's Balanced Salt Solution (HBSS, cat. No 24020-117, Invitrogen).
6. Sterile 1× phosphate-buffered saline (PBS).
7. Neurobasal medium (cat. no. 21103-49, Invitrogen).
8. B-27 Serum-Free Supplement® (B-27, cat. No. LTI17504-044, Invitrogen).
9. 5-Fluoro-2′-deoxyuridine (FDU, cat. No. 46875 Sigma-Aldrich).
10. Glucose (cat. No. G6152, Sigma-Aldrich).
11. Pyruvate (cat. no. 11360-039, Invitrogen).
12. 7S Nerve Growth Factor (NGF, cat. No. 480354, Merck).
13. L-Glutamine (cat. No. 25030-024, Invitrogen).
14. Penicillin/Streptomycin (P/S, cat. No. L0022-100, BioWest).

Medium formulation: Neurobasal medium supplemented with 2 % (v/v) B-27, 60 mM FDU, 25 mM glucose, 1 mM pyruvate, 50 ng.ml^{-1} NGF, 2 mM glutamine, and 1 % P/S.

2.3.2 Osteoblasts Culture

1. MC3T3-E1 pre-osteoblastic cell-line (ECACC, reference 99072810).
2. Alpha-minimal essential medium (α-MEM, cat. No. 11900, Invitrogen).
3. Heat-inactivated fetal bovine serum (FBS, cat. No. 10270, Invitrogen).

   Medium formulation: α-MEM supplemented with 10 % (v/v) FBS, and 1 % P/S.
4. Trypsin solution (cat. No. T4799, Sigma-Aldrich) (0.25 % (w/v) trypsin, 0.1 % (w/v) glucose, and 0.05 % (w/v) ethylenediaminetetracetic acid in PBS).
5. Centrifuge.
6. Incubator (humidified atmosphere with 5 % CO_2).
7. Functionalized glycyl-arginyl-glycyl-aspartyl-seryl-proline (GRGDSP referred as RGD) alginate (NOVATACH MVG GRGDSP coupled high G high MW alginate, cat. No. 4270129, Novamatrix, FMC Biopolymers).
8. 36.7 mg.ml^{-1} calcium carbonate suspension ($CaCO_3$, cat. No. 481807, Sigma-Aldrich). Autoclave prior to use.
9. 58.1 mg ml^{-1} glucone delta-lactone solution (GDL, cat. No. G4750, Sigma-Aldrich). Prepare fresh.
10. 0.9 % (w/v) sodium chloride (NaCl, cat. No. S7653, Sigma-Aldrich).
11. 0.22 μm filters (cat. No. SLGV013SL, Millipore).
12. Positive displacement pipettes and tips.

2.4 Analysis and Characterization of the Coculture System

1. Immunocytochemistry:
   (a) Tris buffered saline solution 1× (TBS, cat. No. T5912, Sigma-Aldrich).
   (b) 2 % (v/v) paraformaldehyde (PFA, cat. No. 30525-89, Merck) in TBS. Danger, fixative reagent.
   (c) 4 % (v/v) PFA and 4 % (v/v) sucrose (cat. No. S9378, Sigma-Aldrich) in TBS.
   (d) 0.25 % (v/v) Triton X-100 (cat. No. 234729, Sigma-Aldrich) in TBS.
   (e) Blocking solution (5 % (v/v) normal goat serum (cat. No. PCN5000, Invitrogen) and 5 % (v/v) FBS, in TBS).
   (f) Antibodies: β3-tubulin (cat. No. G7121, Promega), calcitonin gene-related peptide (CGRP, cat. No. C8198, Sigma-Aldrich), Synapsin (cat. No. AB1543, Millipore), Alexa Fluor 568 (cat. No. A11011, Invitrogen), Alexa Fluor-Phalloidin 488 (cat No. A12379, Invitrogen),

4′,6-diamidino-2-phenylindole (DAPI, cat. No. D9542, Invitrogen).

(g) mQ water.

(h) Confocal laser scanning microscope.

2. Scanning electron microscopy:

(a) 2.5 % glutaraldehyde (cat. No. 16210, Electron Microscopy Sciences) in 0.1 M cacodylate buffer, pH 7.4 (cat. No. 11650, Electron Microscopy Sciences).

(b) Ethanol grade (50, 60, 70, 80, 90 % and absolute).

(c) Critical point drier (CPD7501, Polaron Range).

3 Methods

Microfluidic compartmentalized devices have strong potential for applications at the interface of neuroscience, developmental cell biology, advanced biomaterials and microfluidic technology [2]. Indeed, an increasing number of coculture studies were published [7, 8], reporting novel discoveries in cell biology such as the study of endothelial and mesenchymal stem cells' migration [9], epithelial cells and fibroblasts interactions [10], as well as in mesenchymal stem cells and neuronal cells transdifferentiation studies [11]. Furthermore, and concerning neuronal cells, cell culture in compartmentalized microfluidic devices has been performed, not only with several central nervous system neurons but also with peripheral neurons [1, 2, 12–16].

The methods described here provide instructions to perform a coculture in microfluidic devices comprising neurons and osteoblasts. As experiments require the use of animals, the experimental protocol must be approved by the animal care authorities.

3.1 Coating of Glass Coverslips

The washing and coating of glass coverslips are crucial steps to improve cell attachment in the main channel during incubation, which can significantly affect the number of adherent and quality of axons that are produced and, thus, able to cross the microgrooves during differentiation. The coating must be adequate to the cell type in culture. In this protocol we used poly-D-lysine and laminin for sensory neurons and referred collagen as substrate for osteoblasts (*see* Sect. 4, **Note 7**).

1. Preparation of the glass coverslips:

(a) Wash glass coverslips with nitric acid (65 %) overnight under constant agitation.

(b) Rinse thoroughly with water until reach the neutral pH.

(c) Sonicate the coverslips in mQ water for 30–60 min.

(d) Rinse briefly with mQ water.

(e) Thoroughly soak the glass in 70 % ethanol for at least 5 min (*see* **Note 1**).

(f) Transfer the slides to a laminar flow hood (all subsequent steps should be performed in sterile conditions).

(g) Aspirate the ethanol and let the coverslips dry completely. The cleaned glass can be stored at this point in an enclosed container.

2. Glass coverslips coating:

(a) Coat glass coverslips with 0.1 $mg.ml^{-1}$ PDL solution, overnight at 37 °C (*see* **Note 2**).

(b) Wash several times with mQ water.

(c) Dry the PDL-coated glass coverslips in the flow hood for 2 h (*see* **Note 3**). The coating, at this time point, preceding the device assembling, ensures a homogeneous coating of the all coverslip.

3.2 Assembling of Microfluidic Devices

1. The assembling of the microfluidic devices was performed accordingly to manufacturer's instructions (cat. No AX45005, Millipore). Open the pouch containing the microfluidic chambers inside a laminar flow hood.
2. Using sterile forceps remove the devices and place them in a sterile petri dish.
3. Add ethanol 70 %, making sure it completely covers all the surfaces of the devices.
4. Incubate for 5 min.
5. Aspirate the ethanol and let air-dry (30–60 min) (*see* **Note 4**).
6. The device is now ready for use (ideally it should be used within 24 h after sterilization).
7. Transfer a PDL-coated coverslip and place in a 6-well plate (*see* **Note 5**).
8. Using forceps, pick up one microfluidic device and place it upon the coverslip, ensuring that the imprinted side is faced down (examine the device carefully while reflecting light off of it).
9. With the help of the forceps, apply firm but gentle pressure to several points all along the top of the device to make sure it is properly adherent (*see* **Note 6**).
10. To coat the channels and microgrooves with laminin, pipette 150 µl of laminin solution (5 $\mu g.ml^{-1}$) into the two main channels (represented by numbers 1 and 2 in Fig. 1). Fill the remaining reservoirs with 150 µl of laminin solution (represented with letters from A-D in Fig. 1).
11. Check the device on an inverted microscope to confirm that the media has passed through the microgrooves and that there are no air bubbles inside the channels (*see* **Note 7**).

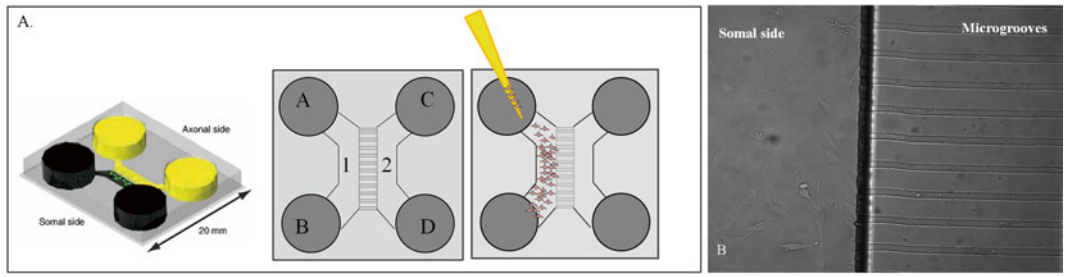

Fig. 1 (**a**) Schematic representation of microfluidic device (*left*) and representation of dissociated cells seeding (*right*). Adapted by permission from Macmillan Publishers Ltd: Nature Methods [1], copyright 2005. (**b**) Phase contrast image showing dissociated cells seeded on the channel of the microfluidic chamber. It is possible to see the microgrooves which link the both channels. Adapted from [3] with permission from The Royal Society of Chemistry

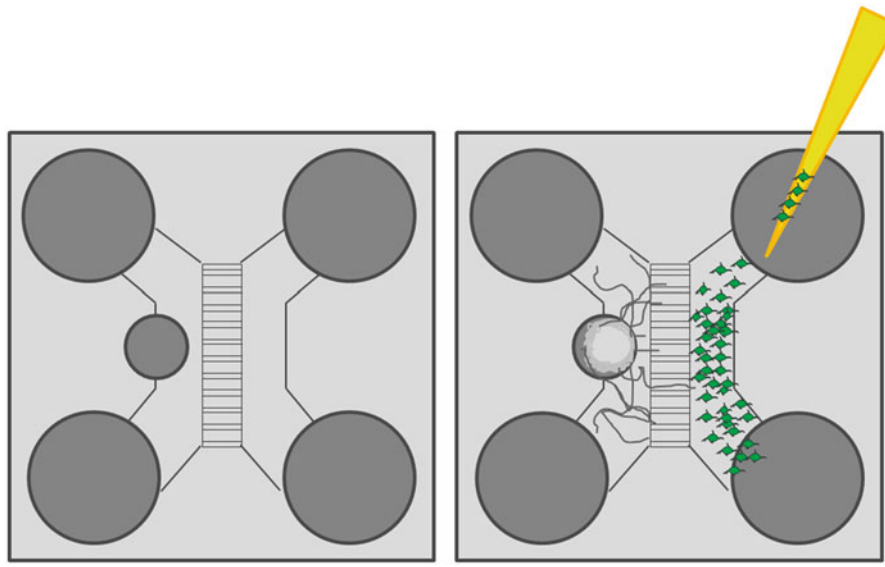

Fig. 2 Schematic representation of adapted microfluidic device (*left*) and representation of dissociated cells seeding after entire DRG culture (*right*). Adapted from [3] with permission from The Royal Society of Chemistry

12. Incubate for 2 h at 37 °C.
13. Afterwards replace the laminin solution for complete appropriated medium for cell culture (*see* **Note 8**).

To perform the coculture with the whole entire ganglion, a small adaptation was performed in the original microfluidic device in order to allow the entire DRG to align with the microgroove axis and restrain its area of adhesion.

1. Use a punch key (2 mm diameter) to perforate the microfluidic chambers, in the somal side (*see* Fig. 2).

2. In order to remove possible PDMS debris from the surface, that can impair the appropriate sealing of the device, clean the device with Scotch tape. Afterwards proceed with the sterilization process (Sect. 3.2, step 2).

3.3 Cell Coculture

All experiments requiring samples from animal sources should be conducted following approved protocols by the Ethics Committee on Animal Welfare and Experimentation.

3.3.1 Dissociated Cell Seeding in the Somal Side: Sensory Neurons

1. Euthanize pregnant Wistar rat by CO_2 inhalation and retrieve the embryos.
2. Embryonic sensory neurons were obtained from embryos E18, under the stereoscope (*see* **Note 9**).
3. Digest the removed DRG with trypsin for 15 min at 37 °C.
4. Mechanically dissociate the DRG using a flamed Pasteur pipette up and down.
5. Centrifuge (1 min, 1000 rpm) to deposit neuronal cells.
6. Discard the supernatant and add complete Neurobasal medium to the cells.
7. Aspirate all media from reservoirs but not from main channels and immediately add 10 µl of cell suspension (4×10^4 cells per channel) into one of the reservoirs. Take care to add the cells next to the channel so that the cell suspension is drawn into the channel by capillary action (*see* Fig. 1). The rest of the cells will settle and attach in the reservoirs (*see* **Note 10**).
8. Incubate at 37 °C, in a humidified atmosphere with 5 % CO_2 (30–40 min) for cell adhesion.
9. Add 150 µl of Neurobasal medium to the reservoirs.

3.3.2 Organotypic Culture in the Somal Side: Dorsal Root Ganglion

1. Euthanize 6–8 weeks old mouse and retrieve the column (*see* **Note 11**).
2. Clean the conjunctive tissue from the excised column.
3. With a thin scissor, cut the vertebrae (*see* **Note 12**).
4. Entire DRG can be retrieved from the cut spine of mouse, under the stereoscope.
5. After removal, keep the DRG in HBSS on ice until plating.
6. Plate the DRG through the punched hole in the microfluidic chambers (*see* **Note 13**) (*see* Fig. 2).
7. Incubate at 37 °C, in a humidified atmosphere with 5 % CO_2.
8. Leave undisturbed until adhesion.
9. Change the medium every 2–3 days and check daily the medium volume (*see* **Note 14**).

3.3.3 Dissociated Cell Seeding in the Axonal Side: Osteoblasts

1. After sensory neurons adhesion and establishment of neurite network, harvest the MC3T3-E1 cells with trypsin solution.
2. Seed the cell suspension into the axonal side of the microfluidic chambers at a density of 1.5×10^5 cells cm^{-2} (1.5×10^4 cells per microdevice) (*see* **Note 15**) (*see* Fig. 2).
3. Let the coculture rest for 3 days before monitoring cell morphology (*see* **Note 16**).

3.3.4 Enrichment of the Axonal Side with Three-Dimensional Microenvironment

1. Alginate preparation
   (a) Prepare the RGD- alginate allowing to dissolve in NaCl 0.9 % (w/v) to yield a 1.8 % solution overnight, at 4 °C (*see* **Note 17**).
   (b) Sterilize the final alginate solution by filtration (0.22 μm).
2. Cell entrapment
   (a) Harvest the MC3T3-E1 cells with trypsin solution.
   (b) Count the cells and centrifuge the needed number of cells into 1.5 ml tubes (1100 rpm, 5 min).
   (c) Reserve the cells.
   (d) Add the suspension of $CaCO_3$ in 0.9 % NaCl to the alginate solution at a $CaCO_3$/COOH molar ratio of 1.6 and mix thoroughly.
   (e) Add fresh immediately prepared and filtered GDL solution to alginate solution at a $CaCO_3$/GDL molar ratio of 0.125 and mix (*see* **Note 18**).
   (f) Add the final alginate solution to the cell pellet at 1×10^4 cells.ml^{-1} and mix carefully.
   (g) Pipette the solution to the microfluidic channel and allow to polymerize for 1 h in the incubator (*see* **Note 19**).
   (h) Add cell culture medium.

3.4 Analysis and Characterization of the Coculture System

1. Immunocytochemistry:
   (a) Fix the cells with 2 % PFA in TBS for 10 min, at 37 °C (*see* **Note 20**).
   (b) Replace the first PFA solution for 4 % PFA with 4 % sucrose in TBS.
   (c) Incubate for 10 min at 37 °C.
   (d) Wash the cells with TBS.
   (e) Permeabilize the cells with 0.25 % (v/v) Triton X-100 in TBS.
   (f) Incubate with blocking solution for 30 min at room temperature.
   (g) For neuronal staining, incubate the cells with anti-βIII tubulin (1:2000), anti-calcitonin gene-related peptide

(CGRP, 1:8000), or synapsin (1:1000) in blocking solution, overnight at 4 °C (*see* **Note 21**).

(h) Wash the cells with TBS.

(i) Incubate with secondary antibody (Alexa Fluor 568) for 1 h at room temperature. At the same time stain osteoblastic cells with Alexa Fluor-Phalloidin 488.

(j) Wash the cells with TBS.

(k) Incubate with DAPI for 5 min and wash (*see* **Note 22**).

(l) Capture the images using an inverted microscope or a confocal laser scanning microscope equipped with image acquisition software (*see* Figs. 3, 4, and 5).

2. Scanning electron microscopy:

(a) Fix the cells at 4 °C for 2 h with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4).

(b) Dehydrate in a graded ethanol series (50, 60, 70, 80, 90 % and absolute), 10 min each.

(c) Perform the critical point dried to your samples (*see* **Note 23**).

(d) Mount the samples upon metal support with carbon tape and sputter-coat the samples with gold and palladium.

(e) Observe with a JEOL JSM-6301F scanning electron microscope, at 10 kV and amplifications from 1000× to 30000× (*see* Fig. 6).

3.5 Automatic Quantification of Neurite Outgrowth

It has become mandatory to combine experimental readouts with computational models, in order to predict, analyse and quantify data with reproducible image analysis. Quantification of the axonal growth regarding the different conditions can be performed using the fluorescence images collected from the morphological analysis in order to quantify the axonal outgrowth.

1. Acquire images using low magnification or making use of software tools such as mosaic/grid acquisition (*see* Fig. 7).
2. Isolate the images related to the channel of neuronal marker.
3. Rotate and crop the image, if necessary, in order to obtain a final image containing part of both somal and axonal channels with the microgrooves parallel to the image (*see* **Note 24**).
4. Convert your image to 8-bit and save.

All analysis regarding the quantification of the axonal outgrowth can be performed in MATLAB (R2010a, MathWorks, Natick, Massachusetts, USA).

Using the algorithm, images are first preprocessed to remove background gradients through the use of morphological structuring elements. The algorithm to quantify the axonal density spatial

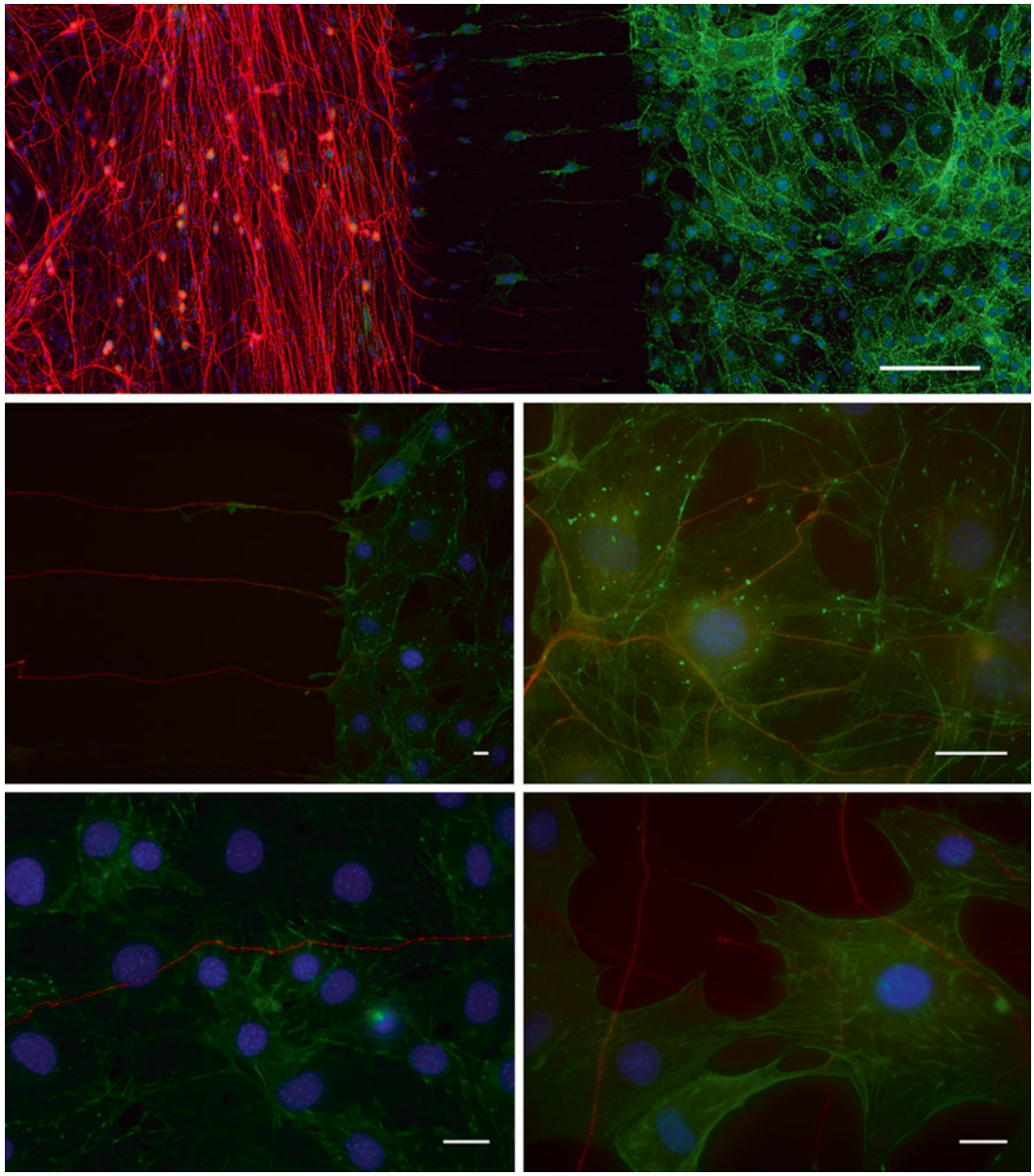

Fig. 3 Representative images of 3-day coculture system regarding MC3T3-E1 cells plus dissociated embryonic DRG (low magnification on *top* and higher magnifications on center), and MC3T3-E1 cells plus entire DRG (*bottom*). MC3T3-E1 cells were stained in *green* for F-actin, neurons in *red* for βIII tubulin, and nuclei in *blue* with DAPI. Scale bar 500 μm, *upper* image and 20 μm *center* and *bottom*. Reproduced from [3] with permission from The Royal Society of Chemistry

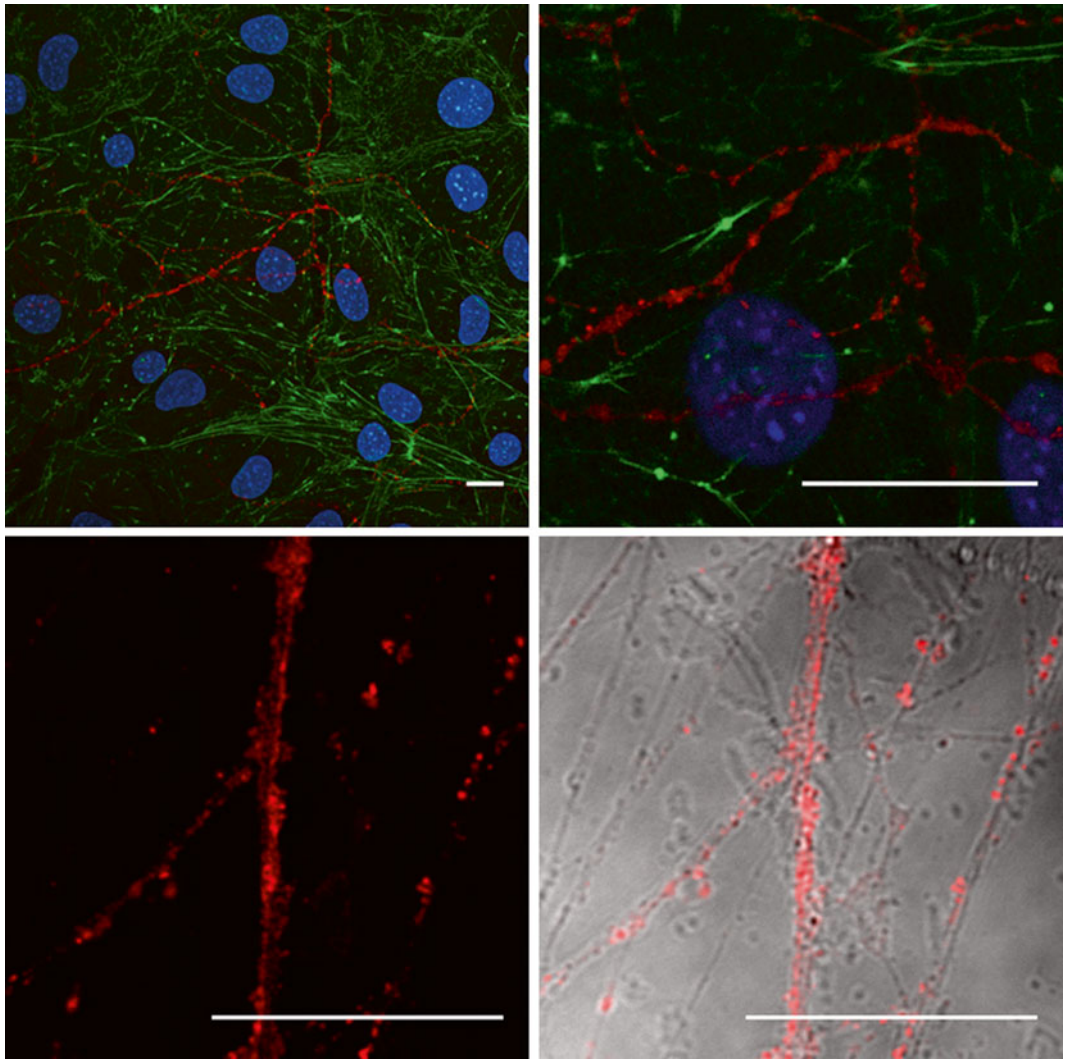

Fig. 4 Immunocytochemistry against synapsin in coculture of MC3T3-E1 cells and entire DRG (*top*). MC3T3-E1 cells were stained in *green* for F-actin, neurons in *red* for synapsin, and nuclei in *blue* with DAPI. Scale bar 100 μm. Immunocytochemistry against calcitonin gene-related peptide (CGRP) in coculture of MC3T3-E1 cells and entire DRG (*bottom*). CGRP marker of sensory neurons in *red* and the merged with bright-field images. Scale bar 20 μm. Reproduced from [3] with permission from The Royal Society of Chemistry

dependence uses a moving column travelling across the longitudinal axis of the image. The column has a predefined width, which balances the desired spatial resolution of the measurement with the spatial scale of heterogeneities.

The algorithm works on a binary mask of the axonal plexus. The binary masks are obtained using composite morphological operations in order to convert the axonal plexus into a wireframe with a single pixel diameter. Under these conditions, the total amount of non-zero pixels in a column provides a reasonable estimate of the total length of axonal fibers.

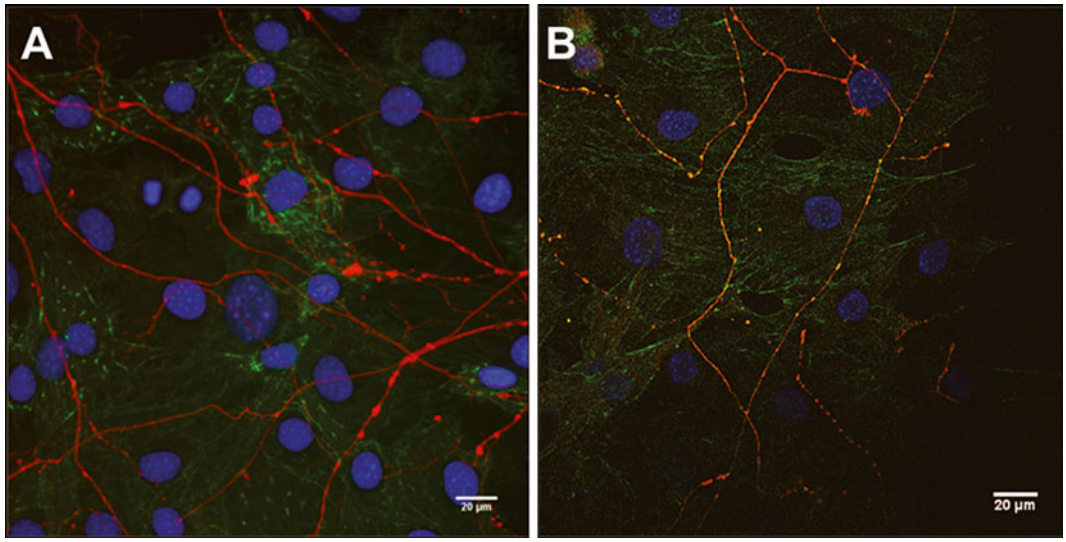

Fig. 5 Neurite network within MC3T3-E1 cells in axonal side. Representative images of MC3T3-E1 cells cocultured with entire DRG upon a collagen layer (**a**) or within functionalized alginate matrix (**b**). MC3T3-E1 cells were stained in *green* for F-actin, neurons in *red* for βIII tubulin, and nuclei in *blue* with DAPI. Scale bar 20 μm. Reproduced from [3] with permission from The Royal Society of Chemistry

The AxoFluidic software is available at tinyurl.com/AxoFluidic [3].

To run the standalone it is necessary to have the MATLAB™ Component Runtime (MCR) installed. This is a free package from MathWorks.

The spatial profile produced in this case provides a complementary assessment in conditions where large sprouting may occur. For the analysis the mean amplitude of the measurement in the somal side is used for normalizing the spatial profile amplitude. This normalization provides the means to compare the transition from somal side to axonal side between different experiments (*see* Fig. 8).

4 Notes

1. Alternatively, glass coverslips can be sterilized by autoclaving. To prepare several slides simultaneously, incubate them inside sterile petri dishes, between filter paper.
2. By coating the two surfaces of the glass coverslip, you will not take the risk of using the uncoated side. Again, to prepare several at once, put the glass coverslips in a petri dish and incubate with PDL solution, overnight under agitation.
3. PDL-coated coverslips can be stored in a sterile container up to 1 month.

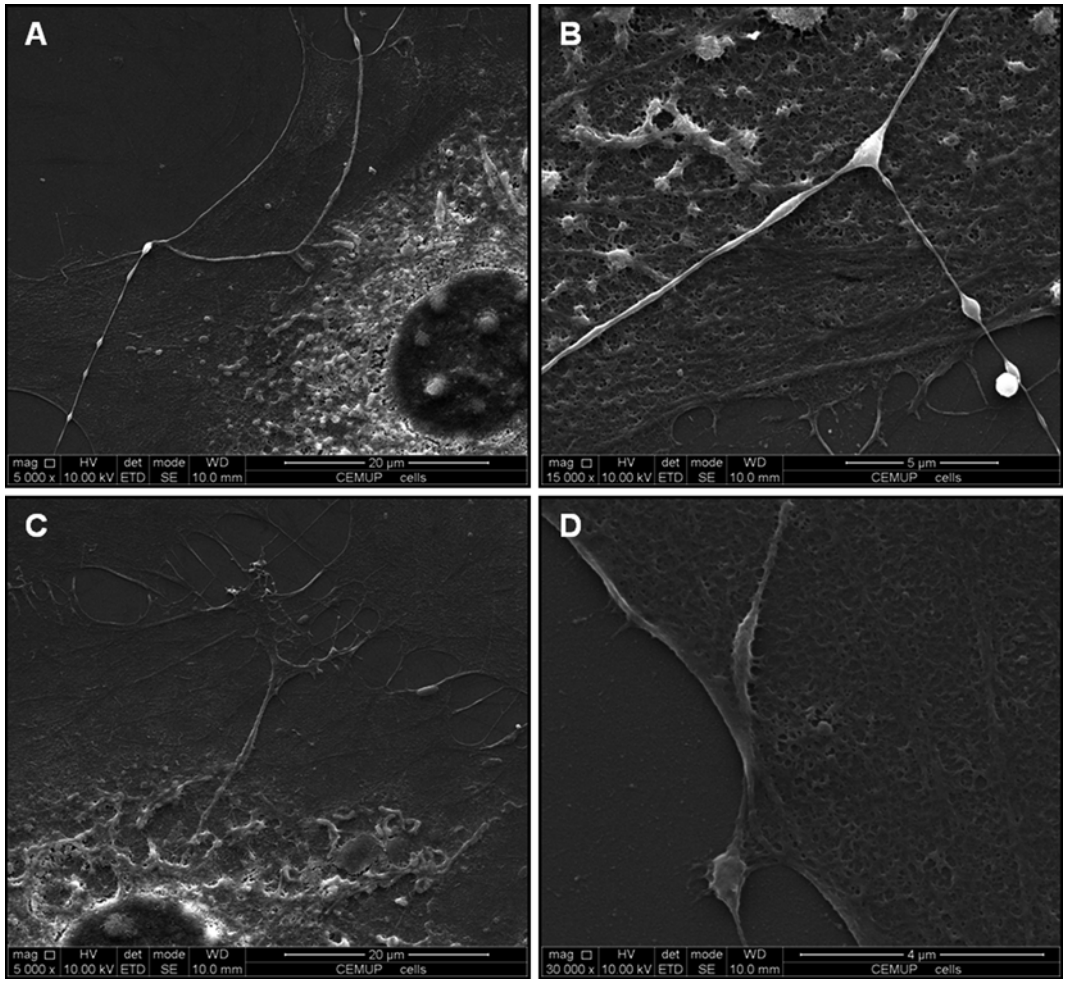

Fig. 6 Images of scanning electron microscopy (SEM) of MC3T3-E1 cells and entire DRG coculture. (**a**) Semi-continuous layer of MC3T3-E1 cells traversed by the neurites extending projections. (**b**) Thin filaments varying size and ramifying suggesting the presence of varicosities associated to synaptic vesicles accumulation. (**c**) Growth cones in the distal and leading end, forming flattened anchoring extensions to the substrate. (**d**) Cytoplasmic projections in the membrane of MC3T3-E1 cells were observed in contact with nerve fibers. Images acquired at 10 kV and amplifications from 1000× to 30000×. Reproduced from [3] with permission from The Royal Society of Chemistry

4. Certify that both the glass coverslips and microfluidic devices are completely dried. Small water drops can compromise the sealing of the devices against the glass.
5. To allow proper conditions for cell culture and maintenance, the microfluidic devices can be assembled in 6-well culture dishes (1 device per well). To better hold the coverslips to the bottom of the well, just pour 1 µl of sterile water on it before placing the coverslips.
6. When sealing the PDMS to substrate, do not press around the microgrooves region as they can collapse and may become blocked when excessive pressure is applied.

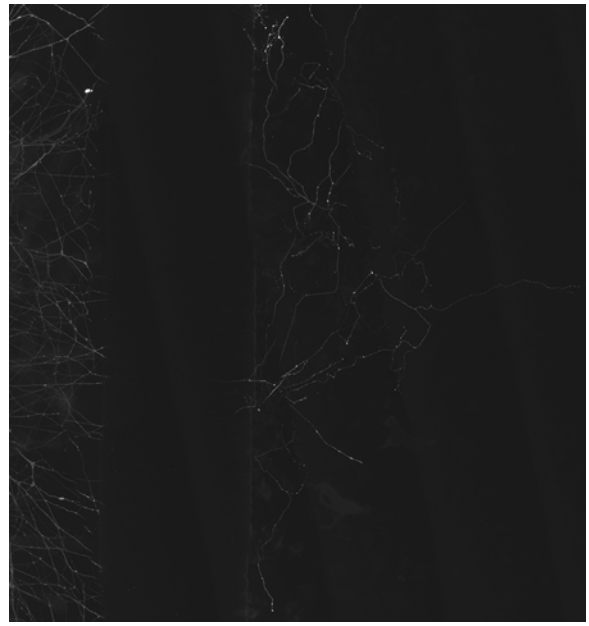

Fig. 7 Representative image used for quantification. Axons in *white* stained against βIII tubulin. Adapted from [3] with permission from The Royal Society of Chemistry

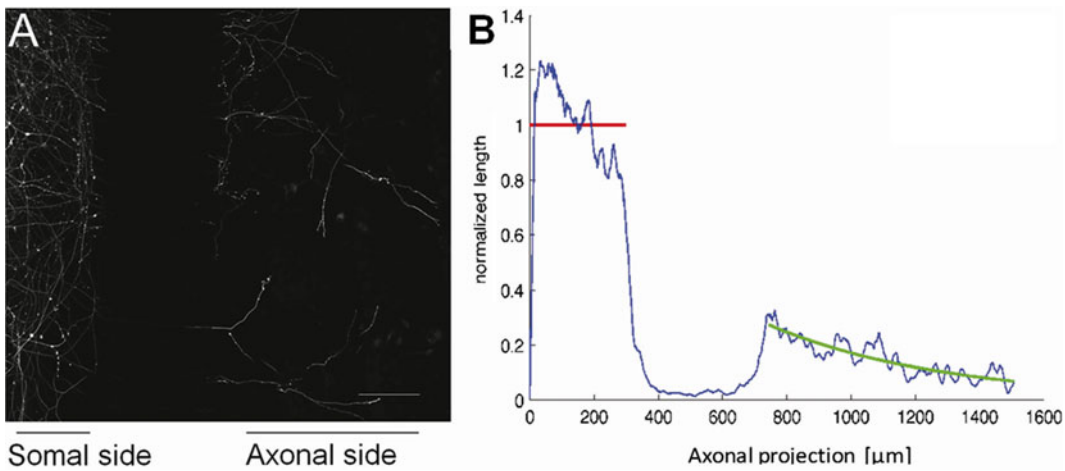

Fig. 8 Output of the quantification method. Representative images used for quantification. (**a**) Axons in *white* stained against βIII tubulin. Scale bar 100 μm. (**b**) Profile along the *xx* axis of the microfluidic device showing the area occupied by the axons (*blue line*), the area normalized to the somal side (*red line*) and the projected axons with the exponential fit (*green line*) in the axonal side. Adapted from [3] with permission from The Royal Society of Chemistry (color figure online)

7. Make sure that the microgrooves connecting the compartments (somal and axonal sides) are totally filled. To do so, take the microfluidic chambers to a vacuum system/desiccator for 5 min. Any air bubbles that might exist inside the microgrooves will move to the reservoirs and should then be removed.

Take special attention not to push the tip too hard under the device to avoid its dislodgement from the coverslip.

Higher concentrations of coating solution might be required for effective cell attachment and due to the flow characteristics of the microfluidic devices. Other researchers have tested: poly-D-lysine at 0.5 $mg.ml^{-1}$; laminin at 20 $\mu g.ml^{-1}$; collagen at 50 $\mu g.ml^{-1}$; and poly-L-ornithine at 0.5 $mg.ml^{-1}$ [13]. We also tested collagen coating in the axonal side for osteoblasts, with the purpose of mimicking the properties of naturally occurring tissue extracellular matrices, which provide mechanical and biochemical signalling factors. For that we have prepared 0.01 % (w/v) collagen solution (powder, cat. No C7661, Sigma-Aldrich) in acetic acid 0.1 M (Sigma-Aldrich) and incubate overnight at 4 °C [3]. The remaining solution was removed and the channels were air-dried.

8. After filling the channel with medium you should never remove the medium directly from them.
9. Other neuronal cells have been successfully cultured on microfluidic devices. Prepare embryonic day (E) 18 rat cortical and/or hippocampal dissociated cell suspension with an approximate density of 3×10^6 $cells.ml^{-1}$. Other neuronal cells such as embryonic DRG neurons, P1 DRG neurons, P6 DRG neurons, E18 septal neurons, P0 hippocampal neurons, and cholinergic neurons have also been successfully cultured in the microfluidic device.
10. Resuspend in a small volume 10 μl so that the cell suspension is kept in the channels. Different cells have different time to adhere, but be aware of the medium evaporation and do not let the cells without medium. Filling an empty well with water will decrease the evaporation rate.
11. For euthanasia, deeply anesthetize the animal proceeding with the CO_2 gas inhalation. It is not recommendable to perform cervical displacement so the column stays intact.
12. You must be careful to open the column exactly in two equal parts to reach easily the DRG.
13. Make sure that DRG stays completely submersed in contact with the coated glass to ensure their adherence and growth. Push them down carefully with a needle without injuring them.
14. Be careful not to aspirate the media with the pipet tip turned to the main channel, nor when pipetting the new media, otherwise cell damage may be caused.

    Given that the volume of media per well is low and can evaporate during incubation, the use of 6-well culture dishes allows to counter this possibility by adding sterile water to the

spaces between the wells. In this case, add the water as soon as the cells are cultured.

15. Depending on the cell proliferation rate, the cell density might be adjusted (area of each channel = 1.5 mm wide × 7 mm long).
16. Check the medium level every day due to the high evaporation rate. Once again, to reduce the evaporation you might fill a well with sterile water to increase the humidity of the plate.
17. An initial 1.8 % (w/v) solution is prepared and a final concentration of alginate of 1 % (w/v) is achieved after addition of the cross-linking agents ($CaCO_3$ and GDL) and cell pellet. Volumes added should be accounted for the decrease in final concentration [17–19].
18. Solution of GDL should be prepared and filtered immediately before use since it hydrolyses progressively to gluconic acid. Thus enables the progressive acidification of the solution of alginate and $CaCO_3$, releasing calcium ions to cross-link alginate.
19. All procedures must be done as quickly as possible since the gelation process will start as soon as the cross-linking agents get in contact with the alginate solution. Use positive displacement pipettes when working with alginate.
20. TBS should be used instead of PBS when dealing with alginate due to the phosphate salts that leads to dissolution of the hydrogel.
21. Add 60 μl of antibody solution to one of the channels and 40 μl to the other channel. This volume difference will make the solution flow though the microgrooves.
22. To better detach the coverslip from the well, place some water around the device. After few minutes it will be easier to detach the glass coverslip from the plate even without disassembling the microfluidic device.
23. Samples can be prepared for electron microscopy using chemical agents. For hexamethyldisilazane (HMDS) drying samples, that had been chemically fixed and dehydrated to 100 % ethanol and stored a minimum of 96 h, transfer to 100 % HMDS through a graded series (100 % ethanol; 3:1; 1:1; 1:3; 100 % HMDS). Samples must be soaked for 15–20 min in each stage of the series, and then air dried from HMDS under a fume hood overnight [20].
24. Normalization is important to make comparison among different conditions, and alignment is important to set the different regions.

Acknowledgments

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Chapter 11

A Novel In Vitro Primary Culture Model of the Lower Motor Neuron–Neuromuscular Junction Circuit

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Abstract

Modelling the complex process of neuromuscular signalling is key to understanding not only normal circuit function but also importantly the mechanisms underpinning a range of degenerative diseases. Here, we describe a compartmented in vitro model of the lower motor neuron–neuromuscular junction circuit, incorporating primary spinal motor neurons, supporting glia and skeletal muscle. This culture model is designed to spatially mimic the unique anatomical and cellular interactions of this circuit in compartmented microfluidic devices, such that the glial cells are located with motor neuron cell bodies in the cell body chamber and motor neuron axons extend to a distal chamber containing skeletal muscle cells whilst simultaneously allowing targeted intervention.

Key words Microfluidic, Motor neuron, Muscle, Neuromuscular junction, Cell culture

1 Introduction

The neuromuscular junction is a highly specialized synapse within the peripheral nervous system, forming a connection between the spinal motor neuron and the skeletal muscle. The cells which form the lower motor neuron–neuromuscular junction circuit are highly compartmentalized and spatially organized in vivo, with the motor neuron soma located within the spinal cord surrounded by glial cells and the skeletal muscle located distally. Signalling at the neuromuscular junction is a two-way process involving anterograde electrochemical signalling which results in muscle contraction and determination of muscle fiber type [1]. Additionally, retrograde neurotrophic signalling from the skeletal muscle to motor neuron terminals assists in maintenance of neuronal health [2]. Thus, the health and function of lower motor neurons and the muscle tissue that they innervate are dependent on each other.

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Disruption of neuromuscular signalling is often a key component of many motor neuron diseases and myopathies. In motor neuron disease, dysfunction and degeneration of the spinal motor neuron results in muscular atrophy. Whilst motor neuron dysfunction is assumed to occur prior to denervation and muscular atrophy proposed to occur secondary to neuronal dysfunction, the role of the skeletal muscle in these diseases is being increasingly explored [3–5]. In muscular dystrophy, repeated cycles of muscle necrosis and attempted repair ultimately affect the health of the motor neuron and function of the neuromuscular system [6]. Modelling the unique cellular interactions is critical to uncovering the underpinning mechanisms of a wide range of neuromuscular disorders.

Cultured motor neurons readily form rudimentary neuromuscular junctions on cocultured myotubes, as indicated by colocalization of acetylcholine receptors (AChRs) on the myotube surface with presynaptic motor terminals [7, 8]. While these models demonstrate that cultured cells can interact to form synaptic structures, they do not allow more physiologically relevant questions to be probed that address cellular interactions. To overcome this limitation, neuromuscular junction formation in vitro can be achieved using spinal cord explants placed such that motor neuron axons extend towards cultured muscle [9, 10]. Such models are able to recapitulate some of the unique spatial organization between motor neurons and glial/muscle cells, however they do not allow fluidic isolation within the culture. Growth factors and extracellular matrix components typically present in specific locations in vivo are mixed or uniformly present within the cultures. Furthermore, targeted experimental treatment to motor neuron soma or axon/NMJ is not possible.

In this chapter we describe a novel method for coculture of primary murine spinal motor neurons, spinal glial cells and skeletal muscle using microfluidic chamber devices [11]. Using these techniques, cocultures are developed with timely addition of each cell type such that spinal motor neuron soma are supported by spinal glial cells to allow optimal growth of motor neurons, and distally located skeletal myocytes providing synaptic targets, evidenced by NMJ formation [12] (Fig. 1). These preparations allow not only robust growth of cultured motor neurons, but allow for specific experimental targeting of either the somal/proximal or axonal/distal components of the motor neuron [13]. This coculture preparation is achieved using commercially available microfluidic chambers (Xona Microfluidics) in combination with precise experimental timing and may be easily modified to use relevant cells from a wide range of sources, including cell lines or cells derived from human induced pluripotent stem cells [14, 15].

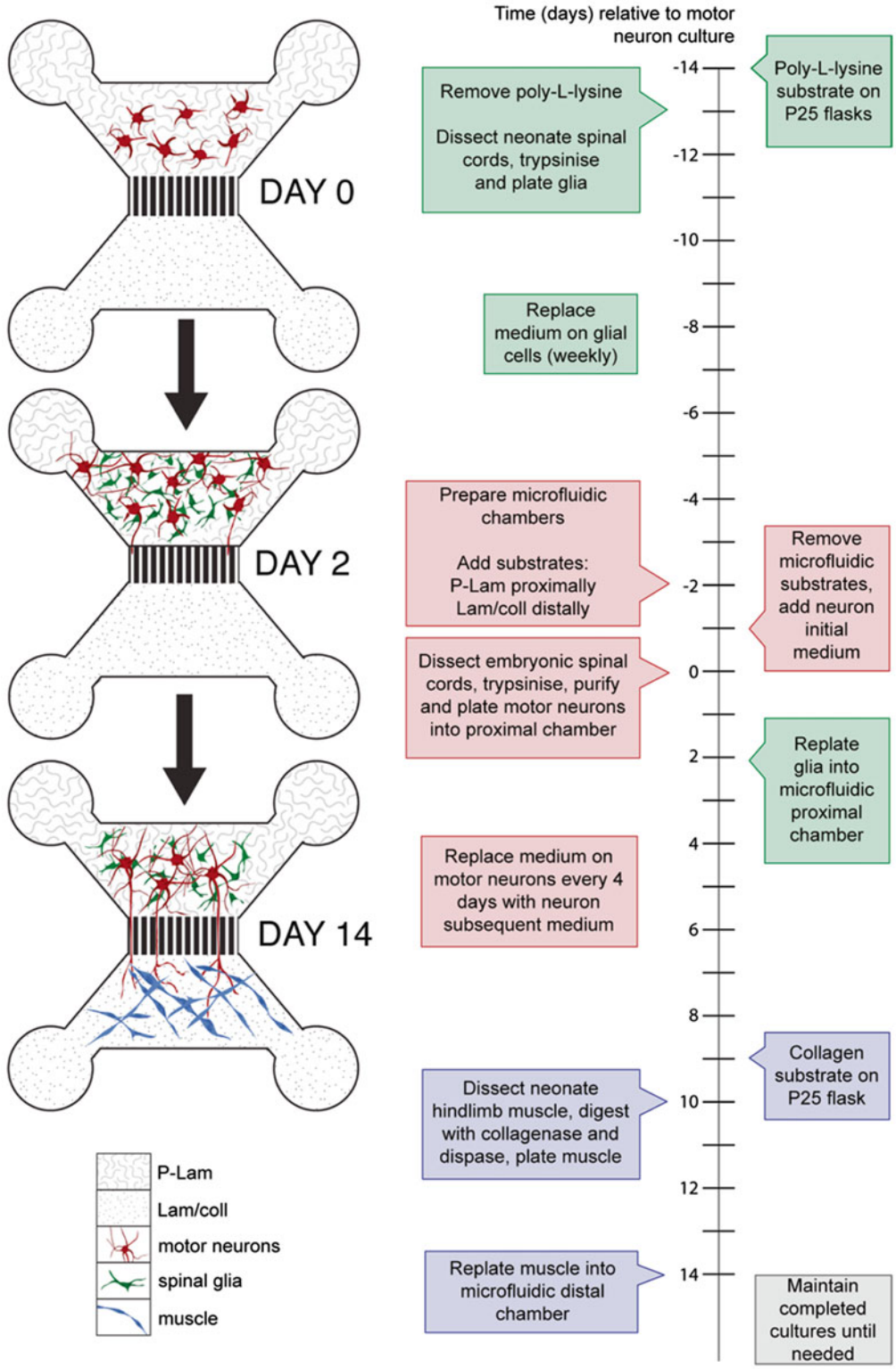

Fig. 1 Schematic and timeline of culture assembly. Primary cultures of spinal glial cells (*green*), motor neurons (*red*), and skeletal muscle (*blue*) are prepared in a timely manner for addition to compartmented culture devices (color figure online)

2 Materials

2.1 Equipment

- Preparation of cell culture devices: Biosafety or laminar flow cabinet, sterile forceps, adhesive tape.
- Primary cell dissection: Laminar-flow cabinet, stereodissecting microscope fitted with a cold-light source, water bath (37 °C), sterile dissection instruments (suggested array: 2× fine #5 forceps, 1× #3 forceps, 1 pair spring scissors (8 mm blades), 1 pair large scissors for neonates), centrifuge, hemocytometer.
- Neuron culture: Humidified 5 % CO_2 incubator, inverted cell culture phase-contrast microscope.

2.2 Materials

2.2.1 Preparation of Cell Culture Devices

- Microfluidic chambers with 450 μm microgrooves (Xona Microfluidics).
- Tissue-culture grade glass 22 mm^2 square coverslips.
- 100 and 70 % ethanol (EtOH).
- 6-well culture plate.

2.2.2 Solutions for Primary Cell Culture

- Poly-L-lysine (PLL) (0.01 % in PBS, Sigma-Aldrich). Store 4 °C, use within 1 month.
- PBS-Laminin (P-Lam): Laminin (5 μg/ml, Sigma-Aldrich) in PBS. Do not refreeze.
- Poly-L-lysine/laminin (PLL-L): Laminin (5 μg/ml, Sigma-Aldrich) in PLL. Do not refreeze.
- Laminin/collagen (Lam/coll): Calf-skin collagen (5 μg/ml, Sigma-Aldrich) in P-Lam. Do not re-freeze.
- Collagen: Calf-skin collagen (1 %, Sigma-Aldrich) in PBS. Store 4 °C, use within 1 month.
- Neuron initial medium: Neurobasal medium (Invitrogen), heat-inactivated fetal bovine serum (10 %, Invitrogen), B27 supplement (2 %, Invitrogen), L-glutamine (0.5 mM, Gibco), L-glutamic acid (25 μM, Gibco), penicillin/streptomycin (1 %, Invitrogen). Store 4 °C, use within 1 month.
- Neuron subsequent medium: Neurobasal medium (Invitrogen), B27 supplement (2 %, Invitrogen), L-glutamine (0.5 mM, Gibco), penicillin/streptomycin (1 %, Invitrogen).
- DMEM: Dulbeccos modified Eagle medium (Invitrogen), heat-inactivated fetal bovine serum (10 %, Invitrogen), penicillin/streptomycin (1 % Invitrogen). Store 4 °C, use within 1 month.
- Primary myoblast medium: Hams F-10 medium (Invitrogen), heat-inactivated fetal bovine serum (20 %, Invitrogen), fibroblast growth factor (2.5 ng/ml). Store 4 °C, use within 1 month.

- Fibroblast growth factor: basic human fibroblast growth factor (25 μg/ml, Promega) in BSA (0.5 %, Sigma).
- Trypsin (0.25 % stock, Invitrogen). Store aliquots –20 °C, thaw before use, do not freeze/thaw.
- Trypsin–EDTA: Trypsin (0.05 %, Invitrogen) and EDTA (0.5 M, BDH). Store aliquots –20 °C, thaw before use, do not freeze/thaw.
- Collagenase: Collagenase (1 mg/ml, Sigma-Aldrich) in PBS. Store 250 μl aliquots –20 °C, thaw before use, do not freeze/thaw.
- Dispase I: Dispase I (2.4 U/ml, Sigma-Aldrich) in PBS. Store 250 μl aliquots –20 °C, thaw before use, do not freeze-thaw.
- $CaCl_2$: $CaCl_2$ (100 mM, BDH) in Milli-Q water. Sterilize and store 4 °C.
- 0.01 M PBS.
- BSA: Bovine serum albumin (3.5 %, Sigma-Aldrich) in HBSS. Use immediately.
- 1.06 g/l OptiPrep: OptiPrep™ (1 ml, Sigma-Aldrich) in 4 ml HBSS. Mix well and use immediately.
- Trypan blue (Sigma-Aldrich).

2.2.3 Primary Cell Culture

- Hank's balanced salt solution (HBSS; Invitrogen).
- P25 and P75 culture flasks (Corning).
- 10 or 15 ml centrifuge tubes (Corning).
- 70 μm cell strainer (BD Biosciences).
- Razor blades (VWR International).
- Sterile plastic petri dishes (VWR International).

3 Methods

3.1 Preparation of Cell Culture Surfaces

3.1.1 Sterilization and Preparation of Microfluidic Devices

1. Clean surface particles from microfluidic devices using adhesive tape.
2. Sterilize devices for 2–3 h in 70 % EtOH. Perform subsequent steps under sterile conditions.
3. Rinse once in 100 % EtOH and let stand upright to dry completely (*see* **Note 1**).
4. Place sterile 22 mm^2 coverslips individually in 6-well trays, ensure sterility by UV exposure for 15 min.
5. Attach microfluidic devices to coverslips, ensuring microchannels are in contact with coverslips (*see* **Note 2**). Use blunt forceps to firmly press devices onto coverslips without breaking glass (*see* **Note 3**).

6. Pipette 200 µl PLL-L into one side of the devices (chamber into which neurons will be plated) and 200 µl L lam/coll into the other side (distal/muscle chamber) (*see* **Note 4**).
7. Incubate overnight at room temperature.
8. Remove both substrates and fill chambers with neuron initial plating medium. Allow to equilibrate in cell culture incubator for a minimum of 2 h prior to addition of cells (*see* **Note 5**).

*3.1.2 Preparation of Flasks for Glial and Myocyte Culture (See **Note 6**)*

1. Coat the surface of P25 flasks with sufficient substrates with PLL for glial cultures or collagen for myocytes. Use enough to coat entire surface (2–3 ml).
2. Incubate overnight at room temperature.
3. Remove PLL and allow flask to dry. Remove collagen and rinse flask 1× with PBS. Do not allow to dry.

3.2 Primary Cell Culture

3.2.1 Spinal Glial Cells

1. Decapitate P2 (postnatal day 2) rat pups. Sterilize skin with 70 % ethanol.
2. Place body back-side up in sterile petri dish and use spring-loaded scissors to open the skin along the midline of the back. Hold body by inserting tweezers through the body of the pups across the spinal cord.
3. Cut horizontally at the base of the tail to sever the spinal column. Make a small incision vertically into the spinal column.
4. Hold the body firmly and use strong forceps or spring scissors to cut along spinal column to one side of the midline from the tail to the neck to release the spinal cord.
5. Use forceps to gently remove spinal cord in sections if necessary and collect tissue in a small drop (100–200 µl) HBSS at room temperature.
6. Using fine forceps, remove meninges from spinal cords and transfer cleaned spinal cords to 2.5 ml HBSS on ice.
7. Repeat dissection steps for additional neonates (*see* **Note 7**) and volumes optimized for 2× neonates).
8. Add trypsin to final concentration 0.0125 % (125 µl trypsin for 2.5 ml HBSS), flick tube gently to mix.
9. Incubate tissue with trypsin for 5 min at 37 °C.
10. Remove trypsin solution and add 2 ml glial cell growth medium at 37 °C. Gently triturate spinal cords with a 1 ml pipette to dissociate cells. Triturate 10–20 times and avoid introducing air bubbles to the cells.
11. Plate cell suspension into prepared P25 flasks.
12. Replace medium completely the next day to remove cell debris. Replace half the medium once weekly to maintain cultures (*see* **Note 8**).

3.2.2 Spinal Motor Neurons

1. Sacrifice pregnant E14.5-15.5 rat (finding of a positive vaginal plug is defined E0.5) by CO_2 suffocation and carefully remove embryos from the uterus. Decapitate embryos and place bodies on ice (*see* **Note 9**).
2. Place embryos back side up. Hold the embryo with forceps and remove the skin overlying the spinal cord using another pair of forceps.
3. Gently slide forceps along the spinal column to release the spinal cord from the vertebral column. Turn the embryo around and repeat along the other side, and remove the tail using the forceps (*see* **Note 10**).
4. Place spinal cord in a small drop of HBSS on ice. Repeat steps 2–3 with remaining embryos (*see* **Note 11**).
5. Transfer one isolated spinal cord to a fresh drop of HBSS and use fine forceps to remove dorsal root ganglia and meningeal layers. Transfer cleaned spinal cords to 5 ml HBSS on ice. Repeat step for remaining spinal cords.
6. Add trypsin to final concentration 0.00625 % (125 μl trypsin for 5 ml HBSS), flick tube gently to mix.
7. Incubate with trypsin for 5 min at 37 °C.
8. Remove trypsin solution from cells and add 2 ml HBSS. Gently triturate cells 10–20 times with a 1 ml pipette tip until no visible cell clumps are present, avoid introducing air bubbles during trituration.
9. Divide cell suspension equally between two tubes each containing 2 ml 3.5 % BSA (*see* **Note 12**).
10. Pellet cells at $120 \times g$ for 10 min.
11. Resuspend cells in 1 ml HBSS per tube, gently layer onto prepared OptiPrep™ in 10 ml tubes, spin at $400 \times g$ for 25 min (*see* **Note 13**).
12. Remove motor neuron layer from OptiPrep™ (should be visible as a cloudy layer 1 ml from top of 10 ml tube). Transfer motor neurons to fresh tubes each containing 1 ml HBSS and pellet at $700 \times g$ for 10 min (*see* **Note 14**).
13. Resuspend motor neurons in 200 μl neuron initial plating medium and perform cell count using a trypan blue dye exclusion assay (or similar). If necessary, adjust volume of cell suspension to achieve 5×10^6 cells per ml (*see* **Note 15**).
14. Refer to Sect. 3.3.1 for plating of motor neurons into microfluidic devices.

*3.2.3 Skeletal Myocytes (See **Note 7**)*

1. Sacrifice P2 neonates (refer to Sect. 3.2.1). Remove hind limbs from the rest of the body at the top of the hip. Cut off the feet and remove the skin. Discard feet and skin.

2. Use strong forceps and scissors if required to remove muscle tissue from legs and collect muscle tissue in a sterile petri dish (*see* **Note 16**).
3. Use spring-loaded dissection scissors to finely mince tissue. Transfer tissue to 2 ml HBSS (*see* **Note 17**).
4. Mix 250 µl collagenase, 250 µl dispase, and 12.5 µl 100 mM $CaCl_2$, add enzyme mix to 2 ml HBSS. Mix tissue well with enzyme mix. Incubate at 37 °C for 45 min.
5. Gently triturate cells with a 1 ml pipette with tip aseptically removed (*see* **Note 18**).
6. Add 2 ml 37 °C primary myoblast growth medium and strain cells through 70 µm mesh to remove undigested tissue.
7. Pellet cells at 350 × *g* for 10 min. Resuspend in 2 ml 37 °C primary myoblast growth medium, plate cells into a collagen coated P25 flask.
8. Replace medium completely the next day to remove cell debris. Return to incubator and allow myoblasts to become 50 % confluent (approximately 2–4 days) (*see* **Note 19**).
9. Refer to Sect. 3.3.3 for plating of myocytes to microfluidic chambers.

3.3 Assembly of Cocultures

3.3.1 Addition of Spinal Motor Neurons to Microfluidic Chambers

1. Remove equilibrating culture medium from prepared devices.
2. Gently pipette 10 µl of cell suspension to one reservoir of the cell body side of each device, by pointing the tip of the pipette to the entrance of the cell chamber (*see* **Note 20**).
3. Return chambers to the incubator for 10–15 min to allow cell attachment.
4. Fill medium reservoirs with warmed neuron initial plating medium and return cells to the incubator (*see* **Note 21**).
5. Leave for 2 days undisturbed growth.

3.3.2 Addition of Spinal Glial Cells

1. Spinal glial cells can be added to the cell body chamber of the microfluidic devices from motor neuron 2-4DIV.
2. Detach confluent spinal glial cells from P25 flasks by removing glial cell growth medium. Add 2 ml DMEM without added FCS and incubate for 10 min to wash cells.
3. Remove wash and add 1 ml trypsin–EDTA at 37 °C. Return to incubator for 5 min (*see* **Note 22**).
4. Dislodge cells by hitting the flask firmly with the heel of the hand. Observe cell detachment.
5. Add 2 ml glial cell growth medium at 37 °C. Pellet cells at 300 × *g* for 5 min.

6. Resuspend cells in 500 µl 37 °C neuron initial plating medium. Perform cell count and adjust volume to achieve 3×10^6 glial cells per ml.
7. Remove medium from motor neuron chambers of microfluidics and plate 10 µl glial cell suspension into each device.
8. Incubate for 10 min in the incubator to facilitate cell adhesion.
9. Fill medium reservoirs with warmed neuron initial plating medium and return devices to the incubator.

*3.3.3 Addition of Skeletal Myocytes (See **Note 23**)*

1. Remove 50 % confluent myoblasts from flasks with trypsin–EDTA (as for glial cells, refer Sect. 3.3.2), rinsing in serum-free primary myoblast medium prior to addition of trypsin–EDTA (*see* **Note 24**).
2. Add 2 ml warmed primary myoblast growth medium to inactivate trypsin. Pellet cells at $350 \times g$ for 10 min.
3. Resuspend myoblasts in 500 µl warmed neuron initial plating medium. Count cells and adjust volume to achieve 8×10^6 cells per ml.
4. Remove growth medium from distal chambers of microfluidic devices, add 10 µl myoblasts to the distal chamber and incubate for 10 min to allow cell attachment (*see* **Note 25**).
5. Fill chambers with warmed neuron initial plating medium and leave for 2 days undisturbed growth.

3.3.4 Maintenance of Compartmented Cultures

1. Once skeletal myoblasts begin to fuse and differentiate into myotubes (approximately 2–3 days after plating), completely replace all medium with neuron subsequent growth medium.
2. Maintain with weekly medium (neuron subsequent) changes.

3.4 Applications for Compartmented Cultures

Live cultures may be used for live cell imaging and experiments utilizing localized treatments. During live cell treatment or staining, generate fluidic isolation by manipulating fluid levels in each compartment, maintaining the untreated compartment fluid level higher than that of the untreated compartment. For analysis of cell types present, neuronal growth, and formation of neuromuscular junctions, immunocytochemistry can be performed (Fig. 2).

3.4.1 Targeted Proximal or Axonal Treatment

1. Dilute desired treatment or stain in warmed neuron subsequent medium (200 µl for each treated device).
2. Aspirate culture medium from compartment to be treated.
3. Add 200 µl dilute treatment to one well only of treated compartment and allow to flow through cell chamber (*see* **Note 26**).
4. Ensure culture medium in untreated compartment is 400 µl to maintain fluidic isolation between compartments (*see* **Note 27**).

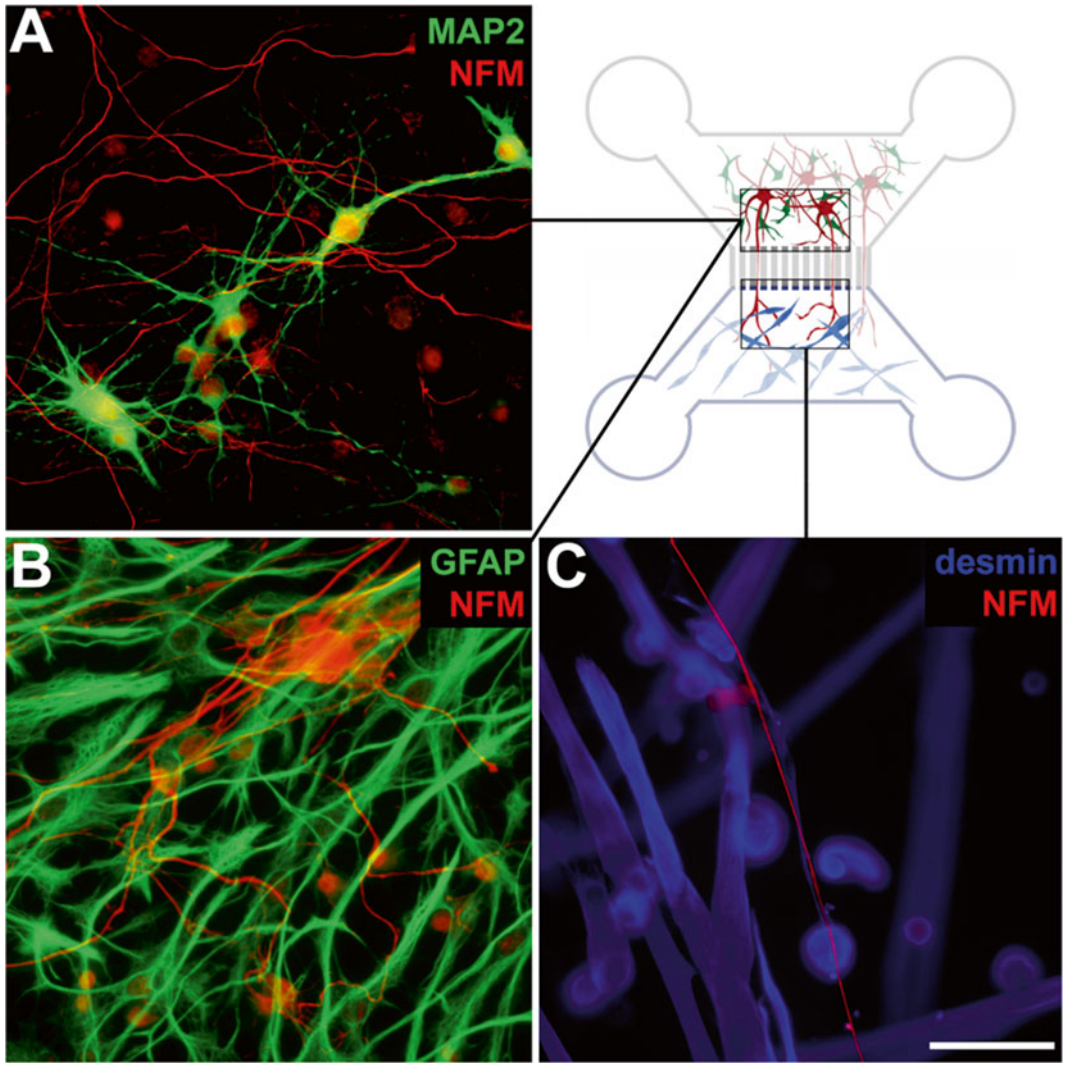

Fig. 2 Immunocytochemical labelling of completed cultures. (**a**) Indicates neuronal axons (NFM, *red*) and dendrites (MAP2, *green*) within proximal compartment. (**b**) Spinal glial cells (GFAP, *green*) are present in the proximal compartment with neuronal axons (NFM, *red*). (**c**) Illustrates neuronal axons (NFM, *red*) alongside differentiated skeletal myocytes (desmin, *blue*). Scale = 50 μm

3.4.2 Fixation and Immunocytochemistry

1. Aspirate culture medium from all wells.
2. Fill one well on each side with fixative (e.g., 4 % paraformaldehyde) (*see* **Note 28**).
3. Incubate on orbital shaker during fixation.
4. Remove fixative and cover devices with PBS to wash.
5. Use forceps to carefully remove devices from coverslips. Wash coverslips well and use for immunocytochemistry or staining.

3.4.3 Reuse of Microfluidic Device

1. Wash for 10 min with laboratory detergent (e.g., Pyroneg).
2. Scrub chambers gently with toothbrush to remove cell debris.
3. Rinse well (3× 10 min) washes in Milli-Q®. Allow to dry completely. Refer to Sect. 3.1.1 for sterilization.

4 Notes

1. Do not expose chambers to UV radiation; however, do make sure they are completely dry before attaching to coverslips.
2. Make sure devices are completely sealed to the coverslip to avoid leakage.
3. Optional plasma treatment. Coverslips may be plasma cleaned prior to attachment of chambers to enhance cell attachment. Place coverslips on a flat, sterile surface (e.g., inside of culture dish lids) and evenly expose to oxygen plasma cleaner held 3–4 cm above six coverslips for 30 s, ensuing all coverslips are equally treated. For best results perform plasma treatment in batches of six and complete device attachment and substrate addition within 2 min.
4. Minimize confusion between side of devices by marking with permanent marker on underside of coverslip. Minimize bubbles when adding substrates.
5. It can be left in incubator for up to 1 week; however, take care devices do not dry out.
6. Flasks for myocyte and glial culture can be prepared up to 1 month in advance.
7. Volumes, flasks and number of cells optimized for using 2× P2 neonates. If a larger culture is needed, perform dissections in batches of two and keep tissue no longer than 1 h without proceeding to next step.
8. Failure to remove debris from culture will result in slower glial growth and may kill the culture.
9. Using embryos <E14.5 or >E15.5 will result in dramatically reduced cell yield and reduced cell viability.
10. Run tweezers along the side of the spinal cord several times to ensure cord is free before lifting from spinal column.
11. Minimize cell loss by performing dissection within 90 min of obtaining tissue. Keep tissue on ice as much as possible to minimize tissue degradation.
12. Make sure tissue is well dissociated prior to addition of BSA. Low cell yields often occur from inadequate trituration.
13. Prepare OptiPrep™ immediately prior to use in culture. Invert four to five times to ensure thoroughly mixed. Improperly

prepared OptiPrep™ is the main reason for low cell yields. If ≥6 embryos are used, cell layer should be obvious following OptiPrep™ spin.

14. Ensure cell layer is taken with minimal contamination from surrounding OptiPrep™.
15. Lower cell yields are common; try resuspending in a smaller volume or plating a smaller amount (e.g., 7 μl).
16. Remove as much visible adipose tissue as possible and ensure both tibia and fibular bones are removed.
17. Take sufficient time to fully mince tissue—allows for shorter enzyme incubation and better cell yield.
18. Gently triturate tissue using a 1 ml pipette with the tip aseptically removed to check on digestion progress. Enzyme digestion is complete when >95 % of cell suspension can pass through an unaltered 1 ml pipette tip.
19. Allowing myocyte cultures to reach confluency promotes fibroblast proliferation.
20. Cells should be visible flowing through plating chamber within first few minutes of addition. Cells still readily flowing >10 min after addition indicates insufficient substrate coating.
21. Adding medium prior to cell attachment will flush cells from growth chamber.
22. Incubating for >5 min in trypsin–EDTA will reduce cell viability.
23. Ensure distal axons are present within distal chamber before adding skeletal myocytes.
24. Myocyte cultures detach more readily than glial cultures; trypsin–EDTA for 2 min is usually sufficient to dislodge from flask.
25. Add myocytes very gently to avoid disturbance to axons.
26. Adding treatments to only one well allows treated medium to contact all cells during diffusion throughout cell chamber. This ensures all cells come into contact with the treatment.
27. If both compartments are being simultaneously treated/stained, make volumes on each side identical.
28. Ensure appropriate care is taken with cell fixatives, and use within fume hood or use appropriate PPE.

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Part V

Pharmacological Manipulation and Screening

Chapter 12

Compartmentalized Microfluidics for In Vitro Alzheimer's Disease Studies

Yufei Ren, Anja Kunze, and Philippe Renaud

Abstract

Compartmentalized microfluidic devices are designed to engineer the cellular environment for cell cultures. The practical use of the compartmentalized chambers can be expanded to induce co-pathological cell cultures, where one cell population expresses a specific disease state, while being in direct-cell or metabolic contact to a second or third unaffected cell population. A typical example for co-pathological cell states in the brain is the well-known neurodegenerative Alzheimer's disease (AD), which still lacks effective treatment approaches. In the brain, AD shows specific disease progression patterns from one functional brain region to another. However, in normal dissociated neuron cultures using petri dishes, the extraction of the progression patterns is very difficult. In this chapter, we describe the methodology to design and fabricate a compartmentalized microfluidic device and apply it to an in vitro AD model to mimic the key pathological hallmarks of AD, allowing us to study disease progression patterns from a "diseased" towards a "healthy" cell population. This derived co-pathological model of AD provides the ability to monitor time-variant changes in cell network morphology and electrophysiology during disease progression and may potentially be used for pharmaceutical tests.

Key words Alzheimer's disease, Co-pathology, Disease progression, Compartmentalized microfluidic device, Micro-electrode arrays (MEAs), Neural cell culture

1 Background and Historical Overview

The first symptoms for Alzheimer's disease (AD) were described at a conference in Germany by Dr. Alois Alzheimer in 1906. Later, in 1910, these symptoms were named "Alzheimer's disease" by Emil Kraepelin [1]. Alzheimer's disease is a progressive neurodegenerative disease in the brain. The disease starts from the transentorhinal cortex, spreads to the hippocampus and invades the entire cerebral cortex in its final disease state [2]. Often, AD lesions propagate through the patient's brains for years before any symptoms appear. As the disease develops, impaired judgment, disorientation, confusion, abnormal behavior, and difficulties in speaking, swallowing, and walking start to appear [3]. Gradually, AD makes patients have

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more troubles in daily life with cognitive and functional abilities declining and finally leading to death [3].

During Alzheimer's disease propagation, two major pathologies play an important role in the brain: the deposition of extracellular amyloid-beta (Aβ) plaques, and intracellular hyperphosphorylated tau neurofibrillary tangles (NFTs) [4]. Newer hypotheses explore the role of tau oligomers and their toxic effects [5], or study the correlation between Aβ and tau protein [6]. Based on the theories of Aβ and NFTs, neuroscientists are focusing on promising therapeutic approaches such as anti-amyloid or tau based drug development to reduce amyloid-beta production, prevent amyloid-beta aggregation, and promote amyloid-beta clearance [7]. Until today, however, no pharmaceutical company has succeeded in developing a fully effective treatment against the progression of AD symptoms, which puts a need into developing novel experimental approaches to better understand Alzheimer's disease.

In vivo studies are in a position to provide the complexity of the brain structure and function; however, the functional and molecular pathways still cannot be clearly understood. To better understand structural connections and the diverse range of brain functions during neurodegeneration processes, in vitro models are indispensable. Usually nowadays studies on Alzheimer's disease are achieved by conventional cell culture tools such as plastic petri dishes or multi-well plates for a great quantity of experiments. As microtechnology has progressed, microfluidics, a new in vitro method based on microfabrication technology, has become a powerful and practical tool for biological studies (Fig. 1).

The first in vitro compartmentalized device for sympathetic neuron culture was described by Robert B. Campenot in 1977 [8]. In this three-chamber neuron culture system, neurites from the sympathetic neuron somata grow through almost fluid-impermeable barriers to other chambers guided by a high concentration of nerve growth factor (NGF), so that the neuron soma and their neurites still keep their physical connection, but are cultured in different environments [9]. Campenot's isolated chamber model gave the possibility of culturing neurites and somata separately in compartmentalized environments in the scale of millimeters to micrometers. In recent years, microfabrication technology has advanced rapidly, including microfluidic technology, which opens a new research field and serves as a highly efficient and multifunctional tool for in vitro neuroscience research. The potential of both physical and biochemical environments are tightly controlled both temporally and spatially at the microscale [8, 10]. Microfluidic devices can provide simple and practical disease models at the microscale, by mimicking brain specific patterns of AD environments in cell studies.

Besides the morphological compartmentalization, which we can obtain from microfluidic devices, electrophysiology is just as

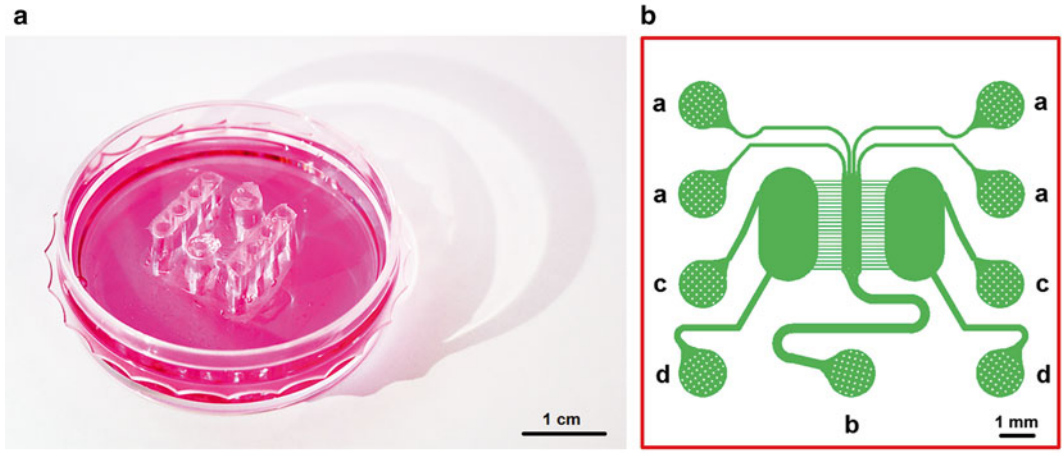

Fig. 1 An example of a PDMS microfluidic device and its design. (**a**) A PDMS microfluidic device is cultured inside a 35 mm^2 petri dish filled with neural cell culture medium. (**b**) The pattern at the bottom of the microfluidic device which was designed by CleWin software. *Green*: the sunken area from the PDMS surface to form channels and chambers. *Black*: notes. (a: Round reservoir and inlet channels for the middle chamber. b: Round reservoir and outlet for the middle chamber. c: Round reservoir and inlet for the lateral chamber which is connected to the middle chamber by many junction channels. d: Round reservoir and outlet for the lateral chamber)

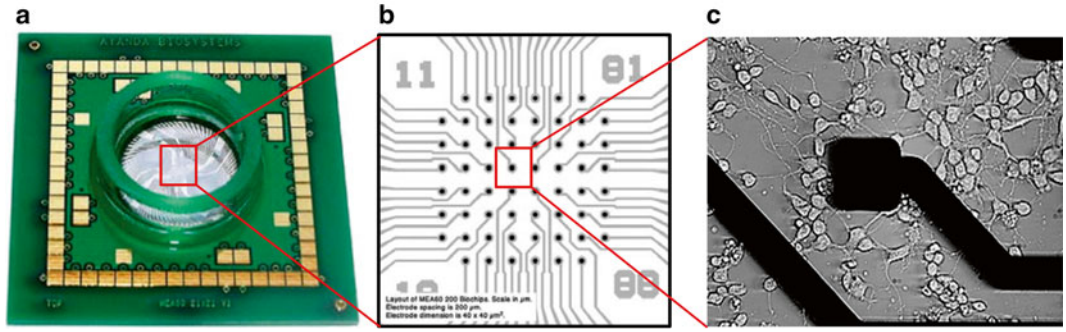

Fig. 2 An example of MEA device with 60 electrodes and an image of neural culture on the MEA surface. (**a**) Image of a MEA device. (**b**) The microelectrode design contains of 60 electrodes, each electrode is connected to external wires. (**c**) Microscope image of a neural cell population seeded and cultured on top of the microelectrodes

important for the characterization of neuronal networks. For simultaneous recording of millions of neurons in vitro, planar micro-electrode arrays (MEAs) have been developed (Fig. 2). A MEA, which has multi microscale electrodes, allows for extracellular simultaneous recording and stimulation. The first trial of using planar multi-electrode arrays for recording was in 1972 for dorsal root ganglion neuron culture [11]. Today, MEAs are widely used in neuroscience laboratories to characterize electrophysiological signals from either natural brain slices, or in vitro grown neural

networks from dissociated neurons. MEA recorded data can be analyzed to extract functional connectivity and functional links in in vitro neural networks using statistical algorithms [12]. The compatibility of MEA and microfluidic fabrication processes allows us to integrate neural electrophysiological studies into compartmentalized microfluidic devices [13].

In this chapter, we present details about the fabrication of our compartmentalized microfluidics platform and the method we used to study the progression of hyperphosphorylated tau on the cellular level within our culture. Previously shown results demonstrate the propagation of phosphorylated tau states from diseased neurons towards adjacent healthy neurons. In addition, we present some information on the micro-electrode array (MEA) technology and its integration in the multicompartment microfluidics system. This integrated microfluidics and MEA system allows us to observe time-dependent alterations in neural morphology and electrophysiology during AD progression.

2 Equipment, Materials, and Setup

2.1 Dissociated Brain Cells

Our method allows culturing a variety of cells of different types (e.g., astrocyte, primary neuron) [14], from different regions of the brain (e.g., hippocampus, cortex), or from different transgenic animal models (e.g., Amyloid precursor protein transgenic mouse models, or Tau transgenic mouse models). In our experimental procedure we cultured dissociated neurons from the cerebral cortex of embryonic rats at day 19 (E19).

2.2 Bulk Material for Microfluidic Device

For microfluidic device fabrication, a variety of techniques can be applied, such as silicon-based, polymer-based hard lithography and soft patterning with soft lithography [15]. Among these alternative materials, a typical material for compartmentalized microfluidic device fabrication is Poly(dimethylsiloxane) (PDMS), a soft polymeric material [16]. PDMS is cell compatible [17] and is highly gas permeable, which allows oxygen and carbon dioxide to exchange [18]. Furthermore, PDMS is also optically transparent and has low autofluorescence [19]. With its favorable biocompatibilities for cell culture and optical properties for cell imaging, PDMS has become a widely used material in soft lithography fabrication technology. We choose PDMS as the material for our microfluidic device based cell culture.

2.3 Setup for MEA Recording

For MEA based extracellular neural activity recording, the MEA device is first inserted into an in vitro recording system with an integrated amplifier and a stimulus generator, and this system is further connected to a computer for data visualization and analysis. The neural activities from the neurons cultured in the PDMS-MEA

device were recorded by the in vitro MEA data acquisition System (Multi Channel Systems, Germany), which has 60 recording channels set at 10 or 20 kHz sampling frequency and 10–3,000 Hz hardware bandpass filter. The MC_Rack software (Multi Channel Systems, Germany) was installed in the computer and was used for data acquisition and analysis.

2.4 Setup for Cell Loading into the Microchannels

To load cells into the channels of the microfluidic device, sometimes micro-syringes and pumps can be used, although not necessary. Controlling the flow rate during cell loading through micro-syringes and pumps allows us to automate this process. The cell loading setup is shown in Fig. 3. Our micro-syringe setup is used to load cells and perfuse cell culture medium to refresh, or to perfuse drug-enriched medium through the microfluidic channels over a long period of time (more than 2 h). Another benefit of the micro-syringe setup is that we can also generate cell patterns inside the chamber. For example, different contents can be brought into the microfluidic system from different channels and then generate a special layer-pattern through the laminar flow regime [20].

3 Procedures/Methods

3.1 Microfluidic Device Fabrication

PDMS serves as a very suitable material to fabricate compartmentalized microfluidic device. The pattern of chambers and channels on the bottom surface from the PDMS device is molded by using a silicon wafer (Fig. 4).

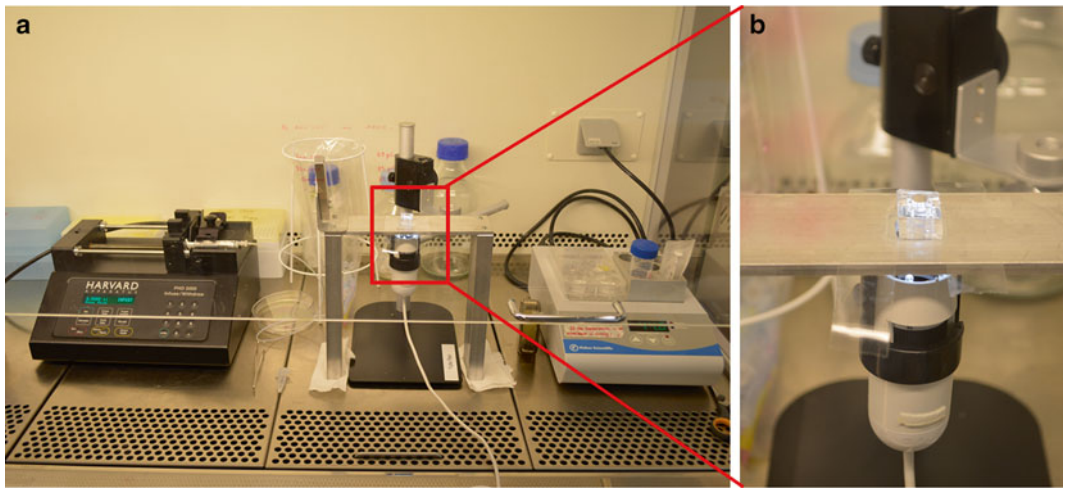

Fig. 3 The cell seeding setups for cell culture in a microfluidic device in a sterile environment. The setup consists of a flow controlling syringe pump, a digital microscope and a heating system. (**a**) A syringe was attached on the top of a syringe pump and should be connected to a reservoir from the PDMS device. PDMS device was fixed by scotch tape on a holder. A digital microscope which was connected to a laptop was placed under the device. Medium was always kept in the heater. (**b**) Zoomed view shows the PDMS microfluidic device and the digital microscope

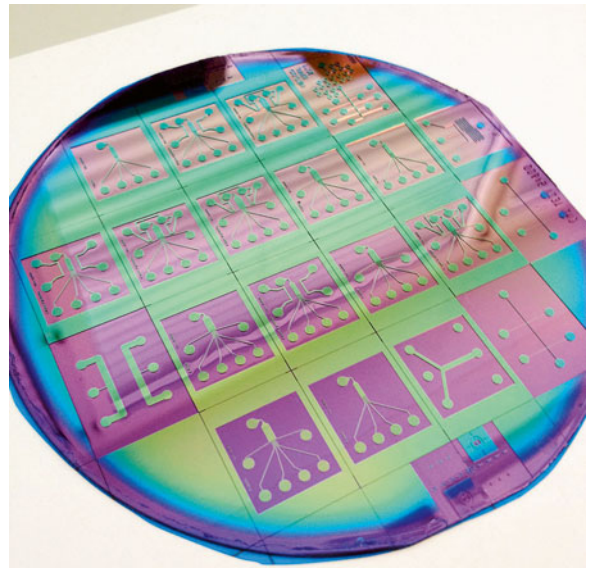

Fig. 4 A representative microstructured silicon wafer contains multiple microfluidic designs for PDMS master molding

The silicon wafer is fabricated in a clean room, where two types of wafer fabrication processes can be used (Fig. 5), depending on whether different heights are necessary for the channels. In order to isolate the neurites, we need to use different heights of channels to make the barriers, for example some low height junction channels (e.g., 10 μm in height which is smaller than the neuron soma size), to block the neuron soma and let the neurites to grow through to other compartments in the device. In this case, junction channels require a different height compared to normal channels, only to let the neurites to grow through. A two-step dry etching process can be used to fabricate the wafer to mold PDMS device which requires different heights (Fig. 5b). After the first etching step, the channels are 10 μm high for the junction channel. With the second etching step, another 90 μm high etching is made so that the total channel height becomes 100 μm. Otherwise, if junction channels are not applied, one-step dry etching process is enough and all the channels are in the same height (Fig. 5a).

For the junction channels, the channel length is very important for building specific neural networks for different experiment purposes. For instance, with 150 μm junction channels, we are able to isolate both axons and dendrites; with 500 μm junction channels, we can isolate axons only and build axon-connections networks. Furthermore, the channel width is 20 μm, the approximate size of the diameter of a neuron's body and the channel height is 10 μm, so that cell bodies are blocked by the junction channels instead of passing through the junction channels into the neurites or axon only middle chamber during the cell planting (Fig. 6a–c).

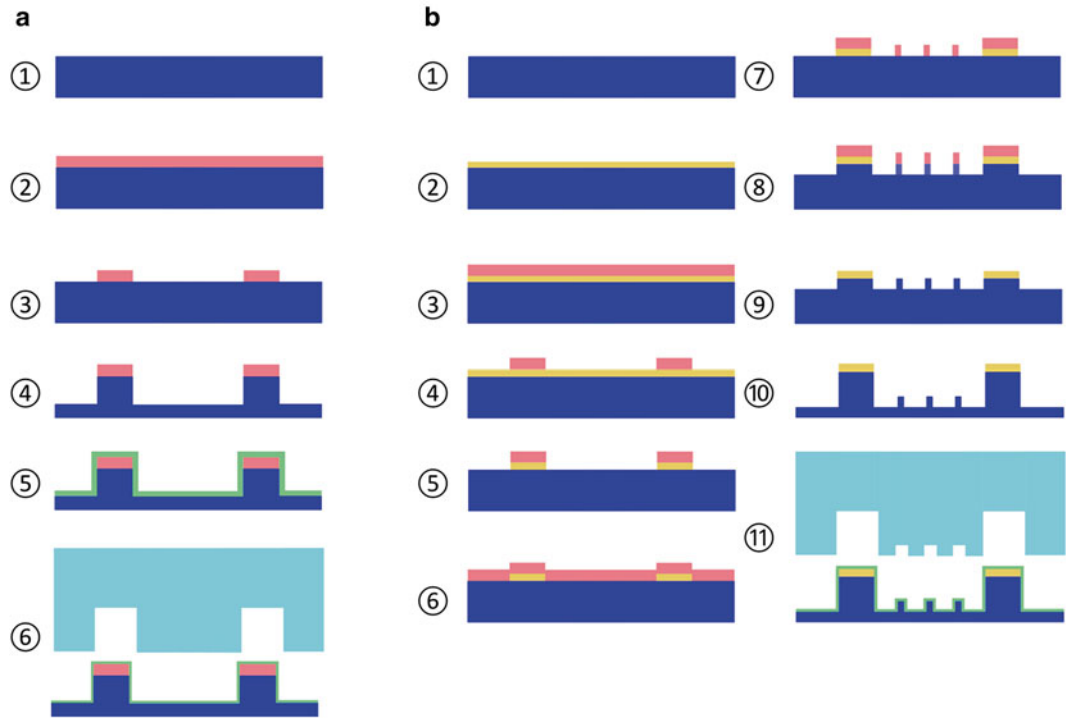

Fig. 5 Process flow for microstructuring PDMS devices in the clean room. (**a**) One depth channel PDMS device fabrication process flow: ① Silicon wafer, 10 mm ② Photoresist coating, 5 μm ③ Photolithography ④ Si DRIE etching, 100 μm ⑤ Wet oxidation, 300 nm ⑥ PDMS molding. (**b**) Two different depths of channels PDMS device fabrication process flow: ① Silicon wafer, 10 mm ② SiO_2 deposition, 1 μm ③ First photoresist coating, 2 μm ④ First photolithography ⑤ SiO_2 RIE etching ⑥ Second photoresist coating, 5 μm ⑦ Second photolithography ⑧ Si RIE etching, 10 μm ⑨ Remove photoresist ⑩ Si DRIE etching, 90 μm ⑪ Wet oxidation, 300 nm and PDMS molding (*RIE* reactive ion etching, *DRIE* deep reactive ion etching)

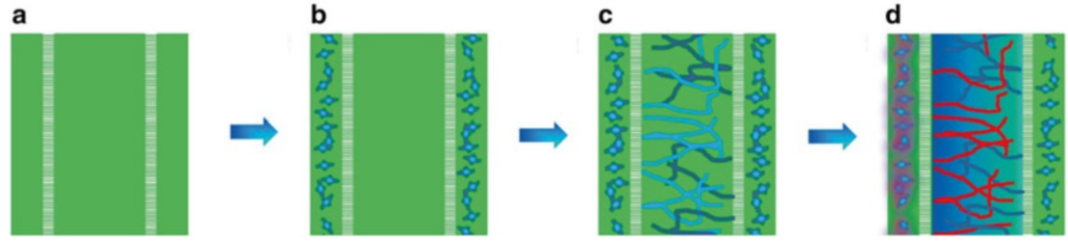

Fig. 6 The schematic pictures on the experimental region inside a microfluidic device to show the steps to achieve a co-pathological "diseased" and "healthy" neural network. (**a**) PDMS microfluidic device with three compartments connected by junction channels. (**b**) Neurons were injected inside the two lateral chambers. (**c**) After 1 week continuous culture, the neurites grew through the junction channels and build up a neurite network in the middle chamber. (**d**) The left neuronal cell population was exposed to a drug and this will generate a drug gradient in the middle chamber by diffusion through the junction channels, while keeping the right cell population unaffected

Once the silicon wafer is fabricated, it is used as a mold for making PDMS devices. Before we use it for molding, we deposit a thin layer of SiO_2 to make a protection layer on top of the silicon wafer to allow for longer usage as a mold for PDMS device (Fig. 5b ⑪) PDMS sticks to the silicon wafer's surface and the PDMS cannot be peeled easily from the silicon wafer. To solve this problem, a silanization should be done for the silicon wafer surface before the first use (*see* **Note 1**). This makes the wafer's surface hydrophobic and allows for the PDMS to be easily unmolded from the silicon wafer.

After the silicon wafer is ready for PDMS device molding, the next step is to prepare the PDMS mixture. The silicone elastomer (PDMS base) and curing agent are mixed using a 10:1 ratio (*see* **Note 2**); pour the base onto the silicon wafer with the pattern, up to a height of 5 mm or even higher. The PDMS elastomer can be cured at 80 °C for 1 h (*see* **Note 3**). Then the design from the silicon master is molded at the bottom surface of the PDMS device. Some opening reservoirs from the device which connect microchannels to the outside environment are created by holes puncher tools. Glass coverslips of 0.17 mm thickness are applied to close the channels and chambers for the PDMS device and to obtain better biological imaging quality. This coverslip bonding is done by an Oxygen Plasma treatment for both the PDMS device's and coverslip's surfaces at 40 Pa (300 mTorr), 50 W for 45 s (*see* **Note 4**). Immediately after this treatment, both parts are pressed against each other to complete the bonding process and kept in the oven for 3 min (*see* **Note 5**). Then, the PDMS device is filled with 70 % ethanol for sterile cleaning for 20 min, and then rinsed by sterile water three times after ethanol to wash away residual ethanol on the surfaces inside the PDMS device. UV exposure for 20 min is the last step to sterilize the PDMS device and dry the chamber inside the device. In summary, after the fabrication of the PDMS device, it and coverslip need to be sterilized by ethanol cleaning or by an autoclave machine (*see* **Note 6**), water washing several times and UV exposure.

3.2 Cell Specific Surface Coating

Before we seed cells into the PDMS device, we need to treat the substrate inside the microfluidic device with biochemical adhesion factors or physical guidance cues, which is an important process for substrate biocompatibility, biostability, cell differentiation, and cell fate [21]. For this surface coating we used the same chemical as for normal neuronal cell cultures in the plastic multi-well plates.

There are a variety of surface coating agents that can be used for this coating step. For instance, poly-lysine-coated chemicals such as poly-L-lysine (PLL), poly-D-lysine (PDL), and polyethyleneimine (PEI) provide much faster neuronal adhesion and more homogenous cell distribution in the culture. On the other hand, laminin (LN) and Matrigel (MG) coatings can facilitate neuritogenesis more than poly-lysine coatings [22]. Here, we used 0.05 %

(v/v) polyethyleneimine (PEI) coating solution. It is injected into the microfluidic channels from the reservoirs using a syringe. The device is then kept for 2 h at 37 °C, and subsequently needs to be rinsed with sterile water or PBS buffer (*see* **Note 7**). The channel contents are then replaced with a neural cell culture medium and incubated in a 37 °C incubator. After all these steps, the microfluidic device is ready for cell plating.

3.3 Cell Dissociation

Cortical neurons are dissected from rat embryonic brains and be kept inside petri dishes with PBS solution. Embryonic brain tissue is cut into smaller pieces to avoid clustering after digestion. The enzymatic digestion solution (2 ml Segal's medium with phenol red as pH indicator, add 200 μl papain and drops of NaOH for adjusting pH to 7.3) needs to be heated up to 37 °C, intermixed with brain tissue at 37 °C for 15 min and swirled lightly by hand every 5 min. After the brain tissue settles down, the supernatant is removed and 1–3 ml protease inhibitor solution (10 % horse serum and 90 % Neurobasal culture medium, adding 2 % B27 supplement and 1 % antibiotics) is added. The brain tissue is then pipetted through a 1,000 μl pipette tip five times to triturate the tissue and the supernatants containing suspended cells are transferred into a fresh falcon tube through the strainer. Afterwards, the cell suspension is flowed through a 40 μm cell strainer to remove the debris, and some medium is then added and centrifuged at 0.6 μ/min for 6 min. The supernatant is then discarded and cells are transferred into an eppendorf tube to count cell density. In the end, the cells are transferred into a falcon tube, some 37 °C pre-warmed cell culture medium (Neurobasal medium, 2 % (v/v) B27, and 1 % (v/v) GlutaMAX) are added and finally can be diluted into the desired concentration for the experiment.

3.4 Cell Injection into the Microfluidic Compartments

For cell injection, we load the cells into the chambers through the opening reservoirs from the device which connect microchannels to the outside environment. When the reservoir hole is big, cells can be injected inside the PDMS device directly by pipetting with normal plastic tips. When the reservoir hole is small (e.g., 10 mm height and 2 mm diameter) and the height of the PDMS device is high, then a special gel loading tip which has a long tip and a small diameter tip is needed, because standard tips with larger diameters on the tip cannot be inserted inside the reservoir. Cell injection can also be performed with a syringe and a pump as we mentioned in Sect. 2 or we can apply a connector between the syringe and the reservoir to inject the cells by applying some pressure on the top of the reservoir. Cells are injected to the lateral chambers (Fig. 6a, b).

3.5 Culture and Maintenance

After planting cells inside the chambers, the PDMS device is immediately transferred into an incubator (37 °C; 95 % air, 5 % CO_2; and 65 % relative humidity). Chambers inside the microfluidic device

usually are on the micron-scale, so that the microfluidic device contains a very small volume, in total of around 100 μl medium per device. The medium can evaporate and this has an effect on the concentration as the medium composition may change. Since the size of one PDMS device is smaller than one well from a six well plate, we keep the PDMS device in a six well plate and surround it with 2 ml fresh cell culture medium to help to maintain humidity and osmolarity around the device and to slow down the liquid evaporation in the device (*see* **Note 8**) The medium in the device chambers was changed every other day by replacing 50 % of the media (*see* **Note 9**).

3.6 Optimal Culture Time Lengths

After 2 days of cell culture in PDMS microfluidic devices in vitro, we observed the growth of neurites grow from lateral chambers through the junction channels (10 μm in height, 150 μm in length, and 20 μm in width) and extended into the middle chamber. From 5 days in vitro (DIV), neurites from the two lateral chambers were growing through the junction channels and became saturated in the middle chamber. After several days of culture in the incubator, the neurites network is built up inside the middle chamber, meaning that they can be used for a drug treatment (*see* **Note 10**). For instance, with the 150 μm length junction channel design for the neurites extraction experiment, on 8 DIV, when the neural network becomes saturated, 600 nM OA is induced and the morphological observation needs to be continued.

For the microfluidic device that is integrated with MEA recording device, the cell culture needs to be kept for a longer time inside the microfluidic device to be able to obtain a mature cell culture for neural electrophysiological activity recording. Normally we start the recording on the 14 DIV continuously until the 28 DIV to observe the spikes and bursts before and after the treatment for analyzing the changes.

3.7 Local Phosphorylation of Tau Through OA Gradients

In order to build "healthy" and "diseased" cell populations in separated compartments in the microfluidic system, we need to induce the left cell population to become a "diseased" population. The generic approach to do this in our compartmentalized microfluidic device is shown in Fig. 6. The microfluidic device consists of three parallel chambers, which are connected by some small junction channels (Fig. 6a). Neurons are plated in the two lateral chambers (Fig. 6b) and the axons grow through the junction channels into the middle chamber forming here a neural network (Fig. 6c). After, the cell population from the left chamber is drug induced to build the "diseased" model. Before loading the treatment drug inside one reservoir, we first empty the two inlet and outlet reservoirs. Then we inject drug enriched medium in one left reservoir and fresh medium in one right reservoir. A time variant flow generates inside the lateral chambers because of the pressure difference

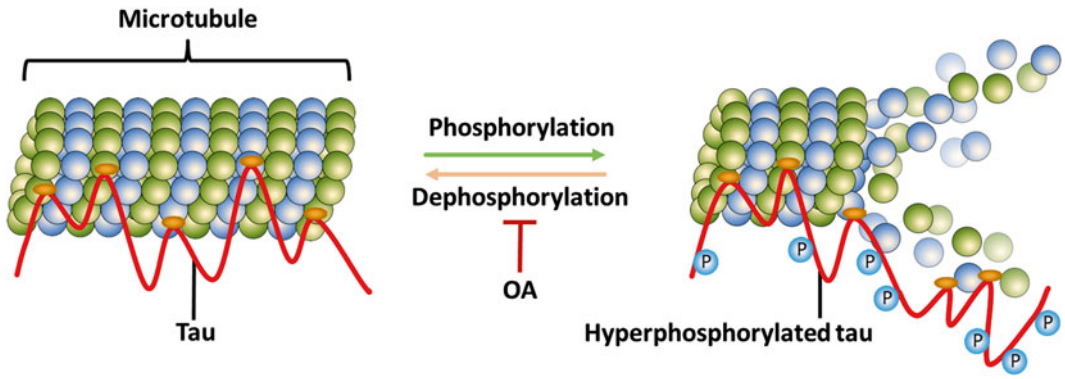

Fig. 7 Microtubule-associated tau protein equilibrium in the brain. By phosphorylation, tau protein becomes phosphorylated tau, and after dephosphorylation, it comes back to be normal tau protein. When OA is induced to the cells, it inhibits the dephosphorylation of the phosphorylated tau. Then the phosphorylated tau becomes hyperphosphorylated tau and highly accumulated inside the brain which is a hallmark of AD. Reproduced from ref. [23]

between the two connected reservoirs (Fig. 6d). During the time variant flow a concentration gradient establishes across the main chamber and through the junction channels. The drug treatment lasts for 75 min. After, the drug is removed from the device through gently washing the lateral chambers with fresh media.

For an AD model, there are varieties of drugs that can be used to induce diseased models, either by over depositing amyloid-beta (Aβ) to form senile plaques or generating neurofibrillary tangles (NFTs), which are composed of highly phosphorylated microtubule-associated tau protein (Fig. 7). In our model we chose okadaic acid (OA), a phosphatase inhibitor, to induce the "diseased" cell population in the left chamber. OA locally induces hyper-phosphorylation of tau proteins by inhibiting the tau de-phosphorylation process. Exposing neurons in one compartment to OA generates a diseased cell population [23]. To visually detect an effect after 75 min treatment time a concentration of 600 nM is found deterministically in control cultures.

3.8 Immunostaining and Imaging

When it is time for sample fixation, the neural cell culture inside the microfluidic device is first incubated with 3 % BSA solution (in 0.1 % Triton/PBS) for 45 min before the immunostaining. After the incubation, the solution is aspirated from the device and then replaced by the primary antibody (Phospho-Tau Ser262 primary antibody) and the device is kept at 4 °C overnight. Then the sample is washed by PBST three times, to wash away the primary antibody that is unbound. We inject the secondary antibody into the channels and keep the device for 2 h at 4 °C. This process needs to be done in the dark and the devices should be packed in

aluminum foil to avoid light exposure. On the second day, the secondary antibody should be removed and the channels should be washed with PBST three times. With these steps complete, the immunostaining is finished and the samples are ready for imaging (*see* **Note 11**). As imaging technology is advancing fast, we are able to get better quality images with different types of microscopes and to obtain more details from our samples.

The microfluidic device is compatible with some current optical microscopy characterization techniques, for example phase-contrast microscopy, fluorescence microscopy, confocal microscopy, and multi-photon microscopy. These different types of techniques have their advantages and disadvantages. For example, the contrast and resolution can be degraded by scattering in wide-field fluorescence microscopy, while confocal microscopy can overcome some effects of scattering and has better contrast and resolution [24]. Two-photon microscopy can improve the detection of signal photons per excitation event, especially for imaging deep (in highly) scattering and absorption environments [24]. Depending on the experimental purpose, choosing a proper microscopy method is very important to acquire good quality images for further data analysis (*see* Table 1). After imaging the microfluidic device data can be analyzed.

4 Experimental Variables

The present method is mainly based on general method for 2D neural culture in vitro by using compartmentalized microfluidic device. This system provides the neuroscientists a special tool to study Alzheimer's disease comparing to traditional culture methods. But there is still some aspects need to be considered.

For microfluidic systems, the material of the microfluidic device itself is very important since it is used for long time cell culture experiment. PDMS, as a porous polymer, is a highly permeable materials compared to other materials. But at the same time, PDMS enables small molecules to diffuse into the bulk of the polymer, allowing them potentially to alter solution concentrations inside the channels. Molecules which are lost from the cell culture medium and treatment solutions which stay inside the PDMS bulk could reduce cell growth and cause cell culture morphological abnormalities [18]. Overall, compared to other materials, PDMS still plays a key role in microfluidic device applications with its excellent properties.

For easier manipulation of the PDMS device in the incubator during the drug treatment time period, a flow can be created by filling only one entrance reservoir of the channel instead of two. By pressure difference, a flow from the filled reservoir to the empty one takes place until the medium height level is equally balanced.

Table 1
Comparison of microscopy characterization methods for neuroscience studies (Reproduced from [15])

Microscope type	Characteristics	Sample preparation	Application
Light microscopy	2D	No treatment	To measure soma size and shape, neurites quantity and density, neurites branching
Phase contrast (PC) and Differential interference contrast (DIC) microscopy	2D, contrast enhanced, high resolution, transparent sample	No treatment	
Fluorescence microscopy	2D, multi-proteins can be located	Fixation, fluorescent staining fluorescent vector	To quantify proteins in soma or neurites
Confocal microscopy	2D and 3D, multiple stacks provide high resolution for thick tissue, but time consuming	Fixation, fluorescent staining fluorescent vector	To study neurites pathways, image electrical activity, neurogenesis, physiology
Two-photon microscopy	2D, tissue are less damaged, sensitive to noise	Fixation, fluorescent staining fluorescent vector	To realize molecule imaging, neurite network imaging in live tissue
Scanning electron microscopy (SEM)	3D, high resolution	Fixation, discharging metal deposition	To observe neural cells, neurites, axons, dendrites, synapses, and synaptic vesicles

As long as the treatment molecular transfer through the continuous flow is higher than the diffusion effect through the junction channels, the gradient of the drug is maintained in the middle chamber where the neurite network is built. The diffusion depends on the diffusion coefficient of the molecules in delivery, with small molecules (100–500 Da) having high diffusion coefficients (400–800 μm^2) [14]. We can also compute the biochemical gradient changes in COMSOL (Multiphysics software). Operating with a high flow rate (>0.1 μl/s) in the lateral chamber can generate crossflow through the junction channels which can have an effect on the gradient formation in the middle chamber [14]. However, in this situation inside the microfluidic device, the maximum flowrate we use in the lateral chamber is around 20 nl/s. This flowrate is much lower than the flowrate that can give a big impact on the gradient profile in the middle chamber, so the effect is neglected.

5 Typical/Anticipated Results

A typical experiment using compartmentalized microfluidic devices for Alzheimer's disease study in vitro is culturing the cell bodies as well as neurites or axons in compartmentalized chambers under different conditions, which allows us to observe a more distinct neural or axonal network with the cell bodies excluded (Fig. 8).

As the experiment we detailed in Sect. 3.7, a local treatment of OA was done on the cell body part. By diffusion through the junction channels, the drug gradient can be maintained in the middle chamber and different phosphorylation states of the neurites were represented in correlation with OA gradient (Fig. 9).

From the result, the cell number from both chambers remained more or less constant, but the number of neurites per cell in the right chamber decreased to 68 % after OA treatment within the time we observed (Fig. 10c, d) In the left chamber where cells were treated by OA, we observed hyperphosphorylated Tau aggregation in the neurites and accumulation in the soma on the diseased cell population side using Ser262-Tau immunostaining (Fig. 10a). On the contrary, in the right chamber, the tau distribution in the cells has lower fluorescence intensity and no accumulation (Fig. 10b). For longer time cell culture like 1 month, we are able to see how the diseased cell population induced by OA has an effect on the other cell population which is healthy originally. This can help neuroscientists to observe how

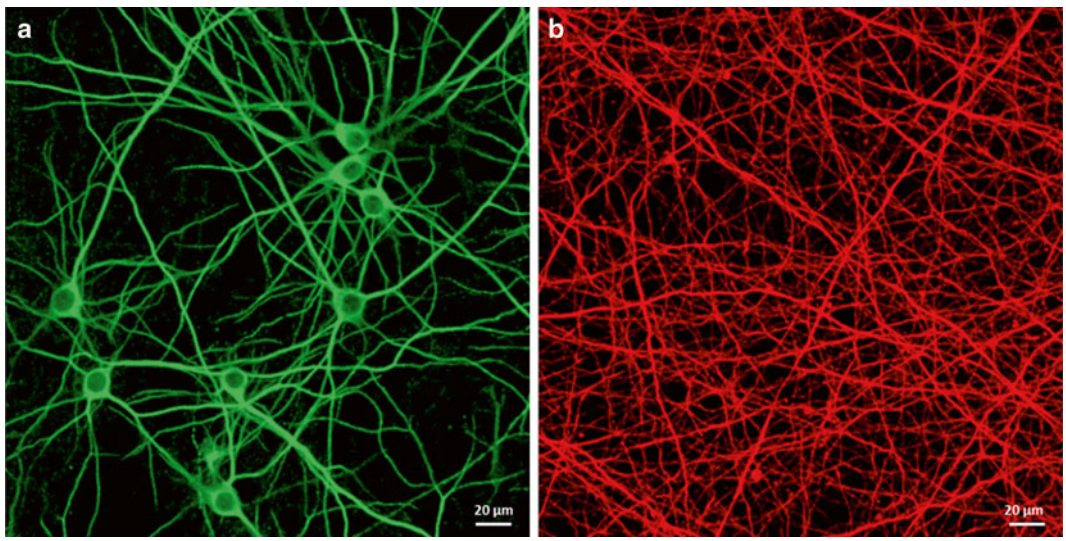

Fig. 8 Immunostaining images of 2D primary cortical neural culture from compartmentalized microfluidic device taken by confocal microscope. (**a**) Fixed sample image from lateral chamber from the device (*Green*: MAP2, soma and dendrites). (**b**) Fixed sample image from the axonal network middle chamber in a device (*Red*: SMI-312, axon)

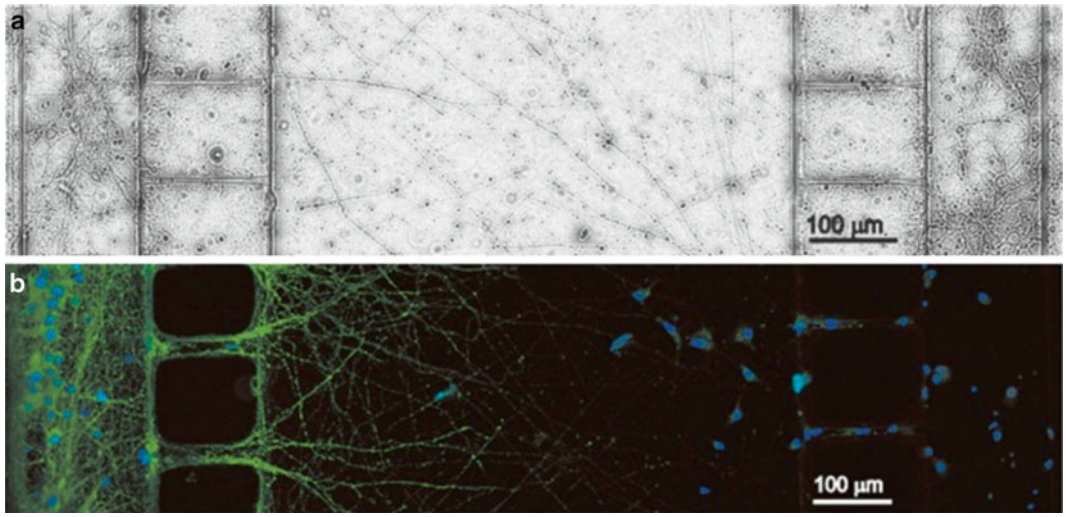

Fig. 9 Images of primary cortical neural network in compartmentalized PDMS microfluidic device after OA treatment. (**a**) Differential interference contrast (DIC) image of the primary cell culture in the microfluidic device before OA treatment. (**b**) Immunocytochemical staining image of primary cortical neurons in the microfluidic device after OA treatment (600 nM) for 75 min on the left cell population. (*Green*: CY-2, *blue*: DAPI) Reproduced from ref. [23]

the disease spreads through the neural network and understand more about the mechanisms of neurological disease.

Except the morphological result we obtained by following the method we explained in Sect. 3, we have electrophysiological result with the integrated microfluidic and MEA device following the same protocol as we present in Sect. 3. Instead of using glass coverslip to make closed chamber with PDMS device, we combined a PDMS device to a MEA device and we are able to record the signal of the soma and the neurites separately, benefitting from the compartmentalized design. The cell culture inside this device can be applied for long time observation. It is inevitable that dissociated neuronal networks lack of a lot of features of the real brain, but it still can develop organotypic synaptic connections and exhibit a rich variety of distributed patterns of electrical activity [25]. We observed the neural activity changes before and after the treatment and we also tried to analyze how the two cell populations correlated with each other (Fig. 11).

6 Conclusion

Overall, there are many benefits for neuroscientists to study neural network mechanisms of Alzheimer's disease propagation in vitro using the presented compartmentalized microfluidic device, developed with microtechnology. It provides more control over experiments in

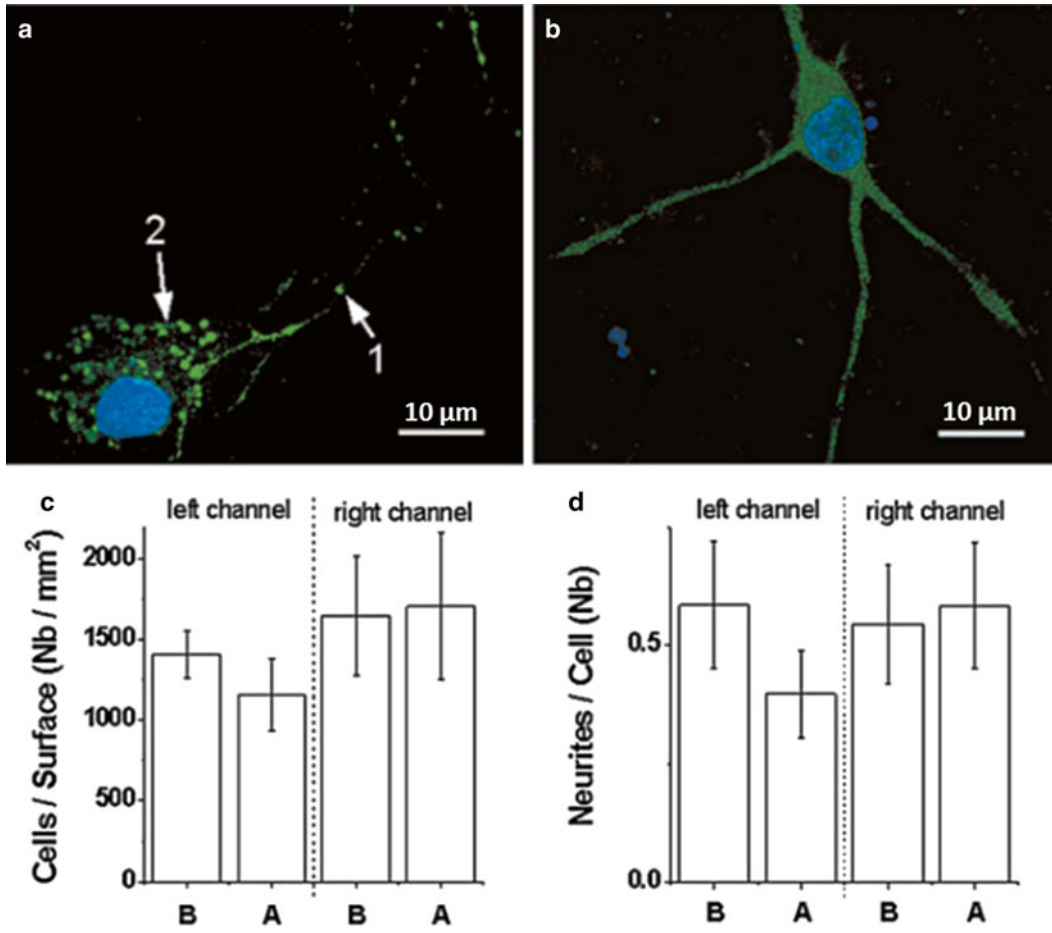

Fig. 10 Fluorescent images of healthy and diseased cells after OA treatment. Diagrams of comparison of cell number and neurites number per cell from healthy and diseased cell populations. (**a**) Diseased cell from left compartment and (**b**) Healthy cell from right compartment from same microfluidic device with Immunocytochemical staining. (*Green*: Ser262-Tau, *blue*: DAPI). Diseased neurons presented accumulation of phosphorylated Tau clusters in neurites (*arrow 1*) and soma (*arrow 2*). Healthy neurons had homogeneous distribution of Tau. (**c**) Cell numbers from both two lateral chambers, B = before treatment, A = after treatment, $n = 5$. (**d**) Neurites per cell before and after OA treatment, $n = 5$. Reproduced from ref. [23]

order to achieve more stable and accurate results at the microscale as we described. Compared to normal cell culture methods, microfluidic PDMS device can be integrated with micro-electrode arrays (MEAs) for electrophysiological study. Furthermore, from an ethical point of view, microfluidic devices, with their micron-sized channels and chambers, require much less cell quantities than petri dishes, which means less mice or rats need to be sacrificed for experiments. At the same time, the biological experiment cost can be reduced significantly, because of the lower quantities of proteins, antibodies and other expensive solutions necessary for experimentation. On the whole, this

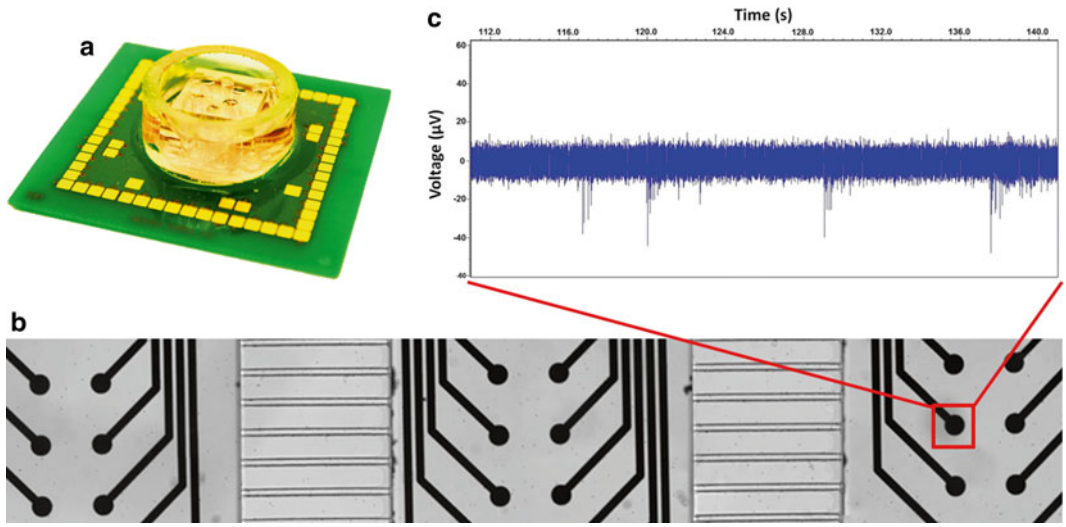

Fig. 11 (**a**) Picture of an integrated microfluidic and MEA device. (**b**) Picture of the partial interface between PDMS device and MEA device including junction channels from PDMS device and electrodes from MEA device. (**c**) An example of 30 s continuous recording result from one electrode among the 60 electrodes simultaneous recording from a microfluidic and MEA device with about 4 weeks cortical neural culture

compartmentalized microfluidic system allows us to mimic the key pathological hallmarks of AD in depth and can be used to observe the long term disease propagation at the microscale.

7 Notes

1. Silane related chemicals can be used for the silanization process for the silicon wafer, such as trimethylchlorosilane and 1H, 1H, 2H, 2H perfluoro-octyl-trichloro-silanes.
2. Other ratios of the PDMS base and curing agent can also be used for different types of experiment. But this will change the properties of the PDMS device. For example, the bigger ratio the PDMS base has, the softer the PDMS device becomes.
3. The silicon wafer with the PDMS mixture should be degassed in a desiccator before curing it in an oven, in order to avoid the generation of air bubbles by the heat during the curing step.
4. The pressure for pressing PDMS chip and glass coverslips should be carefully applied, because the PDMS deformation can happen since it is a relatively soft material compare to glass coverslips. The deformation can change the channel dimensions and has an effect on the experiment.
5. All the parameters should be optimized and fixed for the general process in order to make the experiments repeatable, for example in the case of O_2 plasma treatment for the bonding.

6. After the autoclaving process, the PDMS chip can become non-transparent. Because the water vapor generated from the autoclave machine during the process can go into the porous PDMS material, liquefaction happens when the temperature cools down inside the autoclave machine and the water stays inside the PDMS material. This problem can be solved by keeping the PDMS device inside a metal autoclave box and putting it into an 80 °C oven for a few minutes. The PDMS device will become transparent again.
7. For the surface treatment of a PDMS device, the channels after any injection needs to be checked to make sure that all channels and chambers are covered in solutions, in order to properly apply the surface treatment process. Sometimes, problems such as spontaneous cell death can happen because of nonuniformly coated surfaces. During the experiment, no unnecessary movement should be made since the flow can be very sensitive to shaking.
8. Enough water must be kept inside the water container in the incubator to maintain the humidity for the devices.
9. We suggest not replacing all the media inside the device. Some fresh media can be added in the reservoir to reach the original media level like it was before could be enough.
10. The duration that is needed to wait for the neurites or axon network to saturate depends on the cell density and the junction channel length. The longer the junction channel is, the longer time it takes for the neurites to grow to the middle chamber and reach a high neurites or axon density.
11. The concentrations of the primary and secondary antibodies need to be tested, in order to get the best quality images from the microscope.

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Chapter 13

Selective Biochemical Manipulation of Twin Neuronal Networks on Microelectrode Arrays

Giulia Regalia, Emilia Biffi, Marco Rasponi*, and Alessandra Pedrocchi*

Abstract

Comparability of responses to neuroactive compounds and spatially and temporally resolved delivery of soluble factors are two major key features for pharmacological assays. Here, we describe the fabrication and the use of a device for long-term growth of twin neuronal networks and for their controlled biochemical stimulation. The device is formed by a PDMS microfluidic chamber coupled to a flat Microelectrode Array (MEA), which provides the electrophysiological readout of the pharmacological stimulation. A partial physical barrier divides the chamber in two sub-compartments, where two functionally independent but fluidically connected neuronal networks can be grown. This platform improves biological comparability between cultures and allows to perform selective and temporally controlled stimulations to neurons, running parallel pharmacological tests on the same device.

Key words Microfluidics, Drug delivery, Microelectrode arrays, Neuronal culture, Neuropharmacology, Microlithography, Controlled biochemical stimulation

1 Introduction

In vitro neuronal cultures are widely used as a model for acquiring a basic understanding of network functionality both in physiological and pathological conditions. When coupled to Microelectrode arrays (MEAs), they constitute a reliable model for noninvasive and long-term electrophysiological studies of the effects of neuroactive compounds [1–4].

In the context of neuropharmacological investigations, a major technical requirement is to assure the comparability of data obtained from different MEA chips, in order to connect the electrophysiological readout solely to the drug effect, and not to any external factor. Culture-to-culture variability is conventionally decreased by seeding cultures at the same time and subjecting them to the same feeding schedules and protocols [5]. To further

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increase comparability, nowadays MEA chips can house different cultures in a multi-well structure laid onto the same substrate, thus allowing simultaneous investigations of treated and control cultures [5]. Another relevant demand for pharmacological assays is to provide a spatially and temporally resolved delivery of substances, which allows a localized pharmacological intervention [6]. For this purpose, microfluidic devices are the key technology to deliver microscale volumes of drugs to the desired neurons and to precisely define the biochemical cell microenvironment [7–10].

The device presented in this chapter is a MEA-based microfluidic platform able to selectively and locally stimulate networks of neurons grown and maintained in identical environmental conditions for pharmacological and toxicological tests [11]. Here, we provide a description of the device fabrication and of a simple protocol to seed, maintain, and pharmacologically stimulate hippocampal neurons on the device. However, the device can be used with any other type of neuronal cells.

Specifically, the platform is formed by a Polydimethylsiloxane (PDMS) micro-chamber for cell culturing (100 μm thick), equipped with a thin wall (100 μm wide), which partially divides the cell culture region into two sub-compartments (1,050 μm wide and 7 mm long). Each compartment is linked to a dedicated well for liquid addition/removal (400 μl volume) through a microfluidic channel (500 μm wide). Also, a common well for liquid addition/removal is present, which serves as a fluidic link between the two compartments. The layout of the three wells, the micro-channels, and the micro-chamber is presented in Fig. 1a. The micro-chamber is irreversibly bonded to a flat MEA chip and used to seed primary neurons which originate two parallel neuronal networks, each one monitored by a symmetric half of MEA electrodes. The fabrication technique of the PDMS device with the embedded micro-channel structure (mainly based on micro-lithography and spin coating) allows the microsystems to be prepared with reduced fabrication time.

This device can be used to culture two networks at the same time, which can be similar in cell density and functionality but are not functionally connected. Indeed, thanks to the partial separation, the two networks are independent (i.e., physically separated and electrically uncorrelated). At the same time, they share the same medium in static conditions (through the common well), which allows them to experience the same environmental and biochemical changes during the entire growth period, thus reducing the experimental variability. Thanks to the microfluidic design and to the creation of medium level gradients, the "twin" networks can be fluidically disconnected during pharmacological stimulations. Indeed, they can be independently stimulated in a spatial and temporal controlled manner, through the selection of appropriate flow rates for drug delivery.

The twin network configuration here described reduces biological variability, improves comparability of compounds tested,

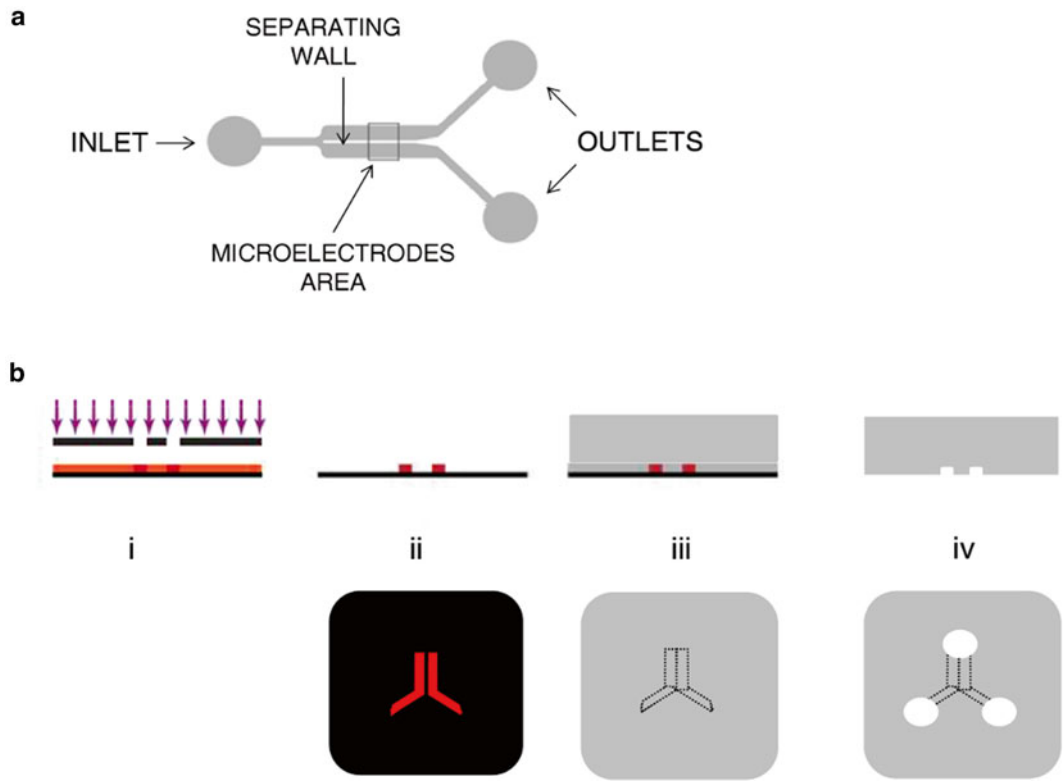

Fig. 1 (**a**) Configuration of the three wells, the micro-channels and the micro-chamber which constitute the microfluidic device. (**b**) Steps for the realization of the PDMS structure embedding microfluidic channels. *Top*: section of the device as seen from the front view. *Bottom*: top view

allows including positive and negative controls in parallel on the same device and shortens the overall experimental timescale. The design can be easily modified to house an increased number of compartments, in order to achieve a higher-throughput microfluidic platform.

2 Equipment and Materials

2.1 Equipment

- Device fabrication:
  - Desktop CNC (Computer Numerical Control) milling machine (MDX40, Roland DG) with an 800 μm in diameter carbide end mill.
  - Clean room facility: mask aligner (MA56, Karl Suss).
  - Clean room facility: two flat hot plates.
  - Clean room facility: fume-hood.
  - Clean room facility: spin coater.
  - Clean room facility: optical microscope.

  - Clean room facility: nitrogen gas.
  - Plasma cleaner (Harrick Plasma Inc.) (*see* **Note 1**).
  - Oven.
  - Vacuum chamber.
- Neuron culture and electrophysiology:
  - Humidified 5 % CO_2 incubator.
  - Recording setup: 60 channel pre-amplifier stage (MEA-1060-Inv-BC-Standard, gain: 55, bandwidth: 0.02 Hz–8.5 kHz, Multi Channel Systems, MCS GmbH); amplification and filtering stage (FA64, gain: 20, bandwidth: 10 Hz–3 kHz, MCS GmbH); data acquisition system (USB-ME64, 16 bit, MCS GmbH); Mc_Rack software (MCS GmbH) (*see* **Note 2**).
  - Syringe pump PHD2000 programmable Harvard Apparatus (*see* **Note 3**).

2.2 Materials

2.2.1 Device Fabrication

- 4-in. polished silicon wafers.
- Negative photoresist (SU-8 50, Microchem).
- SU-8 developer (Microchem).
- Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning).
- Ethanol, isopropyl alcohol (IPA), acetone, chloroform.
- Sharpened biopsy punchers (Diameters 8 mm and 500 μm).
- Scalpel.
- Tape (Magic Tape, 3 M).
- Steel spatula.
- Disposable petri dish (100 mm, VWR International).
- 8 mm thick poly(methyl methacrylate) (PMMA) sheet.
- Flat Microelectrode Array (60MEA200/30iR-Ti-w/o, MCS, GmbH) (*see* **Note 4**).
- Tweezers for silicon wafer handling.

2.2.2 Neuronal Culture

- Plating medium (before cell seeding and 4 h after): Neurobasal medium (Invitrogen), 10 % Fetal Bovine Serum (FBS; Lonza), 1 % Penicillin and Streptomycin (Gibco).
- Culturing medium (from 4 h after the cell seeding to the end of the culture): Neurobasal medium (Invitrogen), B-27 1× (Invitrogen), GlutaMAX 1 mM (Invitrogen), 1 % Penicillin and Streptomycin (Gibco).
- Bidistilled and autoclaved water (dH_2O).
- Poly-L-lysine (Sigma).

3 Methods

3.1 Device Preparation

3.1.1 Device Mold Fabrication

1. Design chip layout through CAD software (AutoCAD, Autodesk) (Fig. 1a).
2. Print out layout mask at high-resolution (greater than 20,000 dpi) on a transparency sheet (deep black for unexposed regions) (*see* **Note 5**).
3. Clean room operations:
   (a) Clean a new wafer by rinsing with ethanol, IPA, and acetone, followed by drying with nitrogen gas.
   (b) Spin-coat SU-8 50 to the desired thickness (a thickness of 100 μm use a spin time of 30 s and a spin rate of 1,100 rpm) (*see* **Note 6**).
   (c) Soft-bake the wafer through a two-step (two hot plate) procedure. Move the coated wafer with tweezers on a 65 °C preheated hot plate. After 10 min, move rapidly the wafer on a 95 °C preheated hot plate. Bake it for at least 30 min and let it cool down to room temperature.
   (d) Cut the mask around the designed wafer with a square shape, slightly greater than wafer diameter, and tape it upside-down to a quartz glass compatible with the mask holder of the mask aligner.
   (e) Place the wafer in the mask aligner, move it against the mask in soft-contact mode and expose to a dose of 500 mJ/cm^2 in the i-line region (*see* **Note 7**) (Fig. 1b*i*).
   (f) Post-bake the wafer in a similar fashion of the pre-bake: bake for 1 min at 65 °C and 10 min at 95 °C.
   (g) Develop the wafer in a SU-8 developer bath for about 10 min (Fig. 1b*ii*).
   (h) Rinse briefly the wafer with IPA then dry it with a gentle stream of nitrogen gas.
4. Place the wafer (final mold) in a petri dish and tie it with tape for further use.

3.1.2 Device Fabrication

1. Mix the PDMS-prepolymer and curing agent in ratio 10:1 (w/w) using a steel spatula.
2. Place in the vacuum chamber and degas the mixture for at least 30 min.
3. Spin the PDMS on the mold with the spin coater up to a thickness of 8 mm (Fig. 1b*iii*).
4. Place in a vacuum chamber until all bubbles are removed.
5. Partially cure in oven at 80 °C for 15 min.
6. Create input and output wells with the puncher (volume of each well 400 μl), as in Fig. 1b*iv* (*see* **Note 8**).

3.1.3 Conical Caps Mold Fabrication

1. Use a 3D CAD modeler to design an array of truncated cones with average diameter equal to the biopsy puncher diameter (8 mm) and a height of 5 mm.
2. Machine out with the CNC milling machine an 8 mm thick PMMA sheet to the final depth of 5 mm.

3.1.4 Conical Caps Fabrication

1. Mix the PDMS-prepolymer and curing agent in ratio 10:1 (w/w).
2. Degas the mixture for about 30 min.
3. Pour the PDMS mixture into the PMMA mold.
4. Place the mold in the vacuum chamber and degas it until no bubbles are visible.
5. Cure in oven at 80 °C for 120 min.
6. Retrieve the caps from the molds and create an opening in the middle of each cap through biopsy puncher 500 μm.

3.2 Device Assembly and Preparation for Cell Seeding

1. Place a MEA inside a petri dish.
2. Place it in the plasma cleaner.
3. Turn on the vacuum pump and wait for 2–5 min to reach vacuum pressure sufficient to generate plasma, as specified by the manufacturer.
4. Turn on the RF power to ignite the plasma and perfuse for 8 min to improve cell adhesion (*see* **Note 1**). Then, leave the MEA in the plasma cleaner.
5. Remove the tape from the PDMS device and place the device inside a petri dish, with channels upside.
6. Place it in the plasma cleaner.
7. Turn on the vacuum pump and wait for 2–5 min to reach vacuum pressure sufficient to generate plasma, as specified by the manufacturer.
8. Turn on the RF power to ignite the plasma and perfuse the MEA and the PDMS device for 60 s (Fig. 2a).
9. After the plasma treatment, lean the PDMS structure with channels downward onto the MEA under a microscope, placing the thin wall in order to separate the 60 MEA electrodes in two subgroups of 30 electrodes (*see* **Notes 9** and **10**) (Fig. 2b).
10. Put the assembled device into a petri dish.
11. Cure in oven at 80 °C for 10 min or more.
12. Fill the device from the inlet with 100 % EtOH for 10 min.
13. Aspirate EtOH from the outlets.

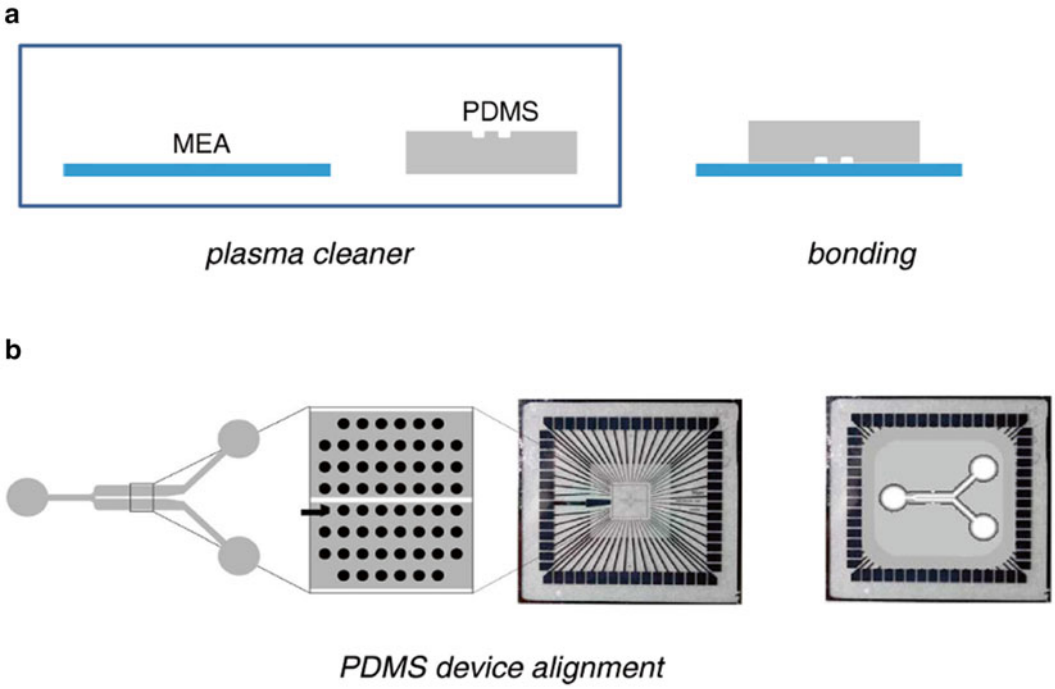

Fig. 2 (**a**) Orientation of the MEA substrate and the PDMS structure during plasma cleaning (*left*) and during bonding (*right*). (**b**) Alignment of the PDMS device on the MEA

14. Rinse the device three times with dH_2O by adding dH_2O into the inlet and aspirating from both the outlets (refer to Fig. 3 from point 14 to point 25).
15. Aspirate water from both the outlets and slowly add 1 ml of plating medium to the inlet.
16. Place the device inside a humidified 37 °C incubator for 2 h.
17. Prepare 2 mg/ml poly-L-lysine (Sigma) in 100 mM Borate Buffer pH 8.5.
18. Aspirate plating medium from the outlets (*see* **Note 11**).
19. Slowly add 400 µl of poly-L-lysine to the inlet device. Aspirate 150 µl from each outlet well (*see* **Note 12**).
20. Place the device in a humidified incubator at 37 °C overnight.
21. Aspirate poly-L-lysine and thoroughly rinse with dH_2O three times with 5 min interval.
22. Place the device, completely filled with dH_2O, in the incubator for 6 h (*see* **Note 13**).
23. Rinse again three times with dH_2O.
24. Fill the device with the plating medium (as described above).
25. Place the device in a humidified incubator at 37 °C until cell seeding (*see* **Note 14**).

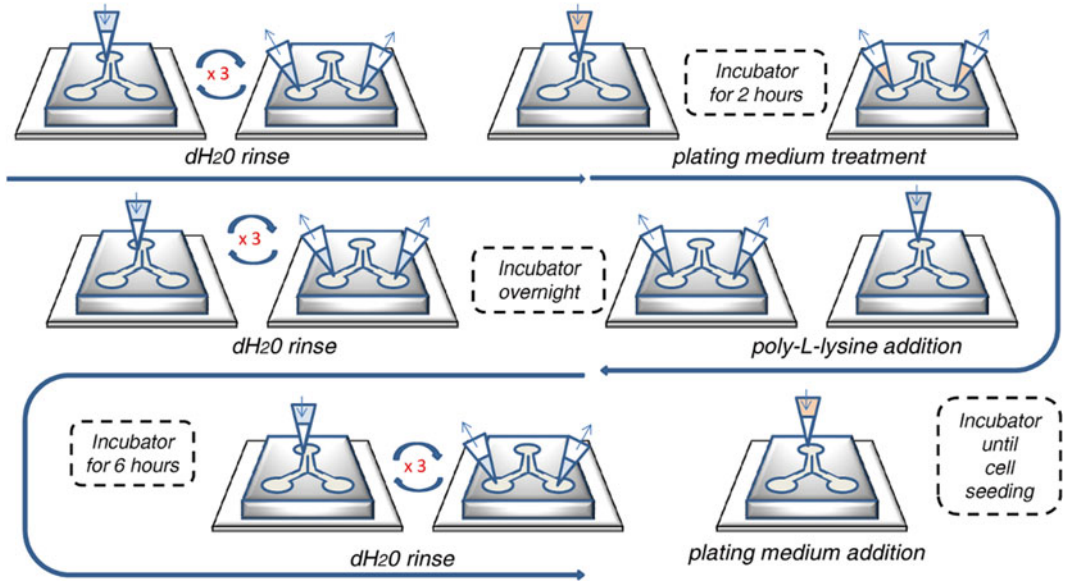

Fig. 3 Steps for the preparation and sterilization of the devices before cell seeding

3.3 Neuronal Culture

1. Sacrifice CD1 mice by inhalation of CO_2 (*see* **Note 15**).
2. Dissect brains in cold HBSS (Gibco) supplemented with Glucose 0.6 % and 5 mM Hepes pH 7.4 (Sigma) and extract hippocampi (*see* **Note 16**).
3. Wash hippocampi in cold HBSS twice, with a 2 min interval.
4. Treat hippocampi with Trypsin (0.25 %, Sigma), in a HBSS solution, for 10 min at 37 °C.
5. Wash again hippocampi in cold HBSS, twice.
6. Aspirate HBSS and add 2 ml of plating medium (*see* **Note 17**).
7. Mechanically dissociate hippocampi using pipettes since no tissue residuals are observed.
8. Count living cells filling a cell counting chamber with 20 μl of trypan blue and 20 μl of cell suspension.
9. Concentrate cells to 2×10^6 cells/ml by adding plating medium as necessary.
10. Aspirate plating medium from the outlets of the device (*see* **Note 11**).
11. Load 30 μl of cell suspension in the inlet of device by gently pipetting close to the compartment entrance (*see* **Notes 18** and **19**) (Fig. 4*i*).

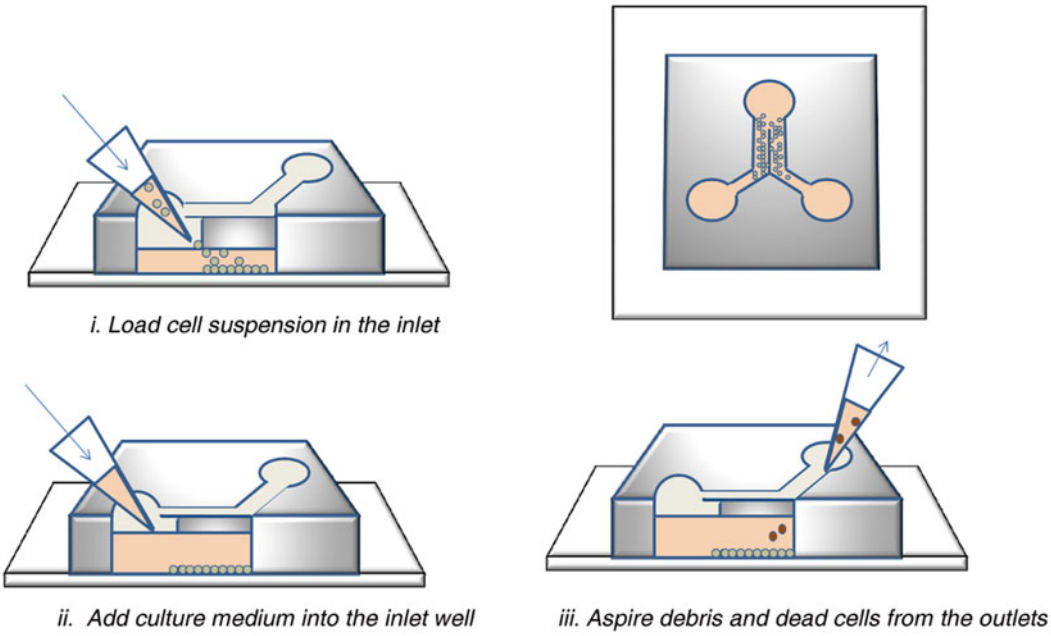

Fig. 4 Steps describing the procedure of cell seeding on the device

12. Place the device in a humidified incubator (37 °C, 5 % CO_2) for 4 h to allow cell adhesion.
13. Add 400 µl of culture medium into the inlet well (Fig. 4*ii*).
14. Aspirate culture medium from the outlets to remove debris and dead cells (*see* **Note 11**) (Fig. 4*iii*).
15. Add culture medium in the inlet until the device is completely filled.
16. Change medium the first day after plating and every 24 h by gently aspirating 200 µl of culture medium from both outlets and adding 400 µl of pre-warmed fresh culture medium into the inlet (*see* **Note 20**).

3.4 Electrophysiological Recordings and Data Analysis (See Note 21) (Fig. 5)

1. Place the device into the recording setup, as suggested by the manufacturer.
2. Start a recording 10 min after the movement of the device from the incubator to the recording setup to allow the stabilization of the electrical signals.
3. Perform the recording continuously during the biochemical stimulation (*see* **Note 22**).
4. Use Mc_Rack software to detect spikes (*see* **Note 23**).
5. Analyze spikes trains, spiking behavior, and bursting behavior with a commercial or custom software (*see* **Note 24**).

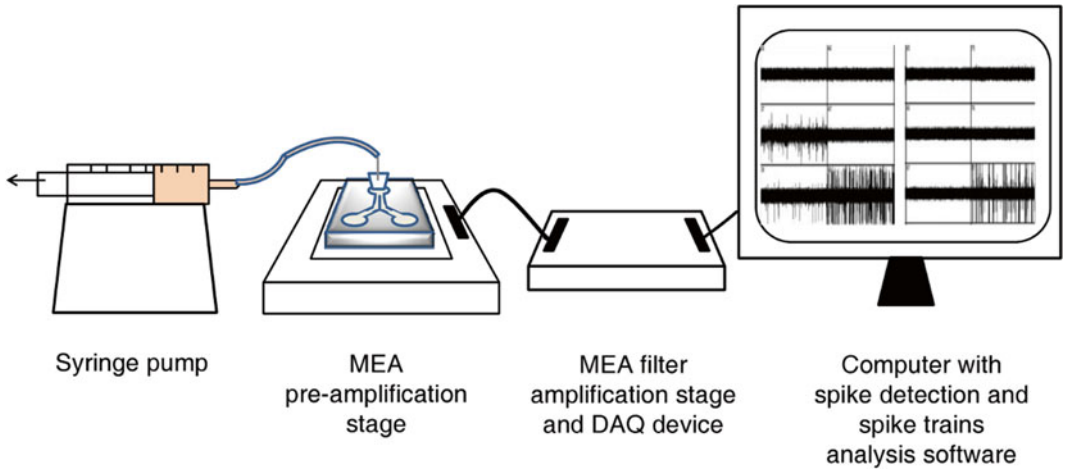

Fig. 5 Schematic representation of the experimental setup to perform biochemical stimulation of the cultures and electrophysiological recordings from MEAs

3.5 Biochemical Stimulation of a Twin Network (Fig. 6)

3.5.1 Left Twin Network Treatment

1. Aspirate culture medium from the inlet and the outlets (*see* **Note 8**).
2. Plug a conical PDMS cap inside the inlet port of the device and connect it to a syringe pump (*see* **Note 25**).
3. Add 200 µl of culture medium to the right outlet port.
4. Dilute the right quantity of selected compound with 200 µl of culture medium to obtain the desired concentration and add it to the left outlet port.
5. Activate the pump in aspiration mode at 10 µl/min for 15 s, the time necessary to completely fill the compartments with the compound (*see* **Note 26**).
6. To stop the biochemical stimulation, add fresh culture medium to both outlet ports to perform the wash-out (*see* **Note 27**).

3.5.2 Right Twin Network Treatment

1. Aspirate culture medium from the inlet and outlet ports (*see* **Note 8**).
2. Plug a conical PDMS cap inside the input port of the device and connect it to a syringe pump.
3. Add 200 µl of culture medium to the left outlet port.
4. Dilute the right quantity of selected compound with 200 µl of culture medium to obtain the desired concentration and add it to the right outlet port.
5. Activate the pump in aspiration mode at 10 µl/min for 15 s (*see* **Note 26**).
6. To stop the biochemical stimulation, add fresh culture medium to both outlet ports to perform the wash-out (*see* **Note 27**).

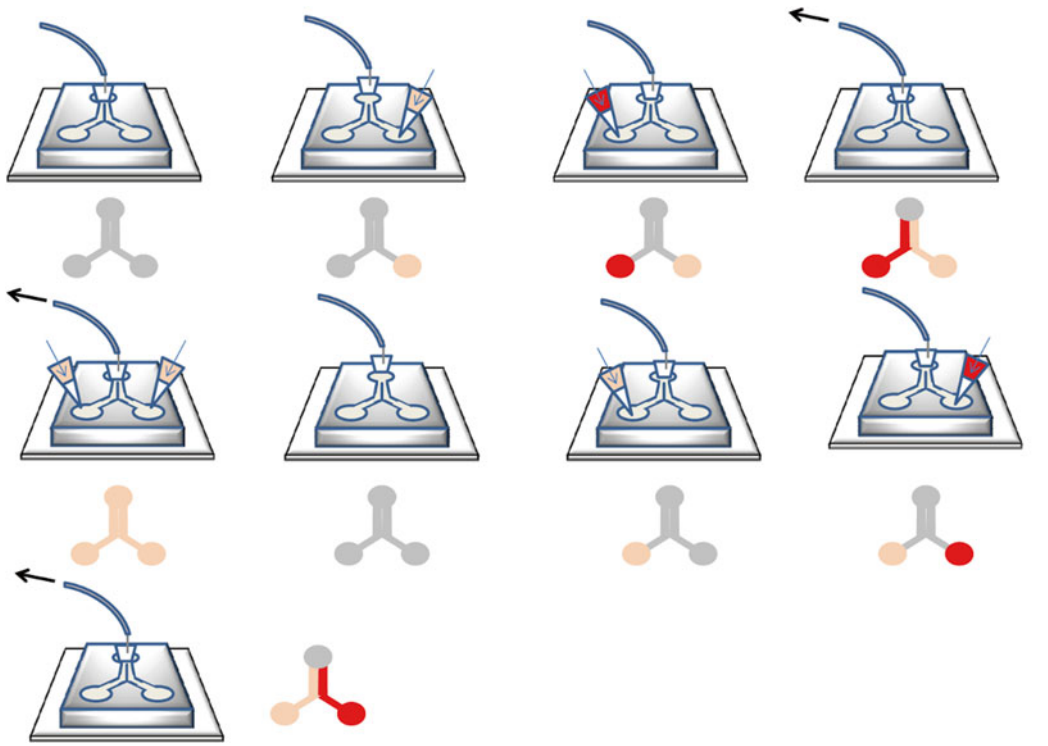

Fig. 6 Procedure of biochemical stimulation of the networks (first the left network and then the right network, in this example). Below each step, a top view of the microfluidic compartments and wells is reported (*red*= neuropharmacological compound diluted with culturing medium; *pink*= culturing medium) (color figure online)

4 Notes

1. Either an air or an oxygen plasma cleaner can be used. Oxygen plasma is effective in less time than air plasma treatment.
2. Any in vitro multichannel recording system can be used, provided that it can be interfaced with flat Microelectrode Arrays.
3. Any syringe pump is suitable on condition that it can set a flow rate lower than 100 μl/min. If the pump is equipped with one syringe, it is used to aspirate the medium from the inlet port of the device (Sect. 3.5). If a dual channel pump is available, it can be used to inject medium/treatment into the outlet ports or to aspirate the medium from the inlet port of the device.
4. Any Microelectrode Array is suitable if it has a flat surface, i.e., there is no well to contain cells and medium over the matrix of electrodes.
5. Outsourcing services for mask productions are available at low cost, e.g., Micro Lithography Services Ltd.

6. In case of bubbles after spincoating, degas through vacuum, if available.
7. To minimize the risk of cracks in the SU-8, it is advisable to provide the required energy dose in 3–4 exposure periods rather than a single one.
8. During device preparation, cell seeding, and culture maintenance, any liquid medium is added to the common well (inlet, in Fig. 1) and aspirated from the two outlets. Vice versa, during selective chemical stimulation of the two networks the common well is used as outlet and the two separated wells serve as inputs. In the following, the common well is named as inlet and the two separated wells as outlets.
9. Whatever is the number of electrodes, they have to be aligned on the substrates so that they can be separated in two symmetrical groups. In this way, the sampling of the electrophysiological activity is comparable between the two networks.
10. It is important to realize the irreversible bonding immediately after the plasma treatment. After bonding, the assembled device can be reusable through washing with 1 g/100 ml Tergazyme (Sigma) followed by autoclave.
11. Be careful to avoid removing all of the liquid: the main channel must remain filled.
12. Because of poly-L-lysine viscosity, it is necessary to aspirate it from the outlets in order to completely fill the two channels.
13. This allows any potential debris or toxic residue to be removed.
14. The device can stay in the incubator for a maximum of 2 days before the cell plating.
15. The example here provided regards the preparation and seeding of hippocampal cultures following standard protocols established in our lab. Any other cell plating protocol may be suitable, provided that the final cell density is the same and the same procedures to seed cells in the compartments are used.
16. Other cell types can be used. Outsourcing company for embryos or brain parts sale are available (e.g., Embryotech Laboratories Inc., Neuromics Inc., AMS Biotechnology Ltd).
17. This volume is adequate when the number of hippocampi is higher than 5. If less hippocampi are available, the volume can be reduced to 500 μl.
18. This procedure has to be performed very gently. Cell passage in the compartments is obtained by adding cells in the inlet well and exploiting the liquid level gradient between the inlet and the outlets. If the volume esteem is not accurate or the pipetting force is too high, cells may sediment at the outlet of the compartments, and not over the electrodes.

19. In this example, a total amount of 60×10^3 cells are plated on the device, which results in a cell density of nearly 4,000 cells/mm^2 in each compartment, given the area of the culturing regions. Note that the final cell density influences network development and thus should be considered when planning the experiment [12].
20. In general, for DIV15 (and older) cultures, less media should be removed when changing the media, since mature neurons get more stressed with high frequency media changing and large volumes of fresh medium.
21. Electrophysiological recordings can be carried out in static fluidic conditions, to measure spontaneous electrical activity of the two networks, or they can be performed during the biochemical stimulation described in Sect. 3.5.
22. Write down the time points of drug adding or washout.
23. Any other spike detection software can be used.
24. Commercial software include McRack (MCS GmbH), NeuroExplorer® (Nex Technologies), Offline Sorter (Plexon Inc.). Custom algorithms are commonly developed in Matlab® (The MathWorks Inc.).
25. PDMS conical caps can also be plugged into the other wells during the experiment in order to preserve culture sterility.
26. Other flow rate values can be set. The lower the flow rate and the higher is the time to stimulate the network (e.g., it is possible to gradually stimulate a growing portion of the network). Higher flow rates decrease the stimulation time, but they do not have to exceed the maximum share stress borne by the culture. Moreover, they can perturb cell responses compared to static conditions, thus potentially altering the electrical activity.
27. Some biochemical compounds strongly bond to the network receptors and higher flow rates (e.g., 300 µl/min) may be necessary to perform a complete washout.

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Chapter 14

Compartmentalized Synapse Microarray for High-Throughput Screening

Amol D. Jadhav, Wei Li, Zhen Xu, and Peng Shi

Abstract

Dissociated primary neuronal cell culture remains an indispensible approach for neurobiology research in order to investigate basic mechanisms underlying diverse neuronal functions, including synaptogenesis. Synaptic function is affected in many brain diseases and disorders. The bidirectional nature of synaptic signaling and the presence of a multitude of transsynaptic signals make it complicated to study the direct effects of regulatory factors during synapse assembly. Neuron–fibroblast cocultures have proven to be a powerful technique to study several aspects of synaptogenesis; however, they suffer from low throughput and limited quantitative outcome. The development of high-throughput technologies for genetic and chemical screening can be significantly advanced by miniaturization. The recent development in the area of microfabrication and microfluidics has enabled creation of microscale-compartmentalized devices for neurobiology. These devices are cheap, are easy to manufacture, require reduced sample volumes, enable precise control over the cellular microenvironment both spatially and temporally, and permit high-throughput testing. In this chapter, we describe the protocol and methodological considerations for developing synapse microarray that enables ultrasensitive, high-throughput and quantitative screening of small molecules involved in synaptogenesis.

Key words Compartmentalized assay, Synapse microarray, Neuron cell culture, In vitro models, High-throughput screening, Synaptogenesis

1 Background and Historical Overview

The human brain undergoes remarkable self-organization and development during its developmental cycle and understanding the mechanism governing these functional aspects has huge implication from basic biology to treatment of brain disorders. The motive behind development of analytical tools and techniques for neurobiology has thus been to advance the understanding of the nervous system from molecular to systems level. Typically, neurons and their projected extensions that travel over considerable distances are subjected to varying chemical, physical and fluidic microenvironment making in vitro investigation a major challenge [1]. Isolated primary neurons are widely used for

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experimental investigation and have served an important role in the understanding of mechanisms governing diverse neuronal functions such as axon guidance and pathfinding [2–10], synaptogenesis [11–15], and plasticity [16–23].

Synaptogenesis can be defined as the entire process that leads to a fully functional synapse, including cell–cell contact, differentiation of nascent presynaptic and postsynaptic terminals, development of morphological specializations, and ultimately the organization of mature synaptic inputs. Synapse formation requires the assembly of a highly ordered protein complex containing receptors, signaling molecules, and scaffolding proteins. The synaptic development is intricately regulated by various signals including neurotransmission, soluble factors secreted by neurons or glia, and direct cell–cell contacts [24, 25]. The bidirectional synaptic signaling and the presence of a multitude of transsynaptic signals make it difficult to discriminate the direct effects of regulatory factors from the indirect effects on synapse assembly [26]. This impasse constitutes a major challenge for the development of screening methods for various diseases that involve specific synaptic proteins. Furthermore, most synaptic adhesion proteins require membrane anchoring and lateral interactions to function normally so direct addition of purified proteins to primary neuron cultures is not suitable [27]. Neuron–fibroblast cocultures enable significant control over the proteins involved in transsynaptic signaling, and thus greatly reduce the complexity in dissecting the transsynaptic signaling. Such coculture assays have been used to discover several adhesion proteins that modulate the synaptic function [28–32], and molecules or drugs that modulate synaptogenesis.

Despite their proven potential, existing coculture assays are not conducive to high-throughput screening for several reasons. First, the random distribution and occurrence of neuron–fibroblast and neuron–neuron interactions on culture substrates makes it difficult to identify and analyze the numerous synaptogenic events. Second, the fibroblast cells typically exhibit irregular morphologies, spreading or clustering, thus severely hindering automated analysis. Furthermore, the spatial variations in the density of randomly growing neurites on substrates create significant fluctuations in the quantification of synaptogenic events induced when neurites contact fibroblast cells, thereby reducing assay sensitivity. As a result, large numbers of cells are required in order to make statistically significant measurements, and subtle effects can be lost within the experimental noise. Finally, in the traditional coculture neuronal somas that are located within proximity to the fibroblast cells may provide neurotrophic or other factors that may affect the synapse formation and could indirectly bias the effects of the fibroblast secreted synaptic proteins [33, 34].

The application of microfabricated culture devices enables us to overcome most of the limitations in traditional neuronal

cultures, and enabling local manipulation of the physical and biochemical environment. The integrated devices are easy to assembly, offer the fabrication flexibility enabling neuronal compartmentalization, microfluidic transport, multiplexed chemical stimulation capability and have been used for both peripheral nervous system (PNS) and central nervous system (CNS) derived neurons. The prominent feature of most compartmentalized microfluidic devices is parallel multi-groove topography, the ends of these groove channels open into a larger channel that serves either to seed the neurons or stimulate the growth of axons. Since the first demonstration by Noo Li Jeon's group [35], there have been a few variations in the design of microfluidic compartmentalized devices, including a three compartment design [36, 37], devices that enable electrode placement [38, 39], and with circular layout [40–42]. Some of these devices have also been used for coculture CNS axon–glia interaction studies [40–42].

As one of the first demonstrations, we developed a compartmentalized synapse microarray device to screen small molecules that enhance synaptogenesis [43] (Fig. 1). Technologies for large-scale synapse assays can potentially facilitate identification of novel drug leads. The synapse microarrays consist of two main

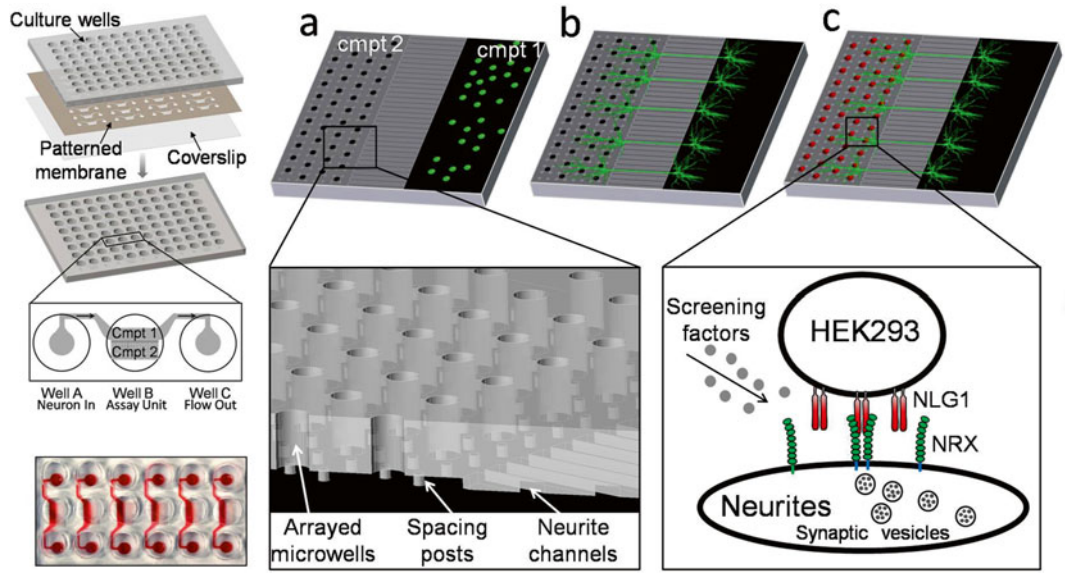

Fig. 1 Schematic of the synapse microarray technology. *Left*, Schematic showing the complete layout of a single synapse microarray device. (**a**) Neuronal cells are loaded and cultured in compartment (cmpt) 1. (**b**) Neurites extend through the microchannels (325 μm long and 10 μm wide) and form a dense network in cmpt 2. Cmpt 2 is covered by a layer of 80-μm thick PDMS membrane containing 30-μm diameter through holes (*see inset* in **a**). The membrane is held 3 μm above the substrate via dispersed 10-μm diameter posts (*see inset* in **a**). (**c**) HEK293 cells expressing NLG1 are then seeded into the through-holes, and cocultured with neurons for 1–2 days in the presence of screening factors before immunostaining for synaptic markers. In *inset*, NRX represents neurexin (Reproduced with permission from Shi et al., 2011.)

compartments connected by parallel microchannels (325 μm long, 10 μm wide, and 3 μm high); which have been previously shown to effectively isolate axons from neuronal somata. Dissociated neurons are plated and cultured in compartment 1 (Fig. 1a). The neural processes originating from the somata grow through the microchannels and extend into compartment 2 (Fig. 1b), which is covered by a thin (80 μm) PDMS membrane held 3 μm above the substrate by small posts (10 μm diameter), which provide space for axonal outgrowth. The membrane also contains an array of through-holes (microwells with 30 μm diameter). This platform was able to induce synapses in regular arrays by precisely controlling the position of NLG1-expressing HEK293 cells and allowed neurites to grow freely around them (Fig. 1c). This platform enables tenfold increase in sensitivity as compared to the traditional assays, and simultaneously decreases the time required to capture synaptogenic events by an order of magnitude. Using this technology, a chemical library was screened and novel histone deacetylase (HDAC) inhibitors that improve neuroligin-1-induced synaptogenesis were identified.

In this chapter, we describe the complete protocol for developing a "Synapse Microarray" that is capable of investigating the differential effects of small molecule inhibitors on synaptogenesis in a high-throughput manner. The major advantage of this platform is that it affords the ability to rapidly image and quantify the synaptogenic events induced by specific proteins. The high sensitivity of this device can also enable identifying subtle changes in synaptic function; also it requires much lower amount of samples than traditional screening. With further modification this platform can also be easily employed for studying dendritic and postsynaptic development.

2 Equipment, Materials

2.1 Equipment

A standard list of equipment required for cell culture can be found in a regular bioengineering laboratory. Access to a clean-room or sourcing to a fabrication foundry (e.g., Stanford Microfluidics Foundry) can meet the minimal microfabrication requirements for implementing this protocol.

1. Plasma cleaner
2. Photolithography equipment, Mask Aligner (Karl Suss MA6)
3. Spin Coater
4. Vacuum desiccator
5. Temperature controlled oven and hot plates
6. Cell culture CO_2 incubator (ThermoScientific)
7. Biological safety cabinet

8. Olympus IX81 fully automated microscope
9. On stage culture chamber for conducting time-lapse experiment (Pathology Labs, MD)

2.2 Materials

The synapse microarray device is made from polydimethylsiloxane (PDMS), a biocompatible silicone material. The process for making a master mold for casting the devices requires materials as listed below.

2.2.1 Microfabrication

1. Silicon wafer (4 in.)
2. 20,000 dpi transparency masks (CAD Art Services, Inc.)
3. SU-8 2002 photoresist (Microchem)
4. SU-8 2050 photoresist (Microchem)
5. SU-8 developer (Microchem)
6. PDMS (Sylgard 184, Dow Corning)
7. Isopropanol (Sigma-Aldrich)
8. Ethanol (Sigma-Aldrich)
9. tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (Sigma-Aldrich)
10. 0.1 % Pluronic F127 solution (Sigma-Aldrich)
11. Laminin (Sigma-Aldrich)

For our experimental investigation we have used the standard cell culture protocols. The N-terminal HA-tagged murine NLG1 construct and the negative control construct, HA-tagged acetylcholinesterase (AChE) were a gift from Prof. Peter Scheiffele (U. Basel).

2.2.2 Cell Culture

1. CMF-HBSS (Calcium bicarbonate-, magnesium bicarbonate-free Hank's balanced salt solution (BSS)) buffer with 10 mM HEPES, pH 7.3 10× Hank's BSS (Invitrogen) and 1 M HEPES buffer, pH 7.3 (Invitrogen)
2. Dulbecco's Modified Eagle Medium (DMEM) DMEM, glutamine, penicillin–streptomycin (Gibco), fetal bovine serum (FBS) (Hyclone)
3. Neurobasal medium, glutamine, B27 serum-free supplement, penicillin–streptomycin (All from Gibco)
4. German glass coverslips (25 mm, Bellco Glass)
5. Poly-L-lysine (PLL) 70 kmol wt (Sigma-Aldrich)
6. Bovine Serum Albumin (BSA) (Sigma-Aldrich)
7. Phosphate Buffer saline (PBS) 10× (Life Technologies)
8. Hippocampal neurons E18 Sprague Dawley rats
9. Stable HEK293 cell line expressing N-terminal HA tagged murine NLG1 or HA-tagged AChE.

10. Lipofectamine 2000 (Life technologies)
11. Paraformaldehyde (Sigma-Aldrich)
12. Papain (Sigma-Aldrich)
13. Mouse natural Laminin (Life Technologies)
14. Antibodies to βIII-tubulin (R&D) and synapsin (Millipore)

3 Procedure

3.1 Fabrication of Compartmentalized Culture Device

Typically, the devices are fabricated using a technique called soft lithography, a modified fabrication strategy borrowed from the semiconductor industry now employed for making microfluidic devices. A standard fabrication workflow for making a synapse microarray compartmentalized device involves three steps, first fabricating a master mold, then casting the PDMS device and finally assembling it with the glass coverslip and culture wells.

3.1.1 Fabrication of Master

Step I: SU-8 2002 Layer Deposition

1. The wafer is exposed to plasma for 5 min to clean the wafer.
2. Program the spin coater for a two-step process (a) 10 s at 500 rpm and (b) 30 s at 1,500 rpm with a ramp speed of 100 rpm/s.
3. Working in a fume hood, carefully load the SU-8 onto the wafer. We normally use about 5 ml of SU-8 2002 for a 4-in. wafer (1 ml/in).
4. Place the wafer at the center of the spin coater and run the program.
5. Carefully place the wafer on the 65 °C hot plate for 1 min and then transfer the wafer on the 95 °C plate for 3 min (**Note 1**).
6. Wait for 5 min to cool the coated wafer.

Step II: Exposure

1. Use a transparency mask as in this protocol (or soda-lime glass mask) with the high-resolution printed microstructure features.
2. Place the mask on the mask holder.
3. Place the wafer at the center of the chuck and apply vacuum to secure the mask in place.
4. Align the wafer close to the mask and perform the exposure in "Contact mode."
5. Expose under UV for certain period of time depending on the calibrated power of the UV lamp (**Note 2**).
6. Post-exposure bake the wafer on 65 °C hot plate for 1 min, followed by 95 °C for 1 min.

Step III: Resist Development

1. Immerse the wafer in SU8 developer followed by gentle agitation for a few minutes.
2. Rinse the wafer thoroughly with isopropanol after development.
3. Air-dry the wafer (**Note 3**).

Step IV–VI: Second SU8 Layer Deposition

Follow all the steps in the first layer fabrication to deposit a second layer of 60 μm photoresist using SU-8 2050. The exact parameters for spin coating, pre-bake, post-bake, exposure can be found in the instruction for SU-8 on Microchem, Inc. (**Note 4**).

3.1.2 Replica Molding of the Compartmentalized Device

Step I: Preparing the Master

1. Place the patterned wafer in a vacuum desiccator.
2. Place a small open vial with few drops of tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane in a desiccator and evacuate the air. Keep airtight overnight (**Note 5**).

Step II: Molding the PDMS device

1. Mix PDMS prepolymer and catalyst in the ration 10:1 (w/w). Thoroughly mix the and then place in a vacuum desiccator for 15 min (**Note 6**).
2. Place the Master mold on an aluminum plate covered with an aluminum foil.
3. Pour (5 ml) of the PDMS mixture on the master, a plastic transparency is lowered onto it to cover the PDMS mixture, avoiding any bubbles.
4. A second plain wafer and then another aluminum plate are placed on the transparency sequentially to gently squeeze out extra PDMS prepolymer between the transparency and the master mold.
5. Clamp the master mold–prepolymer–transparency–plain wafer stack between the two flat metal (aluminum) plates, and apply high pressure to further squeeze excess prepolymer (**Note 7**).
6. Carefully place the assembly in a temperature-controlled oven and cure for at least 12 h at 85 °C. Upon curing the PDMS will look like a transparent, solid material.
7. Remove the assembly and allow it to cool; a thin layer of PDMS membrane is left on the master mold.
8. Attach the premade multiwell formatted culture wells to the membrane and gently peel it off from the master mold.

3.1.3 Assembly of the Synapse Microarray

Step I: Preparing the Coverslips

1. Clean the coverslips with a jet of air. Handle the coverslips using forceps; place them in concentrated sulfuric acid for at least 12 h.
2. Rinse the coverslips extensively with distilled water and air-dry them.

3. At least 1 day prior to tissue dissection, coat the coverslips with polylysine at a concentration of 100 μg/ml for at least 4 h (**Note 8**).
4. Rinse off excess polylysine solution with DI water, air-dry, and store the coated glass coverslips in a 4 °C refrigerator.

Step II: Device Assembly

1. Place the multiwell culture chamber (covered with a PDMS membrane on the bottom side) device onto the polylysine coated glass coverslip, usually a gentle pressure should be enough to achieve conformal contact and sealing. The adhesion between PDMS and the coverslip surface is tight enough to prevent solution leaking out.
2. The assembled device is then coated with 0.1 % Pluronic F127 solution to prevent protein absorption onto PMDS surfaces, and is further soaked with 10 μg/ml laminin solution for at least 2 h before use.

4 Cell Culture and Testing

4.1 Neuronal Cell Culture

4.1.1 Step I: Dissection of Hippocampal Neurons

1. A pregnant E18 Sprague Dawley rat is sacrificed using an approved method of euthanasia, dissect out the uterus and place it in a sterile Petri dish.
2. Working in a laminar flow hood, remove the fetuses from the uterus, extract out their brains, and place them in calcium free Hank's balanced solution (HBSS). The hippocampi are extracted by dissection under a stereoscope according to the standard protocol.
3. The hippocampi are collected and transferred into a tube containing papain solution (20 units/ml), and digested at 37 °C for 30 min.
4. After digestion, get rid of the suspension, add 2 ml Dulbecco's Modified Eagle Medium (DMEM) medium and then break the tissue into single cell solution by gentle trituration using a 1 ml pipette tip.
5. Determine the cell density by adding a drop of the cell suspension to a hemocytometer. Also determine the total yield, which should be 400,000–500,000 cells per hippocampus.

4.1.2 Step II: Culturing Neurons on Synapse Microarray Device

1. Using a micropipette, add 10 μl of the cell solution (neuron density 3×10^6 ml^{-1}) into the cell body compartment of the synapse microarray.
2. After 2–3 h, examine the compartment to ensure that most of the cells have settled and attached to the surface of the coverslip, and then replace the medium with Neurobasal medium supplemented with B27, 0.5 mM L-glutamine and antibiotics.

3. It is a good practice to replace half volume of the old medium with fresh medium every 3–4 days, wait for 7 days before coculturing with the HEK293 cells (**Note 9**).

4.2 HEK293 Cell Culture

4.2.1 Step I: HEK 293 Culture

1. Thaw the vial containing frozen HEK 293 cells by placing it in a 37 °C water bath. In order to sterilize, cleaned the outside of the vial with 70 % ethanol.
2. Warm up the DMEM
3. Transfer the cells (in 10 ml of DMEM medium) to a 100-mm culture plate.
4. HEK 293 cells can be transfected with NLG1 or AChE plasmid constructs using transfection reagent Lipofectamine 2 days after they are transferred to the culture plate.
5. HEK 293 cells are maintained in DMEM medium supplemented with 10 % FBS, 1 % penicillin and streptomycin, 2 mM L-glutamine, and geneticin. Cultures are passed every 3–4 days once they reach 80–90 % confluency.

4.3 Neuron–HEK293 Coculture and Testing on Synapse Microarray

1. Add 100 μl of transfected HEK293 cell suspension to the Well B of the synapse microarray.
2. Briefly centrifuge the plate at 150 rpm for 1 min. The cells should settle down within the microwells in compartment 2.
3. Rinse off the extra cells via medium exchange.
4. The coculture is maintained in Neurobasal medium for 24–48 h.
5. The small molecules test compounds are added directly to the medium at required final concentrations (0.1 μM, 1 μM, 10 μM) (**Note 10**).

4.4 Immunocytochemistry

1. The coculture was fixed for 30 min in 4 % paraformaldehyde in PBS buffer
2. The cells are permeablized using 0.25 % Triton X-100 for 20 min
3. Block with 4 % BSA in PBS for 2 h at room temperature (or overnight at 4 °C)
4. Incubate with primary antibodies (anti-βIII-tubulin and anti-synapsin) in 4 % BSA for 2 h at room temperature. Rinse with PBS (**Note 11**).
5. Finally, incubate with secondary antibody for 1 h and rinse with PBS prior to imaging.

4.5 Automated Image Acquisition and Quantification

1. Place the stained samples on the microscope and image using appropriate filters.
2. Take 10–15 images within 500 μm from the microgrooves guiding neurite outgrowth.

3. To quantify synapsin fluorescence, the microwell's filled with HEK293 cells should be selected as regions-of-interest (**Note 12**).

5 Typical Anticipated Results

1. The length of the microgrooves effectively isolates the soma from the axons (Fig. 1), so that effects of postsynaptic protein can be studied on isolated presynaptic terminal.
2. The contact area between the neurites and the HEK293 cell remains constant, and is defined by the diameter of the microwells. Synapses are expected to form in an array format (Fig. 2).
3. The confinement of the HEK293 cells should not influence the Neuroligin-1 expression levels in the trapped HEK293 cells.
4. The synapse microarrays are expected to identify regulatory factors for the synaptogenesis process in a dose dependent manner. A linear dose response is expected. Refer to published results [43] from our experiment with HDAC inhibitor, trichostatin A (TSA) to check sensitivity analysis on the synapse microarray. We estimate that the sensitivity should be ~10-fold higher than regular neuron–fibroblast cocultures.

6 Troubleshooting

There are various critical experimental steps that can lead to device failure or suboptimal performance during the actual testing with neurons. This section describes several practical recommendations that may help the researchers to obtain more reliable, and reproducible data. It is also advised that the user refers to manufacturer's instructions and published protocols to optimize their experiments.

6.1 Device Fabrication and Assembly

6.1.1 Fabrication Process

1. During the SU8 process there is a possibility of trapping bubbles in the thin film that is spun. The possible reason for this is manual handling while pouring the photoresist. If the facility has an automated dispensing system (e.g., Brewer CEE 6000 automated spin coat), this issue can be usually avoided.
2. The soft-bake prior to exposure is a critical step; failure to sufficiently remove the solvent will affect the resist profile; on the other hand excessive baking destroys photoactive compound and reduces sensitivity. While we have optimized the process, it is recommended to refer to the company manual and test different parameters for desired thickness.
3. If the patterned features are invisible post-exposure the reason might be under exposure or inappropriate dosage. This issue

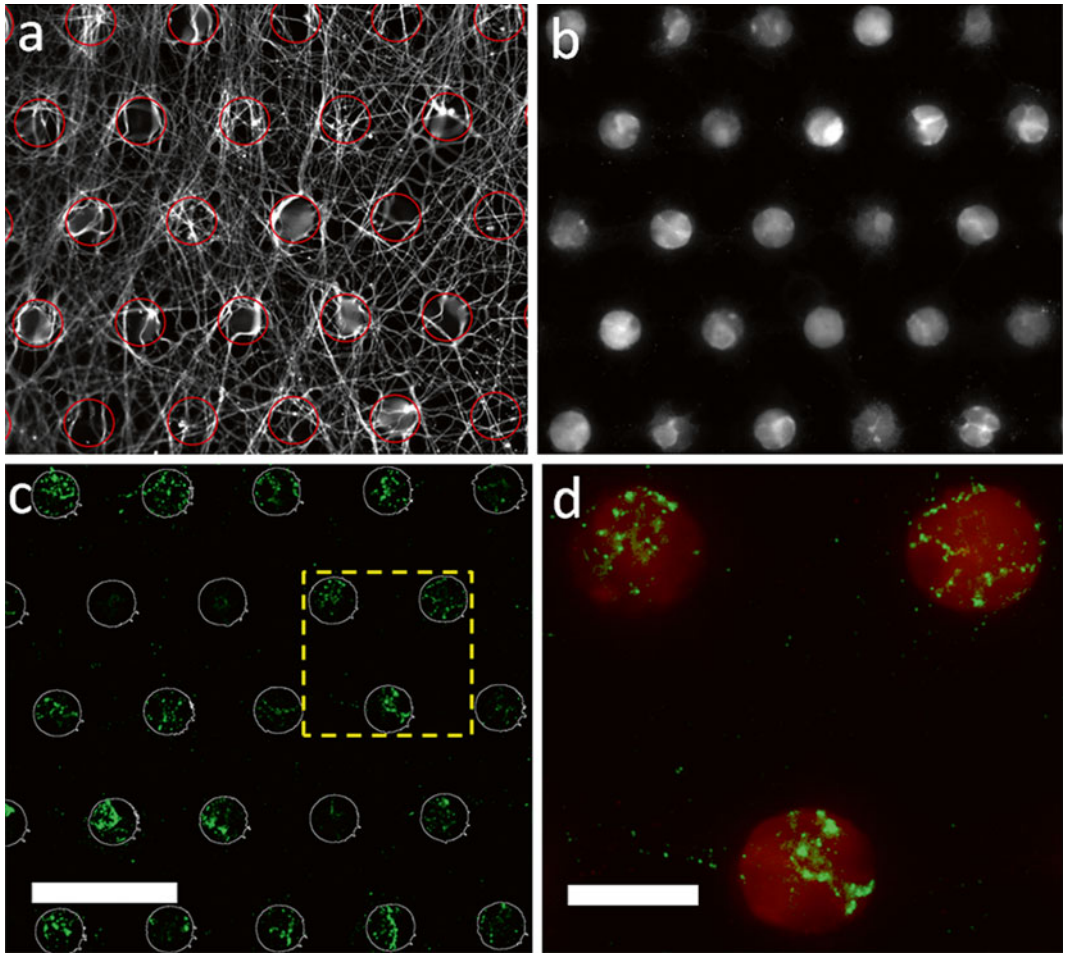

Fig. 2 Precise control of the neuron–fibroblast coculture by synapse microarrays. (**a**) βIII-tubulin fluorescence image of neurites in the synapse microarray. The microwells are circled in *red*. (**b**) Fluorescence image showing neuroligin-1 expressing HEK293 cells in microarray format. (**c**) Fluorescence image of synapsin clustering in microwells (*white circles*) filled with HEK293 cells, scale bar, 100 μm. (**d**) Enlarged view of the *boxed* region in panel (**c**) (synapsin, *red*; HA–NLG1, *green*), scale bar, 30 μm (Reproduced with permission from Shi et al., 2011.)

can be avoided by increasing the energy dose or the exposure time. If the solvent level is reduced (by using longer soft-bake) the user may need to increase their exposure dose.

6.1.2 Device Assembly

1. Leaking is a common issue during the device assembly; this is usually caused by nonuniform bonding of the PDMS with the glass substrate. Clean the PDMS device properly and remove any debris that can be detrimental to the bonding process.
2. Always use clean tweezers and wear gloves while handling the glass coverslips and the PDMS device. Avoid touching the PDMS surface facing glass substrate.

6.1.3 Cell Culture

1. It is critical to have healthy, non-contaminated cells for good experimental results, make sure all of the steps are performed in the tissue-culture laminar flow hood.
2. While working with neurons, the cell bodies may clump together. This might be the result of improper cleaning of the coverslips or long-term storage prior to use. It is recommended to thoroughly clean and fresh PLL coated coverslips.
3. When working with HEK293 cells, it is a good practice to passage the cells while they are 80–90 % confluent. If the cells become too confluent, they can change biochemically and may not be optimal for a critical experiment.

6.1.4 Image Acquisition and Analysis

1. Threshold selection cannot be overemphasized, since further processing and analysis of the images entirely depends on the quality of the segmentation. For quantifying synapsin fluorescence, all images taken in a single experiment should be thresholded equally. We set manual threshold, but for larger set of images automated-segmentation can be employed.
2. If a lot of variation is observed in the quantification of synapsin, try to quantify total neurite length within each microwell using the βIII-tubulin fluorescence images. If the axon density also shows much variation, it means that the neuron culture is not good enough.

7 Notes

1. The wafer should be exactly located at the center of the spin coater. The final thickness of SU-8 is determined by the SU-8 viscosity and spin rate. The thickness and correlated spin speed curves are available for the supplier Microchem.
2. Information about the optimal exposure dose can be obtained from Microchem, Inc.
3. This step will fabricate the first SU-8 layer (3 μm thick) that contains negative features for the supporting posts and the microchannels.
4. It is critical to align the two layer of photoresist under the mask aligner before exposing the second layer. Good alignment guarantees precise placement of the microscale features on different layers.
5. The silanization process enables easy removal of the molded PDMS structures
6. If the mixture is not degassed properly the bubbles will be formed and trapped in the PDMS device.

7. Avoid lateral relative movement between the master mold and the transparency; it will otherwise destroy the features on the mold.
8. The polylysine solution should spread evenly over the entire surface of the well-cleaned coverslips.
9. Changing the medium completely will result in neuronal cell death. A culture on plain coverslip should be included as a general control to verify the culture status. After 7 days, significant axons should have passed through the microgrooves forming network in compartment B.
10. The diluted compounds are added to both the cell body and axon compartments.
11. anti-βIII-tubulin is used to visualize neuron morphology and anti-synapsin is used to indicate synaptogentic activities.
12. The fluorescence intensity in the microwells should be quantified and normalized to negative-control HEK293 cells (e.g., AChE-transfected or non-transfected).

8 Conclusion

The ability to control the microenvironment and directing growth of neurons in a spatial and temporal manner makes microfluidic and compartmentalized devices a versatile tool for neurobiological research. The "Synapse microarray" platform is relatively cheap and simple to assemble, test. The major advantage of the platform is the ability to rapidly image and analyze the synaptogenesis mechanism with sufficiently high sensitivity. A quick comparison shows that this platform takes approximately 7–8-fold less time as compared to a traditional coculture. Secondly, given the high assay sensitivity, subtle changes of the synaptogenic activity can be readily detected, and it also requires much lower concentration of the test compound than in the traditional screening. Finally, slight modifications to the synapse microarray can enable studying dendritic as well as postsynaptic development and help gain deeper insights into the synaptogenesis mechanism.

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